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Evaluation of ISCOM matrices clearance from rabbit nasal cavity by gamma scintigraphy

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ABSTRACT

Immune stimulating complexes and/or ISCOM matrices (adjuvant nanoparticles without antigen as a structural component) found potential applications as nasal vaccine adjuvant/delivery system owing to virus like particulate structure and saponin as potent Th1 adjuvant. One of important limiting factor for nasal vaccine delivery is the limited time available for absorption within the nasal cavity due to mucociliary clearance. In this report the clearance rate of ISCOM matrices from nasal cavity of rabbit was determined by gamma scintigraphy. ISCOM matrices were radiolabelled with ^{99m}Tc by direct labelling method using stannous chloride as a reducing agent. ^{99m}Tc labelled ISCOM matrices were administered into the nostril of female New Zealand rabbits and 1 min static views were repeated each 15 min until 4 h. Clearance rate of ISCOM matrices from nasal cavity was calculated after applying the physical decay corrections. The mean labelling efficiency for ISCOM matrices were calculated as ~58.4%. ISCOM matrices showed slower clearance rate compared to sodium pertechnetate control solution ($p < 0.005$) from nasal cavity that may be due to particulate and hydrophobic characters of ISCOM particles even though it was also cleared within 4 h from nasal cavity. Mucoadhesive ISCOM formulations that retain in nasal cavity for longer duration of time may reduce the dose/frequency of vaccine for nasal immunization.

1. Introduction

Nasal delivery of vaccines is gaining prominence as a preferred mode of immunization due to improved safety, ease of use over needles and better patient compliance due to painless administration especially among the elderly and children. Further, with the use of appropriate adjuvant/delivery system, nasal vaccination elicits mucosal as well as systemic immunity, a feature that is difficult to obtain with needle-based vaccinations (Slütter et al., 2008; Csaba et al., 2009). In addition, potent immune responses in the respiratory and genital tracts could be induced by intranasal immunization as a consequence of the common mucosal immune system (Sharma et al., 2009).

However, nasal delivery of vaccine is impaired by the mucociliary clearance, which is movement of mucus from peripheral airways to larynx by ciliary activity of the underlying epithelium. It removes inhaled substances like dust, bacteria and viruses entrapped in mucus from the nasal cavity towards the throat preventing those potentially harmful substances to penetrate the nasal epithelium (Yang et al., 2008). This mucociliary transport system is essential for the protection of the conducting airway surfaces from ambient irritants and infectious agents and for maintenance of airway patency.

Among possible mucosal delivery systems, nanoparticles hold great promise because of their capacity to protect encapsulated antigens, to promote interaction with mucosae and to direct antigens towards lymphoid tissues as potential inductive sites (Csaba et al., 2009). One of potential mucosal adjuvant/delivery system is immune stimulating complexes (ISCOMs) (Sun et al., 2009). These 40 nm 'cage-like' particles are composed of phospholipid, cholesterol, saponin and incorporate antigen by virtue of hydrophobic interactions via membrane anchor sequences of viral envelope glycoproteins. An alternative form of this adjuvant system is ISCOMATRIX adjuvant, formed by the combination of saponin, phospholipid and cholesterol to give similar 'cage-like' structures without the inclusion of antigen in its structure. When this inherently more convenient ISCOMATRIX adjuvant is mixed with the appropriate antigen prior to immunization, the immunological outcomes are similar to those observed for ISCOM vaccines (Pearse and Drane, 2005). Antigens formulated as ISCOMs or physically mixed with ISCOM matrices induce augmented antigen-specific responses after either parenteral or mucosal immunization in animal models (Sun et al., 2009) or following parenteral administration to humans (Sanders et al., 2005). This adjuvant technology also found great

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potential as nasal delivery system for vaccines where humoral, cellular as well as mucosal responses are required especially in situations after bacterial and viral pathogens invade the host via the mucosal surface (Hu et al., 2001).

Gamma scintigraphy imaging technique provides clearance data from the entire airway and being non-invasive includes the capability to restudy animal subjects when time course and/or prolonged treatment(s) are being considered. This technique relies on the use of radioactive tracers included into the medicament and selected so as to enable an optimum detection by a gamma camera. It has proved valuable and versatile in the assessment transit times of nasal sprays and drops (Bryant et al., 1999; Di Giuda et al., 2000), deposition patterns of nasal sprays (Harris et al., 1988; Suman et al., 1999; Eyles et al., 2001) and bioadhesive behaviour (Illum et al., 1987; Soane et al., 1999).

The aim of the work presented here was to study the nasal deposition and clearance characteristics of ISCOM matrices from rabbit nasal mucosa using external gamma scintigraphy to monitor the deposition pattern and clearance rate.

2. Materials and methods

2.1. Materials

Quil A (purified fraction of saponin mixture from Chilean tree *Quillaja saponaria* Molina) was kindly provided by Branntag Biosector, Frederikssund, Denmark. Cholesterol, L-α-phosphatidylcholine (PC) from egg yolk, MEGA-10, stannous chloride dehydrate ($SnCl_2{\cdot}2H_2O$) and sephadex G-25 were purchased from Sigma–Aldrich Private Ltd. (St. Louis, MO). All other chemicals and solvents were of analytical reagent grade and were used without further purification. Distilled deionized water (18 Ω A Milli-Q™ Water system, Millipore Corporation, Massachusetts, USA) was used throughout the study.

2.2. Experimental methods

2.2.1. Preparation of ISCOM matrices

ISCOM matrices were prepared by lipid hydration method (Demana et al., 2004). Briefly, 8 mg PC and 4 mg cholesterol were dissolved in 1 ml chloroform. The solution was evaporated to dryness at 45 °C for 1 h using rotary evaporator (Rotavapor 210 R, Büchi, Switzerland). 4 mg of Quil A dissolved in 3 ml of PBS, pH 7.0 (phosphate buffered saline contained 1.7 mM KH_2PO_4, 7.9 mM Na_2HPO_4, 2.7 mM KCl, 250 mM NaCl, pH 7.0) was then added to the dried lipid films. It was stirred for 3 h at 4 °C to ensure complete hydration. Formulations were then freeze-dried (Heto Drywinner, Germany) overnight followed by rehydration with 3 ml PBS, pH 7.0. Particles formed were purified by sucrose density gradient (10–60%) ultracentrifugation at 200 000 × *g* for 6 h (L-7 Ultracentrifuge, Beckman Coulter, USA) followed by dialysis for 48 h at 4 °C against PBS, pH 7.0 to separate the sucrose. It was concentrated to 2 ml by ultrafiltration using a 10,000-molecular weight cut-off membrane (Millipore, USA) and a 10 ml filtration cell (Amicon, Beverly, Massachusetts, USA) pressurized to 200 kPa.

2.2.2. Characterization of ISCOM matrices

The morphology of ISCOM matrices was determined by transmission electron microscopy (Philips EM268D, The Netherlands). One drop of aqueous dispersion was placed over a 400-mesh carbon-coated copper grid followed by negative staining with phosphotungstic acid (3%, w/v, adjusted to pH 4.7 with KOH) and placed at the accelerating voltage of 80 kV. The particle size and size distribution of the ISCOM matrices was determined by the laser diffraction method (Zeta Nano ZS 90, Malvern Instruments Inc., Worcestershire, UK). Nanoparticle suspension (1.0 ml) was dispersed in 4.0 ml ultrapure deionized water. The mean particle size and size distribution were determined at 25 ± 1 °C by scattering the light at 90°. The zeta potential of the ISCOM matrices was determined by laser doppler anemometry using a Zetasizer (Malvern Instruments, UK) following 1:300 dilution in PBS pH 7.0 An electric field of 150 mV was applied to measure the electrophoretic velocity of the particles. All the measurements were made in triplicate.

2.2.3. Gamma scintigraphy

2.2.3.1. Radiolabelling of ISCOM matrices. The prepared ISCOM matrices were labelled with ^{99m}Tc using the stannous reduction method as described previously (Garg et al., 2008). Briefly, 1 ml of the ISCOMATRIX dispersion (diluted to 5 mg/ml with PBS, pH 7.0) was mixed with stannous chloride dihydrate solution (100 μg in 100 μl of 0.10 N HCl). Before dissolving stannous chloride, sterile, pyrogen-free water was bubbled for 30 min with nitrogen in order to expel most of the oxygen to exclude the possibility of the oxidation of tin to the unreactive stannic form. The pH was adjusted to 7.00 ± 0.20 using 50 mM sodium bicarbonate solution. 1 ml of technetium pertechnetate (75–400 MBq) in sterile saline was then added; the mixture was shaken vigorously for 1 min by vortexing at 1200 rpm (Vortex mixer, Fischer Scientific, India) and left to react at room temperature for 30 min with continuous nitrogen purging. The final volume was made up to 2.5 ml using 0.9% (w/v) sodium chloride solution. The effects of incubation time, pH, and stannous chloride concentration on labelling were studied to achieve optimum reaction conditions.

Labelling efficiency of the purified radiolabelled formulations was determined by ascending instant thin layer chromatography (ITLC) using silica gel (SG)-coated fiber sheets of approximately 10 cm in length (Gelman Science Inc., Ann Arber, MI, USA) at room temperature (25 ± 1 °C). The ITLC was performed using 100% acetone as the mobile phase. A tiny drop (2–3 μl) of the radiolabelled formulation was applied at a point of 1 cm from one end of an ITLC-SG strip. The strip was developed in acetone and the solvent front was allowed to reach approximately 8 cm from the origin. The strip was cut into two equal halves and the radioactivity in each segment was determined in a well-type gamma-ray counter (gamma-ray scintillation counter, Type CRS 23C, Electronics Corporation of India Ltd., Mumbai, India). The free $^{99m}TcO4^-$ moved with the solvent (Rf = 0.9) while the radiolabelled formulation remained at the point of application. Percent labelling efficiency was calculated from the formulae:

$$\text{Labelling efficiency}(\%) = \frac{T \times 100}{T + B}$$

where *T* is the counts at top and *B* is the counts at bottom.

In vitro stability of the labelled formulations was determined by incubating 100 μl of the labelled formulation with 2.0 ml of simulated nasal fluid, pH 6.4 (Lorin et al., 1972) at room temperature and change in labelling efficiency was monitored over a period of 6 h by ITLC as described above.

2.2.3.2. Scintigraphic evaluation. In order to study the clearance characteristics, ISCOM matrices were administered to healthy female New Zealand rabbits (*n* = 4 per preparation, weight 3.40 ± 0.20 kg age 90–140 days). Animals were housed at animal house facility of the Institute of Nuclear Medicine and Allied Sciences (INMAS), New Delhi, India. Experiments were performed under strict supervision of associated technical specialist of the same laboratory. All animal studies were carried out under the guidelines compiled by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Culture, Government of India) and all the study protocols were approved by institutional animal ethics committee.