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Hydration of lipid films with an aqueous solution of Quil A: a simple method for the preparation of immune-stimulating complexes

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Abstract

Immune-stimulating complexes (ISCOMs) are stable colloidal complexes of the adjuvant Quil A, cholesterol and phospholipid, which are effective carriers for subunit vaccines. The techniques currently available for the preparation of ISCOMs from the constituent components are rather complex and are based on either centrifugation or dialysis. This note reports a new simple procedure for the preparation of ISCOM matrices based on hydration of a cholesterol/phospholipid film with an aqueous solution of Quil A. It is demonstrated that ISCOM matrices do not form in the absence of phospholipid when prepared by this method. Further, the ratio by weight of phospholipid to either cholesterol or Quil A must be greater than that required for preparation by either dialysis or centrifugation. Photon correlation spectroscopy, negative stain transmission electron microscopy and centrifugation through a sucrose gradient demonstrate that ISCOM matrices can be prepared from cholesterol/lipid films by hydration with an aqueous solution of Quil A when the ratio of phospholipid:cholesterol:Quil A by weight is 6:1:4, respectively. Lower ratios of phospholipid:cholesterol reduce the efficiency of ISCOM formation while higher ratios produce systems containing a mixture of ISCOMs together with liposomes.

Keywords: ISCOM; Immune-stimulating complex; Quil A; Phospholipid; Vaccine; Adjuvant

There is a growing trend to use purified antigen instead of whole pathogens for vaccination in order to overcome many of the negative side-effects associated with the use of whole microorganisms. The use of subunit vaccines (purified antigen), however, generally results in only poor immunogenicity. The immunogenicity of purified antigens can sometimes be enhanced to that of the whole pathogen by the creation of defined multimeric forms, e.g. liposomes or protein micelles (Kersten and Crommelin, 1995). These may possibly mimic the submicroscopic particle of a microorganism or virus having several copies of the surface antigen. Often, even the immunogenicity of antigens presented in whole microorganisms or artificial multimeric forms is insufficient to elicit an adequate immune response and this must be enhanced by the addition of an adjuvant.

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Immune-stimulating complexes (ISCOMs) combine the advantages of a particulate carrier system with the presence of a built-in adjuvant. They are symmetrical colloidal particles with an open cage-like structure in the size range 40–100 nm (Özel et al., 1989; Kersten et al., 1991). In their simplest form, ISCOMs are composed of the adjuvant Quil A and cholesterol held together by hydrophobic interactions (Höglund et al., 1989). Amphipathic proteins together with an additional lipid, e.g. phospholipid can be incorporated to form a highly immunogenic antigen complex. Literature essentially reports two methods for the preparation of ISCOMs: centrifugation and dialysis (Dalsgaard et al., 1989). Both methods of preparation, despite being rather elaborate, propose that ISCOMs form spontaneously under appropriate conditions, which suggests that these structures are thermodynamically stable. This work investigates whether the spontaneously forming nature of ISCOMs can be exploited to prepare ISCOM matrices by simple hydration of a lipid film with an aqueous solution of Quil A.

A control, protein-free ISCOM preparation was prepared according to the standard dialysis method (Dalsgaard et al., 1989). Briefly, a mixture of 2 mg of cholesterol and 2 mg egg derived phosphatidylcholine (both from Sigma, St. Louis, MO) were hydrated with 2 ml of tris pH 7.4 buffer containing 8 mg Quil A (Superphos Biosector, Vedbaek, Denmark) and 80 mg Octyl-β-D-glucopyranoside (Sigma). The mixture was agitated until lipid particles were no longer visible. The resulting solution was dialysed (1000 molecular weight cut-off membrane) for 40 h against several changes of buffer. The sample was then removed from the dialysis sac and centrifuged through a sucrose gradient (10–50% w/w sucrose) for 18 h at $50\,000 \times g$. The ISCOMs appearing as a visible band in the gradient were collected. The isolated ISCOM matrices were then characterised for particle size by photon correlation spectroscopy (PCS, Zetasizer 3000, Malvern) and morphology by transmission electron microscopy (TEM-Philips 410LS). For TEM, samples were negatively stained with 2% aqueous solution of phosphotungstic acid adjusted to pH 5.2.

To investigate whether ISCOMs could be prepared by hydration of the lipid components of the ISCOM matrix, dried thin films of cholesterol (2 mg) and various amounts of phosphatidylcholine (0, 2, 6, 12 and 20 mg) were prepared in a large round-bottomed flask by evaporation of a solution of the lipids in chloroform. The lipid film was then hydrated by addition of 2 ml of an aqueous solution of Quil A (4 mg/ml) with gentle mixing for approximately 20 min at room temperature. The resulting preparation was either fractionated by centrifugation through a 10–50% sucrose gradient or analysed without further treatment. PCS and TEM were used to identify the presence of ISCOM matrices in the preparations.

The control ISCOM matrix sample prepared by dialysis yielded a very distinct peak having a mean size of 54.2 nm when analysed by PCS. When viewed by TEM, the colloidal matrices were seen as symmetrical cage-like structures consistent with that reported in the literature (Lövgren and Morein, 1988; Özel et al., 1989; Kersten et al., 1991). TEM confirmed the size determined by PCS.

The turbidity of the samples prepared by hydration increased with increasing phospholipid content; the phospholipid free samples appearing slightly translucent while the samples prepared from 20 mg phospholipid were milky in appearance. The signal obtained by PCS for the phospholipid free samples was very weak. There was no evidence of particles having a diameter in the range 30–100 nm, taken as evidence for the presence of ISCOM matrices. The count rate, as measured by PCS, increased for samples prepared from 2 and 6 mg phospholipid. However, again there was little definitive evidence of particles having a size of less than 100 nm in either preparation with inconsistent, variable peaks being observed above this diameter up to 1 μm. Further, no structures characteristic of ISCOMs could be identified in these samples when viewed by TEM. Analysis of the samples prepared by hydrating a mixture of 12 mg phospholipid and 2 mg cholesterol showed a bimodal particle size distribution with a peak being observed with a mean of around 60 nm and a second smaller broad peak around 300 nm. This type of profile was typical of

a number of preparations. Negative stain TEM analysis of these samples demonstrated the presence of cage-like structures having a size of around 50 nm, characteristic of ISCOM matrices

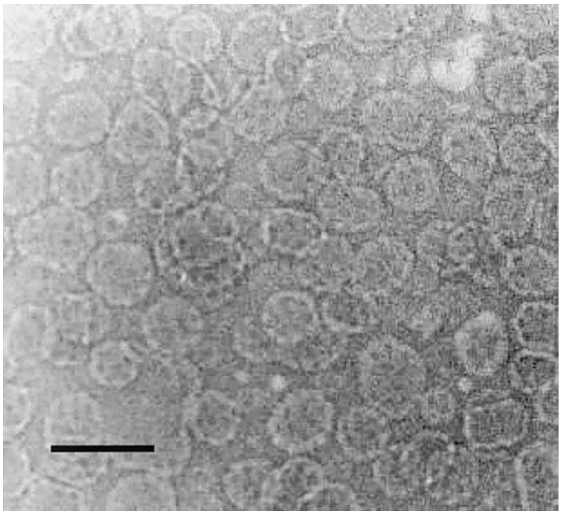

Fig. 1. Negative stain transmission electron microscopy (TEM) of immune-stimulating complex (ISCOM) matrices prepared by hydration of a phospholipid and cholesterol film having a weight ratio of 6:1. Bar = 100 nm.

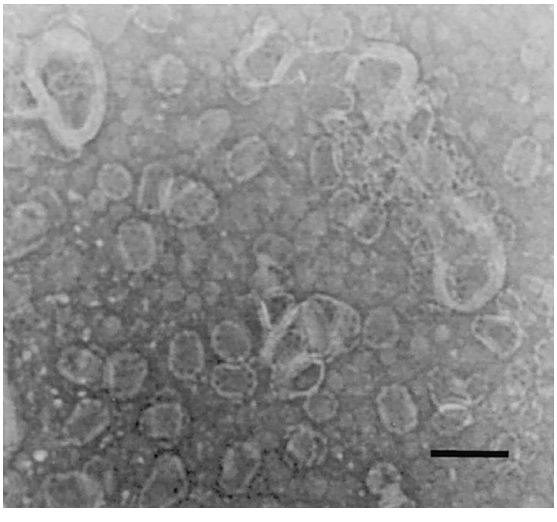

Fig. 2. Negative stain transmission electron microscopy (TEM) of immune-stimulating complex (ISCOM) matrices prepared by hydration of a phospholipid and cholesterol film having a weight ratio of 10:1 showing presence of multilamellar liposomes. Bar = 100 nm.

(Fig. 1). The structures observed were consistent with those seen in the sample prepared by dialysis. The presence of particles in the 100–1000 nm range increased in systems prepared from 20 mg phospholipid. A broad peak spanning from 30 to in excess of 500 nm was commonly observed by PCS. When viewed by TEM, ISCOM matrices could be identified together with larger lipid particles (Fig. 2). The lamellar structure of these lipid particles is clearly visible. For samples prepared from both 12 and 20 mg phospholipid, discrete bands consistent with that noted for ISCOM matrices prepared by dialysis were observed upon centrifugation through a sucrose gradient. Samples prepared from 0, 2 and 6 mg phospholipid yielded bands having higher densities.

The effectiveness of ISCOM matrices as immunoadjuvants is well documented (Höglund et al., 1989; Kersten and Crommelin, 1995). However, the techniques currently documented for their preparation namely, centrifugation and dialysis are rather elaborate, complex and relatively expensive procedures. The first method involves centrifuging the antigen together with the lipid components through a density gradient containing Quil A, typically at a concentration of 0.1%. High forces and long run times are required for this procedure, for example 4 h at $150\,000 \times g$ (Dalsgaard et al., 1989). ISCOMs sediment in a band, the position of which depends on lipid and antigen content (Höglund et al., 1989). Following formation, the ISCOM band is isolated and washed free from low molecular weight substances including Quil A by recentrifugation through 20% sucrose. The alternative method employs a surfactant with a high critical micelle concentration to solubilise the mixture of cholesterol, phospholipid and the amphipathic protein. The solubilised lipids and protein are mixed with a solution of Quil A and dialysed, typically using a molecular weight cut-off membrane of less than 10 000, for at least 24 h to remove the surfactant. ISCOMs form as the surfactant molecules are removed. Again, excess Quil A can be removed by gradient ultracentrifugation. A possibility exists, however, of residual detergent being present in the ISCOM matrix prepared by this method.

The findings of the present study suggest that ISCOM matrices can be simply prepared by the hydration of a dried film of the lipophilic components by an aqueous solution of Quil A. However, it would appear that the relative amounts of each component used in this preparative procedure together with the presence of phospholipid is critical for the formation of ISCOM matrices. Conflicting views regarding the requirement of phospholipid for the formation of the ISCOM matrix is however reported in the literature. Lövgren and Morein (1988) proposed that only cholesterol and Quil A were essential for the formation of the ISCOM matrix and that the inclusion of a phospholipid was only necessary to increase the fluidity of the matrix to enable incorporation of a protein. Kersten et al. (1991), however, proposed that phospholipid was an essential structural component of the ISCOM matrix. In the absence of phospholipid, Quil A/cholesterol micelles are formed or even Quil A/cholesterol lamellae at higher levels of cholesterol. The requirement of phospholipid is supported by the results of the present study as no evidence of ISCOM matrices was obtained upon the hydration of a cholesterol film with an aqueous solution of Quil A in the absence of phospholipid. PCS analysis would suggest that in the absence of phospholipid, Quil A/cholesterol micelles are formed when a cholesterol film is hydrated with an aqueous solution of Quil A in a weight ratio of 1:4, respectively, which is in agreement with the findings of Kersten et al., (1991).

Kersten et al., (1991) reported a typical composition of a protein-free ISCOM matrix to be 1:1.2:6.2 by weight of cholesterol, phospholipid and Quil A, respectively, when prepared by dialysis using octylglucoside as a solubilising surfactant. As mentioned, the dialysis technique employs a surfactant with a high critical micelle concentration and consequently it is used at a concentration greatly in excess of Quil A or the lipid components of the ISCOM matrix. A weight ratio of 1:1:4:40 for cholesterol, phospholipid, Quil A, and octylglucoside was used by Kersten et al. (1991), as was used in the present study for the preparation of ISCOM matrices by dialysis. In the absence of any additional surfactant, however, an increased concentration of phospholipid is required for the formation of the ISCOM matrix. A phospholipid:cholesterol ratio of at least 6:1 is required for their efficient formation by hydration of a lipid film. However, at this ratio, formation of ISCOM matrices from the lipid components is still not complete and evidence of larger particles was obtained by PCS though these could not be readily identified by TEM. The presence of these larger lipid particles could possibly be reduced by increasing the ratio of Quil A in the hydrating solution as proposed by Lövgren and Morein (1988) for the preparation of ISCOM matrices by dialysis. Increasing the phospholipid:cholesterol ratio to 10:1 resulted in the increased appearance of lamellar structures upon hydration which are typical of liposomes. ISCOMs could be isolated from such mixtures by centrifugation through a gradient or through a 20% sucrose system as is done following preparation by centrifugation or dialysis. However, this would add to the complexity of the procedure.

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