Chapter 2

ISCOMs and Immunostimulation with Viral Antigens

Stefan Höglund, Kristian Dalsgaard, Karin Lövgren, Bo Sundquist, Ab Osterhaus, and Bror Morein

1. INTRODUCTION

With the dissection of microorganisms followed by biochemical and immunological characterization, antigens inducing protective immunity became recognized. Early attempts to use these isolated antigens as vaccines, i.e. subunit vaccines, showed that although immunogenic *in situ* as part of the microorganism, they were not immunogenic as purified antigens. Subsequent studies showed that the formation of antigen into defined multimeric forms such as protein micelles or into liposomes made them considerably more immunogenic. In a way, micelles and liposomes mimic a submicroscopic particle of a microorganism with several copies of surface antigens. By contrast, monomeric forms of antigens, e.g., envelope proteins of parainfluenza-3 virus or Semliki forest virus, not only had a low immunogenicity but had a specific suppressive effect on the immune response as well; this was shown when the monomers were given simultaneously

Stefan Höglund Institute of Biochemistry, Biomedical Center, Uppsala, Sweden. **Kristian Dalsgaard** State Veterinary Institute of Virus Research, Lindholm, Kalvehave, Denmark. **Karin Lövgren and Bror Morein** The National Veterinary Institute, Department of Virology, Biomedical Center, Uppsala, Sweden. **Bo Sundquist** The National Veterinary Institute, Division of Vaccine Research, Uppsala, Sweden. **Ab Osterhaus** National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands.

J. R. Harris (ed.), *Virally Infected Cells*

with the same antigen in a micelle (Morein *et al.*, 1982, 1983; Morein and Simons, 1985; Jennings, 1987).

In general, it seems that the immunogenicity of an antigen in a micelle or in a liposome is of the same magnitude as that of a killed microorganism. However, the immunogenicity of antigens in killed microorganisms or in micelles is often insufficient and must be enhanced by the addition of adjuvant. Unfortunately, in order to be effective, conventional adjuvants must be used in high doses, resulting in unacceptable adverse effects (Bomford, 1980).

The *i*mmuno*s*timulating *com*plex (ISCOM) was created to become a well-defined submicroscopic particle, with several copies of the antigen well exposed and with a built-in adjuvant, Quil A (Morein *et al.*, 1984). The binding of Quil A to the antigen complex was performed in an effort to reduce the amount of adjuvant required, thereby avoiding or minimizing the toxic effects connected with conventional adjuvants when used in effective doses. This chapter reviews the composition and assembly of ISCOM, as well as its function from an immunological point of view.

2. QUIL A: THE UNIQUE COMPOUND OF THE ISCOM

2.1. Origin

The adjuvant Quil A* is a component derived from the South American tree *Quillaja saponaria* Molina. Saponins are some of the most common secondary constituents of plants, found in more than 500 plant genera. Their physiological function is not clearly understood, but it has been suggested that they may in some cases protect the plant by exerting antimicrobial activity. They may also contribute to the depot nourishment of the plant by their ability to release monosaccharides. Chemically, they are glycosides with one or more mono- or oligosaccharide groups in glycosidic links with a nonpolar aglycon usually of steroid or triterpenoid structure. *Quillaja* saponins are triterpenoids. They are present in most parts of the tree, but the highest content is found in the bark of the tree. This bark, *Cortex quillajae,* is collected and sold commercially, mainly in Chile and, because of the traditional trade route, it is sometimes referred to as Panama bark. This old pharmaceutical drug is generally not produced for making immunological adjuvants. The bulk of the bark has been used in the past and is still used for making a crude extract, saponin, applied in diverse industries as film emulsions, shampoo, and food and beverages. The common property of the saponin used in these processes is its surface activity, making it useful as a mild detergent,

*Quil A is manufactured by the Danish company Superfos, Vedbaek, as a purified product of a uniform composition. It is available as a 2% sterile aqueous solution or as a lyophilized powder.

emulsifier, and froth-producing agent. The history of saponin as an immunological adjuvant dates back to early experiments with formol toxoid vaccines conducted at the beginning of the nineteenth century. It was discovered early that the formulation of vaccines and the constituents could influence their immunogenic potential quite remarkably. In a search for adjuvants, a whole variety of different substances were tested. It was then discovered that some saponins had immunological activity. The effect was quite unpredictable, however, since all batches of saponin were not active, and some of them produced intense local reactions at the site of injection. Except for a few experimental applications in laboratory animals, saponins did not have any practical use until some decades later. In 1951, R. G. Espinet reported on the application of saponin in foot-and-mouth disease vaccines. Espinet found that the saponin available in Argentina had a beneficial effect on such vaccines. Several years later, saponin was used in foot-and-mouth disease vaccines by the major manufacturers. It remained obscure why some batches of saponin had excellent adjuvant activity whereas some had no measurable effect. The approach for years was then to buy a large batch of a good one for use.

During the early 1970s, in search of the active components, Dalsgaard (1970, 1974) was able to show by thin-layer chromatography (TLC) that all the saponins with documented adjuvant activity were derived from *Quillaja saponaria Molina*. The fact that this tree is indigenous in South America evidently facilitated for Espinet (1951) the choice of this adjuvant for foot-and-mouth disease vaccines. It can by no means be excluded that saponins other than quillaja saponin may be active as immunological adjuvant. However, quillaja bark is certainly the best choice of the commercially available saponin drugs. The adjuvant component of quillaja bark, denoted Quil A (Dalsgaard, 1974), constitutes only about 1% of the bark on a weight basis. However, the supply of bark has been stable for a century, making it an attractive source of raw material for the production of Quil A.

2.2. Isolation

Quil A is freely soluble in water. Solutions of Quil A at a concentration of $\leq$10% can be handled readily. Therefore, isolation procedure is always started with a simple aqueous extraction of the bark. This aqueous extract contains a mixture of different plant constituents: saponins, tannins, sugars, and flavonoid compounds. The most important step in the purification of Quil A is a dialysis of this water extract. Quil A forms micelles and is retained in the dialysis bag whereas the bulk of low-molecular-weight substances will diffuse through the membrane. About 75% of the material extracted from the bark can be removed in this way without significant loss of Quil A. This is because Quil A has a relatively low critical micelle concentration (CMC) of 300 mg/liter. However,

because of the hydrophobic interaction between Quil A and amphipathic molecules, several substances are trapped in the dialysis bag. Using both ion-exchange chromatography and gel filtration, most of the substances of nontriterpenoid nature will be removed (Dalsgaard, 1974). The product Quil A produces a single band in TLC silica gel, representing about five closely related compounds. These compounds were resolved in high-performance TLC (HPTLC) silica gel. The major nonpolar group of these molecules appears to be a triterpenoid with 5–10 oligosaccharide moieties. For comparison, the triterpenoid of quillajic acid is shown in Fig. 1.

2.3. Stability of Quil A

In the dry state, e.g. as a lyophylized powder, Quil A is very stable and can be kept for years at room temperature. In solution and after addition to vaccines stored at 5°C, no loss of Quil A can be detected after 1 year. It is recommended to keep stock solutions frozen at −20°C. The glycosidic bonds in the triterpenoid molecule may be sensitive to extreme pH values, especially low pH; autoclaving should also be avoided. The triterpenoid moiety of Quil A can be assayed by a colorimetric reaction with chlorosulfonic acid (Brieskorn and Briner, 1954). A homogeneous solution of Quil A is best suited for this method, since it is influenced by a variety of other substances. The most sensitive assay of Quil A is based on its hemolytic activity. Used in either radiodiffusion in an agarose gel containing red blood cells (RBC) or a similarly designed electrophoresis rocket hemolysis (Sundqvist *et al.*, 1983), Quil A can be detected in biological systems, including vaccines at a level above 25 μg/ml.

2.4. Use of Quil A as Adjuvant in Vaccines

Only during the last few years has Quil A been used for the formation of ISCOMs. However, in veterinary vaccines it has been commercially available

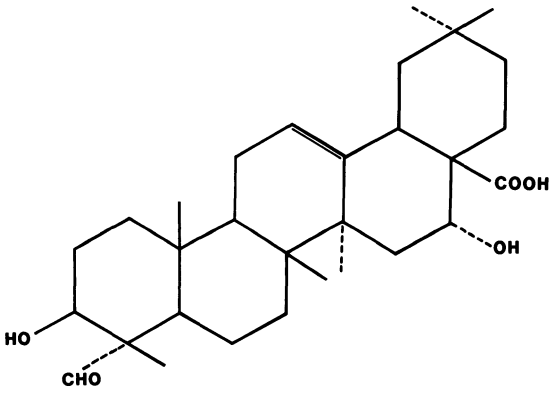

FIGURE 1. Triterpenoid structure of the amphipathic quillajic acid.

for several years and is now being widely used as an adjuvant for a variety of vaccines in domestic animals (Dalsgaard 1970, 1974, 1977, 1978, 1984, and references cited therein).

The assessment of the dose of Quil A as a conventional adjuvant in a given system is a balance between obtaining maximum adjuvant effect and inducing minimum local reaction at the site of injection. Being surface active and hemolytic to RBC, Quil A produces a local reaction or even tissue necrosis if applied in excessive amounts.

In large animals, i.e., cattle, pig, and sheep, a dose of 1 mg Quil A given subcutaneously (SC) is usually well tolerated. It must be emphasized that a dosage in any particular vaccine should be adjusted in each species. With 1 mg Quil A in large animals, vaccination reactions (if any) are usually limited to a small soft swelling accompanied by a short rise in body temperature, both of which disappear in 1 or 2 days, leaving no macroscopic signs at the site of injection. Formulations containing Quil A are registered additives to food and beverage, and as natural constituents of normal human diet; therefore, Quil A should not represent any hazard in the sense of meat residues.

In small animals, the optimum dose of Quil A varies from 10 μg in mice to 50–100 μg in guinea pigs and rabbits. In cats and dogs, 25 μg has been applied successfully. In dogs, this may seem a low dose, at least in the larger species, but because dogs are pet animals, there is great public concern against acceptance of any local reaction from a vaccination. LD_{50} in mice is ~35 mg/kg. Toxicology studies in rats have been reported with ISCOMs containing envelope proteins of measles virus that were compared with a trivalent commercially available vaccine containing diphtheria–pertussis–tetanus–polio (DPT–polio). The ISCOM vaccine gave less local reaction than did the DPT–polio vaccine (Speijers *et al.*, 1987). The amount of Quil A included in ISCOMs may be several hundredfold lower than when used as a conventional adjuvant, provided that free Quil A is removed from the ISCOMs. With this in mind, ISCOM vaccines can be prepared with a very low toxicity; thus, toxicity should not be a serious obstacle to the use of ISCOM vaccines.

3. STRUCTURE AND COMPOSITION OF ISCOMs

Early ISCOM preparations were described as structures containing amphipathic proteins assembled in a multimeric complex through hydrophobic interaction with Quil A (Morein *et al.*, 1984). However, at that time there was limited knowledge about the composition of ISCOMs as well as about the minimal requirements of the components essential for formation of an ISCOM. The self-assembly of amphipathic molecules into complexes in aqueous solution is mainly due to the interaction between available hydrophobic sequences of the amphipathic molecules. Examples of such complexes are lipid vesicles, micelles of

proteins or detergent molecules, and ISCOMs. It turned out that the ISCOMs, maintained by hydrophobic interaction, are energetically stable constructs that, once formed, are not easily dissociated.

A great number of ISCOMs were prepared from glycoproteins from a wide range of virus species. All ISCOMs were shown to be stable structures and moreover had a similar cagelike morphology, regardless of the properties of the protein: glycosylation, size, or tertiary structure (Morein *et al.*, 1984; Höglund *et al.*, 1986). Therefore, it was unlikely that the proteins were determining the structure of ISCOM. In a series of experiments, different lipids, with emphasis laid on cholesterol and phosphatidylcholine, were tested for ISCOM formation (Lövgren and Morein, 1988). It was then shown that an ISCOM matrix in its simplest form is composed of Quil A and cholesterol. Into this ISCOM matrix, amphipathic proteins together with an additional lipid, e.g., phosphatidylcholine, are incorporated by hydrophobic interaction to form a highly immunogenic antigen complex—the ISCOM.

3.1. Requirement of Lipid for the Construction of ISCOM

3.1.1. ISCOM Matrix

Long before ISCOMs were made, it was shown that Quil A has the ability to penetrate and bind to a number of lipids, particularly cholesterol, to form soluble lipid complexes (Bangham *et al.*, 1962; Dourmashkin *et al.*, 1962; Glauert *et al.*, 1962; Lucy *et al.*, 1964). But, to constitute the ISCOM matrix, only cholesterol was indispensable, and none of nine other lipids tested was able to interact with Quil A and replace cholesterol. By contrast, from a mixture of cholesterol and Quil A only, an ISCOM matrix was formed, with a slightly flattened cagelike morphology (Fig. 2). What, then, is the matrix? We have defined the ISCOM matrix as the cagelike structure produced through the interaction between Quil A and lipids with cholesterol being a prerequisite of its assembly. Even though the lipids tested, except cholesterol, would not alone associate with Quil A to constitute the ISCOM matrix, they were readily incorporated into the

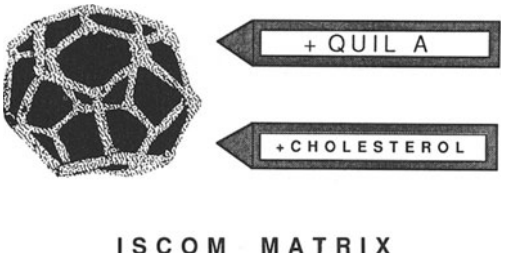

FIGURE 2. Model of the cagelike ISCOM matrix composed of Quil A and lipid, as has been demonstrated by electronmicroscopy.

ISCOM matrix; moreover, such a lipid was prerequisite to include a protein into the ISCOM. Although the ISCOM matrix is composed of Quil A, cholesterol, and lipid, it constitutes only a minor part of the total mass. In a typical ISCOM, 5–10% of the weight is Quil A, 1–5% is composed of cholesterol and phospholipids, and the rest is protein (i.e., the antigen). On a molar basis, the proportion of protein–Quil A–cholesterol will be approximately 1 : 1 : 1.

3.2. Morphology of the ISCOM Particle

The cagelike structure of the ISCOM is a characteristic feature, used for the analysis of ISCOM preparations (Morein *et al.*, 1984; Höglund *et al.*, 1986) (Fig. 3). ISCOMs prepared from viral proteins appear as a rigid isometric particle with a diameter of ~35 nm. The electron-dense negative stain readily penetrates the open structure of the ISCOM, demonstrating a regular pattern of holes in its shell (Fig. 3a,b). Structural subunits of the shell were envisaged with the aid of electronmicroscopic tomography (Skoglund *et al.*, 1986), allowing reconstruction of models of the ISCOM particle (Fig. 4). Interestingly, lyophilization of ISCOMs has proved to preserve a round shape of the ISCOM particle (Fig. 3b).

The construct of the ISCOM particle, by analogy with isometric viral capsids (Liljas, 1986), is probably energetically optimized, thus rendering the ISCOM remarkably stable. The ISCOMs have kept their morphology and immunogenicity for 3 years stored as a sterile suspension in a physiological buffer or preserved by lyophilization (Sundquist *et al.*, 1988a). By different cryopreparation techniques (Özel *et al.*, 1988) a symmetrical array of morphological subunits assembled on the shell of the ISCOM particle, exhibiting five- and six-fold symmetry (Fig. 3b) is envisaged. Interestingly, 20 morphological subunits assembled on a dodecahedral shell (Fig. 7) would mimic the ISCOM particle, as shown by electron microscopy (Höglund *et al.*, 1989; Özel *et al.*, 1989).

4. PREPARATION OF ISCOMs

ISCOMs have been prepared to contain a great variety of amphipathic proteins, including viral envelope proteins from about 20 different viruses, such as influenzavirus, parainfluenza-3 virus, measles virus, bovine viral diarrhea virus, bovine leukemia virus, feline leukemia virus, and simian and human immune deficiency viruses (SIV and HIV) (Morein *et al.*, 1987, and references cited therein). Membrane proteins from bacteria and protozoa were also readily integrated into ISCOMs, and in all cases tested they became highly immunogenic (Uggla *et al.*, 1989).

The detergent solubilization of a membrane involves chemical cooperation

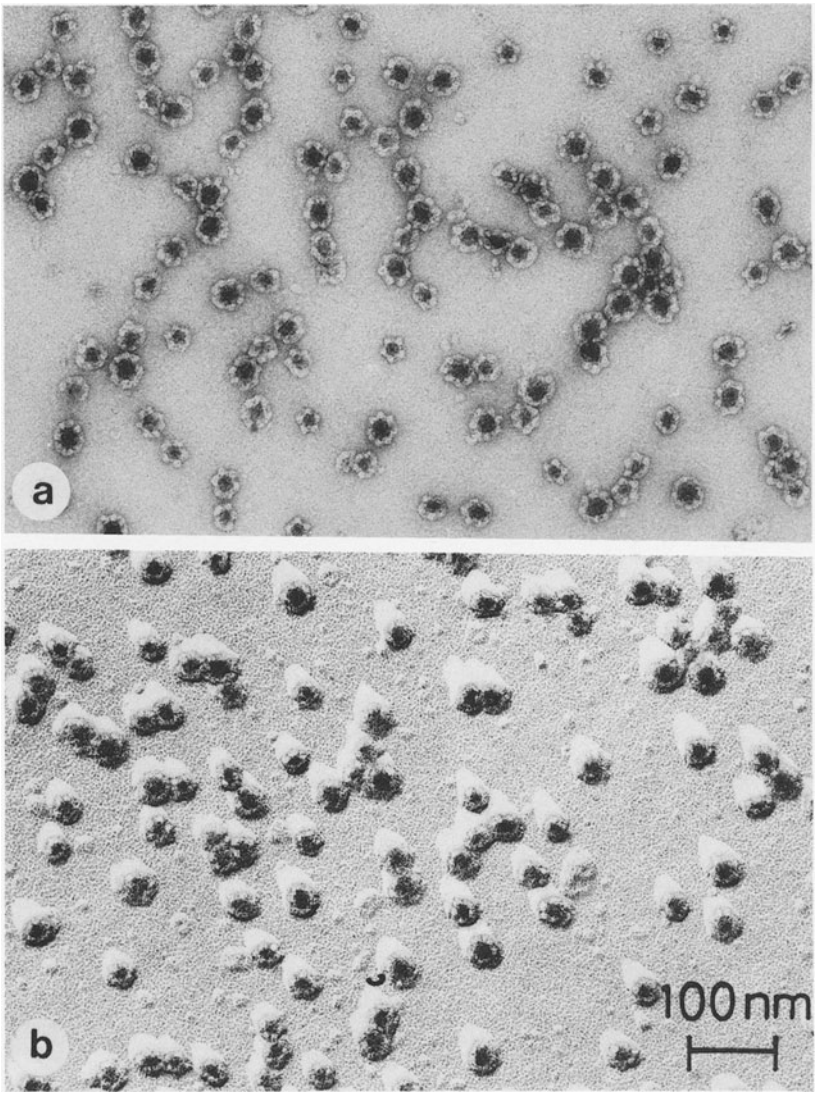

FIGURE 3. (a) Cagelike shell demonstrated on negatively stained ISCOM readily penetrated by the stain. The ISCOMs were preserved by freeze drying. (Courtesy of M. Özel.) (b) Specimen of ISCOMs, prepared by negative staining and shadow casting, showing a population of round ISCOM particles preserved by freeze drying. An assembly of globular morphological subunits is shown on the shell of the ISCOM particle. (Courtesy of M. Özel.)

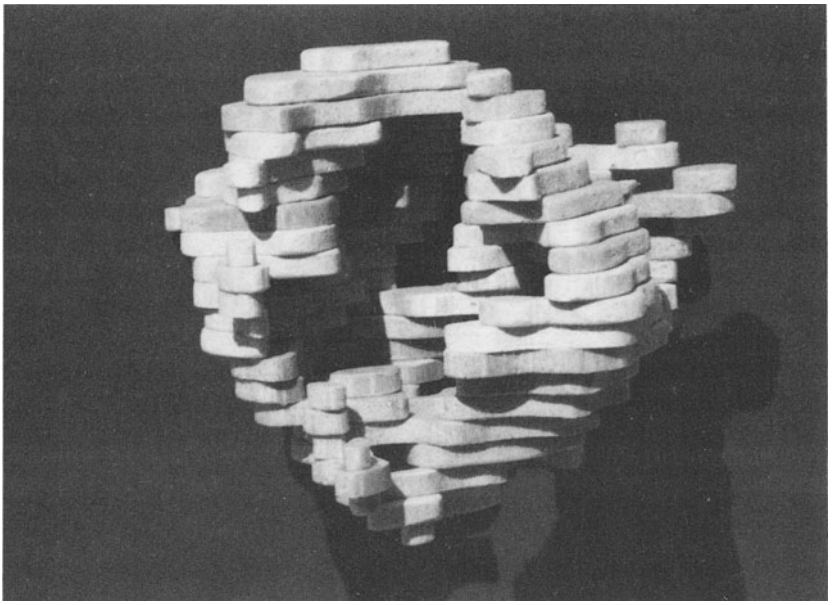

FIGURE 4. Model reconstruction, produced from micrographs of an ISCOM analyzed by electron-microscopic tomography, clearly showing an open shelf composed of structural subunits. (Courtesy of L. G. Öfverstedt and Su Xiao-dong.)

between the essential components, i.e., proteins, lipids, and detergent molecules (Helenius and Simons, 1975; Tanford and Reynolds, 1976). A nonionic detergent is preferable to use, since it has a low or no denaturing effect on the hydrophilic parts of the proteins, which are likely to contain the epitopes inducing protective immunity. For certain methods, its relatively low molecular weight and the fact that it forms micelles at a high concentration, facilitate its removal by the dialysis method. Two such candidates for disintegration of viral envelopes are octylglucoside (Helenius *et al.*, 1979) and MEGA (Hildreth, 1982; Hanatani *et al.*, 1984) (Fig. 5).

4.1. Centrifugation Method

The centrifugation method is suitable for the preparation of ISCOMs containing viral envelope proteins (Morein *et al.*, 1984). Briefly, after harvest from the cell culture, cells and cell debris were removed by differential centrifugation at low speed. The virus is usually concentrated and purified by centrifugation through a buffered sucrose gradient at ~100,000*g* forming a zone of virus or a

pellet. The purified virus is disintegrated with a detergent, preferably a nonionic detergent. The solubilized viral proteins are sedimented through a thin layer of 10% sucrose containing 0.5% detergent, e.g., Triton X-100, on top of a 20–50% sucrose gradient containing 0.1% Quil A (Fig. 6). Following rate zonal centrifugation (McEven, 1967), the ISCOMs can be obtained sedimenting at about 19 S, whereas monomer proteins will stay at the upper part of the gradient material, at approximately 4 S (Fig. 6). Protein micelles of envelope proteins generally have a higher sedimentation coefficient of about 30 S (Morein *et al.*, 1984; Helenius and Bornsdorf, 1976; Sundquist *et al.*, 1988a). The ISCOM matrix has a sedimentation coefficient of 14–18 S, depending on lipid content, which is lower than for the ISCOMs (Lövgren and Morein, 1988).

4.2. Dialysis Method

Basically, the formation of ISCOMs takes place while the detergent molecules are removed from the mixture of protein, lipid, detergent, and, importantly, Quil A (Fig. 6). Therefore, other methods can be used to remove the detergent. For instance, we have used a dialysis method that requires dialyzable detergents, e.g., octylglucoside or MEGA (see Fig. 5). The dialysis is performed extensively, e.g., for 3 days, against a desired buffer. In the dialysis bag, several components, not included in the ISCOMs, may be present. The ISCOMs can then be separated from contaminants by centrifugation through a cushion of 20% sucrose. A thin layer of detergent on top of the sucrose will result in retention of excess lipid and Quil A due to a slower sedimentation through the sucrose than for the ISCOMs. Nonamphipathic proteins, such as nucleoproteins of feline leukemia virus and HIV, will also be removed.

At the preparation of viral ISCOMs, there is a major concern as to how the virus is produced, e.g., what cell line is used for virus propagation, which

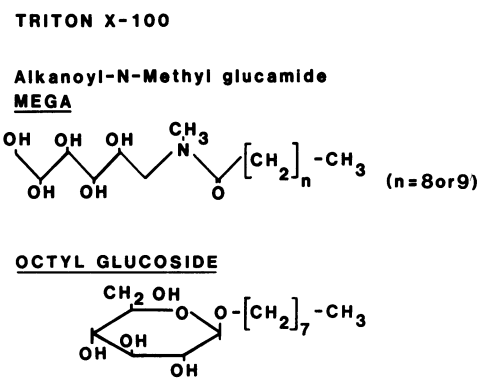

FIGURE 5. The nonionic detergents Triton X-100, MEGA, and octylglucoside.

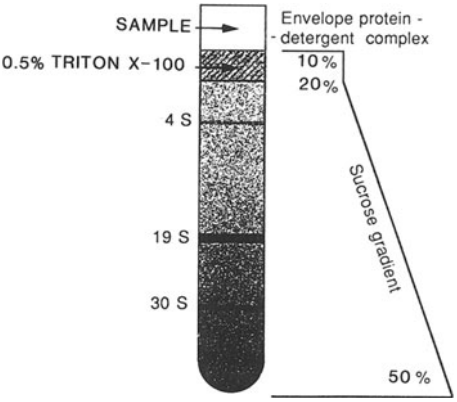

FIGURE 6. Preparation and isolation of ISCOMs by rate zonal centrifugation. The sample of envelope protein–detergent complex (mixed micelles of protein and detergent molecules) applied on 10% sucrose containing 0.5% Triton X-100, which will trap excess of Quil A and lipid ; below is a 20–50% sucrose gradient containing 0.1% Quil A. By centrifugation, 19 S ISCOMs are formed and separated from 4S monomer proteins and 30S micelles.

methods are employed for viral purification and subsequent viral disintegration, and what system is used for the reassembly of viral proteins into the ISCOM. An example is given below to demonstrate problems in preparation of ISCOMs from HIV proteins due to cellular components incorporated in the envelope of HIV (Gelderblom *et al.*, 1987; Henderson *et al.*, 1987; Hoxie *et al.*, 1987) and the loss of relevant antigen (Gelderblom *et al.*, 1985, 1987; Höglund *et al.*, 1986; 1988).

4.3. ISCOM of HIV Antigens

The HIV used was the HTLV IIIB strain grown on the human H9 cell line that expresses the class II transplantation antigen HLA-DR. Like other retroviruses, HIV has two envelope proteins, either loosely connected or completely disconnected, that originate from one intracellular precursor protein gp160. During intracellular transport, gp160 is cleaved by trypsin or a trypsin-like enzyme to the transmembraneous part gp41 and the external part gp120, both being glycosylated. During the budding process, gp120 seems to be detached from the anchoring transmembraneous gp41 (Gelderblom *et al.*, 1987).

Specific requirements have been raised in the production of an HIV vaccine, which have not yet been met in the field of viral vaccination (Barnes, 1988; Newmark, 1988). For example, it is obligatory to prepare the immunogen freed of nucleic acid because of inherent risk of the virus genome to become infectious or to be integrated into the genome of the host cell. A prospective protective antigen of HIV is most likely gp120; however, gp41 and p17 would also be required possibly to induce protective immunity (Barnes, 1988). Gp120 is not readily incorporated into ISCOMs, lacking an accessible hydrophobic region, in contrast to gp41, which has at least three sequences of hydrophobic amino acid

residues (Modrow *et al.*, 1987). During purification of the virus, gp120 is efficiently separated from the virus, leaving a viral particle where more than 95% of gp120 has been detached. Such material has been used for vaccination experiments, e.g., with SIV proteins (Letvin *et al.*, 1987), obviously with a poor result as regards the immune response to gp120.

ISCOMs have been prepared from HIV proteins according to the method described above (Höglund *et al.*, 1989). The virus used was HIV, strain HTLV IIIB, grown in H9 cells. The HLA-DR protein, incorporated in the envelope of HIV, became the major constituent of the ISCOMs, whereas gp120 became a minor component hardly detectable by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and with immunoblotting techniques. Such ISCOMs induced high-antibody titers to HLA-DR in mice, rabbits, and guinea pigs compared with the barely detectable antibody response to gp120. However, purified gp120 integrated into ISCOMs induced a high-antibody response. Antibody response was also evoked to other proteins incorporated into the ISCOMs, e.g., p24 of the nucleocapsid and the myristylated p17 of the envelope (Gelderblom *et al.*, 1987). The protein p24 can be removed from the ISCOMs by centrifugation through a detergent layer, but not the myristylated p17. It is also noteworthy to mention that HLA-DR can be removed from the viral proteins by immunosorbent affinity chromatography using specific monoclonal antibodies (Akerblom and Villacrez, 1987).

In conclusion, it should be emphasized that identification and quantification of the proteins of the ISCOM preparation is desirable to predict since the immune response to the ISCOM reflects the antigens included (Höglund *et al.*, 1989). The quality of the ISCOM preparations is also routinely analyzed by electron microscopy; the morphology and homogeneity of the ISCOM particles are then assayed.

5. INTEGRATION OF NONAMPHIPATHIC PROTEINS INTO ISCOM

Initially, the assembly of proteins into an ISCOM was done with native membrane proteins with a readily recognized transmembraneous region, which is surrounded by lipids *in situ* in the virus or in the cell. However, there is also of great interest to enhance the immunogenicity of a number of nonmembrane proteins. Many of these, perhaps the majority, have domains of hydrophobic amino acid residues hidden in the tertiary structure of the protein. Once made accessible, these hydrophobic amino acids may provide the necessary nonpolar character to attach the proteins to the ISCOM matrix. The possibility of enhancing the immunogenicity also encompasses a number of gene technology products (Moss *et al.*, 1988; Murray, 1988). Compared with the isolation of native mem-

brane proteins, extraction and purification of polypeptides prepared by recombinant DNA (rDNA) techniques will often include harsh methods such as urea and SDS treatments. In particular, proteins cloned in *Escherichia coli* often contain sequences of hydrophobic amino acids, which may or may not be hidden in the tertiary structure of the protein. If not hidden, difficulties in the isolation and purification procedures will arise due to the insolubility of the protein in a polar buffer.

5.1. Model System

In attempts to demonstrate nonaccessible hydrophobic domains, two methods were evaluated: proteins were exposed to acidic pH or to heat treatment. In a model system, bovine serum albumin (BSA) was employed. Acid treatment of BSA at pH 2.5 in the presence of the detergent, MEGA-10 (see Fig. 5), cholesterol, phosphatidylcholine, and Quil A was followed by the removal of the detergent by dialysis. About 15% of BSA then became integrated into ISCOMs, according to the criteria described above. Alternatively, when BSA was exposed to 70°C instead of acid exposure, it also became integrated into ISCOMs. The recovery of integrated BSA ranged between 30% to 50% following heat treatment.

The immunogenicity of both acid- and heat-treated BSA incorporated into the ISCOM was tested in mice. Both preparations were more immunogenic than native and nonintegrated BSA. The serum antibody response was 10-fold higher for ISCOM preparations. Also, the dose of BSA could be reduced by 10-fold, e.g., 100 ng BSA in ISCOMs induced a serum antibody response in mice comparable to or better than 1 μg nonintegrated BSA induced (Morein *et al.*, 1989a).

5.2. Gp120 ISCOM

The experience with acid treatment of BSA for the preparation of ISCOMs was applied to purified gp120 of HIV. The resulting gp120 ISCOMs proved highly immunogenic in rhesus monkeys and chimpanzees. After multiple immunizations with ISCOMs containing gp120 of HIV-1B, a rhesus monkey developed a transient *in vitro* neutralizing response to the HIV-1 isolates RF and MN (Pyle *et al.*, 1989).

5.3. Gene-Technology Products

Proteins cloned in *E. coli* have been incorporated into ISCOMs. Examples are a *gag*-gene product from HIV, an *env*-gene product of HIV corresponding to the carboxy-terminal part (kindly supplied by S. Putney, Repligen). Both prepa-

rations were highly immunogenic in mice. Gp160 of HIV has been cloned in a vaccinia vector and produced by Vero cells, expressed as the uncleaved gp160 antigen (kindly supplied by Immuno AG, Vienna). Thus gp160 has the transmembraneous part, gp41, and does not need any treatment to expose hidden hydrophobic domains to be incorporated into ISCOMs.

5.4. Picornavirus

Picornaviruses do not have envelope proteins with transmembraneous hydrophobic domains. However, hydrophobic amino acid sequences exist. Fohlman *et al.* (1989) integrated three capsid proteins of coxsackievirus (CB3) by treating the virus with SDS and urea and subsequent removal of the chemicals. In a model experiment in mice, 16 ng injected three times completely protected the mice from mortality when challenge-infected with a myocarditic strain of CB3.

In conclusion, nonamphipathic proteins can also be rendered hydrophobic to be integrated into ISCOMs, and an enhanced immunogenicity can thereby be obtained. To be able to make hydrophobic domains accessible, however, partial denaturation is required that might affect crucial antigenic determinants. Since there are alternative methods for partial denaturation, a quality-control system for the important antigenic determinants should be established. Such a system is described by Merza *et al.* (1989) for gp50 of bovine leukemia virus (BLV) using monoclonal antibodies that recognize neutralizing epitopes. With the aid of this quality control, methods were worked out to make BLV ISCOMs with non-destroyed (native) neutralizing epitopes.

6. ISCOMS AS CARRIERS FOR SMALL MOLECULES AND PEPTIDES

The poor immunogenicity of small molecules, including synthetic peptides, is a well-known problem. To improve their immunogenicity, these molecules have been conjugated to carrier molecules, e.g., BSA, keyhole limpet hemocyanin (KLH), or random amino acid co-polymers (Erlanger, 1980; Audibert, 1982; Palfreyman *et al.*, 1984). However, in order to induce a more substantial immune response, it is generally necessary to include an adjuvant, e.g., Freund's complete adjuvant or Freund's incomplete adjuvant (Freund, 1956).

Coupled to a carrier protein, haptens may be considered as epitopes or antigenic determinants of the carrier protein. As shown by Morein and coworkers (1978, 1982, 1984, 1985), multimeric forms have several immunogenic advantages over monomeric proteins. Consequently, a hapten or a small molecule conjugated to a carrier protein included in an ISCOM should be a considerable improvement to the monomeric carrier protein.

Hapten Model

Instead of a peptide, succinyl-activated biotin was used as a hapten model (Lövgren *et al.*, 1987). The coupling reaction is easily monitored, since ^{3}H-labeled biotin is available. The conjugates produced differed only as to the number of biotin molecules coupled per carrier protein in the ISCOM, ranging from 0.1 to 20. It was concluded that at a dose of 1 μg conjugate, approximately 10 biotin molecules per protein were optimal for the antibody response. It is likely that 10 biotin molecules were needed to expose one of them as a highly immunogenic antigen determinant of a protein. In additional experiments, biotin conjugated to different carrier systems were studied as immunogens in mice. The multimeric carriers, i.e., ISCOMs and micelles, were highly immunogenic in contrast to the monomeric BSA. Ten μg of biotin-ISCOMs induced about three times higher antibiotin titers than did the same amount of biotin–micelles or biotin–BSA in Freund's complete adjuvant. Biotin–BSA inoculated without adjuvant induced only a poor antibody response.

In conclusion, these results (Lövgren *et al.*, 1987) clearly indicate that the ISCOM is an efficient carrier system for hapten presentation. This system has also been adapted for efficient presentation of synthetic peptides, including viral peptides. The dose can be kept low, $\leq$10 μg, and no additional adjuvant is required.

7. INDUCTION OF IMMUNE RESPONSE INCLUDING PROTECTION BY ISCOMS

Several reports showed that ISCOMs produced with various antigens induce high antibody response, e.g., against influenzavirus, paramyxovirus, togavirus, and coronavirus (Morein *et al.*, 1987; Sundquist *et al.*, 1988b). Generally, antigens included in ISCOMs induce a 10-fold higher antibody titer than do the corresponding antigen *in situ*, e.g., in the virus particle or in a protein micelle (Morein and Simons, 1985; Morein *et al.*, 1987).

7.1. Influenzavirus

Recently, Lövgren (1988) showed that ISCOMs containing the hemagglutinin and the neuraminidase glycoproteins from the influenzavirus H1N1, PR8 strain induced a classic serum antibody response in mice after both subcutaneous and intranasal immunization with an early immunoglobin M (IgM) response, followed by an IgG response. The antibodies were also shown to be evenly distributed in all IgG isotypes, i.e., IgG1, IgG2a and b, and IgG3. In this connection, we would like to emphasize the fact that the ISCOM efficiently

induced antibody- as well as cell-mediated immunity (CMI) following local intranasal immunization. Cell-mediated immunity was demonstrated by Jones *et al.* (1988), who showed that the H1N1 influenzavirus ISCOMs after intranasal application in mice induced a cytotoxic T-cell memory response, inducing a broadened specificity with reactivity to the subserotype H3N2 of influenzavirus. In contrast to live experimental vaccine, the ISCOMs efficiently boostered the cytotoxic T-cell response.

7.2. Herpesvirus

For the herpesvirus, the T-cell response is considered important for protective immunity but less so for antibody-mediated immunity. Wahren *et al.* (1987) showed that ISCOMs as carriers for cytomegalovirus (CMV) antigens induced high CMI, measured with the lymphocyte-stimulation test in monkeys, in contrast to a whole-virus preparation. This CMI response was of the same magnitude, measured in counts per minute (cpm), of incorporated [^{3}H]thymidine, as that of naturally infected but healthy man. Monkeys immunized with CMV carried by monocytes also showed a CMI response provided a high dose of CMV; i.e., 150 μg was included. By contrast, when CMV antigens were presented by the ISCOMs a low dose of 15 μg was sufficient.

7.3. Measles Virus

In a laboratory animal model system with BALB/c mice, DeVries *et al.* (1988a) studied the activation of measles virus-specific T cells by the F protein incorporated into ISCOMs. It was demonstrated that a measles virus-specific delayed-type hypersensitivity (DTH) response was induced. The mice were immunized with 0.25 or 1.0 μg ISCOMs or micelles containing the fusion (F) protein of measles virus; they were challenged 7 days later with whole measles virus in the ear. A specific ear swelling was measured during the following three days, which was most prominent in the mice immunized with the ISCOM preparation of F protein. The F–ISCOM-immunized animals showed specific swellings of maximal 76 μm or 153 μm, respectively, whereas the F–micelle-immunized animals showed specific swellings of maximal 25 or 75 μm above background values, respectively.

The ability of F–ISCOM to induce F-specific T-cell response in mice was further shown by isolating F-specific T-cell clones from F–ISCOM-immunized BALB/c mice. The clones were shown to be specific both for ultraviolet (UV)-inactivated measles virus and for purified F protein. Most cells from each T-cell clone stained for Thy-1,2, Lyt-1, and L3T4 markers, a pattern characteristic of murine T-helper cells. The functional characterization of these clones showed that they were able to assist B-cells in the production of measles virus-specific antibodies *in vitro* (DeVries *et al.*, 1988a).

7.4. Rabiesvirus

Also in the rabiesvirus system, the activation of virus-specific T cells by viral proteins presented in the ISCOM was shown in a rabiesvirus-specific DTH response (Osterhaus *et al.*, 1986). BALB/c mice immunized with 0.1, 1, or 10 μg rabiesvirus ISCOMs were challenged 7 days later with inactivated rabiesvirus in the ear, and specific ear swelling was measured during the following 3 days. A specific ear swelling to a maximum of about 40% of the ear thickness was observed in the animals immunized with 1 or 10 μg of the ISCOM preparation. The ability of the rabiesvirus ISCOM to stimulate rabiesvirus specific T-cells in man was indicated by proliferative responses of rabiesvirus-specific human T-cell clones of the helper-cell phenotype upon stimulation with the ISCOM in the presence of antigen-presenting cells (Fig. 8). Importantly, CMI response was efficiently induced in different animal species by immunization with ISCOMs containing glycoproteins from cytomegalovirus, rabiesvirus, influenzavirus, and measles virus.

8. PROTECTION

Protection studies, if possible in the host, are the relevant tests of a vaccine. Experimental ISCOM vaccines have been tested in various animal species (Table I) and are summarized below.

8.1. Bovine Viral Diarrhea Virus

Bovine viral diarrhea virus (BVDV) belonging to the *Pestivirus* genus in the Togavirus family is closely related to border disease virus (BDV) of sheep and hog cholera virus (HCV) of swine. The most common form of BVDV is subclinical, but it can occur sporadically with severe clinical signs with low and high morbidity and mucosal disease. Recent investigations have indicated that dams may produce persistently infected offspring and that these individuals are the most important source for the spread of the infection. In susceptible dams or ewes, infection with BVDV or BDV can result in abortion, mummified fetuses, as well as persistently infected offspring. Efficient prophylaxis against BVDV and BDV is needed to protect against reproduction disturbances caused by these viruses in cattle and sheep. Consequently, such animals should be vaccinated before breeding and insemination. For that purpose, an experimental ISCOM vaccine was designed to contain the envelope proteins of a cythopathic BVDV isolate. This vaccine was tested in pregnant sheep seronegative for BVDV (Alenius *et al.*, in press, 1989). After two vaccinations spaced three weeks apart, 15 pregnant ewes responded with high serum-neutralizing antibody with a mean titer of 625 in a microplate test, while 14 nonvaccinated ewes were still

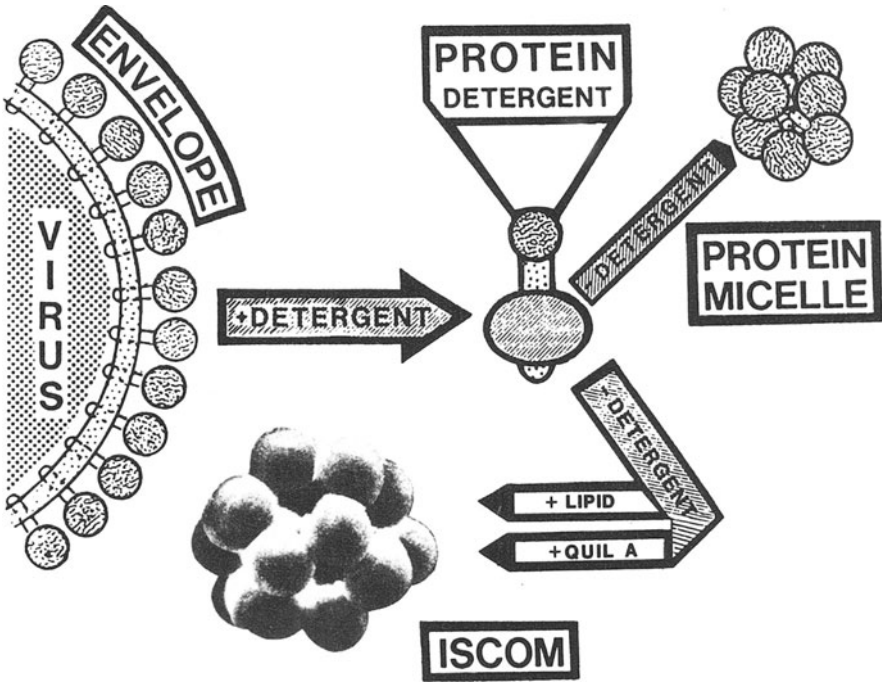

FIGURE 7. Schematic drawing of ISCOM preparation from viral envelope proteins. A detergent causes disintegration of the envelope, so that it will release spike protein–detergent complex. Removal of detergent (e.g., by dialysis) will form protein micelles. Addition of Quil A and lipid, followed by the removal of detergent, will result in the assembly of Quil A, lipid and spike proteins into a symmetrical shell of globular subunits of the ISCOM particle.

seronegative. At that time, 28 and 26 fetuses were detected, respectively, in the two groups of ewes, by ultrasonic technique. The animals were challenge-infected with a recent field isolate 3 weeks after the second vaccination, i.e., between the 48th to 65th days of gestation. The 15 vaccinated ewes lambed 26 liveborn lambs, whereas the BVDV infection resulted in extensive fetal death and the 14 nonvaccinated ewes gave birth to only six liveborn lambs. This experiment demonstrates that an experimental BVDV ISCOM vaccine induced high serum-neutralizing antibody and a complete protection from abortion.

Table I
Protective Immunity Induced by Various ISCOM Preparations

Antigen	Animal	Disease	Reference
Epstein–Barr virus	Tamarin monkey	Lymphoma	Morgan *et al.* (1988)
Pseudorabies virus	Swine	Lethal	Sundquist *et al.* (1988)
Bovine herpesvirus type 1	Cattle	Disease	Trudel *et al.* (1988)
Bovine viral diarrhea virus	Sheep	Abortion	Alenius *et al.* (1989)
Feline leukemia virus, gp70/85	Cat	Viremia	Osterhaus *et al.* (1985)
Hemagglutinin of measles virus	Mouse	Encephalitis	deVries *et al.* (1988a); Varsanyi *et al.* (1987)
Fusion protein of measles virus	Mouse	Encephalitis	deVries *et al.* (1988a); Varsanyi *et al.* (1987)
Hemagglutinin, fusion protein of canine distemper virus	Dog	Viremia	deVries *et al.* (1988b)
Hemagglutinin, neuraminidase (H3N8), equine influenzavirus	Horse	Pneumonia	Mumford *et al.* (1986)
Toxoplasma surface antigen	Mouse	Lethal	Uggla *et al.* (1989)

8.2. Epstein–Barr Virus

There is a compelling evidence that infection with Epstein–Barr virus (EBV), belonging to the Herpesviridae family, is one of the causative events leading to the subsequent development of two important human malignancies, i.e., undifferentiated nasopharyngeal carcinoma, prevalent in China and Burkitt lymphoma, endemic in Africa. Since the former is the most common cause of cancer in men of Southern China, there is a need for a development of a vaccine to prevent or reduce the incidence of these diseases. The major envelope glycoprotein of EBV, gp340, is believed to play an important role in the induction of protective immunity and would therefore be the antigen of choice in a prospective subunit vaccine. To evaluate an EBV vaccine, an animal model has been developed using the cottontop tamarin. ISCOMs were prepared to contain purified gp340 by the dialysis method and were used at a dose level of 2 μg to vaccinate four tamarins (Morgan *et al.*, 1988). The animals were immunized three times with intervals of 2 weeks. Serum antibody was shown to neutralize EBV and EBV-induced transformation of cord blood lymphocytes. When the tamarins were infected with a challenge dose of EBV, no tumor development occurred in the vaccinated animals, although some transient lymph node enlargements were detected. However, the histologic examination showed no evidence of the presence of lymphomatous tissue reaction but of an inflammation consisting of reactive lymphomatous tissue. By contrast, in a group of four nonvaccinated tamarins, which had received the same challenge dose, the monomorphous appearance of lymphomas appeared during the third week following challenge.

These data indicate that microgram doses of gp340 incorporated into ISCOMs can protect tamarins against a complete lymphomagenic challenge of EBV, which would suggest that there is a future possibility for use of EBV vaccine in man.

8.3. Pseudorabies Virus

Pseudorabies or Aujezsky disease is caused by another herpesvirus, pseudorabiesvirus (PRV), which affects the respiratory tract, central nervous system, and reproduction system of swine. Morbidity and mortality rate are high and can often reach 100% in young suckling pigs. Since an eradication program of PRV employing stamping out has almost eliminated PRV in the pig populations in Denmark and England, it is likely that other countries will follow a similar policy. However, for economical reasons, many countries would like to combine an eradication program with a vaccination program. Such a program would require a diagnostic test differentiating PRV-infected from PRV-vaccinated animals. Since the ISCOM technology will selectively choose the envelope proteins, in principle it would not induce antibody to the internal proteins of the virus. In an experiment designed to explore that possibility, at least five proteins found to be present in the whole-virus preparation were lacking in the ISCOM and did not induce antibody in the vaccinated animal (Morein *et al.*, 1989b). This opened a possibility to use a competition enzyme-linked immunosorbent assay (ELISA) directed against one or more of these proteins to differentiate PRV-infected animals from vaccinated ones.

For a potency test of PRV, vaccine pigs are used. In pigs, however, the morbidity rate following experimental infection is variable and is also age restricted, which is noticeable in pigs over 6 weeks of age, at which time the animals hardly show any symptoms. In an alternative potency test model, lambs have been used for efficacy documentation (Morein *et al.*, 1989b). Lambs are extremely sensitive to PRV infection, and the infection is not age restricted, as it is in the pig. The ISCOM vaccine containing the envelope glycoproteins of PRV was tested in Hungary in lambs according to their potency test protocols (T. Soos, Control institute of Veterinary Biology, Budapest, Hungary). After a single vaccination with 3 μg PRV antigen per dose, the lambs were completely protected to challenge infection in contrast to the animals in the nonvaccinated group, in which the infection caused a 100% mortality (Morein *et al.*, 1989b).

8.4. Infectious Bovine Rhinotracheitis Virus

Infectious bovine rhinotracheitis (IBR) virus, or bovine herpes virus type 1, infects the respiratory tract of cattle; a variant of the virus infects the vagina and uterus. It is a major cause of economical losses in the cattle industries. Several

vaccines have been tried and are available on the market, but their efficacy is questionable. Trudel *et al.* (1988) prepared ISCOMs containing the envelope proteins of IBR virus and reported that a dose of 50 μg protein induced protection to challenge infection. In contrast to commercially available vaccines, the ISCOMs conferred complete protection to disease. The animals showed no temperature rise, and there was a 10^5 reduction in virus excretion measured in secretion, collected as nasal swabs, as compared with unvaccinated challenge-infected animals.

8.5. Measles Virus

ISCOMs were prepared to contain the fusion (F) protein of measles virus, isolated from virus by immunosorbent affinity chromatography. These ISCOMs induced anti-F protein antibodies in monkeys shown by hemolysis inhibition and cell-fusion inhibition assays (DeVries *et al.*, 1988a). ISCOMs prepared from a whole virus contained both the F protein as well as a small amount of hemagglutinin. In contrast to the pure F–ISCOM preparation, they also induced hemagglutination-inhibiting (HI) and virus-neutralizing serum antibodies in monkeys and in rats, besides the hemolysis-inhibiting antibodies. The BPL-inactivated measles virus preparation, from which the ISCOM preparation had been produced, was less efficient in inducing virus-neutralizing and hemagglutination-inhibiting antibodies and failed completely to induce hemolysis-inhibiting antibodies in rats (Morein *et al.*, 1984). DeVries *et al.* (1988a) could protect mice against fatal encephalopathy with ISCOMs prepared from whole virus or with F-protein ISCOMs. Varsanyi *et al.* (1987) used ISCOMs containing cell-derived measles virus envelope proteins depleted of either the H protein or the F protein by an immunosorbent technique. Both preparations induced protective immunity in mice to intracerebral challenge of a measles virus strain causing fatal encephalopathy.

In developing countries, measles virus is a major cause of mortality in young children. Therefore, it is desired to find a vaccine that can induce protective immunity, while the child still has maternal antibodies. In an attempt to mimic passive maternal immunity mice were passively given antibodies to measles virus. An antibody response could be induced in the passively immunized mice much more efficiently with a measles virus–ISCOM than with a whole measles virus preparation (DeVries *et al.*, 1988a).

8.6. Canine Distemper Virus

The potential of the ISCOM for the induction of protective immunity against morbillivirus infection was further shown by immunization of dogs with canine distemper virus (CDV)–ISCOMs, which contained both the F protein and

the hemagglutinin of CDV. The dogs developed virus-neutralizing antibodies; in contrast to the nonvaccinated animals, they did not develop viremia or clinical signs upon intranasal challenge with the virulent Snyder–Hill strain of CDV. Immunization of the dogs with measles virus ISCOMs, prepared from either purified F protein or purified virus, resulted in partial protection against disease following challenge infection with CDV (DeVries *et al.*, 1988b).

8.7. Rabies Virus

Rabies virus vaccines are generally tested for efficacy in the National Institutes of Health (NIH) test (Atanasin, 1973; Seligman, 1978). In this test, mice are immunized twice 2 weeks apart. Such an immunization scheme is unfavorable for ISCOM vaccines, as we have found that a considerably longer interval is much better. For instance, with ISCOMs containing gp160 of HIV, an interval of 6 weeks was optimal, while no or hardly any booster effect was observed when the interval was only 2 weeks. In such an NIH test, rabiesvirus ISCOMs, containing the 80-kDa G protein have been tested [F. Makonnen Centers of Disease Control (CDC), Atlanta, personal communication] and by Osterhaus (1986). In a number of independent experiments with ISCOMs prepared from different rabiesvirus strains, it was found that the rabies ISCOM vaccines induced virus-neutralizing antibodies and protection in mice at least as efficient as with whole rabies virus (Osterhaus *et al.*, 1986). In an *in vitro* system for human antibody production against rabies virus, it was shown that ISCOMs were more efficient than the virus at inducing rabiesvirus-specific antibodies (UytdeHaag *et al.*, 1983; Osterhaus *et al.*, 1986). (Fig. 9).

In countries in which rabies virus is endemic, only limited resources are available for mass vaccination; consequently, vaccination often has to be given after suspected exposure to infection. Therefore, mice were pre-exposed to 50–100 LD_{50} doses of rabiesvirus before being subsequently vaccinated with ISCOMs containing 3 μg G protein per dose; 90% of the ISCOM-vaccinated mice survived the challenge infection, while the same dose of commercial rabies vaccine-induced protection at a level of 30%, using the same immunization procedure (Makonnen, personal communication).

Using standard techniques for the preparation of ISCOMs from concentrated virus harvests, it was found that the ISCOM preparations contained levels of contaminating nucleic acids below 10 pg per dose. This observation would in principle make it possible to produce high yields of rabiesvirus in continuous cell lines, e.g., BHK cells, for the production of human rabies vaccines (Osterhaus, personal communication). No nucleic acid-free rabies vaccine is available on the market.

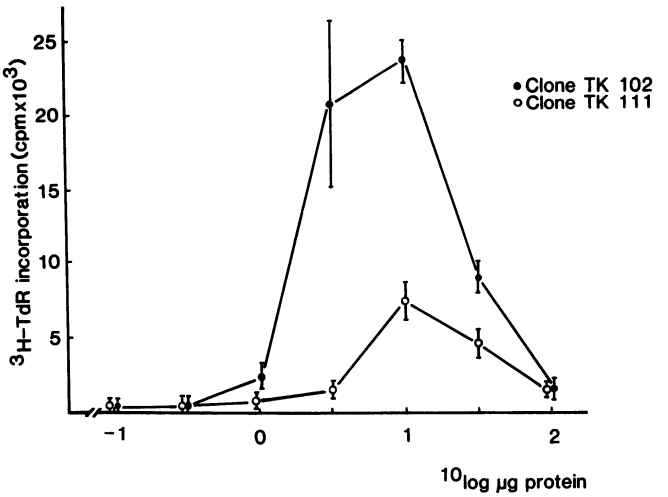

FIGURE 8. Proliferation of rabies virus-specific human T-cell clones upon stimulation with ISCOM.

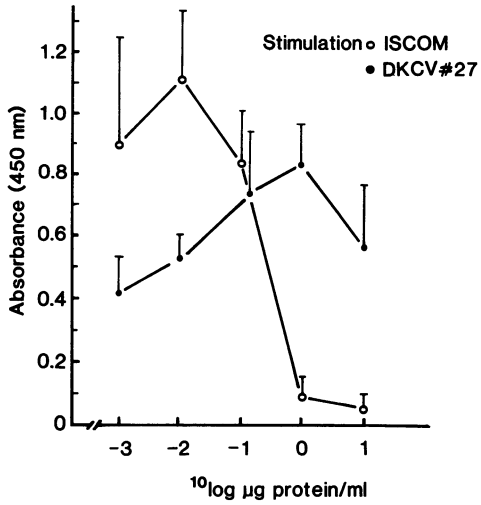

FIGURE 9. Secondary *in vitro* antibody (ELISA) synthesis by human PBL upon stimulation with rabies vaccine.

8.8. Equine Influenzavirus

Equine influenzaviruses, belonging to the Orthomyxoviridae family, are pathogens of the ciliated respiratory tract of horses, like the influenzaviruses infecting man. In susceptible horses, the inhalation and subsequent replication of virus may produce multiple epithelial wounds and disruption of the clearance mechanism of the ciliated epithelial cells. This damage of the epithelial surface might become the site for secondary infections. Two subserotypes infect horses: A/eq 1 (H7N7) and A/eq 2 (H3N8). The latter is the most pathogenic virus, causing worldwide epidemics, mostly among young, but also among old horses. Despite the fact that horses have been vaccinated with available vaccines containing A/eq 2 (H3N8) strains, they became infected, resulting in the development of clinical signs. The conventional vaccines induce a short-lived immune response with protective levels of antibodies lasting less than 6 months or even 4 months. Thus, a need for an improvement of available vaccines is obvious.

In order to study the immunogenicity of influenza virus ISCOMs, a monovalent experimental vaccine was prepared with the envelope glycoproteins hemagglutinin (HA) and neuraminidase (NA) of the equine virus strain A/eq 2/Solvalla/79, which had caused an epidemic among horses in Sweden. Six Welsh Mountain yearling ponies, seronegative for influenzavirus, were vaccinated with 15 μg HA per dose (Mumford *et al.*, 1986). The six ponies received four doses of ISCOMs administered with 8-, 27-, and 52-week intervals between doses. Six nonvaccinated ponies were included in the study as a control. The ISCOM vaccine induced comparatively high hemagglutination-inhibition (HI)-antibody response; these titers were detectable during a 50-week period after the third immunization. In a challenge experiment, a recent A/eq 2 H3N8 isolate A/eq 2/Newmarket/79 was used for challenge infection 10 weeks after the last vaccination. All nonvaccinated animals developed disease symptoms as assessed by measurement of pyrexia and coughing in contrast to all ISCOM-vaccinated animals, which were fully protected from the disease (Hannant *et al.*, 1987; Sundquist *et al.*, 1988b).

8.9. Feline Leukemia Virus

Oncogenic RNA viruses cause leukemia and sarcoma in several animal species. Many attempts have been made to produce vaccines against retroviruses (Morein *et al.*, 1986; Ferdinand *et al.*, 1987; Merza *et al.*, 1989; Åkerblom *et al.*, 1989). One such virus is the feline leukemia virus (FeLV), which causes a transient, persistent, latent viremia in experimentally and naturally infected cats. In a FeLV subunit vaccine, the envelope gp70/85 antigen was incorporated into ISCOMs. Immunization of specific pathogen-free (SPF) cats resulted in virus-neutralizing antibodies. A proportion of these antibodies proved to be directed to a neutralizing epitope on gp70, which was shown in competition ELISA with a

virus-neutralizing monoclonal antibody, which reacts with all three subtypes of FeLV (Osterhaus *et al.*, 1985). The protective effect of FeLV ISCOM was further shown in vaccination experiments in which six 8-week-old SPF cats were immunized with FeLV ISCOMs and subsequently challenge-infected oronasally. All the cats developed virus-neutralizing antibodies, but none of the vaccinated cats developed viremia, while four of the six nonvaccinated control cats became viremic. To assess the potential of the ISCOM further as a vaccine against FeLV, a comparative trial was conducted with a commercial FeLV vaccine containing the same amount of glycoprotein gp70/85 per dose. Three groups of privately owned cats, kept under conventional conditions in single- or multiple-cat households, were vaccinated three times with either the commercial vaccine, FeLV ISCOM, or a control preparation not containing FeLV antigen. Serological responses were measured by ELISA, membrane immunofluorescence, virus neutralization, and Western blotting both before and after vaccination. Less than 6% of the cats vaccinated with the commercial vaccine developed a serological response, and there was no statistical difference from the control group. By contrast, more than 80% of the animals vaccinated with the ISCOM preparation responded in all tests (Osterhaus *et al.*, 1987; Osterhaus, *et al.*, 1989). The experiments reported clearly demonstrated that ISCOMs of viral antigens could induce protection from clinical signs, caused by viral infections, e.g., lethal disease, abortion, pneumonia, and tumorgenesis.

9. ISCOMS AS ANTIGENS IN IMMUNOASSAYS

A likely reason for the enhanced immunogenicity of antigens in the ISCOMs is that the antigens are well exposed on the surface. Consequently, the ISCOM should also be an effective structure for use as antigen in immunoassays. Since the membrane proteins on the surface of microorganisms, e.g., virus envelope, often are species specific, in contrast to many of the internal proteins (Maizels *et al.*, 1982); these proteins are the antigens of choice for specific immunoassays. Within this context, the ISCOM is an interesting structure for immunoassays because the procedure for preparation is selective for membrane proteins. This concept was recently tested for *Toxoplasma gondii* using the surface membrane proteins as antigen in an ELISA to measure serum antibody to this parasite in sheep and in calves. For detection of low levels of antibody to *T. gondii,* the ISCOM ELISA was found to be as sensitive as an immunofluorescence technique (IFAT) employing intact parasites or a conventional antigen ELISA, in which a whole-cell extract of the parasite is used as antigen. However, the ISCOM ELISA was found more specific than a conventional *Toxoplasma* ELISA, as exemplified by the fact that the conventional ELISA recorded antibody to the related parasite *Sarcocystis cruzi.* By contrast, no cross-reactions were observed using the IFAT or the

ISCOM ELISA (Uggla *et al.*, 1989). It was concluded that the ISCOM ELISA will combine the specificity of the IFAT with the simplicity and objectivity of the conventional ELISA. We routinely use the ISCOM technology to produce antigen for ELISA to assay for subserotype-specific response to influenza and para-influenza viruses.

10. EPILOGUE

The ISCOM technology offers a way to enhance both antibody-mediated immunity (AMI) and cell-mediated immunity (CMI). This enhancement results in longer lasting immunity, thereby reducing the required number of immunizations—an important attribute for nonreplicating vaccines. As regards CMI, it is worth noting that the ISCOMS induce cytotoxic T-memory cells; this is unique for a nonreplicating vaccine form. The technology has been considered to have great significance for viral vaccines because of its importance in protective immunity. Another interesting factor is the use of ISCOMs for local application of the nonreplicating antigens that are important for evoking effective local immunity. Until now there have been few alternatives to replicating vaccines for induction of local immunity. This is partly due to a lack of adjuvants or adjuvant systems that enhance immunogenicity in local application on mucus membranes. Defined antigens, regardless of whether they originate from microorganisms propagated conventionally or by the aid of gene technology or chemical synthesis, require a system for antigenic presentation in order to optimally elicit immune response. This immunological response should in most cases include both B- and T-cell response. The construct of the ISCOM can make up the required antigen-presenting system. The first ISCOM vaccine for animal use is already on the market, and it is likely that a vaccine for humans will follow.

11. REFERENCES

Åkerblom, L., and Villacres, M., 1987, HIV iscoms: A vaccine model, in: *Tenth Scandinavian Virus Symposium,* Umeå, p. 72.

Åkerblom, L., Höglund, S., Strömstedt, K., Osterhaus, A., and Morein, B., 1989, Formation and characterization of FeLV iscoms, *Vaccine* (in press).

Alenius, A., Sundquist, B., and Carlsson, U., 1989, Protective effect of an iscom bovine diarrhea (BVD) virus vaccine against an experimental BVD virus infection in pregnant sheep, in preparation.

Atanasin, P., 1973, Quantitative assay and potency test of anti-rabies serum and immunoglobulin, in: *Laboratory Techniques in Rabies* (M. Kaplan and H. Koprowski, eds.), pp. 314–318, World Health Organization, Geneva.

Audibert, F., Jolivet, M., Chedid, L., Arnon, R., and Sela, M., 1982, Successful immunization with a totally synthetic diphtheria vaccine, *Proc. Natl. Acad. Sci. USA* **79**:5042–5046.

Bangham, A. D., and Horne, R. W., 1962, Action of saponin on biological cell membranes, *Nature (London)* **196:**952–953.

Barnes, D. M., 1988, Obstacles to an AIDS vaccine, *Science* **240:**719–721.

Bomford, R., 1980, The comparative selectivity of adjuvants for humoral and cell-mediated immunity. II. Effect of delayed-type hypersensitivity in the mouse and guinea pig, and cell-mediated immunity to tumour antigens in the mouse for Freund's incomplete and complete adjuvants, Alhydrogel, *Corynebacterium parvum, Bordetella pertussis,* muramyl dipeptide and saponin, *Clin. Exp. Immunol.* **39:**435–441.

Brieskorn, C. H., and Briner, M., 1954, On the analysis of triterpenes. Colorimetric determination of the total content of triterpenoid acids in labiatae. *Arch. Pharm. Detsch. Pharm. Ges.* **287:** 429–432.

Dalsgaard, K., 1970, Thin layer chromatographic fingerprinting of commercially available saponins, *Dansk. Tidskr. Farm.* **44:**327–331.

Dalsgaard, K., 1974, Saponin adjuvants. III. Isolation of a substance from *Quillaja saponaria* Molina with adjuvant activity in foot-and-mouth disease vaccines. *Arch. Ges. Virusforsch.* **44:** 243–254.

Dalsgaard, K., 1978, The application of the saponin adjuvant Quil A in foot-and-mouth disease vaccines, *Bull. Off. Int. Epiz.* **89:**963–966.

Dalsgaard, K., 1984, Assessment of the dose of the immunological adjuvant Quil A in mice and guinea pigs using sheep red blood cells as model antigens, *Zbl. Vet. Med.* **B31:**718–720.

Dalsgaard, K., Jensen, M. H., and Sörensen, K. J., 1977, Saponin adjuvants. IV. Evaluation of the adjuvant Quil A in the vaccination of cattle against foot-and-mouth disease, *Acta Vet. Scand.* **18:**349–360.

DeVries, P., Van Binnendijk, R. S., Van der Marel, P., Van Wezel, A. L., Voorma, H. O., Sundquist, B., Uytdehaag, F. G. C. M., and Osterhaus, A. D. M. E., 1988a, Measles virus fusion protein presented in an immune-stimulating complex (Iscom) induces hemolysis-inhibiting and fusion-inhibiting antibodies, virus-specific T cells and protection in mice, *J. Gen. Virol.* **69:**549–559.

De Vries, P., Uytdehaag, F. G. C. M., and Osterhaus, A. D. M. E., 1988b, Canine distemper virus (CDV) iscoms, but not measles virus iscoms, protect dogs against CDV infection, *J. Gen. Virol.* **69:**2071–2083.

Dourmashkin, R. R., Dougherty, R. M., and Harris, R. J., 1962, Electronmicroscopic observations on Rous sarcoma virus and cell membranes, *Nature (Lond.)* **194:**1116–1119.

Erlanger, B. F., 1980, The preparation of antigenic hapten-carrier conjugates; a survey, *Methods Enzymol.* **70:**85–104.

Espinet, E. G., 1951, Nuevo tipo de vacuna antiaftosa a complejo glucovirico, *Gac. Vet.* **74:**1–13.

Ferdinand, F. J., Dorner, F., and Kurth, R., 1987, Perspectives of HIV vaccine developments, *J. Virol. Methods* **17:**63–67.

Fohlman, J., Ilbäck, N. G., Friman, G., and Morein, B., 1989, Vaccination of BALB/c mice against enteroviral mediated myocarditis, *Vaccine* (submitted).

Freund, J., 1956, The mode of action of immunological adjuvants, *Adv. Tuberc. Res.* **7:**130–148.

Gelderblom, H., Reupke, H., and Pauli, G., 1985, Loss of envelope antigens of HTLV-III/LAV, a factor in AIDS pathogenesis, *Lancet* **2:**1016–1017.

Gelderblom, H. R., Hausmann, E. H. S., Özel, M., Pauli, G., and Koch, M. A. K., 1987, Fine structure of human immunodeficiency virus (HIV) and immunolocalization of structural proteins, *Virology* **156:**171–176.

Glauert, A. M., Dingle, J. T., and Lucy, J. A., 1962, Action of saponin on biological cell membranes, *Nature (Lond.)* **196:**953–955.

Hanatani, M., Nishifuji, K., Futai, M., and Tsuchia, T., 1984, Solubilization and reconstitution of membrane proteins of *Escherichia coli* using alkanoyl-*N*-methylglucamides, *J. Biochem.* **95:** 1349–1353.

Hannant, D., Jessett, D. M. O. Neill, T., Sundquist, B., and Mumford, J. A., 1987, Nasopharyngeal, tracheobronchial, and systemic immune responses to vaccination and aerosol infection with equine influenzavirus (H3N8), in: *Seventh Equine Infection Disease Conference, Kentucky.*

Helenius, A., and Simons, K., 1975, Solubilization of membranes by detergents, *Biochim. Biophys. Acta.* **415**:29–80.

Helenius, A., and von Bornsdorf, C. H., 1976, Semliki Forest virus proteins. Preparation and characterization of spike complexes soluble in detergent-free medium, *Biochim. Biophys. Acta* **436**:895–899.

Helenius, A., McCaslin, D. R., Fries, E., and Tanford, C., 1979, Properties of detergents, *Methods Enzymol.* **56**:734–749.

Henderson, L. E., Sowder, R., Copeland, T. D., Oroszlan, S., Arthur, L. O., Robey, W. G., and Fischinger, P. J., 1987, Direct identification of class II histocompatibility DR proteins in preparations of human T-cell lymphotropic virus type III, *J. Virol.* **61**:629–632.

Hildreth, J. E. K., 1982, *N*-D-Gluco-*N*-methylalkanamide compounds, a new class of non-ionic detergents for membrane biochemistry, *Biochem. J.* **207**:363–366.

Höglund, S., Åkerblom, L., Gelderblom, H., Özel, M., 1986a, Characterization of HTLV-III iscom, in: *Proceedings of the International Conference on AIDS* (J. C. Gluckman, eds.), p. 13.

Höglund, S., Morein, B., Sundquist, B., Gelderblom, H., and Özel, M., 1986b, Immunostimulating complex (iscom) of viral envelope, in: *Proceedings of the International Congress of Electron Microscopy* (T. Imura, S. Maruse, and T. Suzuki, eds.), *Kyoto, Japan,* pp. 3403–3404.

Höglund, S., Özel, M., Åkerblom, L., Villacrez, M., Gelderblom, H., and Morein, B., 1989, A construct of HIV antigens: Structural and immunological analysis, submitted.

Hoxie, J. A., Fitzharris, T. P., Youngbar, P. R., Matthews, D. M., Rackowski, J. L., and Radka, S. F., 1987, Nonrandom association of cellular antigens with HTLV-III virions, *Hum. Immunol.* **18**:39–52.

Jennings, R., Pemberton, R. M., Smith, T. L., Amin, T., and Potter, C. W., 1987, Demonstration of an immunosuppressive action of detergent-disrupted influenzavirus on the antibody response to inactivated whole virus vaccine, *J. Gen. Virol.* **68**:441–450.

Jones, P. D., Tha Hla, R., Morein, B., and Ada, G. L., 1988, Cellular immune response in the murine lung to local immunization with influenza A virus glycoproteins in micelles and iscoms, *Scand. J. Immunol.* **27**:645–652.

Letvin, L., Daniel, M. D., Kiyotaki, M., Kannagi, M., Chalifoux, L. V., Seghal, P. K., Desrosiers, R. C., Arthur, L., and Allison, A. C., 1987, AIDS-like disease in macaque monkeys induced by simian immunodeficiency virus: A vaccine trial, *Vaccine* **87**:209–213.

Liljas, L., 1986, The structure of spherical viruses, *Proc. Biophys. Mol. Biol.* **48**:1–36.

Lövgren, K., and Morein, B., 1988, The requirement of lipids for the formation of immunostimulating complexes (iscoms), *Biotechnol. Appl. Biochem.* **10**:161–172.

Lövgren, K., Lindmark, J., Pipkorn, R., and Morein, B., 1987, Antigenic presentation of small molecules and peptides conjugated to a preformed iscom as carrier, *J. Immunol. Methods* **98**: 137–143.

Lövgren, K., Uggla, A., and Morein, B., 1987b, A new approach to the preparation of a Toxoplasma gondii membrane antigen for use in ELISA, *J. Vet. Med.* **34**:274–282.

Lövgren, K., 1988, The serum antibody response distributed in subclasses and isotypes after intranasal and subcutaneous immunization with influenzavirus immunostimulating complexes, *Scand. J. Immunol.* **27**:241–245.

Lucy, J. A., and Glauert, A. M., 1964, Structure and assembly of macromolecular lipid complexes composed of globular micelles, *J. Mol. Biol.* **8**:727–748.

Maizels, R. M., Philipp, M., and Oglive, M. O., 1982, Molecules of the surface of parasitic nematodes as probes of the immune response in infection, *Immunol. Rev.* **61**:109–136.

McEven, C. R., 1967, Tables for estimating sedimentation through linear concentration gradients of sucrose solution, *Anal. Biochem.* **20:**114–149.

Merza, M. S., Linne, T., Höglund, S., Morein, B., Portetelle, D., and Burny, A., 1989, Bovine leukemia virus iscoms: Biochemical characterization, *Vaccine.*

Modrow, S., Hahn, B., Shaw, G. M., Gallo, R. C., Wong-Stahl, F., and Wolf, H., 1987, Computer-assisted analysis of envelope protein sequences of seven human immunodeficiency virus isolates: Prediction of antigenic epitopes in conserved and variable regions, *J. Virol.* **61:**570–578.

Morein, B., and Simons, K., 1985, Subunit vaccines against enveloped viruses: Virosomes, micelles and other protein complexes, *Vaccine* **3:**83–93.

Morein, B., Helenius, A., Simons, K., Pettrson, R., Kääriäinen, L., and Schirrmacher, V., 1978, Effective subunit vaccines against enveloped animal virus, *Nature (Lond.)* **276:**715–718.

Morein, B., Sundquist, B., Höglund, S., Helenius, A., and Simons, K., 1982, Protein micelles and virosomes from the surface glycoproteins of parainfluenza-3 virus, in: *Protides of the Biological Fluids* (H. Peeters, ed.), pp. 101–104, Pergamon Press, Oxford.

Morein, B., Sharp, M., Sundquist, B., and Simons, K., 1983, Protein subunit vaccines of Para-influenza type 3 virus: Immunogenic effect in lambs and mice, *J. Gen. Virol.* **64:**1557–1569.

Morein, B., Sundquist, B., Höglund, S., Dalsgaard, K., and Osterhaus, A., 1984, Iscom, a novel structure for antigenic presentation of membrane proteins from enveloped viruses, *Nature (Lond.)* **308:**457–460.

Morein, B., Merza, M., Höglund, S., and Osterhaus, A., 1986, An approach to retrovirus vaccines, in: *Proceedings of the Retrovirus of Human AIDS and Related Animal Diseases;* "Colloque des Cent Gardes" *Paris*, pp. 168–171, (L. Montagnier), Paris.

Morein, B., Lövgren, K., Höglund, S., and Sundquist, B., 1987, The Iscom: An immunostimulating complex, *Immunol. Today* **8:**333–338.

Morein, B., Elkström, J., and Lörgren, K., 1989a, Inclusion of non-amphipathic protein (BSA) into ISCOMS increased its immunogenicity, submitted for publication.

Morein, B., Soos, T., Rusvai, M., McGwire, B. S., Bognar, K., and Belak, S., 1989b, Efficacy of immunostimulating complexes for controlling pseudorabies (Aujeszky's disease),

Morgan, A. J., Finerty, S., Lövgren, K., Scullion, F. T., and Morein, B., 1988b, Prevention of Epstein–Barr (EB) virus induced lymphoma in cottontop tamarins by vaccination with the EB virus envelope glycoprotein gp340 incorporated into iscoms, *J. Gen. Virol.* **69:**2093–2096.

Moss, B., Fuerst, T. R., Flexner, C., and Hugin, A., 1988, Roles of vaccinia virus in the development of new vaccines, *Vaccine* **6:**161–163.

Mumford, J. A., Cook, R. F., Hannant, D., Jesset, D. M., Wood, J. M., and Sundquist, B., 1986, The evaluation of antigen presentation methods and adjuvants for the enhancement of antigenicity and immunogenicity of equine influenza vaccines, in: *Proceedings of the First International Veterinary Immunology Symposium, Guelph, Canada.*

Murray, K., 1988, Application of recombinant DNA techniques in the development of viral vaccines, *Vaccine* **6:**164–174.

Newmark, P., 1988, Receding hopes of AIDS vaccines, *Nature (Lond.)* **33:**p. 699.

Osterhaus, A., 1988, Induction of anti-FeLV antibodies in cats with vaccinated virus and FeLV iscom,

Osterhaus, A., Weijer, K., Uytdehaag, F., Jarrett, O., Sundquist, B., and Morein, B., 1985, Induction of protective immune response in cats by vaccination with feline leukemia virus iscom, *J. Immunol.* **135:**591–596.

Osterhaus, A. D. M. E., Sundquist, B., Morein, B., Steenis, G., and van Uytdehaag, F., 1986, Comparison of an experimental rabies iscom subunit vaccine with inactivated dog kidney cell vaccine, in: *Proceedings of the First International Veterinary Immunology Symposium, Guelph, Canada.*

Osterhaus, A., Weijer, K., Uytdehaag, F., Knell, P., Jarrett, O., and Morein, B., 1987, Comparison

of serological responses in cats vaccinated with two different FeLV vaccine preparations, *Vet. Rec.* **121**:260.

Özel, M., Pauli, G., and Gelderblom, H., 1988, The organization of the envelope projections on the surface of HIV, *Arch. Virol.*

Özel, M., Höglund, S., Gelderblom, H., and Morein, B., 1989, The construct of iscom, in preparation.

Palfreyman, J. W., Aitcheson, T. C., and Taylor, P., 1984, Guidelines for the production of polypeptide specific antisera using small synthetic oligopeptides as immunogens, *J. Immunol. Methods* **75**:383–393.

Pyle, S. W., Morein, B., Bess, J., Nara, P. L., Nigida, S. M., Lerche, N., Fischinger, P., Arthur, L., 1989, Immune response to immunostimulatory complexes (ISCOMs) prepared from human immunodeficiency virus type 1 (HIV-1) or the HVI-1 external envelope glycoprotein (gp120) *Vaccine* (submitted).

Seligman, E. B., 1978, *Laboratory Techniques in Rabies,* 3rd ed., World Health Organization, Geneva.

Skoglund, U., Andersson, K., Strandberg, B., and Daneholt, B., 1986, Three-dimentional structure of a specific pre-messenger RNP particle established by electron microscope tomography, *Nature* **319**:560–564.

Speijers, G. J. A., Danse, L. H. J. C., Beuvery, J. J. T. W., Strik, A., and Vos, J. G., 1987, Local reactions of the Saponin Quil A and a Quil A containing iscom measles vaccine after intramuscular injection of rats: A comparison with the effect of DPT-polio vaccine, *Fund. Appl. Toxicol.* **10**:425–430.

Sundquist, B., Dalsgaard, K., and Morein, B., 1983, Assay of detergents by rocket electrophoresis in agarose gels containing red blood cells: "Rocket hemolysis," *Biochim. Biophys. Res. Commun.* **114**:699–704.

Sundquist, B., Lövgren, K., Höglund, S., and Morein, B., 1988a, Influenzavirus iscoms: Biochemical characterization, *Vaccine* **6**:44–48.

Sundquist, B., Morein, B., and Jonsson, K., 1988b, Registration file for a horse influenza virus vaccine in Sweden.

Tanford, C., and Reynolds, J. A., 1976, Characterization of membrane proteins in detergent solutions, *Biochim. Biophys. Acta.* **457**:133–170.

Trudel, M., Nadon, F., Seguin, C., Simard, C., and Lussier, G., 1988, Experimental polyvalent iscom subunit vaccine based on the fusion protein induces antibodies that neutralize human and bovine respiratory syncytial virus, *Vaccine.*

Uggla, A., Araujo, F. G., Lunden, A., Lövgren, K., Remington, J. S., and Morein, B., 1989, Immunizing effects in mice of two *Toxoplasma gondii* preparations, *J. Vet. Med.* in press.

Uytdehaag, F. G. C. M., Osterhaus, A. D. M. E., Loggen, H. G., Bakker, R. H. J., Van Aasten, J. A. A. M., Kreeftenberg, J. G., Van der Marel, P., and Van Steenis, G., 1983, Induction of antigen-specific antibody response in human peripheral blood lymphocytes *in vitro* by a dog kidney cell vaccine against rabies virus (DKCV), *J. Immunol.* **131**:1234–1239.

Varsanyi, T. M., Morein, B., Löve, A., and Norrby, E., 1987, Protection against lethal Measles virus infection in mice by immune-stimulating complexes containing the hemagglutinin or fusion protein, *J. Virol.* **61**:3896–3901.

Wahren, B., Nordlund, S., Åkersson, A., Sundquist, B., and Morein, B., 1987, Monocyte and iscom enhancement of cell-mediated response to cytomegalovirus, *Med. Microbiol. Immunol.* **176**:13–17.