

ISCOMs: an adjuvant with multiple functions

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Abstract: Aluminum salts are currently the only widely used adjuvant for human vaccines. Over the past 10–15 years, a large research effort has attempted to find novel adjuvants with ability to induce a broad range of immune responses, including cell-mediated immunity. The immunostimulating complex or ISCOM is one adjuvant with multiple adjuvant properties. ISCOMs are open cage-like complexes typically with a diameter of about 40 nm that are built up by cholesterol, lipid, immunogen, and saponins from the bark of the tree *Quillaia saponaria* Molina. ISCOMs have been demonstrated to promote antibody responses and induce T helper cell as well as cytotoxic T lymphocyte responses in a variety of experimental animal models, and have now progressed to phase I and II human trials. This review describes recent developments in the understanding of the structure, composition, and preparation of ISCOMs and will cover important aspects of the understanding of the adjuvant functions of ISCOMs and how they act on the immune system. *J. Leukoc. Biol.* 64: 713–723; 1998.

Key Words: *Quillaia saponaria* · triterpene glycosides · saponin · immunostimulating complex · antibody and T cell response · vaccine

INTRODUCTION

The novel particles or complexes known as ISCOMs were first described by Morein and co-workers in 1984 [1]. ISCOMs (ISCOM[®] and ISCOMs[®] are trademarks of ISCOTEC AB, Uppsala, Sweden) are composed of saponin, cholesterol, phospholipid, and immunogen, usually protein. They were originally designed to form a vaccine delivery system that combined certain aspects of virus particles such as their size and orientation of surface proteins, with the powerful immunostimulatory activity of saponins [1]. These characteristics gave ISCOMs their ability to induce strong immune responses to a variety of antigens in a number of species. ISCOMs, unlike many other vaccine adjuvants, appear to promote a broad immune response by simultaneously promoting high levels of antibody and strong T cell responses, including enhanced cytokine secretion and activation of cytotoxic T lymphocyte (CTL) responses [2, 3]. The ability to stimulate CTL may be important in generating effective immune responses to virus-

infected cells and possibly cancerous cells. A further attraction of ISCOMs is their reduced toxicity and reactogenicity compared with other saponin-based formulations.

Since their first description, considerable effort has been expended to understand the composition and structure of ISCOMs and to control their method of manufacture. In parallel, a number of studies on their *in vivo* distribution, uptake, presentation, and stimulation of T cell subsets have addressed the way in which ISCOMs mediate their effects *in vivo*. Because ISCOM-based vaccines have now progressed to phase I and II human trials [4], this review documents our present understanding of the structure, preparation, and functional properties of ISCOMs and will describe some modifications that have been made to enable ISCOMs to be suitable for human applications.

THE ISCOM PARTICLE

Two types of ISCOM particle or complex have been described in the literature. The first is the classic ISCOM, formed by the combination of cholesterol, saponin, phospholipid, and amphipathic proteins [1]. The second is essentially the same structure but without the protein, and is usually referred to as Iscom Matrix (also called ISCOMATRIX[®], a trademark of ISCOTEC AB) [5]. Typically, both ISCOMs and Iscom Matrix exist as spherical, hollow, rigid, cage-like particles of about 40 nm in diameter with a strong negative charge [5–9]. However, other structural forms including rings and aggregates can occur with properties essentially identical to the 40-nm particle.

Although both ISCOMs and Iscom Matrix are immunostimulatory, there may be some advantages in incorporating molecules in ISCOMs, either naturally (through an intact transmembrane domain) or by chemically or physically altering the protein structure to obtain insertion or association [3, 10–12]. Possible advantages of associating proteins with ISCOMs include partial purification or enrichment of membrane-bound molecules, correct presentation of proteins for immune recognition, increased uptake of protein [3, 10–13], and enhanced induction of CTL [14].

Abbreviations: BSA, bovine serum albumin; RP-HPLC, reversed-phase high-performance liquid chromatography; OVA, ovalbumin; DTH, delayed-type hypersensitivity; LPS, lipopolysaccharide; APC, antigen-presenting cell.

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THE COMPONENTS OF ISCOMS

Cholesterol, saponin (triterpene glycosides), and phospholipid are essential components of ISCOMs and Iscom Matrix. The saponin source that is generally used for making ISCOMs or Iscom Matrix is derived from the bark of the *Quillaia saponaria* Molina tree. The basis of the unique structure of ISCOMs is the interaction between saponin and cholesterol molecules, which when combined form stable rings in aqueous solutions after the removal of detergents. The individual cholesterol-saponin rings combine to form pentagonal dodecahedrons [9] (much like a soccer ball) which is the typical form of an ISCOM particle. These rings are held together by hydrophobic interactions, steric factors, and possibly hydrogen bonds [15]. Phospholipid (typically egg-derived phosphatidylcholine [16] or phosphatidylethanolamine [7]) is needed when protein is to be incorporated into an ISCOM structure. It has been suggested that this promotes a less rigid structure than saponin and cholesterol alone and thus allows the bulky amphipathic molecules (e.g., viral membrane proteins) to insert into the ISCOM structure [16].

RECENT MODIFICATIONS TO ISCOMS

Most of the early studies with ISCOMs used Quil A as a source of saponin [1, 5, 8]. Quil A is a semi-purified fraction of *Quillaia* saponins with a standardized triterpenoid saponin content and less local toxicity than crude saponin [17, 18]. Although suitable for veterinary applications, Quil A was considered unsatisfactory for human applications because it, and other similar preparations, has been found to be a heterogenous mixture of dozens of closely related saponins [7, 19, 20–25]. The existence of many different saponins that vary in their chemical or biological activities may lead to unpredictable effects *in vivo* if their relative ratios cannot be controlled. Kensil et al. [19, 23] identified 22 fractions in Quil A and reported that adjuvant activity was present in at least 10 of those tested, including the four most abundant saponins, termed QS-7, 17, 18, and 21. Kersten [24] described 23 fractions in Quil A, all of which had adjuvant activity but that differed in other biological properties such as cell lysis and toxicity.

Rönnerberg et al. [26] further examined the characteristics of different Quil A fractions in their free form and when formed into Iscom Matrix. Three fractions of Quil A were isolated by reversed-phase high-performance liquid chromatography (RP-HPLC) corresponding to a series of fractions eluting early (QH-A) and a series of the more hydrophobic fractions termed QH-B and QH-C that eluted later. When adjuvant activity was examined with the purified saponin fractions in their free form, QH-B promoted greater antibody responses to influenza protein than QH-C followed by QH-A. However, when the fractions (or combinations of the fractions) were administered as Iscom Matrix there was little difference in the antibody responses to influenza proteins [26]. A combination of seven parts QH-A, zero parts QH-B, and three parts QH-C (ISCOPREP[®]703) has successfully completed various pre-clinical studies and is the basis of ISCOMs and Iscom Matrix vaccines that have been

tested in phase I and II human clinical studies [4]. QH-B was not included in this preparation because of its higher relative toxicity (see below).

PROTEIN ASSOCIATION WITH ISCOMS AND ISCOM MATRIX

ISCOMs have been made with amphipathic molecules derived from many sources including cell wall and membrane proteins from viruses such as Herpes Simplex type 1 (HSV-1), cytomegalovirus (CMV), Epstein Barr virus (EBV), hepatitis B, rabies, and influenza; bacteria such as *Neisseria gonorrhoeae*, *Escherichia coli*, and *Brucella abortus* and parasites such as *Toxoplasma gondii*, *Plasmodium falciparum*, and *Leishmania major* [2, 3, 7, 13, 27, 28]. Antigens that are derived from purified organisms or cell membranes and have the ability to incorporate into ISCOMs are generally proteins, glycoproteins, or lipoproteins that are normally anchored into the cell or viral membrane by a hydrophobic transmembrane sequence or by lipid [3, 12, 13, 28–30]. When ISCOMs are made, these molecules are extracted by detergent treatment and integrate into the lipid-saponin matrix when the detergent is removed and ISCOM particles form [13, 16]. Regardless of the molecular weight, level of glycosylation, or tertiary structure of incorporated proteins, the ISCOMs that are generated are usually indistinguishable from Iscom Matrix when examined by electron microscopy [5, 8, 16, 29, 31] or their sedimentation rate in sucrose gradients [32]. The degree of incorporation is usually determined indirectly by the coincidence of the selected protein and ISCOM particles in the same sucrose density gradient fraction [29, 31]. Direct visualization of protein and ISCOM particles by immunoelectron microscopy is possible but can be technically difficult, requiring specific antibodies and gold labeling techniques. **Figure 1** shows an example of native *L. major* Parasite Surface Antigen-2 incorporated into ISCOMs (PSA-2-ISCOMs) using this technique.

Although it is possible to insert or at least associate non-amphipathic molecules with ISCOMs, structural modifications such as partial denaturation of proteins with urea and mercaptoethanol [33], exposure to low pH [34–36], or high temperature [31] were required to expose normally internal hydrophobic regions within proteins. Exposure to pH 2.5 buffers permitted 15 and 14% incorporation of purified HIV-1 gp120 [34] and bovine serum albumin (BSA) [35], respectively, into ISCOMs, whereas treatment of ovalbumin (OVA) [36] or BSA [31] at 70°C increased the incorporation level. These methods may lead to a loss of conformationally dependent B cell epitopes. An alternative strategy is to covalently attach fatty acids to soluble proteins. A number of soluble proteins including OVA, cytochrome *C*, Tamm Horsfall glycoprotein, and HIV-1 gp120 have been successfully incorporated into ISCOMs after attachment of palmitic acid through the ϵ -amino groups of lysine [37–40]. Mowat et al. [37] found that only palmitified OVA was incorporated into ISCOMs and only this formulation was capable of inducing delayed-type hypersensitivity (DTH) responses in mice when injected into the footpad. OVA, palmitified OVA, or palmitified OVA mixed with Quil A or Iscom Matrix induced no DTH effect. The addition of

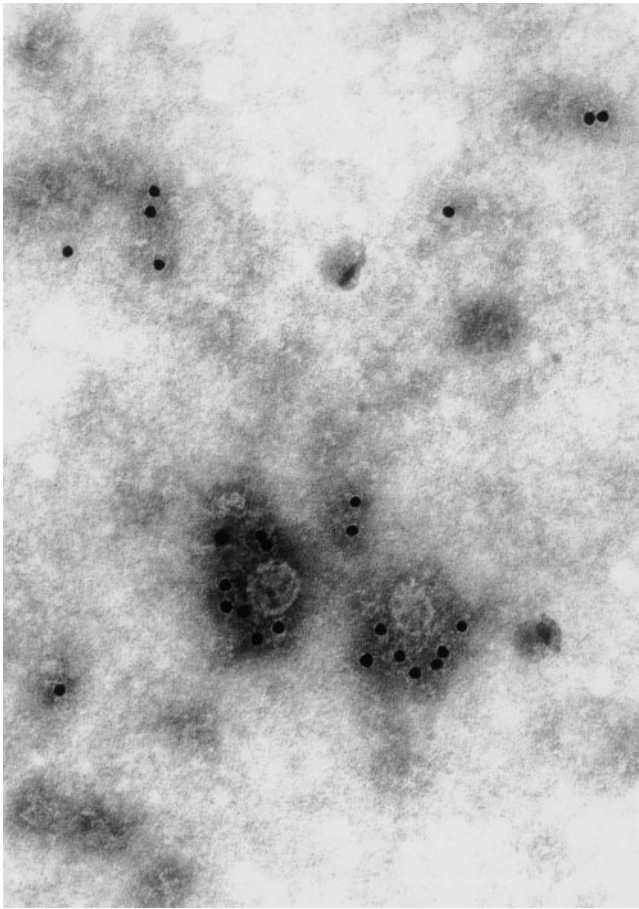


Fig. 1. Immunoelectron microscopy of *L. major* parasite surface antigen-2 incorporated into ISCOMs. The antigen was detected in ISCOMs with the monoclonal antibody 11E5 and rabbit anti-mouse conjugated to colloidal gold.

palmitic acid [41] or myristic acid [11] fatty acid tails during peptide synthesis is relatively simple and allows the incorporation of defined peptides into ISCOMs.

Other methods used to associate molecules with ISCOMs include chemically coupling peptides [42] and proteins to pre-formed Iscom Matrix or ISCOMs containing influenza envelope proteins [42–45]. Weiss et al. [46] chemically coupled (by periodate oxidation) influenza nucleoprotein to *E. coli* lipopolysaccharide (LPS), which allowed association of 10–20% of the nucleoprotein with ISCOMs. The problem of incorporating non-amphipathic molecules into ISCOMs has been approached by utilizing genetic manipulation to re-engineer the protein. In one example [47] a protein was modified to retain a transmembrane region and this protein was subsequently shown to induce higher antibody responses in cats when incorporated into ISCOMs than did the native protein given alone or when combined with Quil A.

HOW DO ISCOMS DIFFER FROM OTHER PARTICULATE DELIVERY SYSTEMS?

Table 1 compares some properties of three particulate vaccine delivery systems, ISCOMs, liposomes, and microparticles. The major difference between these systems is the role of saponin,

which is essential to the formation and action of ISCOMs, but can be optionally used in the other systems. For example, Lipford et al. [48] examined the addition of Quil A to OVA liposomes in an attempt to enhance the immune response. The resulting Quil A-containing liposomes were effective at inducing an MHC Class I restricted CD8⁺ CTL response. Liposomes without Quil A failed to generate any CTL, whereas simply mixing empty liposomes, peptide, and Quil A gave a detectable but lower CTL response [48]. The authors claim that the advantage of this system, compared with ISCOMs, was that the need to add lipid tails to the peptides was removed. This system has also been used with a lysteriolysin peptide from *Listeria monocytogenes*. When formulated in Quil A liposomes specific CTL responses were induced and mice challenged with 10 LD₅₀ of live *L. monocytogenes* organisms showed increased survival [49].

Microspheres have been used to enhance or prolong immune responses and can also benefit by the addition of adjuvants such as saponins. In studies by Cleland et al. [50], recombinant gp120 (HIV-1_{MN} strain) was microencapsulated into polylactide (co-glycolide) microspheres and gave long-lasting antibody responses in guinea pigs after two subcutaneous doses. This response was enhanced fivefold by the addition of 50 µg of the purified saponin QS-21 [19, 23, 51]. These studies have been extended to baboons where high antibody titers and virus neutralization titers were induced using microspheres containing QS-21 [52].

TOXICITY OF SAPONINS, ISCOMS, AND ISCOM MATRIX

The issue of toxicity with saponin-based adjuvants has been raised at various times as a potential impediment to their widespread use in human and veterinary vaccines. In mice and rats, crude saponins appear to only effect weight gain when administered orally [53, 54] but are toxic when administered parenterally. The LD₅₀ for intravenously administered Quil A is 0.67 mg/kg in rats [55] and doses of greater than 25 µg of Quil A given intraperitoneally can be toxic in the B₆D₂F₁ strain of mice [56]. Lethality in other strains of mice has also been reported with intraperitoneal or subcutaneous doses of ISCOMs containing from 10 to 50 µg of Quil A [7, 34, 57]. When examined at autopsy, mice showed liver degeneration [7]. Despite these findings with Quil A and Quil A-ISCOMs in rodents, there has been little toxicity observed with parenterally administered Quil A containing ISCOMs or QS-21 in larger animals such as chickens, cats, rhesus monkeys, dogs, sheep, cattle, or horses

TABLE 1. Comparison of Three Particulate Vaccine Delivery Systems

Formulation	Size (nm) ^a	Composition		Antigen location
		Saponin	Charge ^b	
ISCOMs	30–80	Critical	–	Surface
Liposomes	100–1000	Optional	–, 0, +	Mainly internal
Microspheres	>1000	Optional	0	Mainly internal

^a Normal size range. ^b Charge is designated as: –, negative; 0, neutral; +, positive.

[34, 58–62] or with free Quil A in sheep and cattle [63, 64], although there was some toxicity reported with free Quil A in cats [19]. This is presumably because the doses given to larger animals in milligrams per kilogram are well below those observed to be toxic in rodents. Local toxicity was investigated in rats after the injection of a measles-ISCOM vaccine into the gastrocnemius muscle. Histopathological examination showed a lower inflammatory response to the vaccine when the Quil A saponins were incorporated into ISCOMs compared to free Quil A [65].

In an attempt to reduce toxicity, Kensil et al. [23] fractionated Quil A and tested the major peaks for toxicity and adjuvant activity. Lethality of the saponin fractions was assessed in CD-1 mice given graded doses of up to 500 µg of saponin intradermally. This showed that QS-18 was relatively toxic (even more so than whole Quil A), whereas QS-21 had low toxicity and QS-7 showed no lethality at the doses used (lethality of QS-17 was not stated [23]). Other fractions were identified that were toxic for mice (QA-19a) but appeared to be well tolerated in kittens [19]. Increasing hemolytic activity was seen with fractions QS-17, QS-21, and QS-18, respectively, but QS-7 showed no hemolysis at levels up to 200 µg/mL of saponin [23]. The authors' conclusion was that there was no correlation between hemolytic activity, lethality, and adjuvant activity. No specific IgE was induced with any of these four fractions in contrast to other reports using Quil A [66], *Gypsophila* saponins [67], or saponins from un-named sources [68, 69] all of which have shown increased levels of IgE after the addition of saponin to vaccines.

Using the purified saponin fractions QH-A, QH-B, and QH-C, Rönnerberg et al. [26] found that Iscom Matrix containing various ratios of the three fractions, and even ISCOMs made from Quil A, did not cause significant hemolysis of chicken red blood cells (CRBC) *in vitro* at levels up to 100 µg/mL. Extensive hemolysis was obtained when free Quil A or QH-B and QH-C were used without being incorporated into ISCOMs or Iscom Matrix. QH-A was approximately 10-fold less hemolytic in its free form than QH-B or QH-C. Lethality studies in ICR mice showed no toxicity at the tested levels with QH-A (0/10 deaths at 400 µg), QH-C (0/10 deaths at 400 µg), or Iscom Matrix made from QH-C (0/10 deaths at 800 µg) but there was toxicity associated with QH-B (7/10 deaths at 400 µg) [26]. It should be noted that lethal doses of purified saponins can vary depending on the mouse strain used with inbred strains of mice in general being more sensitive than outbred mice [L. Beezum, personal communication].

Some of the purified saponin fractions like QS-21, ISCOPREP[®]703, and formulations that utilize these saponins (Iscom Matrix and influenza-ISCOMs) have been tested for systemic and local toxicity in rats and rabbits as well as mutagenicity and pyrogenicity as part of their pre-clinical evaluation. In rabbits 200 µg of QS-21 given intramuscularly was well tolerated with little evidence of toxicity [70]. Similar results were obtained in rats and rabbits with ISCOPREP[®]703 in free form or in Iscom Matrix and influenza-ISCOMs [4]. The data generated from these studies provided the confidence for institutional ethic committees to approve safety and efficacy trials in human volunteers.

UPTAKE AND PRESENTATION OF ISCOMS

One important property of adjuvants is their ability to act on antigen-presenting cells (APC). Such activities include increased targeting, uptake, and presentation of antigen, expression of costimulatory molecules, and production of cytokines. Although the mechanisms by which saponins or ISCOMs promote and modulate immune responses are not clearly understood, it is likely that they do interact with APC to induce many of these responses.

ISCOMs have been demonstrated to increase the MHC class II expression on APC, an effect that is likely to result in enhanced presentation of antigens. Mice injected intraperitoneally with influenza-ISCOMs had a significantly greater proportion of Ia-positive peritoneal cells than mice injected with influenza antigen or Iscom Matrix [71]. Bergström-Mollaoglu et al. [72] showed that the frequency of cells expressing MHC class II molecules increased after antigen stimulation *in vitro* of spleen cells primed by influenza-ISCOMs or HIV gp160-ISCOMs. The increase was antigen dose-specific and at least partly dependent on interferon-γ (IFN-γ).

Secretion of cytokines typically produced by activated APC has been demonstrated after both *in vivo* and *in vitro* treatment with ISCOMs. Murine spleen cells produced both soluble and membrane-bound IL-1 after stimulation *in vitro* with influenza-ISCOMs [73]. Cell depletion experiments showed that production of soluble IL-1 was dependent on the presence of adherent cells. ISCOMs containing purified *Quillaja* fractions also induce cytokine secretion *in vitro*. Influenza-ISCOMs prepared with ISCOPREP[®]703 stimulated peritoneal cells to produce IL-6 and induced levels of soluble IL-1 comparable to those obtained with *E. coli* lipopolysaccharide, a potent IL-1 inducer [74, 75]. Immunization of mice with influenza-ISCOMs resulted in increased serum levels of IL-6 and IL-12 [75, 76]. Evidence that antigen in ISCOMs is presented to T cells by APC was reported in a study by Villacres-Eriksson [77]. Naive splenic dendritic cells, B cells, and peritoneal cells were pulsed *in vitro* with influenza-ISCOMs and used to stimulate T cells from mice immunized with the same ISCOM preparation. All populations of APC stimulated the T cells to proliferate and produce IL-2. Dendritic cells were the most efficient cell population in inducing IFN-γ secretion.

Together, these results suggest that ISCOMs are efficiently taken up and presented by APC and induce or enhance a number of functional properties. Indeed, direct adherence and uptake of ISCOMs by APC has been demonstrated. Using electron microscopy, Watson et al. [71] observed influenza-ISCOMs adhered to macrophage cell membranes or contained within phagolysosomes only 30 s after intraperitoneal injection. In a related study, ISCOMs containing rabies virus antigen were labeled with lipophilic fluorescent carbocyanine and their cellular distribution examined after intraperitoneal or intravenous injection [78]. Both rabies antigen and rabies-ISCOMs localized to marginal zone macrophages but the ISCOMs preferentially located to marginal metallophilic macrophages. The authors suggested that the difference in cellular localization may explain why rabies-ISCOMs are able to induce good immune responses at low doses.

In addition to the effect on antigen presentation and APC function, incorporation into ISCOMs may increase the targeting of antigens to lymphoid organs. Intraperitoneal injection of radioactively labeled influenza-ISCOMs resulted in a significantly higher recovery of radioactivity in the spleen compared to injection of influenza antigen alone [79]. After subcutaneous injection, labeled influenza-ISCOMs were more efficiently targeted to the draining lymph nodes than influenza antigen [80]. In agreement with the latter observation, both T and B cell responses were first detected in the draining lymph nodes after subcutaneous immunization with influenza-ISCOMs, whereas the responses in the spleen developed later [81, 82].

MODULATION OF T CELL RESPONSES BY ISCOMS

The main use of adjuvants has been to improve the magnitude of antibody responses. Lately, the ability of adjuvants to alter the nature of immune responses has received considerable attention. For example, the influence of adjuvants on the pattern of cytokines produced by T helper (Th) cells is of great interest because of the importance of different Th cell subsets for generation of protective immunity against certain infectious diseases [83–85]. Several studies in murine models have addressed this issue with regard to ISCOMs.

The potential of ISCOMs to activate murine Th cells to secrete Th1-like cytokines such as IL-2 and IFN- γ has been frequently demonstrated (summarized in **Table 2**). Production of IL-2 was first observed in a study by Fossum et al. [86] and has been demonstrated after immunization with several anti-

gens in ISCOMs, including influenza antigen [77, 80, 82, 86, 87], OVA [88, 89], HSV type 1 glycoproteins [90], and EBV gp340 [91]. Immunization with ISCOMs containing these antigens [77, 80, 82, 87–91], or antigens from the parasites *L. major* [28, 92, 93] or *Toxoplasma gondii* [A. Sjölander et al., unpublished results] have also been shown to activate T cells to secrete IFN- γ . In addition, immunization of mice with influenza-ISCOMs results in levels of IL-12 high enough to be measured in serum by enzyme-linked immunosorbent assay (ELISA) [76]. Spleen cells from mice receiving influenza-ISCOMs produced higher levels of IL-2 and IFN- γ after restimulation than cells primed with influenza antigen in Freund's complete adjuvant [82]. Administration of OVA in ISCOMs resulted in an antigen-specific up-regulation of the secretion of IL-2 and IFN- γ compared to OVA without adjuvant, or in aluminium hydroxide [89]. The results suggest that ISCOMs are potent inducers of murine Th1 cells, an assumption that is supported by the observation that ISCOMs up-regulate the production of IgG2a antibodies and efficiently activate CTL (see below).

The ability of ISCOMs to induce production of Th2-like cytokines is less clear and appears to be dependent on several variables such as experimental system, antigen, the cytokines analyzed, and the assays used to determine cytokine secretion. Immunization with OVA-ISCOMs or PSA-2-ISCOMs induced T cells producing significant amounts of IL-5 [28, 88], whereas administration of antigens in ISCOMs has been reported to both increase [89, 90, 92] and decrease the production of IL-10 [77, 91]. The results from several studies suggest that activation of IL-4-producing T cells by ISCOMs is low or absent, when assessed by measuring the cytokine secretion into cell culture supernatants after antigen stimulation *in vitro* [77, 80, 82, 88, 91, 92]. However, when the ELISPOT technique was used to analyze T cell responses to PSA-2-ISCOMs, high numbers of cells secreting IL-4 were detected despite the fact that the corresponding culture supernatants only contained trace amounts of IL-4 [28]. Moreover, OVA-ISCOMs activated T cells to produce levels of IL-4 after restimulation comparable to those from cells primed with OVA in aluminium hydroxide, an adjuvant with a very strong capacity to induce Th2-like immune responses [89]. It thus appears that ISCOMs, at least with certain antigens, have the ability to induce T cells to secrete IL-4 but that analysis of cytokine secretion in cell culture supernatants may not reflect the response *in vivo*. This difference may be explained by IL-4 being rapidly consumed in *in vitro* cultures [94, 95].

In conclusion, in most experimental murine models analyzed to date, ISCOMs have induced potent T cell responses of a Th1 type. In some cases ISCOMs have also induced a concomitant Th2 response giving rise to T cell responses with mixed Th1/Th2 properties. It should be noted that the importance of IL-4 and IFN- γ for the immunopotentiating properties of ISCOMs was questioned in a recent report using mice deficient in either IL-4 or the IFN- γ receptor [96]. After oral or parenteral immunization with OVA-ISCOMs, the resulting serum IgG and intestinal IgA antibody responses, T cell proliferation and cytokine production and CTL activity in these mice were of the same magnitude as in the corresponding wild-type mice. The significance of these results for the adjuvant properties of ISCOMs remains to be established.

TABLE 2. Cytokine Production After Immunization with ISCOMs

Antigen	Adjuvant	Detected cytokines	Reference
Influenza antigen	Quil A-ISCOMs	IL-2	[86]
	Quil A-ISCOMs	IL-2, IFN- γ	[87]
	Quil A-ISCOMs	IL-2, IL-4, IL-10, IFN- γ	[77]
	Quil A-ISCOMs, Quil A-Iscom Matrix	IL-2, IL-4, IFN- γ	[80]
	Quil A-ISCOMs	IL-2, IL-4, IFN- γ	[82]
	ISCOPEP™703-ISCOMs	IL-6	[75]
	Quil A-ISCOMs	IL-12	[76]
Ovalbumin	Quil A-ISCOMs	IL-2, IL-5, IFN- γ	[88]
	ISCOPEP™703-ISCOMs	IL-2, IL-4, IL-10, IFN- γ	[89]
	Quil A-ISCOMs	IL-5, IFN- γ	[96]
Herpes Simplex Virus type 1 glycoproteins	Quil A-ISCOMs	IL-2, IL-10, IFN- γ	[90]
	Quil A-ISCOMs	IL-2, IL-10, IFN- γ	[90]
Epstein Barr Virus gp340	Quil A-ISCOMs, ISCOPEP™703-Iscom Matrix	IL-2, IL-10, IFN- γ	[91]
<i>L. major</i> PSA-2	ISCOPEP™703-ISCOMs	IL-4, IL-5, IFN- γ	[28]
	ISCOPEP™703-ISCOMs	IL-4, IFN- γ	[93]
	ISCOPEP™703-ISCOMs	IL-4, IFN- γ	[93]
<i>L. major</i> gp63	Quil A-ISCOMs	IL-2, IL-4, IL-10, IFN- γ	[92]

One important question is which cell populations contribute to the production of cytokines after immunization with ISCOMs. Experiments with spleen cells from mice immunized with OVA-ISCOMs or influenza-ISCOMs have shown that depletion of CD4⁺ T cells before antigen stimulation *in vitro* abrogates several antigen-specific cellular activities, including proliferation and secretion of IL-2, IL-10, and IFN- γ [87–89]. Likewise, depletion of CD4⁺ T cells after antigen stimulation of spleen cells primed with PSA-2-ISCOMs removed all IL-4-secreting and 90% of the IFN- γ -secreting cells as determined by ELISPOT [28]. In contrast to these observations, no marked effects on these cellular responses were observed after a corresponding depletion of CD8⁺ T cells. This finding is surprising in view of the strong capacity of ISCOMs to induce CD8⁺ CTL. It is possible that the absence of cytokine production and proliferative responses by CD8⁺ T cells is a result of the experimental conditions used for the *in vitro* restimulation, e.g., stimulation with exogenous antigen and choice of APC. Further studies are required to clarify this issue.

Most studies of T cell responses to ISCOMs have used Quil A as a source of saponin. The recent purification of defined *Quillaia* saponin fractions [26] has raised the question whether ISCOMs prepared from these fractions retain the adjuvant activities of Quil A containing ISCOMs. Recent studies with one of these, ISCOPREP[®]703, suggest that ISCOMs retain their adjuvant properties when prepared with this defined saponin fraction. Thus, injection of OVA-ISCOMs or PSA-2-ISCOMs containing ISCOPREP[®]703 [28, 89] induced T cells that produced a pattern of cytokines similar to that observed after immunization with Quil A-ISCOMs (Table 2). In addition, immunization of mice with influenza vaccine in combination with QH-C in free form induced a serum cytokine profile with high IFN- γ , measurable IL-2, and moderate levels of IL-5 and IL-6 [97]. Furthermore, OVA formulated with QS-21 activated spleen cells that secreted IL-2, IFN- γ , IL-4, and IL-5 after antigen stimulation *in vitro* [70].

The cytokine profiles described above have all been the result of T cell responses induced by parenteral immunization (subcutaneous or intraperitoneal) but induction of cytokine-secreting T cells has also been observed after oral or intranasal administration of ISCOMs. Oral immunization with OVA-ISCOMs induced spleen cells that produced IL-2, IL-5, and IFN- γ in a pattern similar to that seen after subcutaneous injection [88]. Intranasal administration of influenza-ISCOMs generated spleen cells producing high levels of IL-5 and IFN- γ after restimulation [S. Sjölander, personal communication].

ACTIVATION OF CTL RESPONSES

A number of adjuvants have been shown to induce CTL responses under certain conditions, including oil-in-water emulsions [98], aluminium hydroxide [99], MPL-liposomes [100], and lipopeptides [101]. However, these examples are relatively infrequent compared with the numerous reports of the ability of ISCOMs and other saponin-based adjuvants to induce MHC class I restricted CD8⁺ CTL. Activation of CTL responses by ISCOMs was first reported with HIV gp160-ISCOMs and influenza-ISCOMs [102]. Other antigens that have been shown

to induce CTL responses in mice after parenteral administration in ISCOMs include OVA [37, 96, 103], measles virus F-protein [104], RSV [105], mycobacteria hsp60 [106], SIV proteins [107], and HPV E6 and E7 proteins [108]. Intranasal administration of influenza-ISCOMs or ISCOMs containing a CTL epitope of measles virus nucleoprotein linked to a fusion peptide-induced CTL responses [109, 110] as did oral immunization with OVA [96, 103, 111]. Purified *Quillaia* saponin fractions, in free form or incorporated into ISCOMs also induce CTL responses in mice. This has been reported for influenza-ISCOMs containing ISCOPREP[®]703 [14] and QS-21 formulated with OVA [112], HIV gp160 [113], HIV-1 gp160 peptides [114], recombinant human CMV glycoprotein B [115], RSV F protein [116], and ras-expressing tumor cells [117]. SIV envelope glycoprotein (Env) and p27gag protein in ISCOMs and SIV proteins formulated with QS-21 induced CTL activity in non-human primates [118, 119].

ISCOMs have also been demonstrated to sensitize target cells *in vitro* for lysis by CD8⁺ CTL. Binnendijk et al. [104] showed that ISCOMs containing the measles virus F protein sensitized target cells for lysis by CD8⁺ MHC class I-restricted CTL clones. Sensitization was dependent on cytosolic processing of the F protein to generate peptides presented by MHC class I molecules. Tarpey et al. [108] used ISCOMs containing fusion proteins of the E6 and E7 early proteins of HPV type 11 for restimulation *in vitro* of CTL elicited *in vivo* by immunization with the same ISCOMs. Restimulation was found to require low-density spleen cells (an enriched source of dendritic cells [120]) as APC. The results suggest that administration in ISCOMs facilitates the entry of antigen into the cell cytosol and that dendritic cells may play an important role as APC. Further studies are required to delineate these activities.

INDUCTION OF ANTIBODY RESPONSES BY ISCOMS

The adjuvant properties of ISCOMs were first recognized by their strong capacity to induce high antibody responses. A large number of studies have described the ability of ISCOMs to induce antibody responses to antigens of viral, bacterial, or parasite origin using various routes of administration. This information has been extensively reviewed by others [2, 3, 5, 27, 31, 121] and will not be discussed in detail here. Two observations of particular interest are the ability of ISCOMs to induce high levels of antibodies at lower doses of antigen than used with most adjuvants and the isotype pattern of such responses. Subcutaneous immunization of mice with influenza-ISCOMs have been demonstrated to induce good antibody responses at antigen doses as low as 0.01 μ g of influenza protein [14] and ISCOMs containing OVA, *L. major* PSA-2, EBV gp340, or HSV-1 antigen were highly immunogenic at doses ranging from 1 to 5 μ g of protein [28, 89, 91, 122]. It is not clear why such low amounts of these, and other antigens in ISCOMs, are able to induce potent antibody responses, but it is likely that the co-incorporation of adjuvant-active *Quillaia* saponins and antigen in the same ISCOM particle plays a crucial role, possibly by targeting antigen and adjuvant to the same APC. This is supported by the finding that immunization

with influenza-ISCOMs required 6–10 times less Quil A to induce the same magnitude of antibody responses as influenza antigen administered with Iscom Matrix [80].

Results from numerous studies in mice have demonstrated the ability of ISCOMs to induce antibodies of all IgG subclasses. In general, ISCOMs containing Quil A elicit high and comparable levels of IgG1 and IgG2a in mice and a similar isotype profile has also been observed with purified *Quillaia* saponin fractions, both in free form and administered in ISCOMs [reviewed in 51 and 123]. The induction, and in some cases up-regulation of IgG2a responses by ISCOMs is important because these antibodies are most effective at fixing complement [124] and for activation of antibody-dependent cellular toxicity [125, 126].

APPLICATIONS OF ISCOMS

Although the adjuvant properties of ISCOMs have primarily been studied in murine models, ISCOMs have been reported to induce antibody responses and/or protective immunity in a variety of other species. These include guinea pig [127, 128], turkey [129, 130], cat [47, 58], rabbit [39, 41, 43, 131], dog [132], seal [133], sheep [134–136], pig [137], cow [138, 139], and horse [140, 141]. Of particular interest with regard to human applications is the ability of ISCOMs to induce strong immune responses in monkeys. Induction of high antibody responses in monkeys has been reported after immunization with ISCOMs containing antigens from influenza virus, measles virus, Japanese encephalitis virus, HIV-1, SIV, and *Plasmodium falciparum* malaria [32, 34, 62, 118, 131, 142–145]. A rhesus rotavirus ISCOM vaccine increased the levels of IgA and IgG in milk from baboon mothers [146] and measles-ISCOMs elicited high titers of neutralizing antibodies in the presence of passively transferred measles-specific antibodies [147]. Specific proliferative T cell responses were detected in monkeys injected with influenza-ISCOMs [142] or ISCOMs containing CMV antigens [148] or HIV-2 gp125 [149]. Immunization of macaques with a combination of SIV Env ISCOMs, p27gag ISCOMs and three Nef lipopeptides activated CTL precursors against each of these proteins [118]. In addition, ISCOMs have been demonstrated to induce protective immunity in monkeys against several infectious diseases, including influenza [142], SIV [62, 144], HIV-2 [149, 150], and EBV [151].

Although there are a number of experimental and veterinary vaccines that utilize Quil A or crude saponins, only three licensed vaccines use ISCOMs or QS-21. The licensed ISCOM-based vaccine is used to protect horses from equine influenza. The vaccine has been produced by ISCOTEC AB and Mallinckrodt (UK) since 1989; over 1 million doses of the vaccine have been sold in Sweden with no adverse side effects registered [2]. QS-21 has been used in a cat vaccine to prevent feline leukemia (Quilvax-FelV) and in a dog vaccine to prevent canine lyme disease (Quilvax-L™).

Human applications to date have been limited to phase I and II clinical trials using both ISCOMs and Iscom Matrix containing ISCOPREP™703. At this stage only safety data on the local and systemic effects of Iscom Matrix in human volunteers has been published [4]. At doses of 25–200 µg of ISCOPREP™703

in Iscom Matrix some volunteers reported a mild ache/pain at the site of the intramuscular injection, the rate was similar to that reported by volunteers given a placebo (vaccine diluent) [4]. Clinical trials have also been carried out with the purified saponin QS-21 added to a melanoma GM2 ganglioside-KLH vaccine. Doses of the GM2 ganglioside-KLH given with 100 µg of QS-21 subcutaneously induced mild local tenderness and inflammation at the dose site, which lasted for 2–4 days and brief, but low-grade fever and malaise with no significant incapacitation [152]. Clinical trials of QS-21 with HIV-1, HSV, malaria, and influenza vaccines are in progress [51].

CONCLUSIONS

The understanding of the composition, structure, preparation, and functional properties of ISCOMs has made considerable progress since they were first described. The identification and purification of a large number of fractions from *Quillaia* saponin, the critical component of ISCOMs, has allowed manufacturing of defined ISCOMs with consistent composition and reduced toxicity. A combination of *Quillaia* saponin fractions (ISCOPREP™703) has successfully completed pre-clinical studies and is the basis of ISCOMs and Iscom Matrix tested in phase I and II human clinical studies. The requirement of amphipathicity in proteins to be used with ISCOMs has been reduced by the development of several techniques that facilitate the incorporation or association of hydrophilic antigens with ISCOMs. This has increased the range of applications of the ISCOM technology but further work is required to make such techniques suitable for large-scale production.

The adjuvant properties of ISCOMs have now been extensively studied in a broad range of animal models and with a large number of antigens. From these studies it can be concluded that ISCOMs efficiently promote both humoral and cell-mediated immune responses, including induction of high antibody responses, stimulation of both Th1-like and Th2-like T cells, and activation of CTL. It is important to note that ISCOMs containing ISCOPREP™703 appear to have retained the adjuvant activities of conventional Quil A-ISCOMs. The mechanisms by which ISCOMs potentiate and modulate immune responses are not completely understood but targeting to lymphoid organs, effects on APC functional activities, and the delivery of antigen and adjuvant in the same particle to the same APC most likely play key roles.

Presently, the only commercially available ISCOM-based vaccine is a veterinary vaccine against equine influenza. A critical question concerning the use of ISCOMs as a general vaccine adjuvant is whether the results in animal models such as the efficient induction of CTL responses can be reproduced in humans. The potential for ISCOMs to induce significant and sustained levels of CTL activity in humans may be of particular importance to viral vaccines and for the immunotherapy of cancer. Ongoing and future human clinical trials should give results over the next few years which will clarify these issues and determine the potential of ISCOMs for human applications.

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