ELSEVIER

International Journal of Pharmaceutics 241 (2002) 385–389

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

Note

A method for the incorporation of ovalbumin into immune stimulating complexes prepared by the hydration method

Stephanie Könnings, Melissa J. Copland, Nigel M. Davies, Thomas Rades *

Colloidal Drug Delivery Group, School of Pharmacy, University of Otago, P.O. Box 913, Dunedin, New Zealand

Received 12 March 2002; received in revised form 3 May 2002; accepted 3 May 2002

Abstract

This study describes the development of a method for the incorporation of fluorescently labelled ovalbumin (FITC-OVA) into immune stimulating complexes (ISCOMs) prepared by the hydration method. Conjugation of palmitic acid was performed to fluorescently labelled OVA (pFITC-OVA) or to non-labelled OVA, with subsequent conjugation of FITC to the resulting palmitified OVA (FITC-pOVA). Both pFITC-OVA and FITC-pOVA, but not FITC-OVA, could be incorporated into ISCOMs and other non-liposomal colloidal structures. The degree of incorporation of pFITC-OVA or FITC-pOVA in non-liposomal colloidal particles reaches a maximum, if ISCOMs are the predominant colloids in the system.

Keywords: Immune stimulating complexes; Ovalbumin; Colloidal particles; Hydration method; Quil A

1. Introduction

The development of subunit purified antigen vaccines instead of attenuated or inactivated whole pathogens for vaccination offers many advantages. These advantages include lowering the likelihood of adverse or allergic reactions, or the production of so called 'blocking antigens' as an undesired immune response upon administration. Subunit vaccines, therefore, offer an important, cost-effective and safe way to combat a range of diseases, both in the areas of protective and therapeutic vaccination (Raychaudhuri and Rock, 1998; Sjölander et al., 1998).

It has, however, been shown that isolated subunit antigens, whilst immunogenic as part of a microorganism are almost non-immunogenic as purified antigens. The immunogenicity of purified antigens may sometimes be enhanced to that of the whole pathogen by the creation of defined multimeric forms such as the formulation of antigen in liposomes or as protein micelles (Kersten and Crommelin, 1995; Kensil, 1996). It is thought that the colloidal size of the delivery system mimics the submicroscopic particulate nature of a microorganism or virus having several copies of the surface antigen. Often, even the immunogenicity of antigens presented in multimeric form is insufficient to elicit an adequate immune re-

* Corresponding author. Tel.: +64-3-479-5410; fax: +64-3-479-7034

E-mail address: thomas.rades@stonebow.otago.ac.nz (T. Rades).

0378-5173/02/$ - see front matter
PII: S0378-5173(02)00270-3

sponse. In this case the immune response must be enhanced by the inclusion of an adjuvant within the delivery system (Kensil, 1996).

A specific form of colloidal delivery systems, termed immune stimulating complexes (ISCOMs) has received much attention in the immunological field. ISCOMs combine the advantages of a particulate carrier system with the presence of a built-in adjuvant. ISCOMs are symmetrical colloidal particles with an open cage-like structure in the size range of 30–100 nm composed of the saponin-adjuvant Quil A, cholesterol, phospholipids and the antigenic protein. The ISCOM particle is formed by hydrophobic interaction of the components and hence, in order to incorporate an antigen into the structure, it is a prerequisite that the protein has an amphipathic nature. Membrane spanning proteins often used as subunit antigens are therefore especially suited to incorporation in the ISCOM structure. However, much preliminary immunological research utilises ovalbumin as a model antigen and it is therefore desirable to incorporate this protein into the delivery system to facilitate studies of the antigen delivery potential of ISCOMs. Unfortunately, it is not possible to incorporate ovalbumin in ISCOMs due to its hydrophilic nature (Kersten and Crommelin, 1995; Kensil, 1996).

Another frequently used hydrophilic model protein, bovine serum albumin (BSA), has been successfully incorporated into ISCOMs following acid treatment of the protein at pH 2.5 (Höglund et al., 1989). Preliminary experiments in our laboratory utilising acid treatment to incorporate OVA into ISCOMs did not, however, result in inclusion of protein in the matrix.

Incorporation of BSA has also been achieved by covalent binding of fatty acids to the hydrophilic protein, and subsequent formation of ISCOMs in a one pot synthesis utilising the dialysis method (Reid, 1992). The aim of the current study was to develop a method for incorporation of the model antigen, fluorescein isothiocyanate labelled ovalbumin (FITC-OVA) into ISCOMs formed by the hydration method, recently developed in our laboratory (Copland et al., 2000). Additionally we wished to separate the protein-fatty acid conjugation step from the process of incorporation of the antigen into the colloidal structures, so that the modified protein may also be formulated in alternative delivery systems for comparative analysis.

2. Conjugation of fluorescein isothiocyanate to OVA

Twenty milligrams fluorescein isothiocyanate (FITC) (Isomer I, Sigma, St. Louis, MO) was dissolved in 10 ml of carbonate buffer (220 mM, pH 9.5) and 100 mg OVA (grade V, Sigma) was added. The mixture was gently stirred in the dark at 4 °C for 18 h. Buffer salts and unbound FITC were removed by repeated dilution with water and ultrafiltration using a 10 000-molecular weigh cut-off membrane and a 50 ml filtration cell (Amicon, Beverly, MA) pressurised to 200 kPa. The resulting FITC-OVA solution was freeze-dried and subsequently stored protected from light at 4 °C. Yield of FITC-OVA was $>90\%$.

3. Conjugation of palmitic acid to FITC-OVA (preparation of pFITC-OVA)

Thirty milligrams FITC-OVA was dissolved in 30 ml carbonate buffer (50 mM, pH 9), containing 30 mg sodium deoxycholate (Sigma). Four hundred and seventy microlitre of a solution of 5 mg *N*-(palmitoyloxy) succinimide (NPS, Sigma) in 500 μl dimethylsulfoxide was added. The mixture was protected from light and gently stirred for 15 h at 37 °C. Sodium deoxycholate and unbound NPS was then removed by washing and ultrafiltration as described above. The resulting solution was centrifuged at $30\,000 \times g$ for 30 min and the clear supernatant was freeze-dried. Yield of pFITC-OVA was $>95\%$.

4. Conjugation of palmitic acid to OVA and subsequent conjugation with FITC (preparation of FITC-pOVA)

One hundred milligrams OVA was dissolved in 100 ml carbonate buffer (50 mM, pH 9) contain-

ing 100 mg sodium deoxycholate. A solution of 16 mg NPS in 1.6 ml dimethylsulfoxide was added and the resulting mixture treated as described above for pFITC-OVA (pOVA, yield $>99\%$). Ten milligram FITC was dissolved in 5 ml Tris buffer (50 mM, pH 8.5) and 50 mg of pOVA added. The solution was gently stirred, protected from light for 18 h at 4 °C. Unbound FITC was removed by ultrafiltration as above, collected and freeze dried. Yield of FITC-pOVA was $>75\%$.

5. Incorporation of modified protein into ISCOMs

The hydration method was used to prepare ISCOM formulations (Copland et al., 2000). Briefly, 2 mg cholesterol (Sigma) and 12 mg phosphatidylcholine (type XI-E from fresh egg, Sigma) were dissolved in 400 μl $CHCl_3$ and 2 mg of FITC-OVA, pFITC-OVA or FITC-pOVA added. Dried thin films of the resulting mixture were then prepared in round-bottomed flasks by evaporation of the $CHCl_3$. The lipid/protein film was hydrated by the addition of various amounts of Quil A (0, 4, 6, 8, 12, 16 mg) (Superfos Biosector, Vedbæk, Denmark) dissolved in 2 ml of water. Sucrose gradient ultracentrifugation (SGU) was used to isolate the resulting colloidal structures. Layered sucrose gradients were prepared from 10, 20, 30, 40 and 60% w/v sucrose solutions in Tris Buffer (pH 8.0). Solutions were carefully layered in 2 ml quantities in a 12 ml Beckman centrifuge tube. The sample was layered onto the gradient and centrifuged for 18 h at $45\,000 \times g$ (10 °C). Visible bands were isolated in a volume of approximately 1 ml with a fine glass pipette. The collected sample was dispersed in 12 ml Tris buffer (pH 8.0), and centrifuged for 30 min ($45\,000 \times g$ 10 °C). Washing was repeated and the pellet dispersed in 1 ml Tris buffer.

For measurement of incorporated protein, colloidal structures were disrupted by addition of 50 μl of the dispersion to 750 μl Tris buffer (pH 6.6) containing 5% Triton X-100 (BDH Laboratory Supplies, Poole, UK). Fluorescence of the resulting solution was measured (Shimadzu FR 540, ex. 490 nm, em. 520 nm) and entrapped protein expressed as a fraction of incorporation into the liposomal (0 mg Quil A) formulation. Fluorimetric measurements were performed 120 s after addition of the dispersion to the surfactant solution.

6. Characterisation of colloidal structures

Ten micrometers of the colloidal aqueous dispersions were placed on a glow-discharged (Edwards E306A Vacuum Coater) carbon coated copper grid for approximately 1 min. The grid was then blotted with filter paper and dried for approximately 15 s. Ten micrometers of 2.0% phosphotungstic acid (PTA, pH 5.2), were added for 1 min. Excess PTA was removed, and the EM grid was examined by transmission electron microscopy (Phillips CM100 Biotwin, acceleration voltage 100 kV). The size of the colloidal particles was determined from digital images of 100 structures per sample using Analys® software.

7. Results—Incorporation of modified protein into ISCOMs

With increasing amounts of Quil A, SGU showed formation of bands in higher sucrose concentration regions (from 10 to 30% sucrose for 0–8 mg Quil A respectively). This trend was independent of the protein-modification added (FITC-OVA, pFITC-OVA or FITC-pOVA). The density of the colloidal particles was thus increasing upon increasing Quil A concentration (Fig. 1).

TEM revealed that the predominant type of colloidal structures present in the dispersions varied with the amount of added Quil A (Fig. 2): In the absence of Quil A, multi-lamellar liposomes (20 nm to several 100 nm in diameter) were detected, as would be expected. Upon addition of increasing amounts of Quil A the following structures were observed; 2 mg/ml Quil A: ISCOM formation from lipidic particles, helical micelles, liposomes; 3 mg/ml Quil A: ISCOMs and bilayer structures, few helices; 4 mg/ml Quil A: predominantly ISCOMs, few liposomes and helices; 6 mg/ml Quil A: ISCOMs, ring-like and worm-like micelles, no liposomes; 8 mg/ml Quil A: ring- and

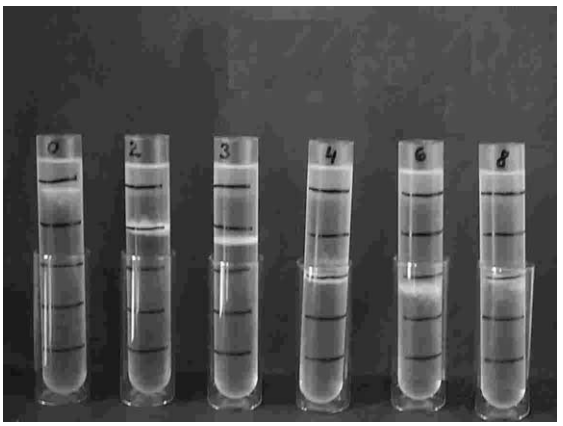

Fig. 1. SGU showing formation of bands in layers with increasing sucrose concentration as a function of Quil A concentration (from left to right: 0–8 mg/ml Quil A).

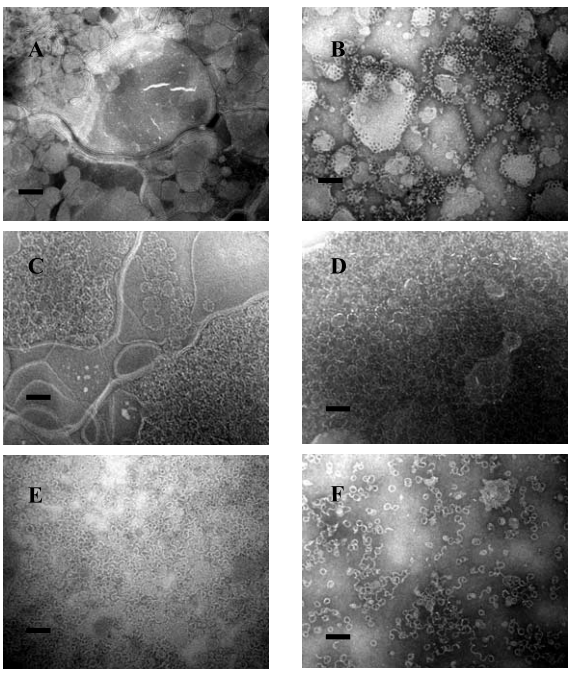

Fig. 2. TEM micrographs showing structures formed when a dried lipid film (2 mg cholesterol, 12 mg phospholipid) was hydrated with various concentrations of Quil A solutions. A = 0 mg/ml Quil A, B = 2 mg/ml Quil A, C = 3 mg/ml Quil A, D = 4 mg/ml Quil A, E = 6 mg/ml Quil A, F = 8 mg/ml Quil A. Bar represents 100 nm.

worm-like micelles, few ISCOMs, no liposomes. With the exception of the bilayer structures the particles were uniform in size; ISCOMs: 35–45 nm,

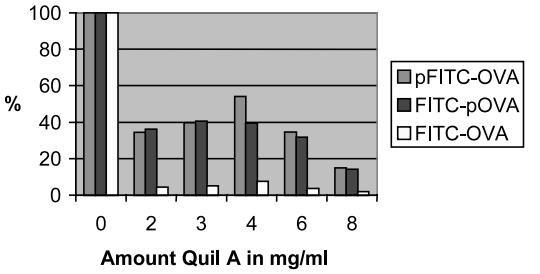

Fig. 3. Relative fluorescence intensity of colloidal dispersions as a function of type of modified protein and concentration of added Quil A.

ring-like micelles: 10–15 nm, helices: 40–200 nm length, 12–15 nm diameter.

Fig. 3 shows that addition of FITC-OVA resulted in a highly fluorescent band (indicative of incorporation of protein in the colloidal particles) only in the absence of Quil A. This demonstrates that FITC-OVA is entrapped in liposomes but is not incorporated in ISCOMs or any other colloidal structure formed. In contrast, the addition of pFITC-OVA or FITC-pOVA also lead to highly fluorescent bands if ISCOMs were the predominant colloid. In systems containing Quil A maximum fluorescence was noted after addition of 4 mg Quil A, which corresponded to systems in which the colloidal structures were predominately ISCOMs.

This study has shown that FITC-conjugated ovalbumin can be incorporated into ISCOMs prepared by the hydration method, following conjugation of the protein with palmitic acid. Whether ovalbumin was palmitified prior to conjugation to FITC (FITC-pOVA) or FITC-OVA was prepared initially and then palmitified (pFITC-OVA) had little effect on incorporation. The degree of incorporation of palmitified ovalbumin in non-liposomal particles reaches a maximum, if ISCOMs are the predominant colloidal particles in the system.

References

Copland, M.J., Rades, T., Davies, N.M., 2000. Hydration of lipid films with and aqueous solution of Quil A. International Journal of Pharmaceutics 196, 135–139.

Höglund, H.S., Dalsgaard, K., Lövgren, K., Sundquist, B., Osterhaus, A., Morein, B., 1989. ISCOM and immunostimulation with viral antigens. In: Harris, R.J. (Ed.), Sub-

cellular Biochemistry: Virally Infected Cells, vol. 15. Plenum Press, New York, pp. 39–68.

Kensil, C.R., 1996. Saponins as vaccine adjuvants. Critical Reviews in Therapeutic Drug Carrier Systems 13, 1–55.

Kersten, G.F.A., Crommelin, D.J.A., 1995. Liposomes and ISCOMs as vaccine formulations. Biochimica et Biophysica Acta 1241, 117–138.

Raychaudhuri, S., Rock, K.R., 1998. Fully mobilizing host defence: building better vaccines. Nature Biotechnology 16, 1025–1031.

Reid, G., 1992. Soluble Proteins incorporate into ISCOMs after covalent attachment of fatty acid. Vaccine 10, 597–600.

Sjölander, A., Cox, J., Barr, I.G., 1998. ISCOMs: an adjuvant with multiple functions. Journal of Leukocyte Biology 64, 713–723.