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Immunostimulating Complexes
Clinical Potential in Vaccine Development

Bror Morein, Karin Lövgren, Bengt Rönnberg, Anders Sjölander and *María Villacrés-Eriksson*

Swedish University of Agricultural Sciences, Faculty of Veterinary Medicine, and National Veterinary Institute, Department of Virology, Biomedical Centre, Uppsala, Sweden

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Summary

An immunostimulating complex (iscom) is a particle containing several copies of an antigen, with a built-in adjuvant. It is constructed to provide a physically optimal presentation of antigen to the immune system. An iscom particle without incorporated antigen is called the iscom matrix, or just matrix, and can be used as a conventional adjuvant that is added to the antigen whose immunogenicity is to be reinforced.

The unique components of the iscom matrix are saponins (triterpenoids) from the tree *Quillaja saponaria*, which exhibit a unique affinity for cholesterol and thereby facilitate the stability of the complex. The triterpenoids can be used as a crude preparation of *Quillaja* saponins or as purified preparations of *Quillaja* triterpenoids. The various triterpenoids have different characteristics, of which some are relevant to vaccine development such as the iscom-forming capacity, the immunomodulatory capacity, a low cell lytic property and low toxicity in general. Consequently, various compositions of triterpenoids, including efficient nontoxic adjuvant formulations or inert carrier formulations, can be made.

The currently used iscom vaccine and experimental vaccines induce a broad immune response, including major histocompatibility complex (MHC) class I

and II T cell responses. The MHC class II response encompasses a prominent response of T helper 1 (T_H1)-like cells, producing interleukin (IL)-2 and interferon-γ and favouring cell-mediated immunity. A T_H2-like response may also be evoked, with cells producing IL-4 and IL-10 and promoting humoral immunity. However, the same influenza virus envelope antigen in a micellar nonadjuvanted form induces a more prominent T_H2 type of response, with cells producing more IL-10.

The iscom particle is also an interesting nonreplicating candidate for induction of mucosal immunity. Iscoms containing different kinds of antigens in various experimental vaccines evoke secretory IgA or cytotoxic T cell responses when administered orally and intranasally. Experimental iscom vaccine formulations have been shown to induce protective immunity to a number of micro-organisms, including viruses and retroviruses, parasites and bacteria, in several species, including primates.

To date, the only adjuvants registered for human use are the aluminium salts, aluminium hydroxide and aluminium phosphate. However, the aluminium salts are effective only for certain antigens and they are not free of adverse effects, since they not infrequently cause local reactions. In contrast, a number of additional adjuvants are used in animals, including various oil emulsions and saponins.

Classically, the effect of an adjuvant has been measured by the increase of the antibody response it stimulates and, to a limited extent, by its capacity to increase a cell-mediated immune response. In a modern view of adjuvants, the concept of immunomodulation has to be included which, in addition to the magnitude of immune response elicited, also encompasses qualitative aspects. These qualitative aspects include:

- the induction of specific antibodies of the desired isotype and IgG subclass
- specific T helper cell responses, as classified by cytokine production and cytotoxic T cell (CTL) response
- the distribution of the immune response to various lymphatic sites, e.g. mucosal surfaces.

Any of these factors may be important to obtain protective immunity, which is the ultimate goal for a vaccine, since one pathogen is different from another and consequently the profile of protective immunity will differ. With such a perspective in mind, it is obvious that there will be no single adjuvant or adjuvant formulation that will cover the diverse needs of immunopotentiation that different vaccines require.

There are certain criteria that vaccines should fulfil for efficient induction of the immune response. Most important is the physical presentation of antigens. In a vaccine the antigens should be presented as several copies in a particle, i.e. in a multimeric form.[1] If a multimeric presentation is not sufficient to convert an antigen into an efficient immunogen in a vaccine, the prospective vaccine has to be supplemented with a suitable adjuvant or adjuvant formulation. If the physical presentation form is combined with an adjuvant-active component, the term adjuvant formulation is used, according to the nomenclature suggested by Allison.[2]

As mentioned above, the aluminium salts are the only adjuvants registered for human use. Other effective adjuvants and adjuvant formulations have been considered unsafe, as judged by the systemic or local reactions they cause. Since adjuvants are often hydrophobic or amphipathic, e.g. saponins, avridine and oil-based formulations,[3] they may interact with cell membranes, thereby causing local adverse effects. Furthermore, adjuvants induce production of various kinds of cytokines, which in high concentrations give rise to toxic reactions.[4] It can, therefore, appear to be an enigma to find an effective adjuvant that is not toxic. This is the rea-

son why effective adjuvants are still not considered safe for registration for human use.

1. Immunostimulating Complexes (Iscoms)

The iscom is formulated as a particle that combines a multimeric presentation of antigen with a built-in adjuvant. Consequently, it can be placed within the category of particulate adjuvant formulations. The iscom particle, first described by Morein et al.,[5] is a cage-like structure of about 40nm (fig. 1).[6,7] It is composed of the saponin adjuvant Quil A, cholesterol, antigen and phospholipids.[8] The iscom with its incorporated antigens is a very stable structure that remains intact after lyophilisation, limited freeze-thawing and prolonged storage at +4°C. The storage limitations are generally determined by the stability of the antigen. In pharmaceutical terms, the iscom can be considered as a carrier for a combination of antigen and adjuvant, aiming at targeting the construct to antigen-presenting cells and the lymphatic system.

1.1 *Quillaja* Saponins: the Adjuvant Component

The backbone or matrix of the iscom is a unique complex formed between certain *Quillaja* saponins and cholesterol. The adjuvant properties of saponins were first described by Ramon,[9] and to a limited extent saponins were used as adjuvants in commercial foot and mouth disease vaccines.[10] During the 1970s, Dalsgaard[11] demonstrated that the adjuvant activity of saponins derived from the South American tree *Quillaja saponaria* Molina is superior to that of other saponins. They isolated a 'homogenous' fraction with a consistent high adjuvant activity, which they denoted Quil A.[11,12] The action of saponins on biological membranes containing cholesterol has been known since the early 1960s.[13,14] However, its practical use, by combining 2 important features for immunoenhancement by formation of an antigen-bearing structure with built-in adjuvant activity, was not discovered until 1984.[5] With the increasing interest in iscoms and Quil A, we and others[15,16] have

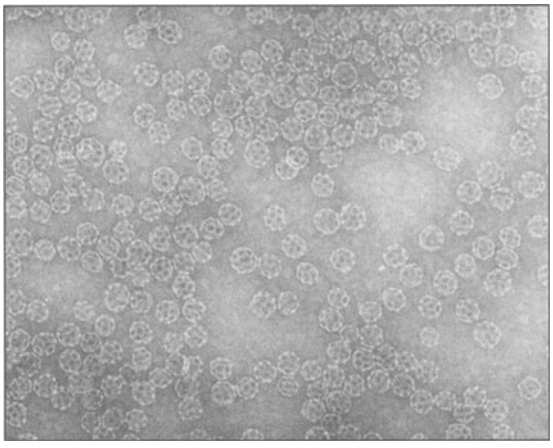

Fig. 1. Negative staining electron micrograph of immunostimulating complexes (iscoms) containing influenza virus envelope antigens (magnification × 90 000).

carried out separation of *Quillaja* saponins and the subsequent characterisation of important and interesting molecules for iscom technology.

The first saponin preparation used for construction of iscoms was the semipurified product Quil A.[12] In Quil A there are a number of related triterpenoids, with small molecular structure differences but as shown later with a number of physical and biological differences. Kensil et al.[15] and Kersten et al.[16] have published procedures to isolate such triterpenoids based on reversed phase high performance liquid chromatography (HPLC). These studies reveal the complexity in composition of *Quillaja* extracts with regard to toxicity and adjuvant activity.

Three groups of *Quillaja* components characterised by reversed phase HPLC (fig. 2) have been identified to be of particular interest for iscoms. These groups of components, named QH-A, QH-B and QH-C, have different characteristics in terms of adjuvant activity, iscom-forming capacity and toxicity.[17]

QH-A is the most complex group of components, consisting of a mixture of triterpenoids (fig. 2, left).[17] QH-A has a low or no adjuvant activity, but it can form iscoms and has a low haemolytic activity. Its cellular toxicity *in vitro* is low, as mea-

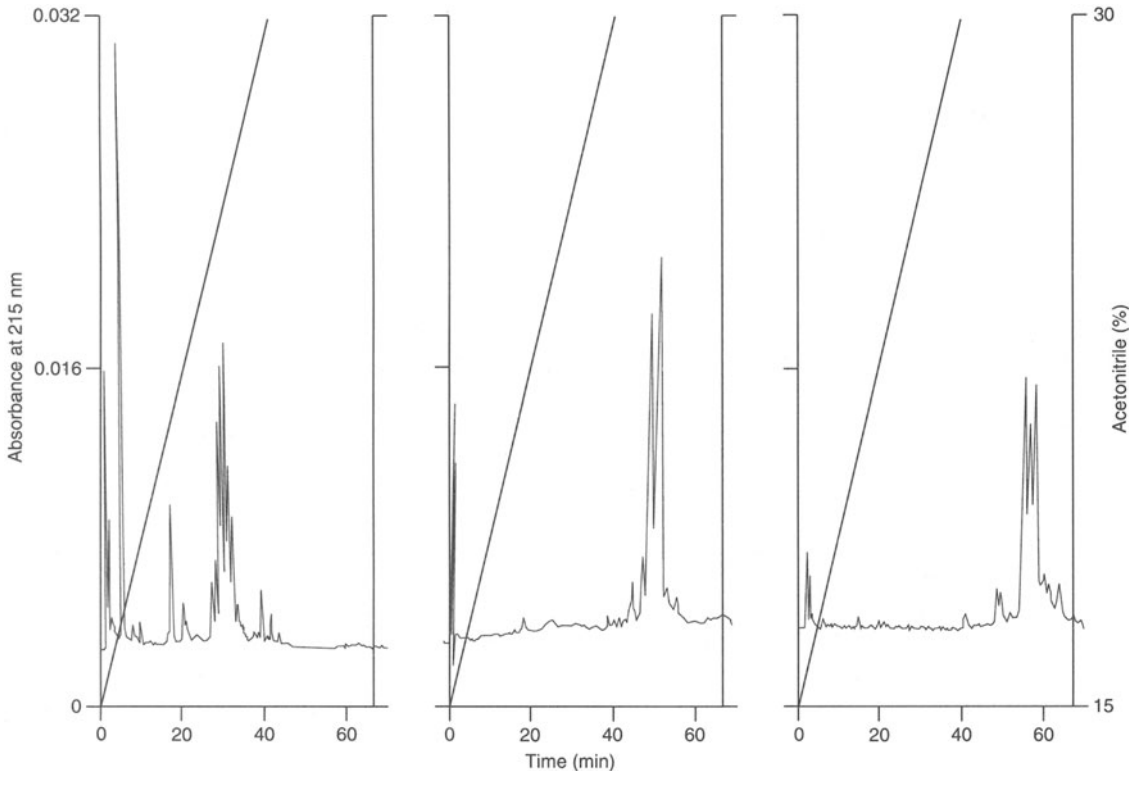

Fig. 2. High performance liquid chromatographic analysis of purified *Quillaja* saponin components QH-A (**left**), QH-B (**middle**) and QH-C (**right**). Column: CTciL-C8, 5μm particle size, 4.6mm × 15cm; mobile phase was 15 to 30% acetonitrile in 17 mmol/L sodium phosphate buffer, pH 6.6, in 40 min. A total of 80μg of purified *Quillaja* saponin was loaded per injection.

sured by inhibition of mitochondrial dehydrogenase activity in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.[17] QH-A is of particular interest as an inert carrier for various molecules, particularly for drugs where the immunoenhancing activity of the *Quillaja* components is not desired. Consequently, QH-A can be used for multimeric presentation of antigens supplemented with adjuvants other than those derived from *Quillaja saponaria* Molina.

QH-B shows a double peak on reversed phase HPLC (fig. 2, centre). It is highly adjuvant-active, but is the most toxic fraction of *Quillaja* saponins, being highly haemolytic and also cell toxic *in vitro* as measured by the MTT assay. It is the dominant adjuvant active component of *Quillaja* saponins, both as measured quantitatively and by immunological activity.

QH-C shows 2 or 3 major peaks in reversed phase HPLC (fig. 2, right). It is highly adjuvant active. It is as haemolytic as QH-B, and inhibits enzyme activity in the MTT assay at the same concentration as QH-B. However, it is substantially less toxic for mice, which survive several-fold higher doses of QH-C (800μg) than of QH-B (50 to 200μg).[17]

1.2 Structure and Construction of Iscoms

The matrix of an iscom is the particle-forming moiety into which the antigens are incorporated. It is a complex between Quil A or selected *Quillaja* components, cholesterol and a phospholipid, for

example phosphatidylcholine. Quil A or the *Quillaja* components and cholesterol are essential for matrix formation. They bind strongly to each other, forming a characteristic complex.[14,18] The phosphatidylcholine seems to facilitate the incorporation of amphipathic proteins and also facilitates particle assembly. The formation of the matrix or iscom is based on solubilisation of its components with a (preferably) non-ionic detergent; the particles are formed as the detergent is removed.

1.2.1 Membrane Antigens

The first iscoms were prepared with membrane-derived proteins from enveloped viruses. Such proteins have a hydrophobic region anchoring the protein *in situ* in the lipid membrane of the virus particle. In order to form an iscom from an enveloped virus, the membrane protein is extracted from the lipid membrane with a detergent. Simultaneously, the membrane lipids are solubilised. However, it is often beneficial to add extra cholesterol and phosphatidylcholine along with the saponin. A common composition of the mixture is 1mg of each antigen, cholesterol and phospholipid plus 5mg of *Quillaja* saponin.

During the removal of the detergent, e.g. by dialysis, ultrafiltration or centrifugation, the iscoms are formed.[19] If the membrane proteins are incompletely solubilised, they form micelles instead of being incorporated into iscoms. It is also important that the lipids are completely solubilised. Non-ionic detergents are preferred because their interaction with the antigen is limited to the hydrophobic part; consequently, they cause less denaturation of the antigen than do ionic detergents.

1.2.2 Nonmembrane Antigens

Essentially nonhydrophobic antigens, e.g. several recombinant DNA products containing hydrophobic amino acid sequences, can be forced by mild partial denaturation to expose hydrophobic domains hidden in the tertiary structure. This can be achieved by exposure of the protein to low pH[20,21] or to chaotropic agents such as urea.[22] The principle is to mix antigen, detergent and iscom constituents (lipid and *Quillaja* saponins) under conditions that expose the hydrophobic domains of the antigen. Removal of the solubilising agent (e.g. detergent) should be started at conditions where the antigen exposes hydrophobic domains ('hydrophobic conditions') so that the antigen is incorporated into the iscom. The buffer can then be changed to more physiological conditions.

1.2.3 Hydrophilic Antigens

Hydrophilic antigens without hydrophobic sequence cannot be incorporated into iscoms by the methods described in sections 1.2.1 and 1.2.2, which rely on hydrophobic interactions. Instead these antigens have to be chemically linked to preformed iscoms[23] or to plain matrix. Synthetic oligopeptides that require a protein carrier can be conjugated to preformed iscoms,[24,25] whereas larger antigens, including T cell epitopes, can be conjugated to matrix.[26,27] The matrix used for conjugation can be specially designed for efficient covalent conjugation by replacing the phosphatidylcholine with an equal amount of phosphatidylethanolamine,[8] which contains an amino group available for covalent linking.

An alternative to covalent coupling of the antigen to preformed matrix is to make the antigen hydrophobic by linkage of a hydrophobic 'tail', for example a fatty acid, to the protein before incorporation into the iscom.[28-30] Oligopeptides can be synthesised with such a hydrophobic tail, enabling their incorporation into iscoms.[31] Some care must be taken not to make short poorly charged peptides too hydrophobic, since such constructs require, in addition to detergent, large amounts of lipid for solubilisation and consequently also consume large amounts of Quil A for incorporation into iscoms, which may lead to unnecessarily toxic preparations. The toxicity can be avoided or drastically reduced by the use of defined *Quillaja* components.

1.2.4 Principles for Construction

There are several parameters to consider when constructing iscoms, since the components building the iscom particles are also the immunologically active ingredients, i.e. antigen and adjuvant. If the antigen requires more lipid for successful incorporation into iscom, the amount of Quil A

must also be increased to match. Some antigens, such as the major outer membrane protein (PI) of *Neisseria gonorrhoea*, require large amounts of lipid for solubilisation and incorporation into iscoms[16] compared with the membrane glycoprotein of influenza virus.[5] A dose of 2.5μg of PI in iscom form contains almost 50μg of Quil A; consequently, such a disproportion may cause toxicity problems. As described in section 1.2.3, these toxicity problems may be overcome by the use of defined *Quillaja* components.[17]

Free forms of *Quillaja* saponins and *Quillaja* components have immunoenhancing capacities comparable with that of matrix. The limiting factor for their use in vaccines has been the local reactions they cause, possibly a reflection of their lytic and cell toxic activities.[17] For instance, it is known from animal studies that the dosage of Quil A in free form is limited by the local reactions it causes. In a recent study, Rönnberg et al.[17] showed that 100 to 200μg of QH-C in the free form causes local reactions at the site of subcutaneous injection in the strain of mice used, whereas QH-C incorporated into matrix and given subcutaneously did not cause local reactions at doses up to 1.6mg (see section 5). It was also shown in cellular systems *in vitro* that by incorporation of QH-B and QH-C into matrix, 10-fold higher doses could be given compared with the free form, i.e. 100 mg/L, before haemolytic activity or cell-toxic activity measured by the MTT test could be observed.

2. Interactions of Iscoms with Cells of the Immune System

2.1 Macrophages as Antigen-Presenting Cells

It is well recognised that antigen-presenting cells stimulate immune competent cells to immune responses by 2 different pathways, involving major histocompatibility complex (MHC) class I or class II molecules, respectively. Differences in handling of the antigen between the 2 pathways are found in the uptake of antigen, its intracellular transport, proteolytic processing and finally in the presentation of processed antigen fragments by MHC class I molecules, leading to a CD8+ T cell response, or by MHC class II molecules, leading to a CD4+ T cell response.

The intracellular transport of antigen decides whether immune responses of MHC class I or MHC class II, or both, will be evoked. In our laboratory we have followed the intracellular distribution of iscom-borne influenza virus antigens in macrophages. By electron microscopy, iscoms containing influenza virus envelope proteins can be seen in vesicles, most likely endosomes and lysosomes, whereas the same antigens in the micelle form cannot be detected. The micelles appear to disintegrate before or soon after uptake by the cell, whereas the iscoms are intact in the cell for a comparatively long period of time.[32] Inside the cell, iscoms seem to adhere to membranes. They will most likely integrate into the lipid membrane and expose antigens to both sides of vesicular membranes, i.e. to the inside of intracellular vesicles but also to the cytosol. In this way antigen is exposed to cytosolic enzymes.

In a morphometric study, 60% of biotin-labelled antigen was found in the cytosol, whereas 40% was found in vesicles or associated with membranes. These results were confirmed in a quantitative enzyme-linked immunoabsorbent assay (ELISA) of cell fractions obtained after differential centrifugation of disintegrated cells.[33] These results partly explain the capacity of iscoms to induce both MHC class I and II responses,[34] since the key to induction of the MHC class I type of immune response is to place the antigen in the cytosol, whereas the MHC class II response requires antigen to be transported via the endosomal pathway to the lysosomes.

However, van Binnendijk et al.[35] provided evidence for a nonclassical, probably cytosolic, route outside the endosomal pathway for induction of MHC class II restricted T cell responses by iscoms. They showed that chloroquine-treated antigen-presenting cells pulsed *in vitro* with iscoms containing the F-protein of measles virus stimulated MHC class II restricted T cells, in contrast to antigen

injected in the form of ultraviolet-killed virus. Chloroquine treatment of cells increases their intralysosomal pH, thereby abrogating their enzymatic activity which is dependent on acid conditions. This results in inhibition of endosomal antigen processing. This alternative route makes it possible for cytosolic enzymes to digest the antigen, possibly creating epitopes for MHC class II presentation different from those created in the lysosomes.

There are several factors (cytokines) secreted by phagocytic mononuclear cells that are important for the initiation of an immune response. The capacity of influenza virus iscoms to induce cytokine production by antigen-presenting cells has been studied in mouse cells. In general, it can be concluded that iscom matrix or influenza virus iscoms stimulate peritoneal or spleen macrophages to produce high levels of both free and cell-bound IL-1, whereas influenza virus micelles only induce production of cell-bound IL-1.[36] Granulocyte-macrophage colony-stimulating factor (GM-CSF) was detected at low levels following stimulation with iscoms, but not with micelles. Monoclonal antibodies neutralising cytokine activity were used to show that iscoms induced macrophages to produce GM-CSF and tumour necrosis factor-α (TNFα) in addition to IL-1 and IL-6.[33] The IL-6 was produced at high levels regardless of whether the macrophage cultures were pulsed with iscoms or micelles, suggesting that nonantigen iscom components do not enhance the secretion of IL-6. None of these cytokines were detected in supernatants from antigen-pulsed unfractionated splenocytes, dendritic cells or B cells.[33]

Antigen initiates the T helper cell response and consequently also the B cell response via the MHC class II pathway. The upregulation of MHC class II expression on antigen-presenting cells is probably an important factor of the immune enhancing activity of iscoms. This was indicated by the demonstration that iscom-borne antigens enhance MHC class II expression on monocytes.[32] Using primed spleen cells, Bergström-Mollaoglu et al.[37] further showed that iscom-borne antigen efficiently enhances MHC class II expression on spleen cells in an IFNγ-dependent way.

2.2 Naive B Cells and Dendritic Cells as Antigen-Presenting Cells

The capacity of B cells from nonprimed mice to function as antigen-presenting cells has been discussed extensively. Models used for studies *in vitro* have generally employed virus-transformed B cells[38] and cloned B lymphomas.[39,40] *In vivo*, B cells are important in the priming of lymphatic T cells.[41] Sanders et al.[42] showed that naive B cells require about 10-fold more antigen than memory B cells to elicit the formation of antigen-specific B cell/T cell conjugates. Our data indicate that naive B cells internalise viral protein antigen in iscoms, process it and present it to primed T cells. These respond with proliferation and secretion of cytokines as IL-2 and, to a lesser extent, IFNγ (table I).

Table I. Concentration of cell-associated antigen in B cells and dendritic cells pulsed with 100ng of immunostimulating complexes (iscoms) or micelles, and their capacity to elicit T cell proliferation and cytokine response in primed T lymphocytes (from Villacrés-Eriksson[33])

Antigen-presenting cell	Antigen formulation	Cell-associated antigen (ng)[a]	T cell responses		
			proliferation (counts/min)[b,c]	interleukin-2 (ng/L)[b,d]	interferon-γ (ng/L)[b,d]
B cells	Iscoms	13.3	24 105	1153	7 609
	Micelles	0.2	15 852	305	3 667
Dendritic cells	Iscoms	16.8	32 104	873	22 831
	Micelles	0.6	18 241	404	2 514

a Cells were pulsed with biotinylated antigen for 1 hour at 37°C.

b Cells were pulsed with antigen for 1 hour at 37°C.

c Maximal values, scored at day 5 of incubation.

d Maximal values, at different time points.

The capacity of dendritic cells to present antigen has been documented both *in vivo* and *in vitro*.[43-45] The mechanism of antigen uptake is unclear, since early reports stated that dendritic cells are, at best, poorly phagocytic.[46] Nevertheless, recent studies indicate that dendritic cells are as active in endocytosis as other cells, but their degradative capacity may be weaker than that of macrophages.[47]

In order to study the capacity of dendritic and B cells to take up iscom-borne antigen, pulse experiments with dendritic cells and B cells with biotinylated influenza antigen in iscoms or as micelles were carried out. Subsequent quantitative detection of cell-associated antigen by immunoassay demonstrated that these cells take up both iscom- and micelle-borne antigen. The amount of antigen taken up by dendritic and B cells, even the low amount taken up after exposure to micelles (table I), is enough to elicit recall proliferative responses and cytokine secretion. As reported previously,[48] dendritic cells are superior to B cells and monocytic cells in eliciting the secretion of IFNγ in secondary responses (table I).

2.3 Activation of Unprimed and Primed Spleen Cells

2.3.1 Unprimed T Cells

It is an interesting result that antigen-presenting cells, including macrophages, dendritic cells and B cells, pulsed with iscoms carrying influenza virus antigen stimulate spleen cells from unprimed mice to proliferate *in vitro*, whereas the same antigen in micelles fails to do this. The proliferative response mediated by iscom-pulsed dendritic cells was enhanced by addition of cell culture fluid from peritoneal macrophages stimulated with iscoms, providing evidence for the synergistic effects of these 2 antigen-presenting cells. This enhancing effect was probably due to cytokines such as IL-1, IL-6, GM-CSF and TNFα, since neutralising monoclonal antibodies to these cytokines partially inhibited the enhancing effect on proliferation (fig. 3). Further work is in progress to determine whether the evoked proliferation is a true primary response *in vitro* or an oligoclonal proliferative response. The fact that iscoms devoid of antigen, i.e. matrix only, did not stimulate a proliferative response *in vitro* points to a primary antigen-dependent stimulation.

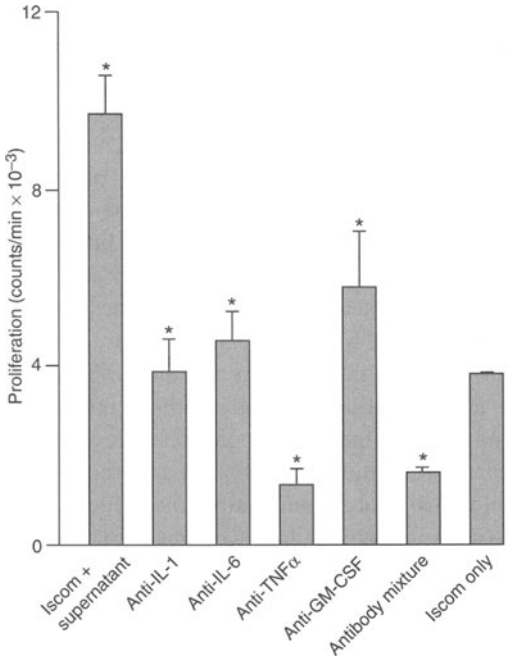

Fig. 3. Supernatants from peritoneal macrophages stimulated with immunostimulating complexes (iscoms) contain factors that enhance the proliferation of cultures containing iscom-pulsed dendritic cells and naive T lymphocytes as measured by [^{3}H]thymidine uptake. Naive T cells were cocultivated with iscom-pulsed dendritic cells alone (right-hand bar) or with iscom-pulsed dendritic cells plus 24-hour culture fluid from peritoneal macrophages previously stimulated with iscoms (left-hand bar) plus antibodies against interleukin (IL)-1, IL-6, tumour necrosis factor-α (TNFα), granulocyte-macrophage colony-stimulating factor (GM-CSF) or a mixture of all the antibodies. *Symbol:* * = $p < 0.05$.

2.3.2 Primed T Cells

Immunisation with iscoms stimulates the development of memory T cells. As expected, the proliferation of primed T cell cultures stimulated with unfractionated splenocytes, dendritic cells or B cells pulsed with iscoms or micelles was higher than that of their naive counterparts. Generally, restimulation with iscoms resulted in higher proliferation than restimulation with micelles.[33]

The supernatants of primed T cell cultures stimulated *in vitro* with iscoms contain IL-2 and IFNγ.[49,50] Overall, cultures stimulated with iscoms contain more IL-2 and IFNγ than those stimulated with micelles. On the other hand, micelles are more efficient in priming T helper memory cells and stimulating them to produce IL-10 *in vitro*.[51] Furthermore, micelles induce IL-4–producing T helper cells as efficiently as do iscoms. B cells, dendritic cells and unfractionated splenocytes pulsed with iscoms mediate more secretion of IL-2 in spleen cell cultures from primed mice than do peritoneal cells. Dendritic cells were superior to other antigen-presenting cells in mediating secretion of IFNγ, provided the cells were pulsed with iscoms (table I). This observation is in agreement with previous studies indicating that dendritic cells are important for the presentation of antigen to cells able to produce IFNγ.[48]

In recent experiments, the adjuvant activity *in vivo* of the QH-C component (see section 1.1) in free form was compared with that of oil adjuvants, muramyl tripeptide and lipid A.[52] The high capacity of the QH-C component to induce T_H1-type cytokines, i.e. IL-2, IFNγ and GM-CSF, *in vivo* was striking. The results clearly indicate that QH-C has similar immunomodulating properties to the crude *Quillaja* saponins generally used in iscoms, and suggest that immunologically efficient iscoms can be produced with a relatively nontoxic purified *Quillaja* component.

3. Immunological Responses to Iscoms

3.1 Kinetics and Organ Distribution

There is surprisingly little information available on the influence of antigen, adjuvant and delivery systems on the kinetics and distribution of immune responses, in view of the importance these factors may have for a protective immune response. To address this question, studies were recently initiated in our laboratory to: (a) analyse the distribution of influenza virus antigen in iscoms and in protein micelles after parenteral immunisation; and (b) study the kinetics and organ distribution of the resulting antibody and T cell responses.

Influenza virus protein in iscoms was rapidly distributed from the subcutaneous injection site throughout the body (A. Sjölander et al., unpublished work). Thus, the immunopotentiating capacity of iscoms is not dependent on the depot mechanism described for aluminium hydroxide and oil emulsion adjuvants. After intraperitoneal administration, influenza virus iscoms are rapidly transported from the site of injection to the spleen.[53] Compared with micelles, higher amounts of influenza virus protein in iscoms are detected in the spleen for longer periods of time following intraperitoneal injection.[53]

We have studied antibody and T cell responses in lymph nodes and spleens taken at different time points from mice immunised subcutaneously with influenza virus iscoms. The T cell response in draining lymph nodes was characterised by a rapid and transient expansion of responding cells, as measured by cell proliferation (A. Sjölander et al., unpublished work). The response in the spleen developed more slowly than in the lymph nodes, but was of long duration, more than 3 months. Primed spleen cells produced high levels of IL-2 and IFNγ after restimulation *in vitro*, whereas the production of IL-4 was approximately 100-fold lower (fig. 4). After a booster injection, there was a rapid expansion of responding cells in the draining lymph nodes. IL-2 production by both lymph node and spleen cells taken after the booster and restimulated *in vitro* decreased as compared with that of cells taken before the booster, whereas the production of IL-4 and IFNγ was enhanced.

The primary antibody response to influenza virus iscoms, recorded as the number of antigen-specific antibody-secreting cells using the enzyme-linked immunospot (ELISPOT) technique,[54] was primarily localised to the draining lymph nodes and reached a peak around day 7 after immunisation (A. Sjölander et al., unpublished work). The serum levels of IgG reacting with influenza virus protein in ELISA increased during the first weeks after immunisation and remained on a plateau level

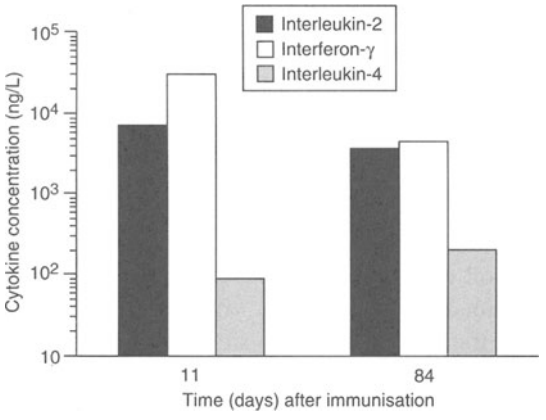

Fig. 4. Secretion of interleukin-2, interleukin-4 and interferon-γ by spleen cells from mice immunised with immunostimulating complexes (iscoms) containing influenza virus antigens. Mice were immunised subcutaneously with 3μg of influenza virus iscoms. After 11 or 84 days, spleen cells (2.5×10^5/well) were restimulated *in vitro* with influenza virus protein 1 mg/L in the form of micelles. Values represent concentrations of cytokines in spleen cell culture supernatants as measured by capture enzyme-linked immunoabsorbent assay. The cytokine concentrations in supernatants from controls (medium only) were below the detection limit.

thereafter. A booster injection resulted in a drastic increase of antibody-secreting cells producing antigen-specific IgG in both the draining lymph nodes and the spleen (fig. 5), and in a strong serum antibody response.

Taken together, these results indicate that functionally different B and T cell activities, including both T_H1- and T_H2-like characteristics, are involved in the immune response to influenza virus iscoms. Moreover, both the activation of regulatory T cell functions as well as the induction of antibody responses vary with regard to kinetics and organ distribution.

3.2 Serum Antibody Responses

A large number of articles have been published showing the capacity of iscoms to induce B and T cell responses.[19,34] The serum antibody response is a classical one, with an initial short-lived IgM response followed by a long-lasting IgG response. Generally, a 10-fold or more enhancement of the antibody response is obtained compared with that induced by the same amount of antigen in a similar size particle, for example as a micelle or *in situ* in a virus particle. Of particular interest is the capacity of iscoms to induce a broad serum antibody response, encompassing both IgG1 and IgG2a in mice. This broad antibody response is seen with a variety of antigens, for example influenza virus antigen, gp160 of HIV-1 or the B subunit of cholera toxin. Conversely, micelles containing influenza virus antigen or cholera toxin B subunit stimulate an antibody response dominated by IgG1.

3.3 Cytotoxic T Cell Responses

The capacity of iscoms as a nonreplicating antigen formulation to induce MHC class I restricted CTL is striking. This was first shown with gp160 of HIV-1 and the envelope proteins of influenza virus.[55] Later, CTL have been induced to a number of other antigens in iscoms, e.g. influenza virus nucleoprotein (D. Burt et al., unpublished work), measles virus F-protein,[35] heat shock protein of mycobacteria[56] and ovalbumin.[30,57,58] After intranasal mucosal administration, influenza virus[59] and respiratory syncytial virus[60] antigens have been shown to induce CTL. Additionally, CTL were induced by oral administration of ovalbumin iscoms.[61]

Likely reasons for the efficiency of induction of CTL by iscoms are their capacity to: (a) deliver antigen to the cytosol of antigen-presenting cells, leading to antigen presentation on class I MHC molecules and a CD8+ T cell response; and (b) induce the production of the relevant cytokines, e.g. IL-1, GM-CSF, IL-2 and IFNγ, by T cells.

3.4 Mucosal Responses

It is well established that it is difficult to evoke mucosal immunity with nonreplicating antigens, since mucosal immune responses are preferentially evoked at the site where the antigen is applied. However, oral administration of antigen has been shown to induce a specific secretory IgA response in milk via the gut-associated lymphoid tissue (GALT). Similarly, bronchus-associated lymphoid

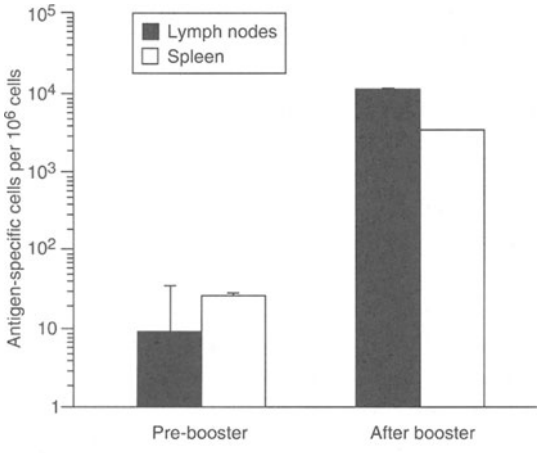

Fig. 5. Booster effect of immunostimulating complexes (iscoms) on the production of antigen-specific IgG. Mice were immunised subcutaneously with 3μg of iscoms containing influenza virus antigens; 6 weeks later some of them received a booster immunisation with the same iscom preparation. Serial dilutions of lymph node and spleen cells taken at the time of and after the booster injection were tested in triplicate by enzyme-linked immunospot assay[54] for production of IgG reactive with influenza virus protein. Values represent means ± S.E. of the number of antigen-specific IgG-producing cells/10^6 lymph node or spleen cells. No IgG-producing cells were detected in wells incubated with lymph node and spleen cells from nonimmunised mice.

tissue (BALT) has been reported to distribute the immune response to mucosal surfaces other than at the site of administration of the antigen.[62] Consequently, parenteral administration may not be an efficient route of immunisation for induction of local mucosal immunity.

However, there are now data claiming that iscoms carrying the H and N antigens of influenza virus induce a strong secretory IgA response in the lung after intramuscular administration similar to that obtained after intranasal immunisation with live virus.[63] Protection is induced in mice[64] and horses[65] after subcutaneous injection or intranasal administration of influenza virus iscoms, and strong serum antibody responses are induced. Various routes of administration of iscoms induce secretory responses in the genital tract of mice.[66] Mowat et al.[57,61,67] showed that oral administration of ovalbumin iscoms induces both antibody responses and MHC class I restricted CTL.

We have conjugated peptides representing the V-3 loop of HIV gp160 to influenza virus envelope proteins in iscoms. In this experimental model, one intranasal immunisation of mice with 5μg of this conjugate induced a clearcut serum antibody response against the peptide (our unpublished work). We have incorporated the recombinantly produced B subunit of cholera toxin into iscoms. The immunological evaluation of these constructs is in progress, in collaboration with Dr J. Holmgren (Department of Microbiology, University of Gothenburg, Sweden). The first results show that clearcut responses are obtained after one oral or intranasal immunisation with a dose of 10μg.

Therefore, the envelope proteins of influenza virus and probably also the B subunit of cholera toxin are able to induce systemic immune responses via the mucosal surfaces. Recently, it was also shown that iscoms containing antigens from protoscoles of *Echinococcus granulosus* induce a serum antibody response in mice after intranasal immunisation (H. Carol et al., unpublished work).

In conclusion, the iscom is an interesting carrier system for local immunisation. Techniques have been partially established for various experimental approaches, but the immunological responses to such constructs need to be further evaluated.

3.5 Protective Responses

Protective immunity induced by iscoms was first demonstrated against influenza virus in mice[64] and horses.[65] The first iscom vaccine available commercially was a veterinary influenza virus vaccine for horses.

Table II lists infections to which protective immunity has been induced by antigens borne on iscoms. Some of these are infections against which vaccines do not presently exist or against which protection has not previously been obtained. It is of particular interest that: (a) an experimental iscom-borne subunit rabies vaccine efficiently prevented disease and death in post-exposure experiments in mice, whereas a commercially available vaccine did not;[68] (b) an experimental iscom vaccine induced full protection against *Trypanosoma*

Table II. Protective immunity induced by immunostimulating complexes (iscoms) containing various microbial antigens

Antigen and micro-organism	Experimental animal	Disease prevented
Haemagglutinin and neuraminidase, influenza virus	Mice	Pneumonia
Haemagglutinin, measles virus	Mice	Encephalitis
Fusion protein, measles virus	Mice	Encephalitis
Haemagglutinin and fusion protein, phoid distemper virus	Seal	Lethal infection
Haemagglutinin and fusion protein, canine distemper virus	Dog	Pneumonia
G protein, rabies virus	Mice	Lethal infection, postexposure immunisation
gp120, simian immunodeficiency virus	Monkey	Lethal infection
gp-125, HIV-2	Monkey	Viraemia
Envelope protein, bovine diarrhoea virus	Sheep	Abortion
gp70, feline leukaemia virus	Cat	Viraemia
gp360, Epstein-Barr virus	Tamarin monkey	Lethal tumour
Surface antigens, *Toxoplasma gondii*	Mice	Lethal infection
Immunoaffinity purified protein, *Trypanosoma cruzi*	Mice	Lethal infection

cruzi in a mouse model;[69] and (c) long-lasting protection has been induced in macaques against simian immunodeficiency virus (P. de Vries et al., unpublished work) and HIV-2.[70]

4. Visions About Immunotherapy

A number of diseases are caused by infectious micro-organisms that persist in the host, for example herpes viruses, retroviruses and various parasites such as African and South American trypanosomes. There are in general no vaccines against these pathogens, which persist in the presence of an immune response evoked by themselves and that is apparently favourable for their survival in the host. To devise a vaccine against such micro-organisms, it is necessary to understand what type of immune response should be evoked.

For several infections, there is increasing evidence that T cell responses of the T_H1 type mediate protective immunity, whereas the T_H2 type of immune response is linked to disease progression.[71,72] This is true for HIV infection and leishmaniasis in humans, or *T. cruzi* infection or murine leprosy in the mouse. Knowledge of this kind suggests that vaccines eventually should be formulated to induce specific types of immune response. For example, in order to cure a persistent infection, it may be necessary to switch the evoked immune response from the T_H2 type to the T_H1 type, or superimpose a T_H1 response on a T_H2 response. It is also claimed that progression towards AIDS after infection with HIV-1 is preceded by a change in immune response from T_H1 to T_H2, or to T_H0, followed by a decrease in the CD4+/CD8+ ratio.[73-75] An efficient induction of IL-12 may cause reversion to a T_H1 response.

An example of a broadening of the immune response has been demonstrated with cholera toxin subunit. Alone, the B subunit of cholera toxin induces an almost pure IgG1 response and no IgG2a response. However, when it is integrated into iscoms or administered with iscom matrix as an adjuvant, a strong IgG2a response is added to the IgG1 response (our unpublished results). The IgG1 subclass is generally evoked in connection with a T_H2 response, and the IgG2a subclass is associated with a T_H1 response. These experiments show that an adjuvant component can superimpose a different immune response than that intrinsically evoked by the antigen alone.

In the case of *T. cruzi* infection, a protein similar to α-fetoprotein exerts an immunosuppressive effect, particularly on T-cell proliferation, IL-2 and interferon-γ production, thus evading the protective mechanisms of the host (D. Hansen et al., unpublished work). This provokes the question of whether an immune response should first be elicited against this immunomodulatory protein, before immunisation with other antigens that may be protective. The outcome could be that a neutralising immune response to the immunomodulatory

protein is evoked, but surprising results in the opposite direction might also occur.

Thus, the prospects for immunotherapy against pathogens causing persistent infections lie partly in being able to modulate the immune response and partly in defining immunomodulating components in the pathogen.

5. Conclusions

The work on separation of various *Quillaja* components has been a major contribution in the progress towards the development of iscom vaccines for human use. Combination of QH-A and QH-C in particular ratios has allowed the optimum balance between immunogenicity and reactogenicity to be obtained with iscom vaccines.

Several candidate iscom vaccines are under development, including one against influenza which CSL Ltd (Australia) expects to take into clinical trials in humans during 1995. Immunological studies in animal models have shown that this and other iscom vaccines produce high serum antibody titres, as well as prominent T_H1 and strong cytotoxic T cell responses. No significant local or systemic toxicity has been observed in animal studies at dose levels many times higher than expected to be effective in humans. The forthcoming clinical trials represent a major advance for iscom technology, and if successful will open the way for other iscom-based vaccines.

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References

1. Morein B, Simons K. Subunit vaccines against enveloped viruses: virosomes, micelles and other protein complexes. Vaccine 1985; 3: 83-93
2. Allison AC. Antigens and adjuvants for a new generation of vaccines. In: Gregoriadis G, Allison AC, Poste G, editors. Immunological adjuvants and vaccines. London: Plenum Publishing, 1989: 153-61
3. Cox J, Coulter A. Advances in adjuvant technology and application. In: WK Yong, editor. Animal parasite control utilizing biotechnology. Boca Raton: CRC Press, 1992: 47-112
4. Beutler B, Milsark IW, Cerami A. Passive immunisation against cachectin/tumour necrosis factor protects mice from lethal effects of endotoxin. Science 1985; 229: 869-71
5. Morein B, Sundquist B, Höglund S, et al. Iscom: a novel structure for antigenic presentation of membrane proteins from enveloped viruses. Nature 1984; 308: 457-60
6. Özel M, Höglund S, Gelderblom H, et al. Quaternary structure of the immunostimulating complex (iscom). J Ultrastruct Mol Struct Res 1989; 102: 240-8
7. Åkerblom L, Strömstedt K, Höglund S, et al. Formation and characterization of FELV ISCOMs. Vaccine 1989; 7: 142-6
8. Lövgren K, Morein B. The requirement of lipids for the formation of immunostimulating complexes (iscoms). Biotechnol Appl Biochem 1988; 10: 161-72
9. Ramon G. Procédés pour accroître la production des antitoxines. Ann Inst Pasteur 1926; 40: 1-10
10. Espinet EG. Nuevo tipo de vacuna antiaftosa a complejo glucovirioco. Gac Vet 1951; 74: 1-13
11. Dalsgaard K. Thin layer chromatographic fingerprinting of commercially available saponins. Dansk Tidskr Farm 1970; 44: 327-31
12. Dalsgaard K. The application of the saponin adjuvant Quil A in foot-and-mouth disease vaccines. Bull Off Int Epiz 1978; 89: 963-6
13. Dourmashkin RF, Dougherty RM, Harris RJ. Electron microscopic observations on Rous sarcoma virus and cell membranes. Nature 1962; 194: 1116-9
14. Lucy JA, Glauert AM. Structure and assembly of macromolecular lipid complexes composed of globular micelles. J Mol Biol 1964; 8: 727-48
15. Kensil C, Patel C, Lennick M, et al. Separation and characterization of saponins with adjuvant activity from *Quillaja saponaria* Molina cortex. J Immunol 1991; 146: 431-7
16. Kersten GFA, Spiekstra A, Beuvery EC, et al. On the structure of immune-stimulating saponin-lipid complexes (iscoms). Biochim Biophys Acta 1991; 1062: 165-71
17. Rönnberg B, Fekadu, M, Morein B. Adjuvant activity of non toxic *Quillaja saponaria* Molina components for use in iscom-matrix. Vaccine 1995; 13: in press
18. Morein B, Höglund S. Subunit vaccines against infection by enveloped viruses. In: Mizrahi A, editor. Viral vaccines. New York: Wiley-Liss, 1990: 69-90
19. Höglund S, Dalsgaard K, Lövgren K, et al. Iscoms and immunostimulation with viral antigens. Subcell Biochem 1989; 15: 39-68
20. Pyle S, Morein B, Bess J, et al. Immune response to immunostimulatory complexes (iscoms) prepared from human immunodeficiency virus type 1 (HIV-1) or the HIV-1 external envelope glycoprotein. Vaccine 1989; 7: 465-73
21. Morein B, Ekström J, Lövgren K. Increased immunogenicity of a non-amphipathic protein (BSA) after inclusion into iscoms. J Immunol Methods 1990; 128: 177-81
22. Åkerblom L, Nara P, Dunlop N, et al. HIV experimental vaccines based on the iscom technology using envelope and *gag* gene products. Biotechnol Ther 1993; 4: 145-61
23. Sjölander A, Lövgren K, Ståhl S, et al. High antibody responses in rabbits immunized with influenza virus iscoms containing a repeated sequence of the *Plasmodium falciparum* antigen Pf155/RESA. Vaccine 1991; 9: 443-50
24. Larsson M, Lövgren K, Morein B. Immunopotentiation of synthetic oligopeptides by chemical conjugation to iscoms. J Immunol Methods 1993; 162: 257-60

25. Lövgren K, Larsson M. Conjugation of synthetic peptides to carrier iscoms: factors affecting the immunogenicity of the conjugate. J Immunol Methods 1994; 173: 237-43
26. Morein B, Villacrés-Eriksson M, Åkerblom L, et al. Initiation of immune response with iscoms. In: Gregoriadis G, et al., editors. New generation vaccines. New York: Plenum Press, 1993: 61-72
27. Lundén A. *Toxoplasma gondii* infection in sheep: studies of epidemiology, food hygiene and vaccination [dissertation]. Uppsala: Swedish University of Agricultural Sciences, 1994
28. Reid G. Soluble proteins incorporate into iscoms after covalent attachment of fatty acid. Vaccine 1992; 9: 597-602
29. Browning M, Reid GR, Osborne R, et al. Incorporation of soluble antigens into iscoms: HIV gp120 iscoms induce virus neutralizing antibodies. Vaccine 1992; 9: 585-90
30. Mowat A, Donachie AM, Reid G, et al. Immunostimulating complexes containing QA and protein antigen prime class I MHC-restricted T lymphocytes *in vivo* and are immunogenic by the oral route. Immunology 1991; 72: 317-22
31. Weijer K, Pfauth A, van Herwijnen R, et al. Induction of feline leukaemia virus-neutralizing antibodies by peptides derived from the FeLv env gene. Vaccine 1993; 9: 946-56
32. Watson DL, Watson NA, Fossum C, et al. Interactions between immune-stimulating complexes (ISCOMs) and peritoneal mononuclear leucocytes. Microbiol Immunol 1992; 36: 199-203
33. Villacres-Eriksson M. Induction of immune response by iscoms [dissertation]. Uppsala: Swedish University of Agricultural Sciences, 1993
34. Morein B, Åkerblom, L. The iscom – an approach to subunit vaccines. In: Isaacson RI, editor. Recombinant DNA vaccines. New York: Marcel Dekker, 1992: 369-86
35. van Binnendijk RS, van Baalen CA, Poelen MCM, et al. Measles virus transmembrane fusion protein synthesized *de novo* or presented in immunostimulating complexes is endogenously processed for HLA class I and class II-restricted cytotoxic T-cell recognition. J Exp Med 1992; 176: 119-28
36. Villacrés-Eriksson M, Bergström-Mollagoglu M, Kåberg H, et al. The induction of cell-associated and secreted IL-1 by iscoms, matrix or micelles in murine splenic cells. Clin Exp Immunol 1993; 93: 120-5
37. Bergström-Mollaoglu M, Lövgren K, Åkerblom L, et al. Antigen-specific increases in the number of splenocytes expressing MHC class II molecules following restimulation with antigen in various physical forms. Scand J Immunol 1992; 36: 565-74
38. Lanzavecchia A. Antigen-specific interaction between T and B cells. Nature 1985; 314: 537-9
39. Chesnut RW, Colon SM, Grey HM. Requirements for the processing of antigens by antigen-presenting B cells. J Immunol 1982; 129: 2382-8
40. Shimonkevitz R, Kappler J, Marrack P, et al. Antigen recognition by H-2 restricted T cells. J Exp Med 1983; 158: 303-16
41. Ron Y, Sprent J. B cells as antigen-presenting cells *in vivo*. In: Antigen presenting cells: diversity, differentiation, and regulation. New York: Alan R Liss, 1988: 321
42. Sanders VM, Uhr JW, Vitetta ES. Conjugation of antigen-specific T and B cells: comparison between virgin and memory B cells. In: Antigen presenting cells: diversity, differentiation, and regulation. New York: Alan R Liss, 1988: 331
43. Inaba K, Witmer-Pack MD, Inaba M, et al. The function of Ia+ dendritic cells and Ia– dendritic cell precursors in thymocyte mitogenesis to lectin and lectin plus interleukin 1. J Exp Med 1988; 167: 149-62
44. Crowley M, Inaba K, Steinman RM. Dendritic cells are the principal cells in mouse spleen bearing immunogenic fragments of foreign proteins. J Exp Med 1990; 172: 383-6
45. Sornasse T, Flamand V, De Becker G, et al. Antigen-pulsed dendritic cells can efficiently induce an antibody response *in vivo*. J Exp Med 1992; 175: 15-21
46. Austyn JM. Lymphoid dendritic cells. Immunology 1987; 62: 161-70
47. Levine TP, Chain BM. Endocytosis by antigen presenting cells: dendritic cells are as endocytically active as other antigen presenting cells. Proc Natl Acad Sci USA 1992; 89: 8342-6
48. Ellis J, Chain BM, Davies DH, et al. Antigen presentation by dendritic cells provides optimal stimulation for the production of IL-2, IL-4 and interferon-γ by allogenic T cells. Eur J Immunol 1991; 21: 2803-9
49. Fossum C, Bergström M, Lövgren K, et al. Effect of iscoms and their adjuvant moiety (matrix) on the initial proliferation and IL-2 responses: comparison of spleen cells from mice inoculated with iscoms and/or matrix. Cell Immunol 1990; 129: 414-25
50. Villacrés-Eriksson M, Kåberg H, Mollaoglu M, et al. Involvement of interleukin-2 and interferon-gamma in the immune response induced by influenza virus iscoms. Scand J Immunol 1992; 36: 421-6
51. Villacrés-Eriksson, M. Antigen presentation by macrophages, dendritic cells and B cells to primed T lymphocytes and their cytokine production following exposure to immunostimulating complexes. Clin Exp Immunol. In press
52. Valensi J-P, Carlson JR, Van Nest GA. Systemic cytokine profiles in BALB/c mice immunized with trivalent influenza vaccine containing MF59 oil emulsion and other advanced adjuvants. J Immunol 1994; 153: 4029-39
53. Watson D, Watson N, Fossum C, et al. Inflammatory response and antigen localization following immunization with influenza virus iscoms. Inflammation 1989; 13: 641-9
54. Czerkinsky CC, Nilsson LA, Nygren H, et al. A solid phase enzyme-linked immunspot (ELISPOT) assay for enumeration of specific antibody secreting cells. J Immunol Methods 1983; 65: 109-21
55. Takahashi H, Takeshita T, Morein B, et al. Induction of CD8+ cytotoxic T cells by immunization with purified HIV-1 envelope proteins in iscoms. Nature 1990; 344: 873-5
56. Zügel U, Schoel B, Yamamoto S, et al. Crossrecognition by CD8 T cell receptor αβ cytotoxic T lymphocytes of peptides in the self and the mycobacterial hsp60 which share intermediate sequence homology. Eur J Immunol 1995; 25: 451-8
57. Mowat A, Donachie A. Iscoms – a novel strategy for mucosal immunization. Immunol Today 1991; 12: 383-5
58. Heeg K, Kuon W, Wagner H. Vaccination of class I major histocompatibility complex (MHC)-restricted murine CD8+ cytotoxic T lymphocytes towards soluble antigens: immunostimulating ovalbumin complexes enter and allow sensitization against the immunodominant peptide. Eur J Immunol 1991; 21: 1523-7
59. Jones PD, Tha Hla R, Morein B, et al. Cellular immune responses in the murine lung to local immunization with influenza A virus glycoproteins in micelles and immunostimulating complexes (iscoms). Scand J Immunol 1988; 27: 645-52
60. Trudel M, Nandon F, Séguin C, et al. Initiation of cytotoxic T cell response and protection of Balb/c mice by vaccination with an experimental ISCOMs respiratory syncytial virus subunit vaccine. Vaccine 1992; 10: 107-12

61. Mowat A, Maloy KJ, Donachie AM. Immune-stimulating complexes as adjuvants for inducing local and systemic immunity after oral immunization with protein antigens. Immunology 1993; 80: 527-34
62. McGhee JR, Mestecky J, Elson CO, et al. Regulation of IgA synthesis and immune response by T cells and interleukins. J Clin Immunol 1989; 9: 175-99
63. Ben Ahmeida ETS, Jennings R, Ertürk M, et al. The IgA and subclass IgG responses and protection in mice immunised with influenza antigens administered as iscoms, with FCA, ALH or as infectious virus. Arch Virol 1992; 125: 71-86
64. Lövgren K, Kåberg H, Morein B. An experimental influenza subunit vaccine (ISCOM) – induction of protective immunity to challenge infection in mice after intranasal or subcutaneous administration. Clin Exp Immunol 1990; 82: 435-9
65. Mumford JA, Jessett DM, Rollinson EA, et al. Duration of protective efficacy of equine influenza immunostimulating complex/tetanus vaccines. Vet Record 1994; 134: 158-62
66. Tharpar MA, Parr EL, Bozzola JJ, et al. Secretory immune responses in the mouse vagina after parenteral or intravaginal immunization with an immunostimulating complex (iscom). Vaccine 1991; 9: 129-33
67. Mowat A, Donachie AM, Reid G, et al. Immunostimulating complexes containing QH and protein antigen prime class I MHC-restricted T lymphocytes *in vivo* and are immunogenic by the oral route. Immunology 1991; 72: 317
68. Fekadu M, Shaddock JH, Ekström J, et al. An immune stimulating complex (iscom) subunit rabies vaccine protects dogs and mice against street rabies challenge. Vaccine 1992; 10: 192-7
69. Araujo FG, Morein B. Immunization with *Trypanosoma cruzi* epimastigote antigens incorporated into iscoms protects against lethal challenge in mice. Infect Immun 1991; 59: 2909-14
70. Putkonen P, Björling E, Åkerblom L, et al. Long-standing protection of macaques against cell-free HIV-2 with a HIV-2 iscom vaccine. J AIDS 1994; 7: 551-9
71. Yamamura M, Uyemura K, Deans RJ, et al. Defining protective responses to pathogens: cytokine profiles in leprosy lesions. Science 1991; 254: 277
72. Heinzel FP, Sadick MD, Mutha SS, et al. Production of interferon-γ, IL-2, IL-4 and IL-10 by CD4+ lymphocytes *in vivo* during healing and progressive murine leishmaniasis. Proc Natl Acad Sci USA 1991; 88: 7011
73. Clerici M, Shearer GM. A TH1-TH2 switch is a critical step in the etiology of HIV infection. Immunol Today 1993; 14: 107
74. Clerici M, Shearer GM. The T helper cell shift in AIDS: significance for pharmacotherapy. Clin Immunother 1995; 3 (2): 95-101
75. Romagnani S, Maggi E, Del Prete G. An alternative view of the TH1/TH2 switch hypothesis in HIV infection. Aids Res Human Retrovir 1994; 10: III-IX

Correspondence and reprints: Dr *Bror Morein*, Swedish University of Agricultural Sciences, Faculty of Veterinary Medicine, and National Veterinary Institute, Department of Virology, Biomedical Centre, Box 585, S-751 23 Uppsala, Sweden.