Genomic Medicine, Biomarkers, and Health Sciences (2011) 3, 91—97

Available online at www.sciencedirect.com

SciVerse ScienceDirect

journal homepage: www.e-gmbhs.com

ORIGINAL ARTICLE

Preparation and evaluation of sucrose stabilized tetanus toxoid encapsulated into chitosan microspheres

Saravanakumar Arthanari [a], **Ponnusamy Renukadevi** [a,*], **Kavaretti Raju Mani** [b]

[a] *Biotechnology Centre, Anna University of Technology Coimbatore, Coimbatore, Tamilnadu, India*
[b] *DTP Section, Central Research Institute, Kasauli, Himachal Pradesh, India*

Received 18 July 2011; received in revised form 30 September 2011; accepted 12 October 2011
Available online 22 November 2011

KEYWORDS
adjuvant;
biodegradable polymers;
chitosan microspheres;
tetanus toxoid;
vaccine delivery systems

Abstract Immunization is the most cost effective weapon for disease prevention in developing countries, and advanced molecular and genetic technologies are making new types of vaccines feasible. Here, the utility of both *in vitro* and *in vivo* methods to assess the release pattern of chitosan microspheres containing tetanus toxoid (TT) vaccine were evaluated. TT was stabilized and encapsulated in chitosan (TTCH) with a water-in-oil-in-water (W/O/W) multiple emulsion method using sucrose as a protein stabilizer. The TTCH prepared was smooth and spherical in shape with a diameter of around 10 μm. The *in vitro* release efficiency of TTCH was evaluated by differing stabilizer (sucrose) concentration (5%, 7%, 10% and 12% w/v) for a period of 70 days. The antigen release rates from the microspheres were determined by enzyme-linked immunosorbent assay. In these TTCH microspheres, a 10% w/v sucrose concentration gave good sustained antigen delivery for the period of 70 days. Based on the results of *in vitro* release, the *in vivo* studies were carried out using alum-adsorbed TT (from the Central Research Institute) as the standard. The antibody level was measured after 6 months, 9 months and finally, with one booster dose, after 12 months. In these *in vivo* studies, the TTCH antibody level rose up to 3.5 IU/mL of guinea pig serum; this compared with 2 IU/mL of guinea pig serum using the alum-adsorbed TT after 12 months with a second booster dose. The TTCH approach would be helpful to replace the existing adjuvant alum in the future.

* Corresponding author. Biotechnology Centre, Anna University of Technology Coimbatore, Jothipuram post, Coimbatore - 641047, Tamilnadu, India.
E-mail address: drrenukadevip@gmail.com (P. Renukadevi).

2211-4254/$36 Copyright © 2011, Taiwan Genomic Medicine and Biomarker Society. Published by Elsevier Taiwan LLC. All rights reserved.
doi:10.1016/j.gmbhs.2011.10.001

Introduction

The majority of currently available vaccines require several booster doses to induce effective immunity and this results in significant compliance problems, particularly in the developing world. This is particularly true for immunization with several vaccines; for example, the triple vaccine against diphtheria, pertussis and tetanus, widely used in the Expanded Program on Immunization, demands administration of the vaccines at repeated intervals of 4–6 weeks, three times. These multiple injection patterns have limitations because a percentage of children who receive the first dose do not turn up for the second or third dose, so do not complete the immunization schedule and are not fully immunized.[1]

Currently, vaccine development is experiencing another wave of tremendous progress with the aid of modern immunology techniques and biotechnology. Various vaccine targets have been identified; alongside prophylactic disease resistance, vaccine development is ongoing for a number of cancers and AIDS.[2] Moreover, safe and efficacious vaccines have been developed, such as subunit, live-vector based and DNA vaccines.

Tetanus remains a major killer in developing countries, with more than 500,000 deaths per year from neonatal tetanus alone, largely due to the logistical difficulty of delivering the two to three doses of vaccine required to confer protection for pregnant women, whose immunity can be passively transferred to the fetus.[3]

Aluminum phosphate and aluminum hydroxide are the currently approved adjuvants for human vaccination and are widely used for vaccines at present.[4] In recent years, there have been various attempts to demonstrate new improved techniques to induce a higher level of immunogenicity following parenteral and/or oral administration.[5] One approach has been to use biodegradable polymer technology to simplify a sustained release antigen following parenteral administration, which, it is envisaged, will minimize the number of injections given in the normal regimen.[6] Our main aim in the present research is to develop a single-dose vaccine to reduce the multiple injections administered to infants during the first 3 months [the present tetanus toxoid (TT) vaccination schedule is one dose per month for the first 3 months]. With respect to our primary goal, the single-dose vaccine has been developed successfully and, upon further investigation, reduces even the need for booster doses. The original aim of the developed vaccine delivery was to develop a single dose to replace the initial multiple doses, not the booster dose; the booster dose is to raise the antibody level later on. Our current investigation is focused on reducing, or completely removing, the booster dose by increasing the initial load of antibody.

Chitosan is derived by the deacetylation of chitin, which is a polymer of D-glucosamine and *N*-acetyl-D-glucosamine. Chitosan is well-known for its hydrophilic, biocompatible, biodegradable and non-toxic properties.[7–9] Chitosan suspensions or micro- and nanoparticles have been reported to have immune stimulating activities, such as increasing accumulation and activation of macrophage and polymorphonuclear cells, promoting resistance to infections by microorganisms, and inducing cytokine response.[10] There are many advantages to using chitosan or chitosan microspheres for vaccine delivery. First, chitosan can open the intercellular tight junctions and favor the paracellular transport of macromolecules. Second, chitosan nano- and microspheres are suitable for controlled drug and vaccine release. Third, chitosan nano- and microspheres are most efficiently taken up by phagocytotic cells. Thus chitosan and its derivatives could induce strong systemic and mucosal immune responses against antigens.[8,10] Immunizations with various antigens co-administered with chitosan produce both systemic and local immune responses. In a phase I clinical study, intranasal immunization with an influenza vaccine formulated with soluble chitosan glutamate showed positive effects.[11]

Materials and methods

Chitosan (0.15 Pa.s viscosity grade, 80% deacetylation) was purchased from the Central Institute of Fisheries Technology (Cochin, India). Tween 80 and Span 80 were purchased from Fluka (Sigma Aldrich, Buchs, Switzerland). TT (molecular weight, 150 kDa), with a limit of flocculation (Lf) content of 1250/mL, and the standard tetanus antitoxin were received as gifts from the Central Research Institute (CRI, Kasauli, H.P., India). Sucrose and sodium tripolyphosphate (TPP) were purchased from Sigma Aldrich (St. Louis, MO, USA). All other chemicals and reagents used were of analytical grade.

Preparation of TT encapsulated chitosan microspheres

A water-in-oil-in-water (W/O/W) multiple emulsion technique was used to prepare chitosan microspheres by following the previously described procedure with slight modifications.[12] Four batches of microspheres were prepared by altering the stabilizer (sucrose) concentration (5%, 7%, 10% and 12% w/v). The dispersion phase was prepared by mixing 50 mL of linseed oil and 10% of Tween 80 at 1700 rpm for 10 minutes. To this, 3 mL of 1% stabilized chitosan gel were mixed separately to form a water-in-oil emulsion. To the above emulsion, 2 mL of alum-free TT (1250 Lf units/mL), which was previously stabilized with various concentrations of sucrose, was added and the stirring continued. After 2 hours, 2 mL of 5% w/v of TPP was introduced drop wise and stirring continued for a further 2 hours. Another 2 ml of TPP was then added and the stirring continued for another 2 hours. Finally, the suspension of microspheres was centrifuged at 15,000 rpm to remove the oil layer and the pellets were separated. The pellets were washed five times with 5 mL of toluene then washed three times with 5 mL of acetone. The microspheres were suspended in 10 mL of acetone and dried at room temperature. The dried microspheres were stored in sealed glass vials in a vacuum desiccator.

Physicochemical characterization of the microspheres

The morphological examination of the microspheres was performed using a scanning electron microscope (SEM; JSM-T