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Immuno-stimulating complexes prepared by ethanol injection

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Abstract

This study describes the formulation of immuno-stimulating complexes (ISCOMs) utilising the ethanol injection technique. Cholesterol and phosphatidylcholine were dissolved in ethanol and the resulting solution was rapidly injected into a stirred, aqueous solution of the triterpene–saponin mixture Quil-A. The reversed experiment was also carried out by adding the aqueous Quil-A solution to a solution of cholesterol/phosphatidylcholine dissolved in ethanol. This was done by either rapid injection or dropwise addition of the aqueous Quil-A solution. The colloidal dispersions obtained by ethanol injection and reversed addition were compared with formulations obtained by the dialysis and lipid-film hydration methods. In a further experiment, the preparation of ISCOMs from liposomes as precursor structures was investigated. Transmission electron microscopy was used to analyse the resulting colloidal dispersions. Samples were also compared with respect to homogeneity of obtained particle species. The ethanol injection technique led to formation of ISCOMs in high numbers within 2 h post formulation. The reversed rapid injection resulted in a similar colloidal dispersion, differing from the former mainly due to the presence of some helical micellar structures. The reversed, dropwise addition led to the formation of helices as the predominant colloidal structure. Of the three previously established methods, only dialysis led to the formation of ISCOMs within 48 h. The lipid-film hydration method and the approach using liposomes as precursor structures did not produce ISCOMs under the conditions and within the time periods investigated. However, it is known that dispersions prepared by the hydration method equilibrate towards ISCOMs after longer storage. Ethanol injection and reversed rapid injection are simple, cost-effective and quick methods to produce ISCOMs.

Introduction

Immuno-stimulating complexes (ISCOMs) are spherical, cage-like particles with a typical size of about 40 nm. They are composed of cholesterol, phospholipid and saponins (Sjolander et al 1998). The saponins are derived from the bark of the Chilean tree *Quillaja saponaria* and are essential both for the formation of the particles and in evoking enhanced immune responses to antigens incorporated into them (Barr et al 1998). A semi-purified fraction of saponins, Quil-A, is typically used for making ISCOMs, but more recent reports describe the use of subfractions to reduce toxicity (Ronnberg et al 1995). The ISCOM structure is most likely an aggregate of ring-like micelles formed by saponins and cholesterol, which are held together by hydrophobic interactions, steric factors and hydrogen bonds (Kersten et al 1991). A pseudo-ternary phase diagram with these three components has been established (Demana et al 2004a). The techniques utilised in the preparation of ISCOMs either use micelles or liposomes as the colloidal precursor structure.

The earliest publications on ISCOMs established the micellar approach. A detergent (e.g., MEGA-10 or n-octylglucoside) is used to solubilize the ISCOM components and is thereafter removed by dialysis (Morein & Bengtsson 1999). The greatest disadvantage of dialysis is that the process is time consuming. Liposomal approaches, on the other hand, avoid the use of additional detergent and are less time consuming. An issued patent described the formation of ISCOMs from extruded liposomes as intermediate particles and subsequent addition of *Quillaja* saponins (Friede & Garcon 2000). Formulation of ISCOMs by hydrating thin cholesterol and phospholipid films

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with aqueous solutions of saponins is a 2-in-1 step approach as it leaves out the intermediate, intentional formation of liposomes (Copland et al 2000). This method is a modification of the Bangham method for making liposomes with respect to the types of colloidal structures formed (Demana et al 2004a). The molar composition of saponins, cholesterol and phospholipid in ISCOMs is about 1:1:1, although compositions may vary (Kersten et al 1991). We have discussed previously that with the lipid-film hydration method more phospholipid is necessary to form ISCOMs compared with the micellar approach (Copland et al 2000; Demana et al 2004b). This may lead to the formation of colloidal structures with a higher proportion of phospholipid as by-products, in combination with ISCOMs when equilibration occurs. These colloidal by-products are liposomes, lipidic particles and layered structures (Demana et al 2004a).

In this study, we modified the ethanol injection procedure for making liposomes (Batzri & Korn 1973; Pons et al 1993) in an attempt to produce ISCOMs in a quick, simple and efficient manner by replacing the formulation buffer with aqueous solutions of Quil-A. Formulations prepared by ethanol utilizing techniques were compared with those obtained by lipid-film hydration, dialysis and the intermediate formation of liposomes as precursor structure for ISCOMs.

Materials and Methods

Materials

Quil-A, as lyophilized powder, was purchased from Brenntag Biosector (Frederikssund, Denmark), egg derived phosphatidylcholine (PC) was purchased from Northern Lipids (Vancouver, Canada) and cholesterol (CHOL) and n-octyl-β-glucopyranoside were purchased from Sigma (St Louis, MO). The buffer used in all experiments was 145 mM TRIS-buffered saline pH 7.4 (TBS), with a composition of 65 mM TRIS (BDH, Poole, UK) and 80 mM NaCl (Sigma). Stock solutions of cholesterol and phosphatidylcholine in chloroform (Merck, Darmstadt, Germany) at a concentration of 1 mg mL^{-1} were prepared, respectively. For the ethanol injection method, an ethanolic stock solution containing 6.76 mg mL^{-1} phosphatidylcholine and 3.44 mg mL^{-1} cholesterol was prepared. For dialysis, a membrane with a molecular weight cut-off of 1000 was used (Spectrum Laboratories, Rancho Dominguez, CA).

Conditions used in all preparations and analysis of colloidal dispersions

The molar composition of Quil-A–cholesterol–phosphatidylcholine in all experiments was 1:1:1 and the amount used of each component was approximately 0.002 mmol. The concentration of Quil-A (with an estimated average molecular mass of 2000 (Barr et al 1998)) was 1.33 mg mL^{-1} and the total dispersion volume was 3 mL. The quantities used in each experiment were 1.52 mg phosphatidylcholine (assumed molecular mass of 760 based on palmitoyl-oleoyl phosphatidylcholine) and 0.77 mg cholesterol. All samples were prepared in duplicate. Samples were formulated and stirred for a period of 48 h at 23°C and analysed by transmission electron microscopy (TEM) at 2 and 48 h post formulation. At least 1000 particles in each sample were classified on several micrographs with respect to their morphology (e.g., ISCOMs, liposomes, bigger aggregates), and homogeneity of samples was determined qualitatively. For TEM, samples were coated onto glow-discharged, carbon-coated copper grids, negatively stained with 2% w/v phosphotungstic acid (pH 5.2) and subsequently viewed on a Philips CM 100 at an acceleration voltage of 100 kV and a magnification of 93 000. Images were processed and particle-size analysed using AnalySIS software (Soft Imaging Systems, Reutlingen, Germany). Photon correlation spectroscopy was found to be an unsuitable method for sample analysis as one is dealing with, depending on the sample, a more or less heterogeneous mixture of different particle species with very different morphologies.

Ethanol injection and reversed addition methods

A 0.225-mL volume of ethanolic stock solution of phosphatidylcholine and cholesterol was aspirated into a small syringe and rapidly injected through a fine needle into a vial containing 4 mg Quil-A dissolved in 2.775 mL TBS. The concentration of ethanol was thus limited to 7.5% (v/v). The reversed experimental design included either rapid injection or slow, dropwise addition of the aqueous Quil-A solution into the ethanolic lipid solution. In the latter case, the Quil-A solution was added over a period of 20 min in small drops using a fine needle.

Lipid-film hydration method

Phosphatidylcholine and cholesterol dissolved in chloroform were pipetted into a 150-mL round bottom flask. Chloroform was evaporated off on a rotary evaporator at a water bath temperature of 40°C. The flask was flushed with nitrogen and the lipid film was hydrated with 3 mL TBS containing Quil-A. Glass beads were used during handshaking (10 min). The colloidal dispersions were then transferred into a vial and stirred.

Liposomal precursor method

A lipid film of phosphatidylcholine and cholesterol was prepared as described above. The film was hydrated with 2.6 mL TBS. Glass beads were used during handshaking (10 min). Liposomes were allowed to equilibrate at 23°C for 22 h. Thereafter 4 mg of Quil-A dissolved in 0.4 mL TBS were added and the round-bottom flask was gently swirled to allow mixing of Quil-A and liposomes. The dispersions were then transferred into a vial and stirred.

Dialysis method

Phosphatidylcholine and cholesterol were pipetted into a glass vial and chloroform was evaporated under a nitrogen stream. The lipids were dried overnight under vacuum.

Four milligrams of Quil-A and 40 mg n-octylglucoside dissolved in 3 mL TBS were added and the mixture was stirred for 2 h at room temperature to allow solubilization. The solution was transferred into a dialysis membrane with a molecular weight cut-off of 1000 Da. The samples were dialysed against 1 L TBS with an exchange of buffer every 12 h for a total period of 48 h.

Results

ISCOMs and related colloidal structures were formulated using the methods described above. The morphology of obtained particles was assessed by TEM after 2 h and 48 h of stirring of the colloidal dispersion at room temperature. A molar ratio of 1:1:1 of Quil-A, cholesterol and phosphatidylcholine was used for all methods.

Rapid injection of an ethanolic cholesterol/phosphatidylcholine solution into a stirred, aqueous solution of Quil-A resulted in the formation of ISCOMs in high numbers 2 h post formulation. The ISCOMs had an average diameter of 33.2 ± 4.1 nm ($n = 100$). With this method, some ring-like micelles, worm-like micelles and helices were obtained as additional components (Figure 1A), and only very small numbers of lipidic particles were found. After 48 h of stirring, predominantly ISCOM-like particles were found (43.0 ± 4.9 nm, $n = 50$; Figure 1B). The numbers of ring-like micelles and helices had further decreased from 2 h to 48 h post formulation. The technique resulted in homogenous formulations with only few bigger aggregates being present.

The reversed, rapid injection of an aqueous solution of Quil-A into ethanolic cholesterol/phosphatidylcholine solution resulted in the formation of mainly ISCOM-like particles 2 h post formulation, and morphology of particles had not changed significantly after 48 h. The formulations obtained by reversed, rapid injection differed from the normal ethanol injection described before mainly in the presence of a higher number of helices and worm-like micelles (Figure 1C). Homogeneity was comparable with the samples obtained by normal ethanol injection.

When the aqueous solution of Quil-A was added dropwise to the ethanolic cholesterol/phosphatidylcholine solution over a period of 20 min, helices and ISCOM-like particles were the major colloidal structures 2 h post preparation (Figure 1D). Some ring-like micelles were also observed. After 48 h, the length of helices had increased markedly (Figure 1E) and helices were the major component of the system. Helices had a length of up to 1 μm, a width of about 20 nm and a distance between helical turns of about 5 nm. However, ISCOM-like structures and ring-like structures were also found. The described helices were the only colloidal structures above 100 nm in size.

A mixture of Quil-A, cholesterol and phospholipid solubilised with the non-ionic detergent n-octylglucoside and thereafter dialysed over a period of 48 h against 4 changes of TBS resulted in the formation of mainly ISCOMs. To a lesser extent, helices and few worm-like micelles were found (Figure 1F). The ISCOMs obtained by dialysis (40.1 ± 3.8 nm, $n = 100$) were bigger compared with those obtained by ethanol injection 2 h post formulation (Figure 1A), but similar in size to those obtained 48 h post injection (Figure 1B). The samples were the most homogenous with no bigger particles being present.

With the lipid-film hydration method bigger, disordered aggregates were found in the samples, leading to a more heterogeneous formulation. No ISCOMs were observed 2 h and 48 h post formulation. ISCOM-like structures were only occasionally seen in the colloidal dispersion, whereas worm-like micelles and ring-like micelles constituted the main proportion of structures obtained (Figure 1G). However, we previously reported that at compositions used in this experiment, formation of ISCOMs is promoted after longer storage (Demana et al 2004a).

Addition of Quil-A to pre-formed liposomes resulted in a complete disappearance of the liposomal bilayer structure. No intact liposomes were observed. However, bigger aggregates with an uncharacteristic fine structure were still found 48 h post formulation (Figure 1H), leading to higher heterogeneity. In addition to these structures, worm- and ring-like micelles were found. The samples prepared from liposomes as precursor structures resembled the dispersions obtained by lipid-film hydration, a finding not too surprising as a fairly similar preparative technique was used.

Table 1 summarizes advantages and disadvantages of the various techniques for making ISCOMs. Normal ethanol injection and reversed rapid injection were combined as fairly similar dispersions were obtained. Furthermore, Table 1 does not include the reversed dropwise addition since this procedure led to a very high proportion of helices. Optimized lipid compositions that lead to high concentrations of ISCOMs are assumed in Table 1. For the ethanol injection technique it may be necessary for ethanol to be removed in an additional dialysis step. However, removal of ethanol, if necessary, is a quicker process than removal of a detergent.

Discussion

In this study, the ethanol injection method was introduced as a new technique for preparing ISCOMs and was compared with other established methods. A 1:1:1 molar composition of Quil-A, cholesterol and phosphatidylcholine was used for all different techniques, but the morphology of resulting colloidal structures differed markedly. Dialysis led to the highest number of ISCOMs and produced the most homogenous samples. It can be assumed that the reason for this lies in the homogenous dispersion of all ISCOM components with help of an additional detergent. This detergent is thereafter removed gradually. In the ethanol injection technique a similar principle is utilized since cholesterol and phosphatidylcholine are dissolved in ethanol. The resulting solution is then injected into an aqueous solution of Quil-A through a fine needle. Ethanol injection is known to produce small liposomes in the absence of Quil-A (Batzri & Korn 1973; Pons et al 1993), indicating that the lipids are finely dispersed.

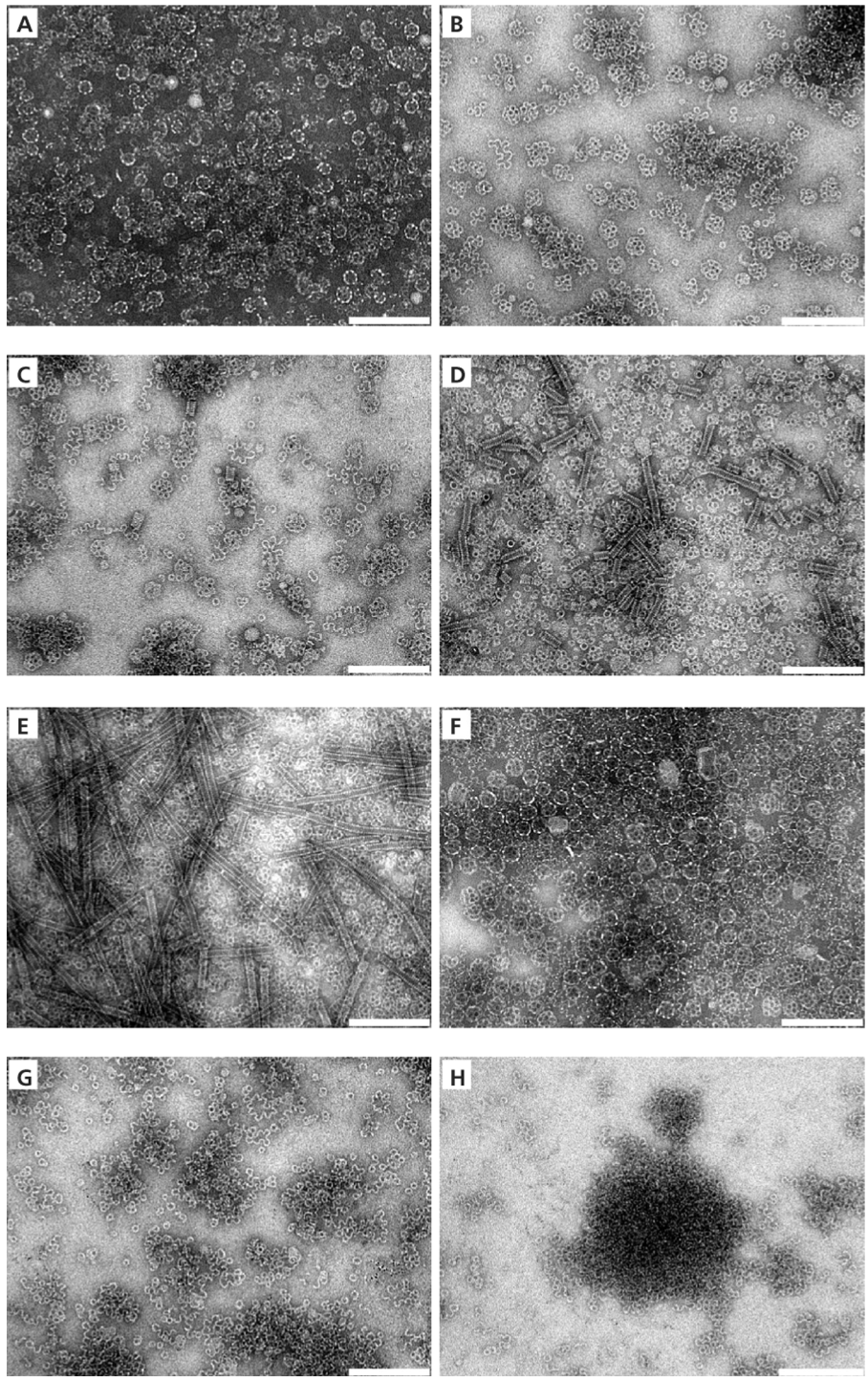

Figure 1 TEM micrographs of samples prepared by different techniques. Bar = 200 nm. A. Ethanol injection method, 2 h post formulation. B. Ethanol injection method, 48 h post formulation. C. Reversed rapid injection, 2 h post formulation. D. Reversed dropwise addition, 2 h post formulation. E. Reversed dropwise addition, 48 h post formulation. F. Dialysis method, 48 h post initiation. G. Lipid-film hydration method, 2 h post formulation. H. Liposomal precursor method, 48 h post formulation.

Preparation of ISCOMs by ethanol injection resulted in relatively homogenous samples with a high number of ISCOMs only two hours post formulation. Thus, the method differs from dialysis in the substitution of detergent with ethanol. The residual concentration of ethanol in the resulting colloidal structures remains to be determined but is expected to be low since ISCOMs are open structures and ethanol is completely miscible with water.

Table 1 Summary of characteristics of the different preparative techniques

	Homogeneity	Ease of preparation	Preparation time	Possible contaminant
Dialysis method	Very high	Easy	Long	Surfactant
Ethanol injection method	High	Very easy	Very short	Ethanol
Liposomal precursor method	Low[a]	Easy	Short	None
Lipid-film hydration method	Low[b]	Very easy	Short	None

[a]The homogeneity of the resulting colloidal particles might be greater if the liposomal precursor is subjected to an extrusion procedure (Friede & Garcon 2000). [b]The homogeneity of the resulting colloidal particles can be increased by longer equilibration times (Demana et al 2004a, b).

Compared with ethanol injection and dialysis, the lipid-film hydration method utilizes no additional solubilizing agents. This generally leads to more heterogeneous colloidal systems and sample equilibration appears to take longer. ISCOMs were not observed in this work using this technique. However, we reported earlier that samples prepared by lipid-film hydration at comparable compositions eventually formed ISCOMs after longer storage times. It is likely that the time frame of this study was too short to make this observation (Demana et al 2004a).

In the liposomal precursor method, bilayered structures completely disappeared within 2 h after addition of Quil-A. However, ISCOM formation was not found. Liposomes were either degraded into separate worm- or ring-like micelles or remained as aggregates with a disordered fine-structure. These results are in contrast with a previous patent application (Friede & Garcon 2000), although these authors used a different lipid composition and extruded liposomes. According to the structural model for ISCOMs suggested by Kersten et al (1991), the organization of lipid molecules in ISCOMs is very different to that of lipids in liposomal bilayers. In a liposomal bilayer, the lipid molecules have an orientation perpendicular to the surface of the liposome, whereas in the suggested ISCOM model the lipophilic tails are oriented almost parallel to the particle's surface. It is conceivable that the transition from liposome to ISCOM is a slow process due to the massive change in molecular organisation. As for the lipid-film hydration method a likely explanation for the absence of ISCOMs is that the time frame of the experiments did not suffice to observe their formation.

The most astonishing finding was that dropwise addition of an aqueous solution of Quil-A to ethanolic cholesterol/phosphatidylcholine solution resulted in the formation of long helices in very high numbers. In earlier reports helices only constituted minor proportions of the particle populations obtained by dialysis and lipid-film hydration (Demana et al 2004a), and the results presented in this work confirmed this. The organisation and composition of individual molecules in the helix might be very similar to that in the ISCOM, so that the two colloidal structures possibly compete with each other. It is conceivable that ISCOMs are predominantly obtained if cholesterol and phospholipid are solubilized into ring-like micelles formed together with Quil-A, whereas random orientation of the lipids (as in the ethanolic solution) leads to a growing contribution of helices upon slow addition of Quil-A. Using the reversed dropwise addition procedure, an interesting possibility arises to use these helices as a vaccine delivery system and this should be followed up in future investigations, as they are colloidal in size, contain Quil-A and might be able to incorporate antigen.

Conclusions

ISCOMs as vaccine delivery systems have been reported to evoke strong immune responses to various antigens. In this work, we were able to demonstrate the formation of ISCOMs by ethanol injection. Application of this technique resulted in a high number of these cage-like structures 2 h post formulation. The advantages of ethanol injection include its potential for scale up, the simplicity of the procedure, low cost and low expenditure of time. Advantages of the established dialysis procedure are homogeneity of the resulting formulation and the high number of ISCOMs it produces. However, ethanol injection may become a simpler, quicker and cheaper alternative to dialysis.

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