

Uptake and adjuvant activity of orally delivered saponin and ISCOMTM vaccines

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Abstract

Saponins are a highly heterogenous group of glycosides which are common in plants and have been known to have adjuvant properties since the 1920s. The immunostimulating complex or ISCOMTM is a particulate adjuvant/antigen delivery system. ISCOMsTM are open cage-like complexes typically with a diameter of about 40 nm which are built up by cholesterol, lipid, immunogen and saponins from the bark of *Quillaia saponaria* Molina (soap bark tree). ISCOMsTM and saponins are used as adjuvants in some commercial veterinary vaccines and have been examined as adjuvants in a large number of human experimental vaccines. This review describes the current status and potential of saponin and ISCOMsTM as adjuvants for orally-administered vaccines with special reference to the induction of local and systemic immune responses and interactions with the intestinal epithelium. The structure and composition of saponins and ISCOMsTM will also be reviewed. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: GALT; M cell; Enterocyte; *Quillaia saponaria*; Triterpene glycosides; Immune response; Oral immunization

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1. Introduction

One of the great dreams of modern vaccinologists is to be able to deliver a range of non-living vaccine antigens by the oral route. The potential advantages are considerable. In human medicine, vaccination would be more acceptable to recipients, especially children, and as a result, vaccine compliance could be expected to rise. Elimination of the syringe and needle for delivery may reduce costs and avoid the risks currently associated with multi-dose containers and needle-stick injury. In veterinary medicine, vaccine delivery would be simplified, risks of accidental self-inoculation avoided and potential carcass damage from vaccine reactions would be eliminated. Finally, control of wildlife diseases such as rabies and tuberculosis would be facilitated if oral vaccination were an option, and the oral delivery of anti-fertility vaccines could permit the non-destructive control of wildlife populations.

Saponins derived from the soap bark tree *Quillaia saponaria* Molina have long been known to be adjuvant active. More recently, Immunostimulating Complex, or ISCOMTM (ISCOM and ISCOMs are trademarks of Iscotec AB, Uppsala, Sweden), vaccines which incorporate saponin components have been developed and both these and the component saponins have been shown to have adjuvant activity in experimental animal models when delivered orally. This review will assess the current and potential impact saponin and ISCOMsTM might have on delivery of vaccines by the oral route.

2. The intestinal mucosal immune system

The intestinal mucosal immune system is composed of scattered, loosely organized lymphoid cells and organized lymphoid tissue. The organized lymphoid tissue consists of Peyer's patches (PP) which are predominantly located in the small intestine and appendix, solitary lymphoid nodules present in the colon and ileum and mesenteric lymph nodes (MLN). Loosely organized lymphoid cells are found in the epithelium and in the lamina propria. Intraepithelial lymphocytes (IEL) are located above the basement membrane between intestinal absorptive

enterocytes whilst B and T lymphocytes together with antigen presenting cells (APC) are disseminated in the subepithelial connective tissue of the lamina propria.

To induce a mucosal immune response, an antigen must be taken up from the gut lumen and delivered to lymphoid cells in the gut-associated lymphoid tissue (GALT). The major inductive sites in the GALT for secreted IgA (sIgA) responses are generally believed to be the organized lymphoid tissue and in particular the PP, which contain a dome region enriched in B and T lymphocytes and APC [1]. The dome area is covered by a special type of intestinal epithelium cell termed microfold (M) or follicle-associated epithelial (FAE) cells which transport luminal antigens and deliver them in an intact form to underlying lymphoid tissue [2–4]. Transported antigen is taken up and processed by APC including dendritic cells (DC), macrophages and B cells. The recent identification of DC in the subepithelial dome and of DC and macrophages in interfollicular T cell regions (IFR) of PP [5] is of particular interest because these populations of APC have been demonstrated to be important for the uptake and presentation of antigens in ISCOMs [6–8]. The T cell-dependent areas of PP contain both CD4⁺ and CD8⁺ T cells and >95% of these T cells use the α/β form of the T cell receptor. Approximately 60% of the T cells that reside in the PP are CD4⁺ T helper (Th) cells [1,4]. Oral immunization with antigen and cholera toxin as adjuvant has demonstrated that PP Th cells can be induced to produce cytokines such as IL-4 and IL-5 which support the shift to production of IgA antibodies [9–12]. Activation of CD8⁺ cytotoxic T lymphocytes has also been demonstrated [13].

Distinct follicles located beneath the dome region of PP contain germinal centres where the majority of surface IgA⁺ B cells are found [14,15]. The shift to production of IgA antibodies and affinity maturation is considered to take place in these germinal centres, a process which most likely is promoted by cytokines produced by T cells in the adjacent T cell dependent areas. Thus, antigens delivered into the PP encounter an environment comprising APC (DC and macrophages) with the ability to process and present antigen, CD4⁺ Th cells displaying properties sup-

porting IgA responses, surface IgA⁺ B cells and CD8⁺ CTL.

Following exposure to antigen, activated B and T cells are transported to the MLN before entering the systemic circulation through the thoracic duct. The lymphocytes then enter the effector sites in the lamina propria where they clonally expand and mature into effector T cells or IgA-secreting plasma cells. The lamina propria is the major intestinal mucosal effector site and primarily contains B cells (20–40%) and T cells (40–60%). IgA secreted by terminally differentiated plasma cells mediates protective immune functions by neutralization of viruses and toxins, inhibition of microorganism adherence to mucosal surfaces and immune exclusion of macromolecules [1]. The majority of T cells in the lamina propria are CD4⁺ Th cells which after activation have been demonstrated to secrete cytokines (IL-4, IL-5, IL-6) which support surface IgA⁺ B cells to differentiate into IgA-secreting plasma cells [16–19]. The phenotype, function and immunoregulation of lamina propria T cells have recently been reviewed in detail [20].

Intestinal epithelial cells (IEC) represent another cell type in the gastrointestinal mucosa which may act as APC. IEC constitutively express major histocompatibility class (MHC) II molecules on their basolateral and apical surfaces [21,22] and have been shown to present soluble peptide antigens and activate primed T cells in vitro [23–25]. In general, IEC preferentially activate CD8⁺ T cells expressing suppressor functions [26,27]. This process has been suggested to involve nonclassical class I molecules (CD1d) [28–30] and possibly other surface molecules on IEC [31–33]. B7-related costimulatory molecules have not been detected on IEC [34,35] suggesting that additional unknown costimulatory pathways may be involved or that antigen presentation by IEC is involved in the induction of tolerance [36]. Antigen presentation in the intestinal mucosa as well as the phenotype and functional properties of intraepithelial lymphocytes have recently been reviewed elsewhere [20,36]. In addition, class II MHC antigen-bearing DC were recently detected within the epithelium of the intestine in rats [37]. Moreover, intestinal lymph-borne DC could acquire orally administered antigen and prime naive T cells in vivo

[38], suggesting an important role of DC in surveillance of mucosal tissues for incoming antigens.

3. Antigen uptake from the gastrointestinal tract

3.1. Cellular and extracellular barriers

Before orally-administered antigens are taken up from the gut lumen and delivered to the mucosal lymphoid tissue, they will be exposed to gastric and intestinal environments which may result in proteolytic degradation, mechanical elimination or neutralization by antibodies. Acidic or alkali conditions and enzymatic digestion [39–41] may result in antigen degradation or loss of epitopes due to denaturation and precipitation in the GI lumen. One factor that can influence the survival of antigens in these conditions is the variability and duration of the GI-transit times, which is related to the gastric-emptying time and may vary from 2 h under fasting conditions to 16 h under fed conditions [42]. The residence time in the small intestine varies from 3 to 5 h and is less variable [43]. Adherence or binding of antigen to the gut surface can be inhibited or decreased by normal clearance mechanisms, for instance entrapment in mucus and antibody complex formation.

The intestinal epithelium consists of a number of cell types such as columnar absorptive cells (enterocytes), mucus-secreting goblet cells and a few endocrine and caveolated cells. Uptake and transport of antigen across the intestinal epithelium is restricted by several mechanisms. The apical surface of the enterocyte, which is the most abundant cell in the intestinal epithelium, is coated with a layer of membrane-anchored, negatively charged glycoproteins, proteoglycans and glycolipids which is called the glycocalyx [44–46]. The glycocalyx, which may extend 0.5–2 μm from the tips of the microvilli, represents both a diffusion barrier and a highly degradative environment due to entrapped proteolytic enzymes [47]. The apical plasma membrane of enterocytes also constitutes a barrier to antigens. This membrane is relatively impermeable to most hydrophilic molecules and fuses with the apical

membrane of adjacent enterocytes to form tight junctions which separate the apical and basolateral sides of the epithelium from each other. Nevertheless, absorptive enterocytes are able to take up and transport antigens [48,49] although M cells present in the FAE are believed to be the major path of entry for luminal antigens [4,49].

3.2. Antigen uptake through M cells

M cells are found in the FAE which separates intestinal lymphoid follicles such as PP from the gut lumen and account for ~10% of the total FAE cell number in humans [4]. M cells are specialized for transepithelial transport of antigens across the epithelial barrier — they have short microvilli, poorly developed glycocalyx, large endocytic domains that function in uptake of macromolecules, particles and microbes and few lysosomes (reviewed in e.g. [4,49]). In addition, PP have a reduced number of goblet cells compared with the surrounding epithelium, suggesting that a low production of mucus facilitates the adhesion of antigens to the surface of M cells [50,51]. The basolateral membrane of M cells is deeply invaginated, forming a pocket region. The pocket results in a small distance between the apical and basolateral membrane which facilitates rapid transcytotic movement of endocytic vesicles and a close association between the pocket membrane and lymphoid cells. The immigrant cells in the M cell pocket have been identified as T lymphocytes (mainly CD4⁺), B cells, small numbers of macrophages and recently also DC [5,52–54].

M cells use multiple endocytotic mechanisms for uptake of macromolecules, particles and microorganisms. Adherent antigens are efficiently taken up by endocytosis via clathrin-coated pits and vesicles [2] and endocytosed antigens can be released at the pocket membrane within 10–15 min. Large adherent particles and bacteria are taken up by phagocytosis [55] whereas nonadherent materials are taken up by fluid-phase endocytosis [3,56].

The foreign material is transported into endosomal tubules and vesicles and large multivesicular bodies and released into the pocket, apparently with little degradation or chemical modification [57,58]. Recently, lysosome-like structures and MHC class II antigen determinants have been detected in M cells

or on M cell membranes, and cathepsin E, a characteristic lysosomal proteinase, is present in M cells [59,60], suggesting that M cells may have the capacity to process endocytosed antigens. However, whether M cells participate in the processing and presentation of antigens *in vivo* remains to be established.

3.3. Antigen uptake through absorptive enterocytes

The absorptive enterocyte is not a uniform cell. The phenotype and the absorptive capacity of enterocytes changes during intestinal development of the fetus and neonate and also during maturation of individual enterocytes [48,61]. The endocytic capacity of individual enterocytes as well as the transport of endocytosed antigens across the epithelial barrier is generally limited. However, the functional effect of such transport may be substantial because of the abundance of enterocytes in the intestinal epithelium [48].

Antigens can cross the absorptive epithelium by active transcellular transport or by the passive paracellular route. Enterocytes have the ability to take up adherent extracellular antigens by receptor-mediated or adsorptive endocytosis [49], whereas non-adherent macromolecules can be taken up by fluid-phase endocytosis [62,63]. The upper size limit of material for uptake by enterocytes has been estimated as 200 nm, although recent studies suggested that the villus epithelium in rats could take up 2- μ m polystyrene microparticles [64–67]. The majority of antigen taken up into endocytic vesicles in enterocytes will usually follow the degradative pathway and is directed to acidic proteolytic compartments such as endosomes and lysosomes where the molecules are extensively degraded. Extensive intracellular degradation of antigen has been demonstrated after uptake by both receptor-mediated and fluid phase endocytosis [62,68]. A fraction of endocytosed macromolecules may follow the non-degradative pathway by a process termed transcytosis [69]. This material will be transported in endocytic vesicles and released on the opposite cell surface as intact or partially degraded antigen by mechanisms which are not clearly understood. Transcytosis of a number of proteins with varying properties has been studied in monolayers of epithelial cells [63,68,70–

74]. It has also been suggested that particulate material with a size of 100–200 nm can cross the epithelium by paracellular transport [75]. Under physiological conditions, paracellular transport is inhibited by the tight junctions. However, the permeability of tight junctions can be regulated by both physiological and non-physiological factors such as osmotic and hydrostatic pressure, nutrients and the action of absorption enhancing agents [76–78].

Enterocytes have been demonstrated to have the ability to function as APC [23–25]. Transport of antigen by enterocytes may therefore stimulate a mucosal immune response in two ways. At least a fraction of the antigen which is transported in enterocytes may be processed into peptides which can interact with nonclassical class I molecules and possibly other molecules to form a complex which can be presented at the cell surface and activate T cells [31]. In addition, intact or processed antigen which is delivered by exocytosis at the basolateral membrane can be taken up by APC such as DC or macrophages and presented to IEL or T cells resident in the LP.

4. Saponins and ISCOMTM

4.1. Nature of *Quillaia* saponins

Saponins are a highly diverse group of glycosides, occurring primarily though not exclusively in plants, and characterised by their ability to form a lather when agitated in water [79]. All saponins contain either a steroidal or triterpenoid aglycone to which one or more sugar chains are attached. Nearly 100 families of plants are known to contain saponins, and where extracts of these plants have been subjected to chemical or physical analysis, considerable chemical heterogeneity has been described.

The adjuvant-active properties of saponins were first recognised in the 1920s [80] and their use in commercial foot and mouth disease vaccines was described in 1951 [81]. Variability in adjuvant activity between batches of saponins led to the discovery [82] that all saponin batches with adjuvant activity were sourced from the soap bark tree, *Quillaia saponaria* Molina. Subsequent studies [83–87] have revealed the expected chemical heterogeneity of

Quillaia saponins and it is likely that the number of molecular structures exceeds 100. The essential features are an olean-12-en triterpene (quillaic acid) with a branched trisaccharide at the C3 position, an oligosaccharide, sometimes branched, at the C28 position, and an aldehyde group at the C4 position. An acyl chain is frequently found attached by ester bond to the fucose at position C28 and when full length (18 carbons), one or two terminal sugars are present. Detailed descriptions of structure/function analysis and nomenclature are available elsewhere [88,89].

Quillaia saponins are generally derived from bark by aqueous extraction. This crude extract will additionally contain considerable quantities of tannin, cresols and flavinoids. Gel filtration and anion exchange chromatography leads to a purified mixture of saponins comprising around 5% of the original extract by weight but retaining all the adjuvant activity [90]. This fraction, known as Quil A, can be further purified by reverse phase HPLC or other procedures to yield defined fractions which may be single or multiple peaks. Typical materials resulting from these separation procedures are QH-A, QH-C and the mixture IscoplepTM703 [91] and QS-21 or StimulonTM [86]. Of these defined components, QS-21 has received the most extensive evaluation as an adjuvant for human vaccines with clinical trials underway and completed for a number of diseases including melanoma, influenza, herpes simplex virus and HIV-1 [89,92–94]. ISCOMATRIXTM and influenza virus ISCOMsTM using the defined fraction IscoplepTM703 have also been in extensive clinical trials ([95] and unpublished results). The use of purified saponins as vaccine adjuvants has recently been reviewed [88,89].

4.2. ISCOMsTM

The high affinity of saponins for cholesterol has long been recognised. This property led to the serendipitous discovery of ISCOMsTM [96] during studies on the formation of virosomes in the presence of saponins. ISCOMsTM are complexes of saponin, cholesterol, lipid and immunogen, which can aggregate into the typical 40-nm open cage-like structure (Fig. 1). Similar complexes form if immunogen is

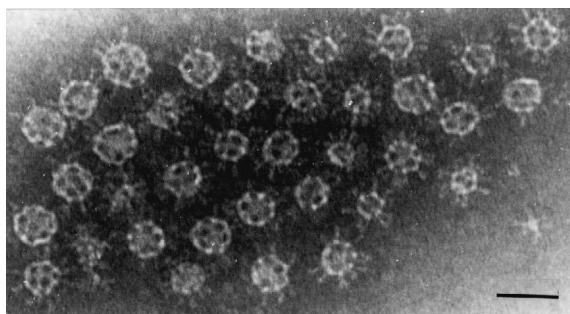


Fig. 1. Transmission electron micrograph of influenza virus ISCOMsTM. The ISCOMsTM were formed at a high haemagglutinin:Quillaia saponin ratio and show the typical ISCOMTM morphology and 'spikes' of haemagglutinin radiating from the surface (bar indicates 0.05 μm).

absent [97] and these are termed ISCOM matrix (also called ISCOMATRIXTM).

ISCOMsTM can be formed using mixtures of *Quillaia* saponins [96,97], defined saponin fractions [91,98] or purified components [99]. Cholesterol is an essential component but may be entirely derived from the preparation of virus or other membrane protein used as immunogen. Inadequate cholesterol concentrations during preparation can lead to ISCOMsTM with high haemolytic activities. The requirement for phospholipid is less certain although its presence does lead to more reproducible ISCOMTM formation [97].

ISCOMsTM and ISCOMATRIXTM are most simply prepared by the dialysis method [100]. A typical reaction mixture will contain 0.8 mg/ml cholesterol, 0.8 mg/ml phospholipid (typically egg phosphatidylcholine, egg phosphatidylethanolamine or dipalmitoylphosphatidylcholine), 4 mg/ml protein (if ISCOMsTM are to be formed) and 4 mg/ml saponin (typically Quil A, ISCOMPREPTM703 or QS-21) in a 16 mg/ml solution of detergent (typically decanoyl-*N*-glucamide (MEGA-10) or *n*-octyl glucoside). Following brief (1 h to overnight) incubation at 25°C, detergent can be removed by dialysis whereupon ISCOMsTM or ISCOMATRIXTM will form. These structures have a net negative charge as a result of glucuronic acid in the saponin structure and are highly stable [101].

5. Oral administration of *Quillaia* saponins

5.1. Interaction and fate of saponins

Although it was postulated almost 70 years ago that the haemolytic activity of saponins derived from their affinity for cell membrane sterols [102], the molecular basis for this interaction is still in dispute. However, current evidence suggests that haemolytic saponins require both amphipathicity, as demonstrated by the ability to form micelles [103] and their normal carbohydrate complement [104]. Chemical modification of the aldehyde group on the quillaic acid abolishes haemolytic activity [103], whilst partial or complete removal of the acyl chain also has a substantial effect upon haemolytic activity [91], possibly as a result of its effect upon the critical micellar concentration. Binding to cell membranes appears to be between the aglycone and cholesterol [105] and is believed to be permanent [106]. Removal of sugar moieties after binding, as a result of β glucosidase activity of cell membranes, does not reverse binding [105]. Certain saponins can also bind to cholesterol-free, phospholipid vesicles [107]. The interaction seems to require phosphatidyl choline and an α -L-arabinofuranose residue on the saponin, a feature of many *Quillaia* saponin derivatives [85].

The activity and fate of saponins delivered orally is even less certain. It is generally believed that saponins do not cross the gastrointestinal wall [108]. However, several studies have shown increased permeability of oral or gastric epithelia following administration of saponins resulting in increased nutrient uptake. Administration of *Quillaia* saponin in association with calcitonin to the oral mucosa of rats caused a significant increase in serum calcium levels [109]. Tea nut saponin was ineffective in this model. Soya saponins were shown to increase the permeability of rabbit jejunal mucosa in vitro to glycinin, a soya-derived protein [110]. It was proposed that soya saponins interacted with the apical membranes of enterocytes, thus permitting glycinin bound to the soya saponin to penetrate the cell.

Various mechanisms are proposed for the fate of orally-delivered saponins. Some saponins will bind directly with cholesterol in the gut to form an insoluble complex too large for absorption [111].

Alternatively, saponins including *Quillaia* saponins can form mixed micelles with bile acids [112] and the resultant micelles can prevent resorption of bile acids, leading to increased hepatic synthesis of bile acid from stored cholesterol. The end result of either of these mechanisms is hypocholesteremia. Saponins are sensitive to acid and alkaline hydrolysis [113] and to attack by bacterially-derived glucosidases in the large intestine [114], as a result of which they will be degraded to the aglycone. Finally, saponins which do bind to mucosal membranes will be rapidly eliminated by the normal process of epithelial replacement.

Long-term feeding of *Quillaia* saponins to rats (0.75% of total diet) showed no effects on food consumption or body weight, although serum triglyceride and cholesterol levels were significantly lowered after 24 weeks [115]. This led to the cautious conclusion that *Quillaia* saponins in the diet may be beneficial rather than detrimental.

5.2. Immunomodulatory effects

Maharaj et al. [116] fed mice *Quillaia* saponins either before, with or both before and with inactivated rabies vaccine. They measured serum antibody virus neutralising titres, resistance to intra-cerebral rabies challenge and passage of radiolabelled antigen into the bloodstream.

Saponins increased responses to all three parameters in a dose-dependent manner. They postulated that the *Quillaia* saponins might interact with the rabies virus surface G protein to protect it from degradation by proteases, complex with virus to present it in an immunogenically more active form and stimulate interaction of antigen with lymphocytes by increasing the permeability of intestinal membranes. Subsequent papers [117,118] showed that mice fed *Quillaia* saponins alone (i.e. without rabies antigen) showed greatly enhanced cell proliferation in both the spleen and mesenteric lymph nodes and an increased and prolonged NK cell activity. Transfer of spleen or thymus cells to unprimed recipient mice gave partial protection to a subsequent intracerebral challenge with live rabies virus. They suggested that saponins acted directly on Th cells of the mucosal immune system, to induce

secretion of soluble mediators which could regulate immune functions systemically. Oral feeding of saponin also increased the efficacy of vaccine antigen delivered by the intraperitoneal route [119], again supporting the proposition that saponins delivered orally can assert a systemic immunomodulatory effect.

6. Immune responses to ISCOMsTM after oral administration

The ability of ISCOMsTM to potentiate both antibody- and cell-mediated immune responses to incorporated antigens after parenteral administration is well established and has been discussed in a number of reviews (for example [88,89,120,121]). The information concerning immune responses to ISCOMsTM after immunisation by oral administration is more limited. However, during recent years several studies have analyzed the induction of local and systemic immune responses to a wide range of antigens following administration of ISCOMsTM by the oral route (summarised in Table 1). The majority of these studies have been performed in mice. Unless otherwise stated, the results below refer to immunisation experiments in mice.

The first indications that ISCOMsTM are active at mucosal surfaces and have adjuvant activity after local administration came from studies using the intranasal route for immunisation [122–125]. In contrast, early attempts to immunise mice orally with ISCOMsTM containing the influenza virus envelope glycoproteins haemagglutinin (HA) and neuraminidase (NA) were unsuccessful when assessed by induction of serum antibody responses [123]. Five µg of influenza virus ISCOMsTM were administered orally twice, 17 weeks apart, and it is possible that the immunisation protocol itself was inappropriate. The first demonstration that ISCOMsTM were adjuvant active when administered by the oral route was reported in 1991 by Mowat et al. [126], who used ISCOMsTM containing palmitified ovalbumin (OVA) as a model antigen (palmitified OVA is required to provide a lipid tail for incorporation of the otherwise hydrophilic OVA into ISCOMsTM). A single oral dose of OVA in ISCOMsTM administered

Table 1
Immunisation by oral administration of ISCOMs™

Antigen	Administration protocol	Immune responses to orally-administered ISCOMs™	Reference
Ovalbumin	One dose of 10, 50 or 100 µg of OVA	Systemic DTH and serum IgG with 50 or 100 µg of OVA	[126]
Ovalbumin	Three or six doses of 10, 50 or 100 µg of OVA	Splenic CTL activity, serum IgG and intestinal sIgA. Prevented oral tolerants	[127]
Ovalbumin	Three to six doses of 50 µg of OVA	Greater CTL responses than with OVA in microparticles	[141]
Ovalbumin	Three doses of 50 µg of OVA	Proliferative responses in the MLN and similar splenic cytokine production as after parenteral immunisation	[130]
Influenza antigen	Two doses of 5 µg of influenza antigen	No detectable serum antibody responses	[123]
Influenza antigen	One or two doses of 0.25 µg of influenza antigen	Serum antibody responses but no IgA in nasal washes. Some protection against challenge	[133]
Influenza nucleoprotein	Three intragastric or two peroral doses of 100 µg of nucleoprotein	Serum and bronchoalveolar fluid IgA and IgG, DTH and protection after peroral but not intragastric immunisation	[134]
Newcastle disease vaccine	Two doses of 125 µg of vaccine delivered in the food	No enhancement of antibody responses compared with vaccine without adjuvant	[138]
<i>E. faeciformis</i> surface antigen P27	Six doses of 20 µg of antigen P27	Systemic IgG and local IgA, proliferative responses of MLN cells and partial protection against infection	[132]
<i>T. spiralis</i> E/S antigen	Two doses of 45 µg of E/S antigen	No significant reduction in worm burdens after infection	[135]
<i>Y. enterocolitica</i> HSP60	Four doses of 100 µg of HSP60	No serum IgG responses or proliferative T cell activity	[136]
Somatostatin-14 (SRIF)-avidin complex	Three doses of 50 µg of SRIF and 2 mg of avidin	Serum IgG and mucosal IgA responses after intraperitoneal priming followed by intragastric immunisation	[137]

with a gavage tube induced serum IgG responses and delayed type hypersensitivity (DTH) whereas OVA without adjuvant was non-immunogenic. However, the responses were poor and required large amounts of ISCOMsTM, ~10-fold more than used for parenteral administration.

These findings were extended in a subsequent study [127], where 3–6 doses of OVA in ISCOMsTM were found to induce strong antibody and cellular responses. This immunisation protocol activated CD8⁺ class I MHC restricted CTL in both the spleen and MLN and induced serum IgG responses as well as production of intestinal IgA. As has been reported for other antigen and adjuvant formulations given orally [12], the induction of intestinal IgA responses by OVA in ISCOMsTM was dependent on repeated administration of antigen. The authors concluded that the frequency of antigen administration, rather than the total dose of OVA given, was the most critical factor in the OVA in ISCOMTM immunisation schedule. Importantly, oral immunisation with OVA in ISCOMsTM did not induce the state of systemic immunological non-responsiveness termed oral tolerance which is frequently seen after oral administration of proteins [20]. Mice fed 100 µg soluble OVA 1 week before parenteral immunisation with OVA in Freund's complete adjuvant had significantly suppressed systemic DTH responses whereas mice fed the same dose of OVA in ISCOMsTM had no evidence of oral tolerance. Moreover, mice immunised with OVA in ISCOMsTM had no evidence of mucosal hypersensitivity when challenged orally with OVA. In situations where oral tolerance has been abrogated by priming with antigen, the mucosa may develop hypersensitivity on refeeding the antigen [128,129]. In addition, oral immunisation with OVA in ISCOMsTM has been shown to prime splenic CD4⁺ Th cells which respond to in vitro stimulation with OVA by secretion of the cytokines IL-2, IL-5 and IFN-γ [130]. The pattern of cytokine production was similar to that observed after parenteral immunisation and indicated that OVA in ISCOMsTM given by the oral route activates T cells of both the Th1 and Th2 type. Thus, ISCOMsTM promote a wide range of local and systemic antibody- and cell-mediated immune responses when administered by the oral route, which may make them useful as a mucosal adjuvant. However, the requirement for

repeated immunisations and large amounts of antigen, together with a relatively short duration of immune responses in animals fed ISCOMsTM, could preclude their practical use [127,131].

Information concerning the ability of ISCOMsTM to function as a mucosal adjuvant with antigens other than OVA is more conflicting (Table 1). Oral administration of ISCOMsTM has been reported to induce both local and systemic immune responses as well as protective immunity in some experimental models, whereas no detectable immune responses were recorded when used with other antigens. Kazanji et al. [132] immunised mice by the oral route with ISCOMsTM containing a purified surface protein (AgP27) from sporozoites of the coccidian parasite *Eimeria falciformis*. Six doses of 20 µg of AgP27 in ISCOMsTM, given at 2-day intervals, induced serum IgG and intestinal IgA responses and primed MLN cells which could be stimulated to proliferate in vitro after antigen stimulation. In agreement with the results with OVA in ISCOMsTM [127], the IgA response induced by AgP27 in ISCOMsTM was of short duration. Immunisation with ISCOMsTM resulted in partial protection against challenge with oocysts as demonstrated by a reduced daily oocyst excretion and significant differences in weight loss and death compared with control mice.

Influenza virus A/Sichuan/2/87 (H3N2) HA and NA in ISCOMsTM also induced a serum antibody response when given orally and provided some protection against a subsequent virus challenge [133]. However, no antigen-specific IgA could be detected in nasal washings and intramuscular injection of the ISCOMsTM was more efficient with respect to the generation of both antibody responses and protection. Because only one or two doses of 0.25 µg of antigen were given, it is possible that repeated administration with higher amounts of antigen could increase the efficiency of influenza virus ISCOMsTM when used by the oral route.

Influenza A virus nucleoprotein (NP) from fowl plague virus (A/FPV/Rostock/34, H7N1) was used to compare peroral and strictly intragastric administration of ISCOMsTM [134]. The hydrophilic NP was incorporated into ISCOMsTM after attachment of bacterial lipopolysaccharide, whose lipid A functioned as a lipophilic anchor. Two to four peroral feedings with 100 µg of NP in ISCOMsTM

induced IgG and IgA responses in both serum and bronchoalveolar fluid and generated strong systemic DTH. Moreover, all perorally vaccinated mice survived an otherwise lethal challenge infection with an unrelated influenza virus subtype, with no severe clinical signs or extensive pathological lesions in the lungs. In contrast, three intragastric administrations of NP-ISCOMs resulted in low levels of serum antibody and no detectable IgA in bronchoalveolar fluid. Compared with control mice, mice vaccinated by the intragastric route showed slightly prolonged survival but no difference in weight loss or development of pulmonary lesions. Partial protection was obtained in chickens that received 100 µg of NP-ISCOMs in the drinking water. Based on these results, it was suggested that the main inductive site in mice immunised perorally with NP in ISCOMsTM was the oro-pharyngeal mucous membrane rather than the gastrointestinal tract.

With some antigens, oral immunisation with ISCOMsTM has been unsuccessful. Vaccination with two oral doses of 45 µg of excretory/secretory (E/S) antigen from the nematode *Trichinella spiralis* in ISCOMsTM had no effect on the worm burdens of infected mice whereas animals vaccinated subcutaneously with the same ISCOMTM preparation had significantly fewer worms than unvaccinated controls [135]. In a similar way, ISCOMsTM containing a heat shock protein (HSP60) from *Yersinia enterocolitica* induced serum IgG and proliferative T cell responses and protective immunity after parenteral immunisation but not after oral administration via a gavage tube [136]. Estrada et al. [137] used biotinylated somatostatin-14 (somatotropin release-inhibiting factor, SRIF) which was conjugated to palmitified avidin and the resulting complex was incorporated into ISCOMsTM. Three intragastric immunisations with the ISCOMsTM (50 µg of SRIF) induced no significant serum or mucosal antibody responses to SRIF. However, intraperitoneal priming followed by intragastric immunisation elicited serum IgG responses and induced IgA-secreting cells in the mesenteric lymph nodes and PP. Finally, oral vaccination with a V4 strain Newcastle disease vaccine in ISCOMsTM administered in the crop did not improve the antibody responses in chickens compared with the vaccine given without adjuvant [138].

Together, these results suggest that ISCOMsTM

can be adjuvant active after oral administration but that their potency appears to vary depending on the antigen and experimental protocol used. The reasons for these differences are not clear but the frequency of ISCOMTM administration and the amount of antigen given is most likely of critical importance. Another variable, which most studies so-far have not addressed, is the amount of *Quillaia* saponin delivered in different ISCOMTM preparations. Saponins may affect the permeability of epithelia [109,110] and contribute to the adjuvant activity of ISCOMsTM [139]. The *Quillaia* saponin content may therefore influence both the uptake and adjuvant properties of orally administered ISCOMsTM. It is also possible that the sensitivity to the harsh environment in the gastrointestinal tract may vary between different ISCOMTM preparations. The observation that the ISCOMTM matrix is extremely resistant to acidic conditions and bile salts [101,140] suggests that the antigen rather than the ISCOMTM particle may be the major target for gastrointestinal degradation and denaturation.

In any event, further studies are necessary to delineate the adjuvant activities of orally-administered ISCOMsTM and such studies may also facilitate the development of experimental protocols suitable for oral immunisation with a broad range of antigens in ISCOMsTM. One important issue which needs to be addressed is how the adjuvant activity of orally-administered ISCOMsTM compares with that of other mucosal adjuvants and delivery systems such as cholera toxin or microparticles. Maloy et al. [141] found that orally-administered OVA in ISCOMsTM induced stronger CTL responses than those observed after oral immunization with OVA in poly(lactide-co-glycolide) (PLG) microparticles. Moreover, three doses of OVA in ISCOMsTM was sufficient to generate strong splenic CTL responses whereas three oral administrations of OVA in microparticles resulted in very low levels of CTL activity.

7. Uptake of ISCOMsTM across the digestive tract

The observation that ISCOMsTM are able to induce a broad range of mucosal and systemic

immune responses after oral administration implies that antigens in ISCOMsTM can be taken up from the gut lumen and transported across the intestinal epithelium with retained immunogenicity. As discussed previously, antigens can reach the GALT via uptake by M cells or absorptive enterocytes. Recent results from in vitro studies indicate that ISCOMsTM can interact with both of these cell types.

Claassen et al. [142] labelled rabies virus antigen in ISCOMsTM and free rabies virus with the fluorochrome DiO (3,3'-diiodododecylcarbocyanine perchlorate; D275) and used fluorescence microscopy to analyse their localisation in segments of the small intestine after in vitro incubation. They found that ISCOMsTM were taken up rapidly and efficiently by M cells in the PP. In contrast, rabies virus did not localise to the PP but was instead found in close association with the surface of epithelial cells. The results suggest that incorporation into ISCOMsTM promotes the targeting of orally-administered rabies virus antigens to M cells (Fig. 2A) and provides one possible explanation of the observation that feeding mice with inactivated rabies vaccine and *Quillaja* saponins increased the transport of antigen into the bloodstream ([116], see section 5.2). However, the

mechanisms by which ISCOMsTM increase the uptake of rabies virus antigen by M cells remain to be established and it is not clear whether this activity represents a general property of orally-administered ISCOMsTM.

The interactions between ISCOMsTM and absorptive enterocytes have been studied in vitro using the human intestinal epithelial cell line Caco-2 as a model of intestinal epithelium. Biotin-labelled ISCOMsTM containing human influenza virus HA and NA were rapidly taken up by Caco-2 cells after apical administration at 37°C where they could be detected in both intracellular vesicles (Fig. 3A) and paracellular spaces (Fig. 3B) [143]. Denaturation of the HA binding site for sialic acid partly inhibited the binding of ISCOMsTM to Caco-2 cells at 4°C but did not influence the transport of ISCOMsTM at 37°C, suggesting that the ISCOMsTM at least partly were transported by fluid-phase endocytosis. This is not surprising because the size of ISCOMsTM (~40 nm) is well below the upper size limit for uptake by enterocytes (200 nm, [64–67]). The transport across Caco-2 monolayers of ¹²⁵I-labelled ISCOMsTM at concentrations where the epithelial integrity and permeability were not affected did not differ sig-

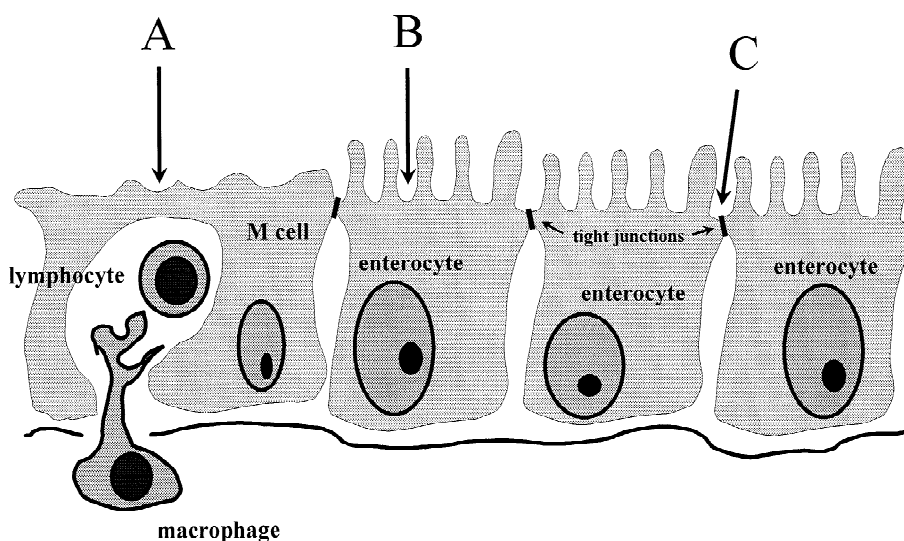


Fig. 2. Hypothetical transport route(s) across the intestinal epithelium of antigens incorporated into ISCOMsTM or delivered with ISCOMATRIXTM. (A) Uptake and transport of ISCOMsTM by M cells in the Peyer's patches [142]; (B) uptake and intracellular transport of ISCOMsTM by enterocytes [143]; (C) paracellular transport of antigen as a result of surfactant-like effects of ISCOMATRIXTM on the epithelial integrity [144].

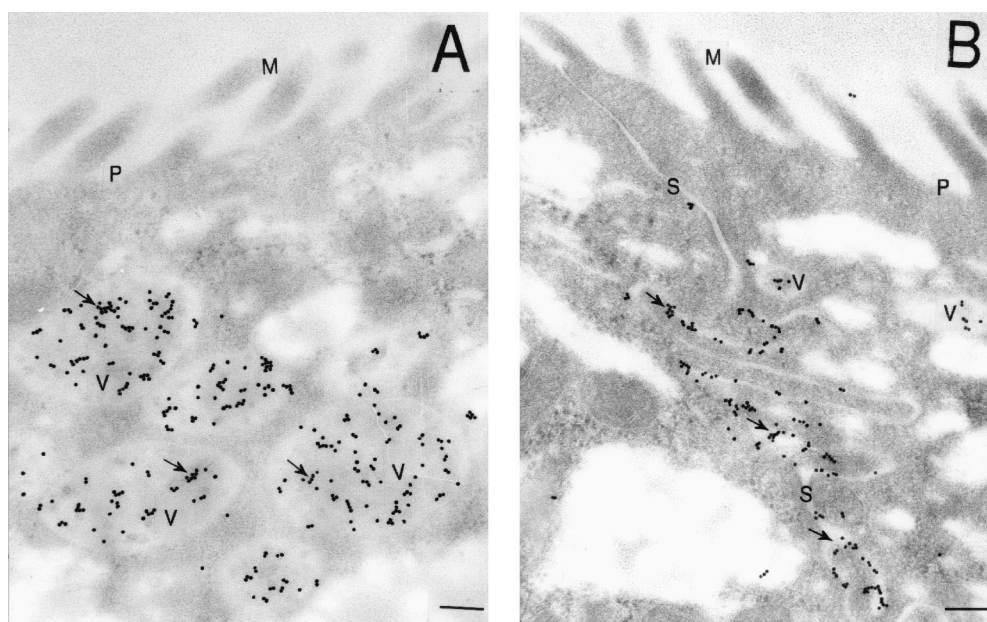


Fig. 3. Internalisation of influenza virus ISCOMsTM by Caco-2 cells. Labelling of intracellular vesicles (A) and paracellular space (B) after apical application of biotin-labelled ISCOMsTM. Caco-2 monolayers were incubated with 100 μ g of ISCOMsTM for 10 min at 37°C and labelled material was visualised with streptavidin-gold and electron microscopy. Arrows indicate gold particles; bar 1 μ m. M, microvilli; P, apical plasma membrane; S, paracellular space; V, intracellular vesicles.

nificantly from that of influenza HA and NA administered in the form of protein micelles. Moreover, both the ISCOMsTM and influenza antigen were extensively degraded during transport in Caco-2 monolayers. However, transported ISCOMsTM, but not influenza antigen, stimulated T cells primed with influenza antigen to proliferate in vitro, an activity which was associated with hydrophobic peptides with a molecular weight less than 3000. The results suggest that antigens in ISCOMsTM can be transported across the absorptive epithelium and released at basolateral surfaces as antigenic peptides (Fig. 2B). However, the mechanisms by which this occurs as well as the in vivo relevance of the results remain to be established. For instance, it is not clear whether ISCOMsTM can penetrate the glycocalyx which covers the apical surface of enterocytes [44–46].

ISCOMATRIXTM has been shown to induce various dose-dependent alterations in Caco-2 monolayers [144]. At concentrations greater than 100 μ g/ml of Quil A, ISCOMATRIXTM displayed depressant effects on the intracellular dehydrogenase activity and transepithelial resistance whereas the short circuit

current and permeability to [¹⁴C]mannitol increased. At similar dose levels, ISCOMATRIXTM enhanced the transport of influenza antigen across Caco-2 monolayers and at 400 μ g/ml of Quil A the transport of high molecular weight influenza antigen was significantly increased. In the latter case, the transported influenza antigen could stimulate influenza antigen-primed T cells to proliferate in vitro. The results suggest that ISCOMATRIXTM affects the epithelial integrity in a dose dependent way, a possibility which is consistent with the observation that Quil A binds to cholesterol in cell membranes and has surfactant-like properties when in its free form [145,146]. In line with this, a semisynthetic saponin, DS-1, prepared by deacylation of the saponin QS-21 enhanced nasal or ocular delivery of aminoglycoside antibiotics and insulin suggesting it may have potential as a transmucosal delivery agent [147–149]. The surfactant-like activity of saponins may also promote the transport of antigens across the intestinal epithelium (Fig. 2C). Some in vivo evidence for this has come from recent results showing that oral administration of ISCOMATRIXTM at

concentrations greater than 100 µg/ml enhances the serum antibody response to keyhole limpet haemocyanin (KLH) to levels comparable to those induced by KLH and cholera toxin as adjuvant (Karin Lövgren Bengtsson, Swedish University of Agricultural Sciences, Uppsala, Sweden, personal communication). Interestingly, cholera toxin, but not ISCOMATRIXTM, also induces a local antibody response to KLH.

To date, all in vivo and in vitro studies of oral applications of ISCOMsTM and ISCOMATRIXTM have been performed using Quil A as a source of saponin. The access to defined *Quillaia saponin* fractions with ability to form ISCOMsTM [88,89,91,98,99] now makes it possible to examine in more detail how different components of Quil A contribute to the interactions between ISCOMsTM and M cells or enterocytes. The results from such studies may also facilitate the design of experimental protocols for immunisation by oral administration of ISCOMsTM.

8. Conclusions and future directions

The extensive use of *Quillaia* saponins in the human beverage industry along with considerable oral toxicity data in laboratory animals attest to their potential safety for the oral delivery of vaccines. Thus, the major issue relates to the paucity of good data to support their usefulness. On the positive side are convincing data that saponins or ISCOMsTM induce appropriate immune responses to rabies virus, influenza virus and OVA in laboratory animals. On the negative side are the high and frequent doses required and the fact that the only convincing data to date has been obtained in mice. The non-specific activity of the saponins raises the possibility that they could induce immune responses to food antigens when used as oral vaccines. However, such outcomes have seemingly not resulted from their use as foodstuffs. Considerable work and progress is required before the approach can be considered as more than a technological curiosity. In particular, work is required to establish the mode of action of orally-administered saponins and their applicability to animals other than mice. Another area of in-

vestigation is the incorporation of targeting molecules either within the ISCOMTM structure or to microencapsulate the ISCOMTM structure with or without additional targeting strategies so that the ISCOMTM can be optimally targeted to M-cells within the gut.

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