Expert Opinion

Starch microparticles as vaccine adjuvant

Niclas Rydell, Linda Stertman & Ingvar Sjöholm†
†*Uppsala University, Department of Pharmacy, Biomedical Centre, PO Box 580, SE-751 23 Uppsala, Sweden*

The demand for new vaccine adjuvants is well documented. New purified antigens from parasites, bacterial or viral pathogens, as well as recombinant subunit antigens and synthetic peptides, are often inherently weak immunogens; therefore, they need some kind of adjuvant to help initiate an immune response. In addition, there are very few adjuvants using the potential of the mucosal immune system, which may play an important role in the defence against air- and food-borne infections. Starch is a natural biocompatible and biodegradable polymer that is suitable for the production of various particulate adjuvant formulations, which can induce mucosal as well as systemic immune responses. This review gives an account of the different starch adjuvants used in immunisation studies. In particular, the properties of polyacryl starch microparticles as an oral vaccine adjuvant that induce protective immune responses in mice challenge experiments are summarised. In addition, a diphtheria booster vaccine has been proposed to be used to proving the concept in man and the possibilities to design an efficient vaccine formulation for human use are discussed.

Keywords: microparticles, mucosal vaccination, oral vaccination, starch, vaccine adjuvant, vaccine formulation

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1. Introduction

The successful use of new vaccine formulations for oral vaccination has long been an attractive goal as an alternative to the present conventional parenteral administration of vaccines. Convenient handling of the vaccines, easier manufacture and, above all, direct access to the mucosal lymphoid-associated tissues (MALT) speak in favour for the oral administration of vaccines. Moreover, there is a need for new kinds of adjuvant alternatives that can be used in combination with protein antigens purified from pathogens or synthesised biotechnologically. New adjuvants are also needed for therapeutic vaccines. So far, only aluminium-based adjuvants are approved for human use by the drug licensing authorities. Therefore, large efforts have been made on the development of new adjuvants suitable for oral vaccination, which is reflected by the large number of reviews that have been published recently on the subject [1-8].

The gastrointestinal mucosa has a decisive importance in oral immunisation, not only because of its large area but also because of the two immune systems active in the mucosa; namely, the adaptive and innate systems. The two systems most probably interact in ways that are not yet completely understood. This present review will focus the discussion on processes of the adaptive part; the reader is referred to other specialised reviews dealing with the innate system [9,10].

This paper discusses the suitability of starch and starch derivatives as a carrier for antigens and their use as adjuvant in vaccination. For a more detailed discussion on the use of other carrier systems (e.g., particles consisting of chitosan or various polylactide, glycolide copolymeric particles), the reader is referred to other recent reviews such as van der Lubben *et al.* [11] and Gupta *et al.* [12]. A discussion on the use of various cyclodextrin complexes as drug carrier is also included.

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2. Starch particle formulations

Starch exists in two forms: the unbranched type called amylose, which is composed of linear glucose residues in α-1,4 linkages; and the branched form called amylopectin, which has approximately one α-1,6 linkage per 30 α-1,4 linkages. Most starches consist of ~ 20% amylose and ~ 80% amylopectin. In its native form, starch is insoluble in water but by hydrolysing it with acid or enzymes (or both) the polymer chains can be sufficiently reduced into a size that is soluble in water. Amylopectin and amylose are readily hydrolysed by α-amylase, which is secreted by the salivary glands and the pancreas. Of the carbohydrate ingestion by humans > 50% consists of starch.

Starch has a variety of uses in the pharmaceutical industry, as diluent, adhesive agents or disintegrating agents, for example. Modification of starch is easily made and can be done in order to decrease swelling or enzymatic degradation [13], or to crosslink the starch into particles, which can be used for entrapment or conjugation of protein molecules [14-16]. Such particles are normally biocompatible, taken up by the macrophages of the reticuloendothelial system and degraded in the lysosomes.

The use of microparticles in pharmaceutical applications and drug delivery has been the subject of extensive research [17], as have the use of microparticles in general as adjuvant and antigen delivery systems, which have been extensively described elsewhere [1,7,18-21]. Considering the widespread acceptance and use of starch as a pharmaceutical excipient along with its abundance and tolerability in humans, there are surprisingly few publications concerning its use in vaccine adjuvants or antigen delivery systems. A summary of the formulations with starch as the main component used as a vaccine adjuvant or antigen carrier is given in Table 1 and will be discussed in greater detail below.

2.1 Spherex®

Spherex® starch microparticles are commonly referred to as degradable starch microparticles in the literature and were introduced by Rothman *et al.* [22] as a means for microembolisation. They were then further developed by Kabi Pharmacia.

Spherex particles are manufactured by emulsion polymerisation of hydrolysed starch, and crosslinked with 1-chloro-2,3-epoxypropane (epichlorhydrin) in alkaline conditions to yield particles of 1 – 500 µm. The microparticles swell in water and exhibit gel-like features with a swollen mean diameter of ~ 45 µm. They are, like the polyacryl starch microparticles, completely degradable *in vivo* essentially by amylase. Spherex particles are intended for use in various applications to facilitate blood flow arrest [23] and have, to our knowledge, not been used in vaccine research or as an adjuvant, but have been tried as a nasal delivery system for insulin [24]. Spherex particles are the only starch microspheres sold commercially. The reason why these particles are scarcely mentioned as vaccine adjuvant probably relates to their comparatively large diameter, which prevents its effective use as a mucosal adjuvant.

2.2 Polyacryl starch microparticles

Polyacryl starch microparticles are prepared from a hydrolysed starch (maltodextrin, MD6; molecular weight ~ 5000 Da), which is acryloylated by allowing glycidyl acrylate to react with the starch to introduce acrylic groups. The degree of derivatisation is defined by the number of acrylic groups per glucose residue [25]. The modified starch can subsequently be polymerised in a water-in-oil emulsion to form microparticles (Figure 1). The degree of derivatisation determines the porosity of the particles, which in turn affects the stability of the particles *in vivo*.

With the manufacturing method used, ~ 90% of the microparticles have a diameter < 3 µm as determined by laser diffraction spectroscopy, with a typical mean diameter of 2 µm based on the number distribution. Protein antigens can be conjugated to the microparticles via their primary amino groups, using the carbonyldiimidazole (CDI) method by Bethell *et al.* [26]. The size of the particles remains unchanged after coupling with protein antigens. The hydrocarbon crosslinks, formed during the preparation of the particles, slow down their degradation by enzymes in saliva, gastric juice and intestines (unpublished observations). Furthermore, it has been shown that particles coupled with human serum albumin (HSA) have strong adjuvant properties parenterally and orally. Microparticles without any conjugated antigen are non-immunogenic but stimulate macrophages weakly to produce IL-1 *in vitro* [27-29].

2.3 Silicone-grafted starch microparticles

Hydrolysed starch is used to entrap antigens in microparticles, rather than covalently conjugate them to the matrix. Hydrophobic polymers are used for coating the microparticles in order to enhance uptake and protect against degradation. The microparticles are manufactured by dissolving the starch in hot dimethylsulfoxide and subsequently allowing it to cool to room temperature. A solution of protein is then added to the starch and the starch-protein solution is emulsified in vegetable cooking oil (Crisco®) by vigorous stirring and sonication. Particles are formed by dropwise addition of the emulsion into acetone containing Tween 80. Alternatively, siliconised grafted particles can be obtained when the acetone contains 3-(triethoxysilyl)propyl-terminated polydimethylsiloxan (TS-PDMS) [15,30]. The microparticles are collected by filtration and have a diameter of 1 – 100 µm, with an arithmetic mean of ~ 4 – 5 µm; antigen content is 5 – 6% (weight/weight) [15]. The silicone-grafted starch microparticles have been used in model studies in mice with entrapped HSA.

2.4 Aminated starch microparticles

Aminated microspheres made by crosslinked starch (80,000 Da) or pullulan (150,000 Da, polymerised maltotriose produced from starch by *Aureobasidium pullulans*) are considered to be potential gene delivery vehicles [31]. Particles were produced by crosslinking the polysaccharides with

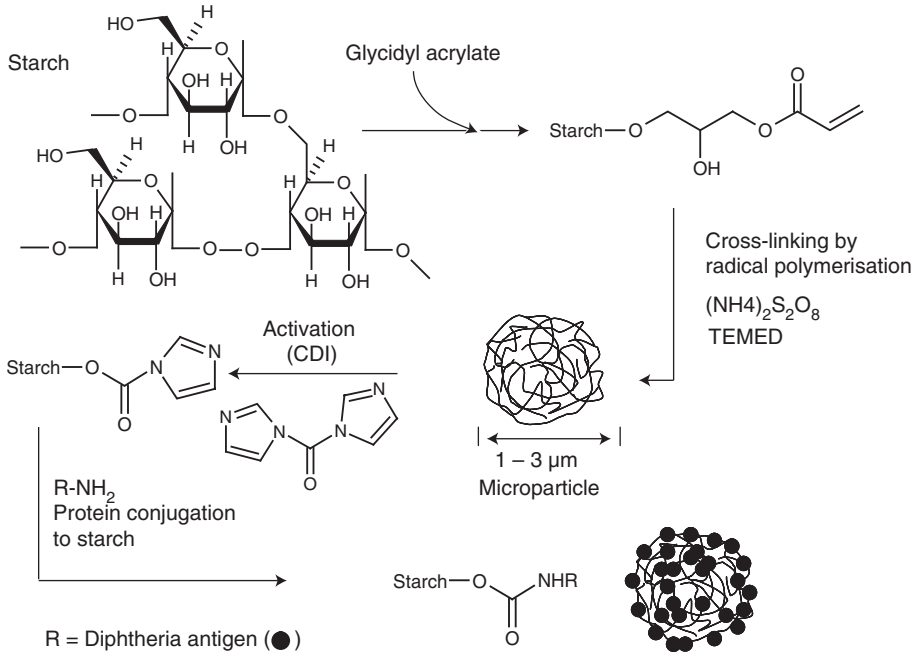

Figure 1. Preparation of polyacryl starch microparticles and conjugation of protein antigens.
CDI: Carbonyldiimidazole; TEMED: 1,1,*N*,*N*,*N*',*N*'-tetramethylethylene-diamine.

Table 1. Starch particles used for protein delivery.

Type of starch microparticle	Method of crosslinking	Modification	Antigen	Mean diameter (µm)	Administration	Ref.
Spherex	Epichlorhydrin	-	Entrapped	20 – 45	Intranasal, intragastric, subcutaneous	[37,112,113,115]
Polyacryl starch	Radical polymerisation, TEMED	-	Covalently conjugated	1 – 3	Intranasal, intragastric, subcutaneous, intramuscular	[14,29,40]
Grafted	-	Silicone grafted	Entrapped	4 – 5	Intranasal, intragastric, intraperitoneal	[15,16,30,139]
Aminated starch/ pullulan	Epichlorhydrin	Aminated	Entrapped	80 – 170	-	[31]

Overview of different starch particle formulations with selected references to publications.
TEMED: 1,1,*N*,*N*,*N*',*N*'-tetramethylethylene-diamine.

epichlorhydrin in an emulsion procedure at 80°C and alkaline pH. Amination (cationisation) was thereafter performed with *N*,*N*-diethyl-2-chloroethyl amine hydrochloride or *N*-glycidyl-*N*,*N*-dimethyl-*N*-methylammonium chloride after reduction with sodium borohydride. The mean diameter of the produced particles is between 80 and 170 µm.

The aminated microspheres have the potential to bind negatively charged polymers and were shown to quantitatively load DNA [31]. The *in vitro* release of DNA showed an initial fast release of DNA (30 min) followed by a slower release rate over 14 days. DNA stability was analysed by agarose gel and showed no degradation 14 days after *in vitro* release. So far, there are, to our knowledge, no publications available in which these particles have been tested in actual vaccination experiments. They are too large to be taken up directly by antigen-presenting cells (APCs) but may function as depot formulations in complex with negatively charged antigens (e.g., intramuscularly).

3. Properties of starch microparticles

3.1 Requirements on adjuvants

A basic requirement that has to be fulfilled by any carrier system to be used *in vivo* is biocompatibility, including biodegradability for systems taken up into any tissue. In addition, carrier systems used as vaccine adjuvants, and not only as a stabilising or delivering particulate system, also have to interact with the immune system. This can be done by activating, stimulating or facilitating the development of the immune response (i.e., by primarily initiating an inflammatory process). At the same time, the adjuvant must not in itself be immunogenic. As previously mentioned, starch as an important endogenous polysaccharide component has all the necessary qualifications to satisfy these demands. In addition, this review will show that the starch microparticles are not only functioning as a stabilising carrier system for the antigen but also as an 'adjuvant' in the sense that they facilitate the delivery of the antigen to the immune-competent cells and have macrophage-activating properties.

3.2 Macrophage activation

Starch cannot function as a carrier system with vaccine adjuvant properties without being modified and transformed into particles. Such modifications may affect the inherent properties of starch; for example, native potato starch is not soluble in cold water, like many other polysaccharides, and has to be partly hydrolysed by acid hydrolysis or enzymes in order to be soluble in water-compatible solvents. Soluble polysaccharides have little effect on macrophages in culture after phagocytosis, however, derivatisation and formation of crosslinked particles stimulate both resident and inflammatory macrophages to take up [^{14}C]glucosamine *in vitro* and to produce and release IL-1 [28,32]. These effects were related to the degree of biodegradability of the particles. As shown in Figure 2, lichenan and mannan particles stimulated a larger glucosamine uptake than starch particles. Soluble starch did not induce IL-1 release from resident macrophages but starch, as well as other polysaccharides in particle form, did so from both resident and inflammatory macrophages [32]. Particles of curdlan (1,3-β-glucan from *Alcaligenes faecalis*) even induced tumour cell toxicity, with the methylcholanthrene-induced mastocytoma P-815 cell line from DBA/2 mice as target cells (Table 2).

The stimulation of the macrophages has also been followed by estimating the release of arachidonic acid metabolites, prostaglandin E_2 (PGE_2) and leukotriene C4 (CysLT), after incubation with starch and β-glucan particles [33]. The release of PGE_2 was inhibited by indomethacin, which is known to be a potent anti-inflammatory agent. Timegadine, inhibiting both the cyclooxygenase and lipooxygenase pathways [34], was shown to totally inhibit the PGE_2 release and ~ 60% of the CysLT release.

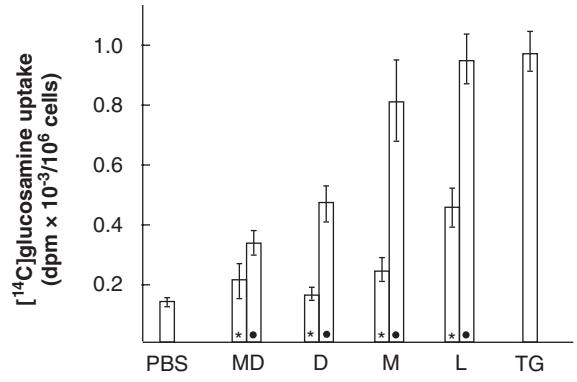

Figure 2. Activation of macrophages by polysaccharides. Uptake of glucosamine by resident macrophages after stimulation with polysaccharides in soluble (*) or particulate (•) form. Reprinted with permission from ARTURSSON P, ARRO E, EDMAN P, ERICSSON JLE, SJÖHOLM I: Biodegradable microspheres V: Stimulation of macrophages with microparticles made of various polysaccharides. *J. Pharm. Sci.* (1987) **76**:127-133 [28].

D: Dextran; Dpm: Disintegrations per minute; L: Lichenan; M: Mannan; MD: Starch; PBS: Phosphate-buffered saline; TG: Thioglycollate-stimulated macrophages.

3.3 Receptor-mediated uptake

Phagocytosis of polyacryl starch microparticles by macrophages is an efficient receptor-mediated uptake. It has been shown that starch microparticles have a substantially shorter half-life in the circulation (< 5 min) than polyacrylamide particles (~ 60 min). The particles are rapidly taken up from the circulation by the reticuloendothelial system mainly in the liver; the uptake is facilitated by opsonins [35]. Specific anticomplement receptor antibodies inhibited the uptake indicating that the complement receptors play an active role. The macrophage uptake *in vitro* is also followed by activation of complement via the alternative pathway. It was also shown that immunoglobulin G crystallisable fragment (IgG-Fc) receptors are active in the uptake [36].

The efficient uptake of the starch microparticles by macrophages in culture and histiocytic macrophages with the subsequent activation may certainly be important factors for the adjuvant capacity of the particles. However, it should be stressed that these effects of the starch particles together with antigens have not yet been shown with other APCs nor in model experiments *in vitro* or *in situ* (e.g., in the immune-competent subepithelial tissues of Peyer's patches). The significance of the inflammatory reaction in relation to the antigen-carrying and -delivering capacity of the microparticles is, therefore, not possible to evaluate at the moment.

Table 2. Expression of various markers by C57/b16 peritoneal macrophages after exposure to polysaccharides in soluble or particulate form.

	Resident macrophages			Inflammatory macrophages*		
Stimulus	Glucosamine	IL-1	Tumour cell cytotoxicity	Glucosamine	IL-1	Tumour cell cytotoxicity
None	-	-	-	-	-	-
Soluble starch, dextran, mannan and curdlan	-/+	-	-	+++	-	-
Microparticulate starch, dextran and mannan	+/++	+++	-	+++	-/+	-
Microparticulate curdlan	+++	+++	+	+++	++	+++

* Inflammatory macrophages were stimulated by thioglycollate [32].

IL: Interleukin.

4. Starch particles as adjuvant in parenteral vaccination

4.1 Immunisation with Spherex®

Montgomery and Rafferty [37] studied the immune response induced in rats after different routes of administration of dinitrophenyl-bovine serum albumin (DNP-BSA) entrapped in Spherex particles. The particles were used as a suspension and injected in the vicinity of the salivary gland with or without IL-5 and -6, or together with alum. They were also used in dry form and applied to the oral cavity. The outcome of the oral experiments is presented in Section 5.5.1. When injected together with the ILs, they induced pronounced serum anti-DNP IgG responses, even if the responses were not as strong as with alum. The salivary IgA responses were moderate. The results indicate that starch particles are a strong adjuvant parenterally. The particle diameter ranged 4 – 95 mm, which means that only the smaller particles can be phagocytosed as such and that the response is induced by DNP-BSA diffusing out of the particles or degraded DNP-BSA particle complexes.

4.2 Polyacryl starch particles as adjuvant

The macrophage-activating properties of the starch microparticles indicate that they may be effective as an immune system-activating adjuvant, which has been confirmed in mouse studies with HSA and mouse serum albumin (MSA) as model antigens [38]. The antigens were immobilised by entrapment (as the antigens were large enough) and injected intravenously. As expected, a specific immune response, shown to be T-cell dependent, was induced against HSA-conjugated microparticles but not against the MSA-conjugated microparticles. The response was confirmed in a haemolytic plaque-forming assay, in which sheep red blood cells, tagged with HSA or MSA, were incubated with guinea-pig complement and spleen cells from immunised mice. As seen from Table 3 [38], MSA particles did not induce any formation of specific antibody-producing spleen cells competent to form any plaque forming cells (PFCs). The results also indicated indirectly that the starch particles as such were not immunogenic in the sense that no haemolysis was induced in the control samples.

4.2.1 *Conjugated low molecular weight drugs: potential haptens*

Experiments with starch microparticles and entrapped HSA and MSA with their relatively poor immunogenicity may be considered as not provocative enough to conclude that starch particles as such are nonimmunogenic. Therefore, to conclude whether an immune response could be evoked against the particles, a strong hapten, the dinitrophenyl group, was conjugated to the particles [39]. DNP was covalently bound to the particles via Lys or Leu-Ala-Lys. Lys–DNP can be released *in vivo* only from the tripeptide arm but not when conjugated directly to the particles. Any DNP-specific immune response was tested for in mice injected intravenously with the two preparations. Lys–DNP particles did not induce any antibody production and the tripeptide conjugate induced only a weak response. Moreover, the antibody response after immunisation with the DNP-tripeptide conjugate was tested in an inhibition enzyme-linked immunosorbent assay (ELISA) and was appropriately inhibited with the Lys–DNP particle conjugate, in which the DNP group is exposed on the surface but only with high concentrations of free Lys–DNP and not at all with unconjugated microparticles. These experiments show that the microparticles can function as carriers for low molecular weight drugs without the production of an immune response to the hapten or to the starch. Some low-avidity anti-DNP antibodies were induced due to the enzymatic release of Lys-DNP from the tripeptide arm, however, no crossreactivity of the antibodies with the starch microparticles was detected.

4.2.2 *Conjugated proteins*

Entrapment of proteins in starch microparticles cannot be used as a general method for a stable immobilisation in the microparticles, as the highly porous structure will not effectively keep smaller proteins (< 60,000 Da; the size of serum albumins) inside the particles. Instead, it is necessary to conjugate the proteins covalently to the starch matrix. The first characterisation of the immune response was made with conjugated HSA as a model antigen after intravenous, intramuscular and intraperitoneal administrations [40]. Intraperitoneal administration induced the strongest humoral

Table 3. The specificity of the immune response in Balb/c mice after immunisation with antigen-containing polyacryl starch microparticles.

Particle-entrapped antigen	SRBC-bound antigen	No. of PFC/10^6 spleen cells*		
		Primary response	Secondary response	Quaternary response
HSA	HSA	180 ± 26	1350 ± 390	1067 ± 143
MSA	MSA	4 ± 2	4 ± 1	2 ± 1
MSA	HSA		6 ± 2	
HSA	MSA		4 ± 1	
HSA/MSA	HSA		1182 ± 253	
HSA/MSA	HSA/MSA		1407 ± 398	
HSA	HSA/MSA		1234 ± 295	
MSA	HSA/MSA		1 ± 1	

The haemolysis of antigen-conjugated SRBCs with the immune sera and guinea-pig complement was measured. The antigens used were HSA and MSA. Reprinted with permission from ARTURSSON P, EDMAN P, SJÖHOLM I: Biodegradable microspheres II: immune response to heterologous and autologous proteins entrapped in polyacryl starch microparticles. *J. Pharm. Exp. Ther.* (1985) **234**:255-260 [38].

*n = 6; mean values ± SEM.

HSA: Human serum albumin; MSA: Mouse serum albumin; PFC: Plaque-forming cell; SEM: Standard error of the mean; SRBC: Sheep red blood cell.

response, which was as large as that induced by HSA in Freund's complete adjuvant also given intraperitoneal. The responses after intramuscular and intravenous administration were lower. In no case did empty starch microparticles inhibit the ELISA response but HSA particles were more effective than free HSA in the ELISA inhibition. The HSA particles also induced significant delayed-type hypersensitivity (DTH) reactions; in particular, after intramuscular immunisation. However, HSA in Freund's adjuvant gave a slightly larger DTH response.

4.2.3 Vaccination with polyacryl starch particles

The first vaccination study of the efficacy of a starch microparticle vaccine after challenge with a live microorganism was made in a visceral leishmaniasis mouse model (Degling Wikingsson L, McMaster WR, Sjöholm I, unpublished data). Leishmaniasis is a fatal disease if not treated, and an incidence rate of 500,000 new cases/year has been reported. Thus, there is a great need for a vaccine, which also has been defined by the World Health Organization [41]. A recombinantly derived surface antigen, glycoprotein 63 (gp63), from *Leishmania donovani* produced in *Escherichia coli,* has been identified as a major antigen recognised by human T cells from infected patients [42,43]. It was conjugated to the particles with and without mouse IL-12 and injected intravenously and intramuscularly. A modest humoral, specific anti gp63 response was induced, but more importantly, a high cellular response, as studied with the DTH test, was detected. The protection against a challenge 2 weeks after booster with live *L. donovani* amastigotes, propagated in hamsters, was followed by counting the number of living parasites in the liver 25 days after infection according to Bradley *et al.* [44]. As seen from Table 4, there was a direct correlation between the DTH reaction and the suppression of the parasite load. The addition of IL-12 to the particles did not further improve the protection after vaccination, whereas IL-12 on its own slightly suppressed the parasite burden.

4.3 Immunisation with silicone-grafted starch particles

The immunogenicity of silicon-grafted or ungrafted starch particles, as prepared by Heritage *et al.* [15], was studied with entrapped HSA and the particles were subsequently administered to mice either intraperitoneally or intragastrically. The results clearly show that the particles can induce a systemic immune response (IgG) following parenteral immunisation with the microparticles. The 3-(triethoxysilyl)-propyl-terminated polydimethylsiloxane-grafted particles (TS-PDMS) induced at least a fivefold (day 63) increase of the IgG response compared with the ungrafted microparticles. The reactive triethoxysilyl groups of TS-PDMS have the capacity of spontaneously binding to the hydroxyl groups of starch. The authors suggest that the grafting of the starch particles is necessary to enhance the immunogenicity of the formulations. Intragastric immunisations were also performed, resulting in a less prominent difference between ungrafted and grafted particles even though the grafted particles elicited both a stronger systemic immune response and a stronger mucosal (IgA) response.

5. Starch microparticles as adjuvant in mucosal vaccination

5.1 The mucosal immune system

The mucosal surface of the human body (gastrointestinal, respiratory and urogenital tracts) has a combined area of ≥ 400 m^2 and represents, together with the skin, the interface

Table 4. Vaccination of mice with gp63 from *Leishmania donovani* conjugated to polyacryl starch microparticles.

Treatment	DTH-response, increase of ear-thickness (%)	Suppression of liver parasite burden (%)
gp63, intramuscular	2.9 ± 1.4	20.2 ± 1.8
gp63, intravenous	3.0 ±1.5	29.1 ± 4.1
gp63 + IL-12, intramuscular	14.8 ± 7.8	31.1 ± 1.4
gp63 + IL-12, intravenous	5.7 ± 3.4	0.3 ± 0.1
gp63-MP, intramuscular	21.0 ± 5.7	62.3 ± 9.4
gp63-MP, intravenous	26.0 ± 7.2	77.3 ± 12.4
gp63-MP + IL-12, intramuscular	28.2 ± 8.3	60.8 ± 7.5
gp63-MP + IL-12, intravenous	20.5 ± 8.1	71.2 ± 11.1
Soluble IL-12, intramuscular	-	42.1 ± 9.0
Soluble IL-12, intravenous	-	22.2 ± 3.1
Empty MP, intramuscular	-	-5.1 ± 0.8
Empty MP, intravenous	-	2.0 ± 0.2

Mice were injected with 0.5-mg microparticles, containing 0.4 µg recombinant gp63/mg, intravenously or intramuscularly with a booster on day 14, with or without IL-12, 1 µg per dose. The DTH reaction was measured 72 h after a challenge in the ears on day 21 with gp63. Challenge with 10^7 *Leishmania donovani* amastigotes was made intravenously on day 28, whereupon the animals were killed on day 53 and the number of parasites calculated according to Bradley *et al.* [44].
DTH: Delayed-type hypersensitivity; gp: Glycoprotein; IL: Interleukin; MP: Microparticle.

between the body and the environment. This makes the mucosal surface the main barrier against various pathogens. In addition, 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 to offer protection from pathogens while simultaneously allowing the absorption of nutrients. This is made possible by a wide range of both innate and adaptive immune defence mechanisms, which have been extensively reviewed [45-49]. The adaptive mucosal immune response will be briefly reviewed here in order to highlight properties relevant to this review.

Vertebrates have evolved a network of organised lymphoid tissues at the mucosal surfaces known as MALT. MALT comprises gut-associated lymphoid tissue (GALT) (Figure 3), the nasal counterpart (NALT) and, in the lower respiratory tract, the bronchus-associated lymphoid tissue. Structurally, these tissues range from loose clusters of lymphoid cells scattered in the mucosal lining to highly organised lymphoid follicles such as the appendix, Peyer's patches and tonsils. These structures function as inductive sites for the mucosal immune response. Together with the effector sites, they form a common mucosal immune system [50].

5.1.1 *Induction of a mucosal immune response*

The inductive site contains the immunocompetent cells necessary for induction of an immune response and the epithelium overlying the organised lymphoid follicles contains M cells (cells with microfolds) specialised in antigen uptake. The most extensively studied inductive sites are the Peyer's patches of the GALT. The surface of the Peyer's patches is covered by a single cell layer called the follicle-associated epithelium (FAE), and is characterised by the presence of a dome-like structure under the FAE that is rich in IgA-committed B cells as well as T cells, macrophages and dendritic cells (DCs). The FAE 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 and endocrine cells [51,52]. These features facilitate closer contact with the luminal antigens, and minimise digestion of the antigens. Most importantly, the FAE exhibits the M cells. 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' in which lymphoid cells are found. The short distance between the apical and basolateral membranes of the cells favours transepithelial transport. As 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, entire microorganisms and particles ≤ 10 µm in diameter [53-55]. As a result of these unique features, M cells have been used as a gateway to deliver microparticles and mucosal vaccines to the mucosal immune system.

After uptake through the M cells (Figure 3), the antigens are processed and presented by APCs, mainly DCs that when encountering an antigen migrate to the T-cell areas and/or B-cell follicles where they can interact with lymphocytes to trigger an immune response.

Alternative pathways for antigen uptake across the epithelium are via DCs and through enterocytes (Figure 3). As a part of the innate immune system, bacterial components, such as flagellin from *Salmonella*, stimulate epithelial cells in the intestines to produce chemokines that attract immune cells, particularly DCs, into the gut mucosa [56]. The DCs can send out their dendrites between the epithelial cells into the

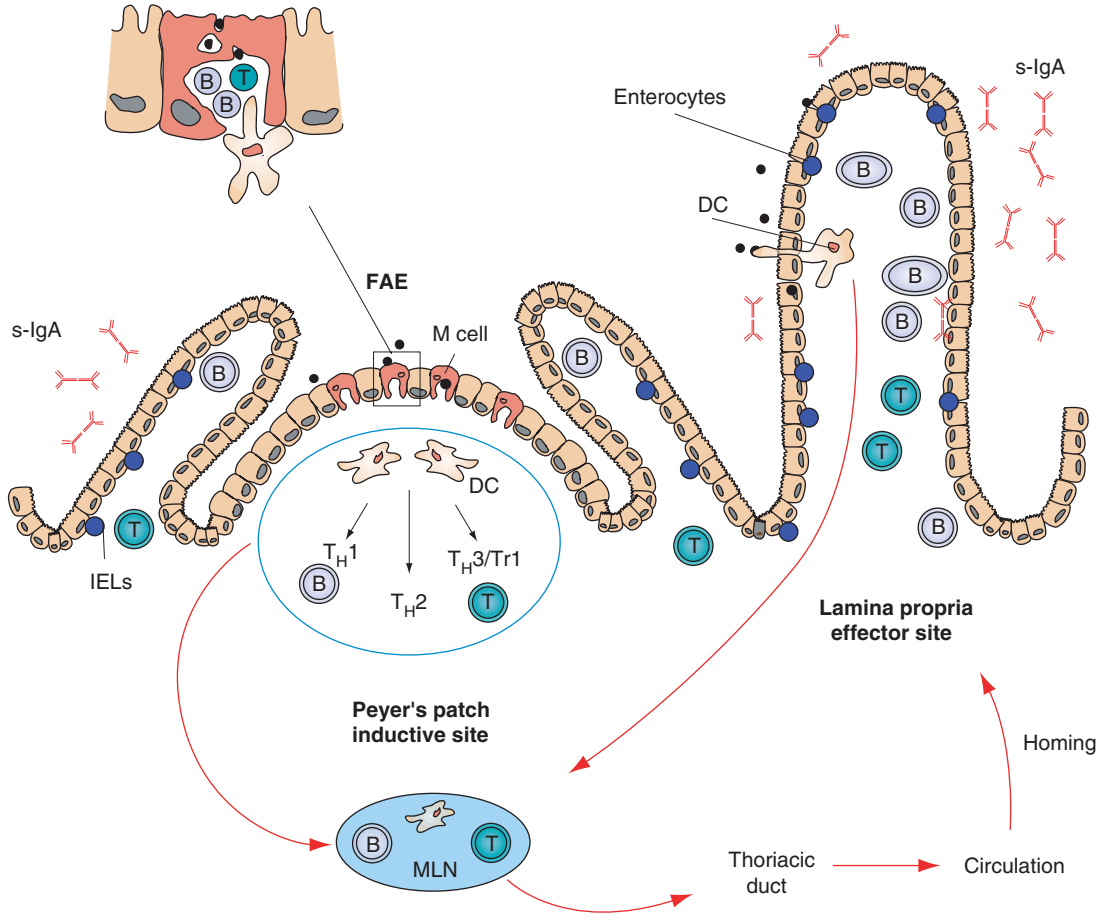

Figure 3. Schematic representation of proposed mechanisms for the induction of an immune response at the intestinal mucosa. The magnification of the FAE shows an M cell with pathogens or particles entering from the gut lumen.
DC: Dendritic cell; Encircled B: B cell; Encircled T: T cell; FAE: Follicle-associated epithelium; IEL: Intraepithelial lymphocyte; MLN: Mesenteric lymph node; s-IgA: secretory immunoglobulin A; $T_H1/2$: T helper cell type 1/2; Tr: T-regulatory.

gut lumen and sample microbial antigens directly from the lumen; they then migrate to the lymphoid follicles where they present the antigens to B and T cells [57].

Enterocytes can also sample soluble antigens through a diverse set of mechanisms from the gut lumen and transport the antigen through the epithelium [58], although the uptake through the M cells is more rapid and with reduced degradation [59]. Moreover, through expressing classical and non-classical major histocompatibility complex (MHC) molecules, enterocytes exhibit the potential to function as unconventional APCs and to take up antigens and present them to adjacent $CD4^+$ T cells. However, it has been proposed that they induce local oral tolerance (a suppressed immune response) rather than activate naïve T cells, as they normally do not express costimulatory signals and secrete immunosuppressive cytokines, including IL-10 and TGF-β [60].

5.1.2 *Effector mechanisms at the mucosal surfaces*

The major effector function at the mucosal surfaces is secretory IgA antibodies (s-IgAs). 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 [61]. Another important effector mechanism of the mucosal surface is the presence of intraepithelial lymphocytes (IELs). 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 [62]. These are thought to be important cytotoxic cells that can eliminate virus-infected epithelial cells, for example [63].

5.1.3 *Homing of lymphocytes to the effector site*

Antigen-sensitised T and/or B cells in the Peyer's patches or in the mesenteric lymph nodes (MLNs) 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) [64]. This homing is dependent on organ-specific surface molecules on the lymphocytes, so-called homing receptors that recognise their counterparts, homing-specific adhesion molecules expressed by endothelial cells in the target tissue. The homing receptors that are expressed on

the surfaces of the lymphocytes thus reflect the mucosal site at which the cells were activated. After oral immunisation, nearly all IgA and IgG antibody-secreting cells express $\alpha_4\beta_7$ integrin, the mucosal homing receptor, whereas only a few of these cells express L-selectin, the peripheral lymph node homing receptor [65-68]. In contrast, intranasal immunisation induced circulating B cells that expressed both $\alpha_4\beta_7$ integrin and l-selectin [67].

The homing molecular expression is the explanation why mucosal vaccination is necessary to induce a mucosal s-IgA response, whereas parenteral vaccination generally does not induce s-IgA responses. Moreover, the microenvironment in the germinal centres of the Peyer's patches, especially the presence of TGF-β [69,70], is instrumental in stimulating B cells to IgA switch [71,72]. These IgA^+ B cells migrate to and enter the lamina propria, where terminal differentiation into IgA-producing plasma cells occurs. Maturation of these B cells into plasma cells is enhanced by cytokines such as IL-5, -6, -10 and -13 [73-77]. These cytokines are distributed throughout the lamina propria, which supports the production of IgA [78,79].

Mucosal vaccination also triggers a systemic immune response. This may be a result of MLN acting as a crossroad between the mucosal and the peripheral recirculation pathways [47]. The antigen presentation to naïve T cells may also take part in the MLN as a result of APC trafficking to the MLN after encountering antigens in the mucosa or Peyer's patches [80-82].

5.1.4 *Regulation of the mucosal immune response*

The mucosal immune system can, to a higher extent than the systemic immune system, induce a suppressive immune response to antigens and particles that are harmless or useful in order to avoid allergic reactions or chronic harmful inflammations. Thus, the microenvironment in the mucosal immune system is more likely able to induce a T helper type 2 (T_H2), T_H3 or a T-regulatory type 1 (Tr1) response than organs such as the spleen. T_H3-type cells are a subset that produces mainly TGF-β, which is a switch factor for IgA production and has suppressive properties on both T_H1 and T_H2 cells. The Tr1 cells are regulatory cells that secrete both IL-10 and TGF-β and have been reported to be able to induce a suppressive response [83,84]. DCs isolated from Peyer's patches or MLN appear to have a particular propensity for producing IL-10 and TGF-β and priming T cells to produce IL-4 and IL-10 (T_H2 cytokines) and TGF-β (T_H3 cytokine) and IFN-γ (T_H1 cytokine), whereas spleen DCs are primed predominantly for IFN-γ secretion [85,86]. Pulmonary DCs produce, after respiratory exposure of antigens, IL-10 but not TGF-β, and induce Tr1 cells that also produce IL-10. Thus, the suppressive response in the respiratory tract seems to be IL-10 dependent [86].

In the subepithelial dome region of Peyer's patches, three different subtypes of DCs, based on their surface markers CD11b and CD8α, have been found [87]. These cells differ according to their location and in their response to stimuli [87-90]. Thus, DCs that secrete IL-10 and induce a T_H2 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 TGF-β on encountering innocuous antigens (food antigens). In contrast, the lymphoid ($CD8\alpha^+$) and double-negative ($CD11b^-/CD8\alpha^-$) DCs both secrete IL-12 and induce T_H1 cells [89,90].

The mucosal surfaces have an immunological milieu that favours IgA production and a T cell suppressive response in order to maintain the homeostasis. However, the mucosal immune system must also be able to respond to pathogens in order to mount an infection with, for example, a T_H1 response. These responses may be induced in the gut by bacterial products such as lipopolysaccharides and polycytosine guanine (CpG) motifs. DCs and several other cells of both the innate and adaptive immune systems, recognise the structures through pattern recognition receptors such as the Toll-like receptors (TLRs). Recognition triggers the 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 [91].

The regulatory mechanisms discussed above have to be taken into consideration when designing a mucosal vaccine. Thus, effective mucosal vaccines will not only require effective delivery through the epithelium but also strategies to trigger a protective immune response. However, the possibility of inducing a suppressive response (tolerance) against mucosal antigens can be a goal when it comes to chronic infections, autoimmune disorders and allergies.

5.2 Uptake of particles at the mucosal surface

Effective oral (mucosal) vaccination requires not only that the antigens are protected during transport to the immune-competent areas of the intestinal mucosa but also that there is effective adherence of the antigen formulation to and uptake over the epithelium. The main pathway for particle uptake is considered to be through the M cells, as long as the particles do not contain or consist of structures that specially interact with distinct components of the gut wall. Although M cells are effective in translocation of particles, the FAE surface area is relatively small compared with the overall intestinal surface. In addition, the M cells only account for 10% of the cells in the FAE in mice, rats and humans. However, particulate delivery holds potential for oral vaccination because translocation over M cells offers direct delivery to inductive sites of the GALT, and large amounts of antigens are normally not needed to activate the immune system.

5.2.1 *Factors of importance for particle uptake*

For particles to be taken up, they first have to diffuse through the mucus layer covering the mucosal surfaces and then initiate contact with enterocytes or M cells. This process is affected by inherent factors of the particles, such as particle size, and surface characteristics, such as hydrophobicity, charge and adhesive properties. Several studies

have been performed in order to study particle binding and uptake in the gastrointestinal tract and have been expertly reviewed elsewhere [3,21,92-96].

Briefly, studies using polystyrene and polylactic-co-glycolic acid particles have shown a higher uptake of smaller particles (nanoparticles) compared with larger ones (microparticles) in the rat intestines [97-99]. Particles in the µm range are preferentially taken up in the Peyer's patches regions [99,100]. In addition, size-dependent restriction of particle uptake in the Peyer's patches has been shown [101-103]. Eldrige *et al.* [103] suggested that the upper limit for particle uptake at the Peyer's patches is a diameter of 10 µm, and that larger particles (5 – 10 µm) remained in the patches, whereas smaller particles (< 5 µm) were transported through the efferent lymphatics. The same group also demonstrated, by using different polymer compositions, that the surface characteristics affected the particle uptake. Thus, hydrophobic particles were found in a higher number in the Peyer's patches than more hydrophilic ones [103]. The reader is referred to a recent review, which expertly covers the effect of the surface properties of biodegradable polymers on the mucosal uptake [104].

There are conflicting reports on the proportion of orally administered microparticles that is taken up over the intestinal epithelium, ranging from 30 to < 0.01% [21,92,105]. The diverging results are probably an effect of lack of standardisation of methodology used including mode of evaluation, animal species or type of particles studied [105]. 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 used in vaccination studies.

5.2.2 *Uptake of polyacryl starch microparticles in the mouse intestines*

The uptake of 2 µm antigen-conjugated polyacryl starch microparticles by the intestinal mucosa has been studied using two different antigens, HSA and recombinant cholera toxin B subunit (rCTB) [106]. HSA was chosen as a neutral antigen with no known specific binding properties and rCTB was chosen for its property to bind to the GM1 receptors. ELISA showed that unlabelled rCTB is present on the surface of the particles after conjugation so that particles can bind to GM1 receptors in the wells of the ELISA plates, whereas subsequent labelling of rCTB conjugated to the particles with a fluorescence dye considerably lowered the efficiency of the receptor interaction.

The uptake of the polyacryl starch microparticles was studied after injecting them into mouse intestinal loops each containing a Peyer's patch. The particles were not fluorescence-labelled prior to injection to avoid the risk that the label changes the surface characteristics of the polyacryl starch microparticles. The *in situ* studies, using confocal laser-scanning microscope and immunohistochemistry with specific fluorescence-labelled antibodies on whole mounted specimens, revealed a qualitative difference in uptake even if a quantitative determination was not possible in the experimental set up used. HSA-conjugated polyacryl microparticles were taken up over the FAE (Figure 4a), most likely by the M cells. The fluorescence-labelled lectin *Ulex europeaus* agglutinin 1 [107] was used as a marker for M cells. rCTB-conjugated polyacryl microparticles, on the other hand, were taken up over both the FAE (Figure 4b) and the villus epithelium (Figure 4c). However, after fluorescence labelling, the rCTB-conjugated particles were found only in or attached to the M cells of the follicles. Thus, the nature of the conjugated antigen did influence the binding and uptake of the particles.

The results indicate that rCTB conjugated to the polyacryl starch microparticles can bind to the GM1 receptor *in vitro* and that CTB also mediates the binding of polyacryl starch microparticles to this receptor *in vivo*. These findings may be regarded as being in conflict with the results obtained by Frey *et al.* [108], who showed that polystyrene particles in the µm range coated with CTB failed to adhere to enterocytes and M cells *in vivo* owing to the barrier function of the intestinal cell glycocalyx. However, the microparticles used here are made of polyacryl starch with a highly porous structure and a very rough surface with protrusions [109] that may have facilitated an interaction between rCTB and the GM1 receptors. The principle that ligand-conjugated particles can be taken up in normal villus epithelium has also been shown by Hussain *et al.* [110], who showed that conjugation of tomato lectin to polystyrene particles (500 nm) resulted in a greater uptake by the villus epithelium than by FAE. In contrast, blocked lectin-conjugated particles were intimately associated with the FAE. The greater binding to and uptake by villous epithelia of tomato lectin is presumably caused by the binding to all types of epithelial cells and that the villous epithelium covers a larger surface area than the FAE [110].

The varying sites of uptake of antigen-containing particles may affect the subsequent antigen processing because the antigens will meet different cytokine environments. This was also shown recently by Grdic *et al.* [111], who studied the immune response induced by ovalbumin (OVA) plus two different oral adjuvants that worked in completely different ways: the immune-stimulating complex (ISCOM) carrier system and cholera toxin (CT). Whereas ISCOMs are probably taken up by nonspecific mechanisms such as phagocytosis, CT is specifically bound to the ganglioside GM1 receptor. Both adjuvants improve the immune response, but it was clearly shown that the adjuvants used different pathways; whereas OVA–ISCOM combinations induce the production of IL-12, the effect of CT was not dependent on IL-12 [111].

5.3 Vaginal immunisation

Only one study on vaginal immunisation with starch particles has been found. O´Hagan *et al.* [112] used Spherex in sheep with lysophosphatidylcholine (LPC) as an adjuvant for influenza-gp fragments. The antigen, administered with LPC mixed with Spherex, induced a much better immune response

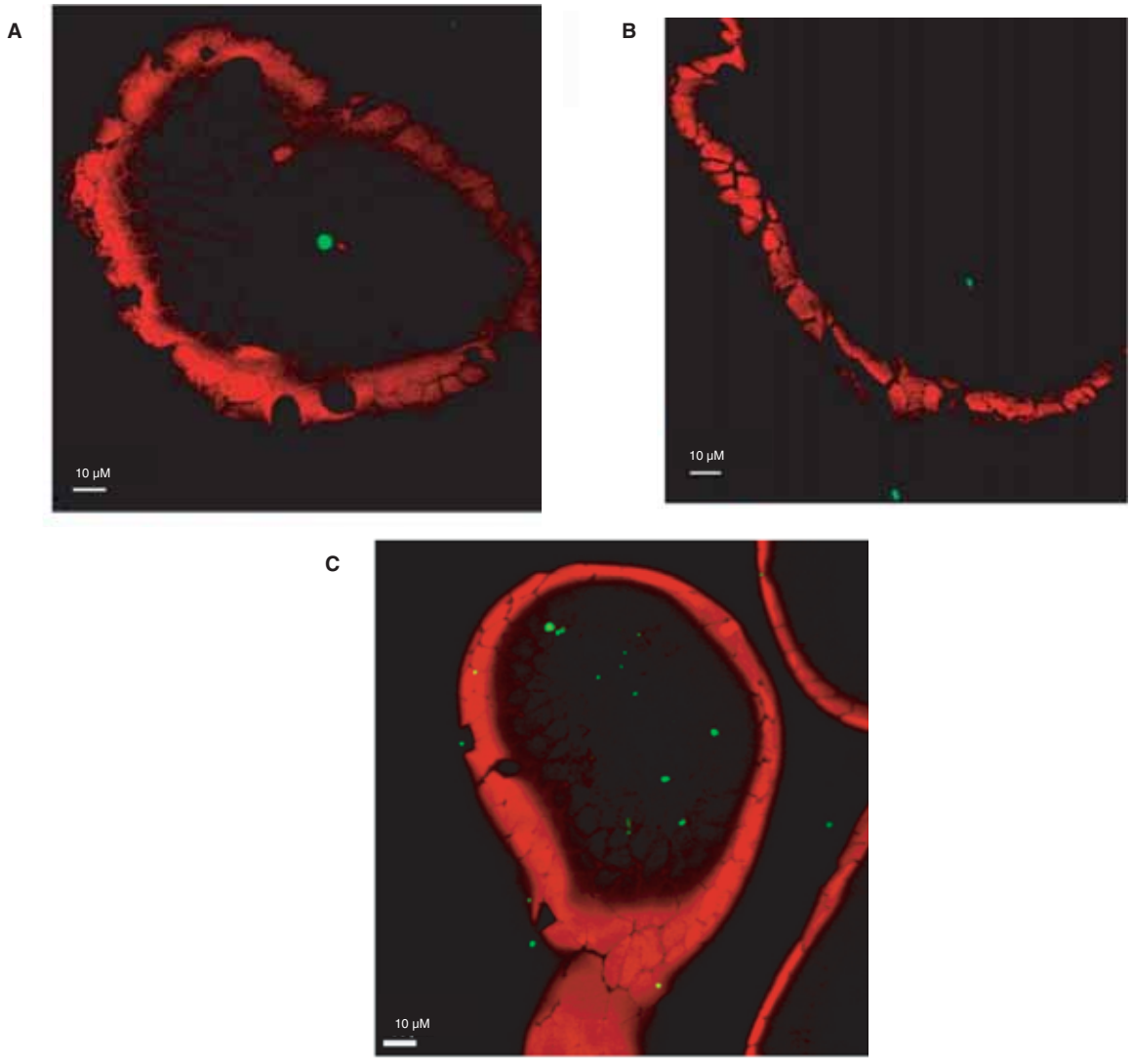

Figure 4. Starch microparticles in intestinal mucosa. The uptake was studied after 40 ± 5 min exposure of particle suspension in mice intestinal loops. A. HSA-conjugated microparticle in a follicle. **B.** rCTB-conjugated microparticles in a follicle. **C**. rCTB-conjugated microparticles in a villus. The specimens were stained after fixation in ice-cold ethanol with rhodamine phalloidine (red) and fluorescein isothiocyanate-conjugated anti-HSA or anti-rCTB antibodies (green). Reprinted with permission from LARHED A, STERTMAN L, EDVARDSSON E, SJÖHOLM I: Starch microparticles as oral vaccine adjuvant: Antigen-dependent uptake in mouse intestinal mucosa. *J. Drug Target.* (2004) **12**(5):289-296 (http://www.tandf.co.uk) [106].
HSA: Human serum albimun; rCTB: Recombinant cholera toxin B subunit.

compared with the antigen mixed without Spherex. However, Spherex was not ascribed any direct adjuvant properties but rather exerted the effect by working as absorption enhancer by maintaining the drug in contact with the mucosal epithelium for a longer period of time.

5.4 Nasal vaccination

5.4.1 Spherex®

Spherex has also been investigated for its intranasal protein- and drug-delivering properties [113,114]. Björk *et al.* [115] have shown that when administering Spherex particles with entrapped insulin as a dry powder (45 µm) intranasally, the particles swell in such a way that the tight junctions may be affected and the paracellular absorption of the insulin is facilitated. Later, *in vitro* studies in Caco-2 cell models showed that the Spherex particles exert reversible tight junction opening and thus may enhance the paracellular transport of proteins, which is a prerequisite for the induction of an immune response [116]. Holmberg *et al.* [117] have also shown that the use of Spherex particles as dry formulation

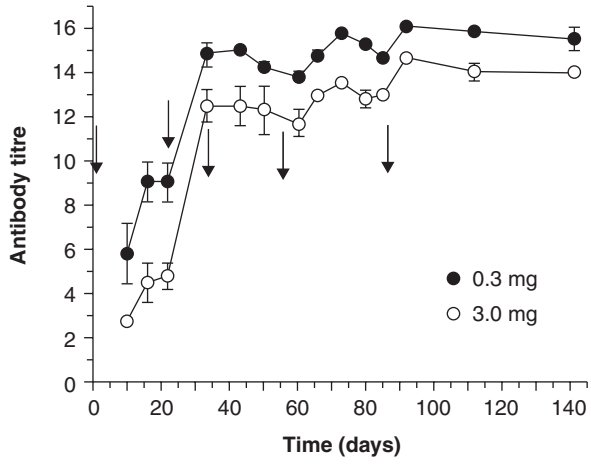

Figure 5. Polyacryl starch microparticles as an oral vaccine adjuvant. Serum IgG/IgM titres after oral immunisation of Balb/c mice with HSA-conjugated polyacryl starch microparticles. The arrows indicate immunisation on 3 consecutive days with two different doses of microparticles containing 156 mg HSA per mg Reprinted from DEGLING WIKINGSSON L, SJÖHOLM I: Polyacryl starch microparticles as adjuvant in oral immunisation, inducing mucosal and systemic immune responses in mice. *Vaccine* (2002) **20**:3355-3363 [29], Copyright (2002), with permission from Elsevier.
HSA: Human serum albumin; Ig: Immunoglobulin.

does not affect the human nasal mucociliary clearance, nor cause any congestion or decongestion of the mucosa in volunteers. However, further studies on the use of Spherex as a vaccine adjuvant are not available.

5.4.2 *Polyacryl starch microparticles*

Several investigations have implied that, in order to induce a successful immunisation with nasal vaccine formulations, some sort of enhancement is needed, such as the addition of substances, which open up tight junctions or chemical modification to make the formulations more sticky and prolong their exposure on the nasal mucosa. Some papers describe such studies with chitosan [118-121]. As far as the authors know, the polyacryl starch microparticles have no characteristics that enhance uptake but dried particles have not yet been studied in the same way as Spherex has been used. As shown above, insulin mixed with Spherex was taken up and decreased the blood glucose concentration [116]. An *in vitro* study showed that only a very small number of polyacryl starch microparticles are taken up when applied as a suspension on pig respiratory mucosa [109]. Only low levels of IgG/M were detected after intranasal immunisation of mice using diphtheria crossreacting material 197 (CRM-197)-conjugated polyacryl starch microparticles [122]. Therefore, it is probable that polyacryl starch particles are not a suitable nasal vaccine adjuvant, at least as long as the antigen is covalently conjugated to the particles.

5.4.3 *Silicone-grafted microparticles*

A strong systemic IgG response was evoked in mice after intranasal immunisation with entrapped HSA in silicone-grafted microparticles (TS-PDMS) but not with soluble HSA [30]. The immune response was not induced by swallowed particles because an enzyme-linked immunospot assay (ELISPOT) showed that no HSA-specific spot-forming cells were activated in Peyer's patches in the gut. A predominance of IL-4 over IFN-γ and IgG1 over IgG2b indicated a T_H2-skewed immune response. No IgA was detected in any lymphoid tissue (not even in nasal secretions or lymphoid tissues) after intranasal administration. The positive results obtained with the silcone-grafted microparticles may be due to the hydrophobic properties of the particles, which allowed contact with the nasal mucosa for a prolonged period of time, thus enabling the entrapped HSA to be taken up by the cells of NALT.

5.5 Oral vaccination

5.5.1 *Spherex®*

Spherex with entrapped DNP–BSA in dried form (see Section 4.1) has been administered to rats either topically at the sublingual epithelium of the oral cavity or by gastric intubation (intragastrically) [37]. Subcutaneous injections in the vicinity of the major salivary glands in the oral cavity were used as comparison and indicated that the particles were effective as an adjuvant parenterally. IL-5 and -6 were either included in the particle formulation or not, in order to try and boost the s-IgA response. The oral formulations were also used together with a penetration enhancer, L-α-lysophosphatidylcholine. Sublingual or intragastric immunisations induced a better s-IgA response against the hapten DNP, both in saliva and tear fluid, when formulated with the penetration enhancer, compared with administration subcutaneously in the vicinity of the salivary glands. The author also noted that the enhancer is needed for a sustained IgA response after mucosal vaccination. The systemic immune response (IgG) was also prolonged when the penetration enhancer was used but was weaker compared with subcutaneous administration. The IgG response did not appear to be affected by the addition of IL-5 or -6. These results are in line with other studies suggesting that the enhancer together with the bioadhesive particles opens up the tight junctions to allow the entrapped DNP–BSA to be taken up into the oral mucosa.

5.5.2 *Polyacryl starch microparticles*

5.5.2.1 *Model studies*

Polyacryl starch microparticles as an oral vaccine adjuvant have been evaluated with HSA as a model antigen in mice [29]. Following oral immunisation, HSA-conjugated microparticles induced a strong, long-lasting humoral serum response and a local s-IgA response (Figure 5 and Table 5, respectively). The maximum IgM/G level was rapidly reached at 30 – 35 days and remained at the same level for the whole studied period (140 days). The antigen-specific IgG1 dominated the subclass profile of the IgG response;

Table 5. Mucosal immune response after oral immunisation of Balb/c mice with HSA-conjugated polyacryl starch microparticles.

HSA-particles used for immunisation	IgA producing cells			IgA antibodies in intestinal flush (ng specific/mg total IgA)
	Total	Antigen specific*	Antigen specific (%)	
0.3 mg (per feeding)	94,900	1160	1.2	5.7 ± 1.5
3 mg (per feeding)	74,600	1440	1.9	15.0 ± 3.0

The same immunisation scheme was used as in Figure 5. The mice were sacrificed on day 92. Reprinted from DEGLING WIKINGSSON L, SJÖHOLM I: Polyacryl starch microparticles as adjuvant in oral immunisation, inducing mucosal and systemic immune responses in mice. *Vaccine* (2002) **20**:3355-3363 [29], Copyright (2002), with permission from Elsevier.

*The number is calculated per 1×10^7 cells prepared from lamina propria.

HSA: Human serum albumin; Ig: Immunoglobulin.

however, the relative content of IgG2a increased with time and with the number of doses.

Antigen-specific IgA response could be detected in faecal samples after the first booster and was further increased after the second booster. ELISPOT assays, with cells from the lamina propria, confirmed that the antigen-specific IgA response found in the faecal samples reflected the locally produced s-IgA and not serum-derived IgA excreted in the bile. The response was dose-dependent; a 10-fold lower dose of the particles induced both a lower IgM/G and lower s-IgA response. However, the epitope density (i.e., the amount of HSA conjugated to the particles) did not seem to influence the response even though the onset of the response was somewhat slower with a 20-fold lower epitope density.

Oral immunisation with the HSA-conjugated microparticles also induced a strong cellular immune response; marked T cell proliferation of primed spleen cells upon activation with free HSA and a strong DTH reaction were detected. These findings indicate that the polyacryl starch microparticles are a promising oral vaccine adjuvant candidate.

5.5.2.2 *Effect of route of administration*

The effect of the route of administration on the character of the immune response has also been studied in various combinations of oral, subcutaneous and intamuscular routes, in which the route of administration was changed after primary immunisation or after a first booster [123]. The HSA-specific IgM/G responses were essentially the same at the end of the immunisation schemes regardless of administration route, even if the response after oral immunisation was slower in onset; however, the route of administration did affect the T_H1/T_H2 balance. Oral and subcutaneous immunisations have similar effects on the T_H1/T_H2 balance, as indicated by the IgG subclass ratios and cytokine analyses, whereas significant differences in the IgG subclass ratios (IgG1/IgG2a + IgG2b) between oral and intramuscular immunisations were seen. Thus, the T_H2 influence was stronger after oral primary immunisation than after intramuscular primary immunisation, whereas oral boosters elicited a comparatively stronger T_H1 response than intramuscular boosters. In agreement with these results, a trend toward a higher DTH response in the groups boosted orally was observed, which indicates a higher T_H1 response [124]. The IgG subclass ratio, as well as the IgE response, was higher after subcutaneous immunisation than after intramuscular immunisation, indicating a higher T_H2 response because the IgE response is coregulated with IgG1 via the T_H2/IL-4 pathway [125].

These results show that the immune response profile obtained with polyacryl 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_H subsets seems to be complex and are not completely understood. It has been established that the differentiation of T_H cells into T_H1 or T_H2 phenotypes is largely controlled by the action of cytokines, in which a T_H1 or T_H2 response is promoted by IL-12 and -4, respectively [126]. Thus, differentiation of the T cells is ultimately dependent on factors that influence which cytokines are present during antigen presentation, such as the type of APC and the microenvironment in which the antigen presentation takes place. Therefore, it is not surprising that the route of administration affects the differentiation of the T cells.

5.5.2.3 *Effect of site of uptake*

The qualitative difference in uptake of the microparticles between the villus epithelium and the FAE (Figure 4) after oral administration may have consequences for the character of the induced immune response. In order to study the possible consequences of these differences in mucosal uptake, rCTB was conjugated together with HSA on the same particles as well as on separate particles given concomitantly [127]. The induced immune responses were compared with the responses induced by immunisation with either HSA- or rCTB-conjugated microparticles; however, there were no differences between the immunised groups after oral immunisation, either quantitatively (as assayed by IgG/M levels, the s-IgA response and the DTH response) or qualitatively (by studying

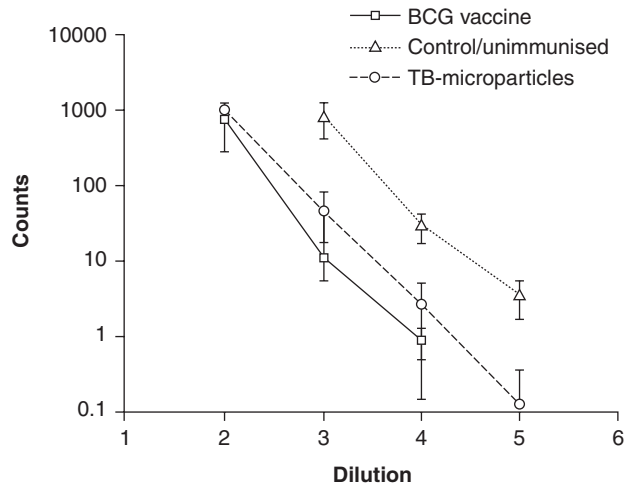

Figure 6. Effect of oral vaccination of mice with antigens from *Mycobacterium tuberculosis* (Harlingen strain) conjugated to polyacryl starch microparticles and BCG-vaccination. The number of colony forming units in different dilutions of lung homogenates was calculated after infection with 5 × 10^6 bacteria 15 days after infection (121 days after the primary vaccination). The mice were immunised with the microparticles in 100 µl physiological saline in four sessions of 3 consecutive days 3 weeks apart (J Berggren, L Degling Wikingsson, D Andersson, I Sjöholm, unpublished data).
BCG: Bacille Calmette Guerin; TB: Tuberculosis.

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. These findings were unexpected; the authors had anticipated that the addition of rCTB to the HSA-conjugated microparticles 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 hypotheses may, however, be proposed.

Particles taken up in the villus epithelium may be processed and presented to adjacent lymphocytes; antigens absorbed apically in intestinal epithelial cells are processed and presented on the basal surface to $CD4^+$ T cells [128]. However, enterocytes express MHC class II but do not normally express co-stimulatory molecules required for T-cell activation [129,130], 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 taken up in the lamina propria by DCs, which subsequently migrate to the MLNs where antigen presentation takes place. However, because the immune response was not improved, suppressed or qualitatively altered, it is more likely that uptake through the villi resulted in local suppression of the immune response in the villus lamina propria: an effect possibly mediated by down-regulating T cells secreting IL-10 and/or TGF-β [131]. Alternatively, the antigen–particle complex may have been hydrolysed and degraded within the highly active lysosomal compartments of the enterocytes, thus losing the immunoactive epitopes and becoming unable to induce an immune response.

5.5.2.4 *Challenge studies*

The first concept study with starch microparticles as an oral adjuvant was performed in mice with secreted antigens from *Mycobacterium tuberculosis* (TB), strain Harlingen, which is highly virulent (Berggren J, Degling Wikingsson L, Andersson D, Sjöholm I, unpublished data). The same immunisation schedule as in the first oral model study with HSA was used, together with controls with intramuscular TB microparticles, intramuscular unconjugated TB antigens and in Freund's incomplete intraperitoneal adjuvant, as well as with a commercial Bacille Calmette Guerin (BCG) vaccine. Both the specific humoral and the DTH responses were strong after vaccination with TB particles, as well as after vaccination with the BCG vaccine and with the antigens in Freund's adjuvant; however, the humoral response after oral administration of the TB particles was lower than that obtained after intramuscular vaccination. On the other hand, protection after challenge with live TB bacteria was better in the group of mice that were treated with the oral vaccine. In this study, the efficacy of vaccination was followed by comparing the remaining colony forming plaques in liver and lung suspension in vaccinated and nonimmunised mice (Figure 6) (Berggren J, Degling Wikingsson L, Andersson D, Sjöholm I, unpublished data). In addition, the weight loss was smallest in the mice vaccinated orally. The results suggest that the cellular response is a better indication on protection than the serum titres, and that oral vaccination with TB microparticles decreases the bacterial burden ~ 80 – 90% compared with unvaccinated mice. This is in line with the view that a cellular response is an important component in a protective response [132,133].

In a series of papers, Strindelius *et al.* [134-136] studied the immune response in mice of secreted antigens, purified flagellin or an atypical recombinant fimbriae construct from *Salmonella enterica serovar Enteritidis* conjugated to starch microparticles. The particles were administered both parenterally (subcutaneously or intramuscularly) and mucosally (intranasally and orally), and the humoral responses were characterised as well as the protection against an oral challenge with live bacteria. In most cases, good response and good protection were obtained as a result of vaccination; however, in this case it was more difficult to draw any conclusions on the role of the starch particles as an oral vaccine adjuvant. The major antigen of the secreted antigens from the *Salmonella* is flagellin and in purified form it induced both a strong humoral response and protection irrespective of whether it was conjugated to the particles or not. Flagellin is known to bind to and activate TLR-5 of the innate immune system of GALT [137]. Indeed, unconjugated flagellin administered orally gave the best protection against challenge; therefore, the alternative innate system plays a significant role in the immune defence against *Salmonella* infections but it is not possible from the limited data available to say to what extent the innate and adaptive system may interact or evaluate their respective quantitative roles.

5.5.3 *Silicone-grafted microparticles*

Silicone grafting was chosen on the basis of the hydrophobicity of silicones. Several different silicones have been compared. They were divided into unfunctionalised (Me_3Si-terminated) and end-functionalised silicones (TS-PDMS). HSA was used as a model antigen in mice with intragastric immunisations, with HSA 50 µg on days 0, 7 and 14. The TS-PDMS-grafted starch microparticles with entrapped HSA induced a superior systemic immune response (IgG) compared with the unfunctionalised microparticles. Antigen release studies showed that almost a total release of HSA was reached within 22 and 24 h in aqueous buffer at pH 7.4 [16].

The authors present a hypothesis that, unlike other microparticle technologies, their approach is facilitating an enhancement of antigenicity rather than merely protecting the antigen against degradation. They propose that the functionalised silicone (TS-PDMS) associated with the antigen appears as 'islands' on the surface of the starch matrix core and, in some unknown way, thereby facilitates the delivery of antigen to the mucosal immune system. Furthermore, they hypothesise that the silicone may alter the native configuration of the antigen which may improve uptake [138].

The group has further shown that intragastric immunisation with HSA entrapped in TS-PDMS-grafted starch microparticles stimulates the immune system via the Peyer's patches with a subsequent proliferation of cells from these regions in mice. The authors reason that the systemic immune response, measured primarily as IgG, may be the result of the migration of stimulated cells from the Peyer's patches to systemic lymphoid compartments. They have also shown a prominent cytokine production of IL-4 and IgG1 in ELISPOT and ELISA, suggesting a T_H2-skewed type of response as a result of immunisation with grafted microparticles [139].

5.6 Diphtheria as a model for proof of principle

The studies performed so far in mice clearly show that polyacryl starch microparticles are an effective vaccine adjuvant after both parenteral and oral administration with model antigens as well as in challenge models. However, to show that starch microparticles are also an effective oral vaccine adjuvant in humans is a more complex problem. There are several aspects that need to be considered to establish proof of principle in man and to satisfy regulatory requirements. The choice of a diphtheria vaccine in a booster model meant that several important obstacles could be circumvented or simplified.

An advantage with a diphtheria vaccine is that the protective effect of the vaccine can be easily monitored in both animal models and man. It is generally accepted that the specific anti-diphtheria toxin (DT) antibody titre fully reflects protection, which means that the effect of vaccination can be followed easily by analysing serum for DT-neutralising antibodies. There is no clear limit defining complete protection against diphtheria but a minimum level of antibodies for protection usually quoted is 0.01 IU/ml [140,141].

Moreover, most countries have national vaccination programmes against diphtheria with priming vaccination parenterally starting a couple of months after birth with several boosters up to the age of ~ 12 years; however, a large proportion of the adult population in the Western world has low levels of DT-neutralising antibodies due to the lack of booster doses in middle age. As a consequence, herd immunity against diphtheria is at risk of compromise in several countries [142-146]. On the other hand, university students at an age ~ 20, who have not received a recent booster dose, constitute a relatively homogeneous group of volunteers, in which the effect of a new oral booster vaccine should be possible to follow.

Furthermore, unlike in a tetanus trial, there is very little risk for participating volunteers to receive a natural boost to their immunity during a study. In order to contract diphtheria, or even to come into contact with the disease, a person would probably require a lengthy stay in the populated areas of developing countries or areas with low herd immunity. Thus, the risk to acquire an increased titre due to natural contacts with the pathogen is low during a clinical study.

In addition, it is easier to detect an immune response to a booster vaccination in an individual with an immunological memory than to induce a primary detectable immunity in naïve volunteers. In summary, the use of a candidate diphtheria vaccine formulation seems to be a convenient way to try to show that starch microparticles can be used as an oral vaccine adjuvant.

Table 6. Serum immune responses (IgG-IgM) with different diphtheria vaccines.

Formulation	Antigen	Formaldehyde treatment	Mean IgG-IgM titre on day 56 (log_2)	
			Orally	Subcutaneously
MP-DT*	Toxin	-	11	11
MP-DTxd‡	Toxin	Yes	9	11
MP-CRM	CRM-197	-	7	11
MP-CRMxd	CRM-197	Yes	10	11
Commercial§	Toxoid	Yes	-	12
Free toxoid	Toxoid	Yes	1.5	-

The immune response is given as titres of diphtheria toxoid-specific IgG-IgM antibodies in mouse serum. Oral doses were given on days 0 – 2, 21 – 23 and 41 – 43, 3 mg MP/dose. Subcutaneous doses were given on days 0, 21 and 41, 1 mg MP/dose.

*This formulation was highly toxic in guinea-pigs. ‡Mice received only one booster. §Adsorbed to alum. The table is an overview of the results presented in [122,152].

CRM: Crossreacting material; CRMxd: Crossreacting material toxoid; DT: Diptheria toxin; DTxd: Diptheria toxoid; Ig: Immunoglobulin; MP: Microparticle.

5.6.1 *Diphtheria antigens*

DT is extremely toxic to humans and can be used in commercial vaccines only after detoxification with formaldehyde. Today, DT toxoid is used in vaccines with various aluminium adjuvants [147]. Formaldehyde reacts with primary amino groups in the DT molecule, blocking at least 30% of them; this removes the toxicity of the protein [148-151]. However, as the conjugation of protein antigens to polyacryl starch microparticles (Figure 1) is also based on the primary amino groups, conjugation of the diphtheria toxoid (with few reactive amino groups due to the formaldehyde treatment) to the particles is less effective than conjugation of naïve DT. Even if it can be done, ≤ 60% more toxoid than native toxin is required to obtain the same antigen concentration in the starch microparticles. Direct covalent conjugation of DT to polyacryl starch microparticles is, however, not enough to detoxify the DT [152].

In the search to find a more effective diphtheria antigen, CRM-197, which is totally devoid of toxicity and already approved by most drug regulatory agencies, was used conjugated to starch microparticles. Although it is a relatively poor immunogen, formaldehyde treatment of CRM-197 appears to offer improvements in both stability and immunogenicity [149,153]. The results indicated that CRM-197 could be used as a potential antigen but needed to be stabilised by formaldehyde. Table 6 summarises the results obtained in mice with different diphtheria antigens conjugated to polyacryl starch microparticles.

5.7 CRM-197-conjugated starch microparticles in man

A CRM-197 formulation optimised with regard to the formaldehyde treatment (0.06% weight/volume, 48 h) [122] has been used in an unpublished human trial (Rydell N, Stertman L, Stålenheim G, Sjöholm I, unpublished data). A total of 20 volunteers were divided into two groups of 10. Group 1 was given 6 mg of the vaccine per dose and group 2 was given 10 mg per dose. The specific anti-DT antibody titres were analysed at the start by a normal ELISA and in a toxin-binding inhibition Vero-cell analysis [154]. The same vaccination protocol and serum sampling procedure as earlier employed in mice were used (i.e., the vaccine was given on an empty stomach on 3 consecutive days in two sessions 3 weeks apart). The sera were analysed for up to 3 months. Despite careful statistical analyses of the results, no increase in IgG antibodies or DT-neutralising antibodies as analysed by the Vero-cell assay were detected. Several reasons for the negative results may be identified and are discussed in the following section.

5.8 Starch microparticles as a vaccine adjuvant in mice and humans: an analysis and comparison

This review has shown that starch microparticles are functioning as a vaccine adjuvant both after parenteral and oral administration. In some cases, the immune responses induced have been shown to be protective after challenge with live microorganisms. Considering the relatively poor mass transport over the gut mucosa [21] and the very efficient degradation of proteins in the alimentary canal, it is surprising that such good immune responses are obtained after oral vaccination in mice. Obviously, the antigen presentation after the delivery of the antigen–particle conjugates to the immune system is effective enough, which indicates that the starch particles have been stable enough during the transport through the stomach and the gut duodenum and ileum and the epithelium (mainly the M cells). However, it seems that there is a delicate balance between the stability of the starch particles during transport and the degradability during the antigen processing in the APC. This balance has been achieved in the mice experiments, but it is evident that it is harder to find the proper balance in man, in which the size of the gut is much larger and the motility, bacterial flora and enzyme content are different. In addition, the density of Peyer's patches in the larger gut volume in man is most probably smaller.

There are several factors that have significant influence on the physicochemical characteristics that control the stability of the starch microparticles. Size, for instance, should be an important factor. Larger particles will probably survive longer during the transit to the Peyer's patches and may be degraded to

a size, which is optimal for uptake through the M cells. When it comes to polyacryl starch particles, the crosslinking, as decided by the degree of derivatisation of the starch with acryloyl groups, will affect the porosity of the particles obtained; the higher derivatisation, the tighter particles and a slower degradation. The porosity will also be lower with increased starch concentration and/or increased protein conjugation with CDI, which should prolong the survival in the gut. Formaldehyde treatment, if used, should have the same effect. However, it is uncertain whether a more stable particle will interact as well with the immune system as the ones used in the animal studies performed so far. At any rate, extensive trials in animal models have to be carried out before it can be concluded that less porous and thus more stable antigen-conjugated starch particles can induce a proper immune response.

Other ways than changing the porosity of the starch particles for use in humans (in order to decrease the degradation during transport) should be considered. Besides using larger particles than in mice, enteric coating of the particles or packing the particles in enterocapsules should be tried. With techniques available today, enteric coating with predetermined dissolution depending on time and pH would be a way to decide where in the ileum the particles are released. Some reports state that the number of Peyer's patches increases in the distal part of the ileum [155], which would possibly improve the uptake of the antigen-carrying particles.

6. Expert opinion and conclusions

The demand for new mucosal vaccine formulations is well defined. Practical and economical arguments speak in favour of oral vaccine adjuvants that can protect antigens during transit through the gastrointestinal tract, to deliver the antigen to the Peyer's patches and the MALT. Starch microparticles as an oral adjuvant have been shown to induce a protective immune response after challenge with live bacteria in mice studies; however, the necessary studies to show that the concept also holds true in man are still missing. The formulations used in mice have to be modified to compensate for the longer transit time in man, which should be possible to achieve, for example, in gastro-resistant formulations. An essential factor, not yet studied enough in this context, may be the possible differences in the immune defence between man and the mouse, including both the innate and the adaptive immune systems and their interaction. Thus, appropriate clinical trials in man remain to be carried out to show that oral vaccination is possible in humans.

Studies with Spherex [115], chitosan [118-121] or siliconised starch microparticles [30] have indicated that proteins/antigens 'entrapped' in such carrier formulations are taken up through nasal mucosa and that the uptake can be improved with the help of enhancers in such a way that both local and systemic immune responses can be elicited. Polyacryl starch microparticles do not seem to be taken up to a sufficient degree for induction of an immune response; therefore, carrier systems with conjugated antigens may not be suitable for nasal vaccination. The problems with low pH, digestive enzymes and relatively high antigen concentrations needed for successful oral vaccination are factors favouring nasal vaccination, if safe tight junction openers or transport enhancers can be found that eliminate the present safety concerns.

The studies performed in mice so far indicate that the profile of the immune response after oral vaccination is similar to the response seen after subcutaneous administration as far as the systemic response is concerned. However, an advantage with oral vaccination is the local IgA response, which should contribute to the defence against food-, water- or air-borne infections. Specific monoclonal IgA antibodies generated by subcutaneous hybridoma grafts have indeed been shown to protect against oral challenge with *Salmonella typhimurium* in mice. However, in this case the antibodies did not protect against an intraperitoneal challenge despite the antibodies were present in the systemic circulation [156]. Similar results were obtained with *Vibrio cholerae* [157]. However, the cited cases describe bacterial infections afflicting the luminal side of the gut epithelium. Challenge studies with bacteria proliferating mainly systemically should, therefore, be carried out to evaluate the relative importance of an adaptive s-IgA response.

Few (if any) mechanistic studies on the development of the immune response after oral vaccinations have been performed so far. It would be of great theoretical interest to learn about the practical consequences of the interaction between the innate and adaptive parts of the immune system. To what extent can the inflammatory reaction to commensal bacteria in the gut have an effect on the development of the adaptive response? Moreover, will the uptake of a vaccine formulation over the absorptive enterocytes of the small intestine (considering the high metabolic activity therein) have any effect on the induction of an immune response? In addition, there is not yet a detailed understanding available of the possible role of tolerance development after oral exposure with particle vaccines, but results in mice have not shown any increased tolerance with polyacryl starch microparticles so far. A factor to study further in this respect is the possible involvement of IELs and their role in the differentiation of the induced immune response.

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Affiliation

Niclas Rydell PhD, Linda Stertman PhD & Ingvar Sjöholm† PhD
†Author for correspondence
Uppsala University, Department of Pharmacy, Biomedical Centre, PO Box 580, SE-751 23 Uppsala, Sweden
Tel: +46 18 471 4467; Fax: +46 18 471 4223;
E-mail: ingvar.sjoholm@farmaci.uu.se