International Journal of Pharmaceutics 475 (2014) 1–8

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical nanotechnology

Rabbit nasal immunization against influenza by dry-powder form of chitosan nanospheres encapsulated with influenza whole virus and adjuvants

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ARTICLE INFO

Article history:
Received 8 June 2014
Received in revised form 14 August 2014
Accepted 17 August 2014
Available online 20 August 2014

Keywords:
Chitosan
Dry powder
Nanospheres
Inactivated influenza virus
Nasal vaccine delivery

ABSTRACT

Influenza virus is one of the main causes of respiratory diseases in human. Although different vaccines have been produced during past decades, there is still a huge demand for a safe influenza vaccine with the ability to induce mucosal immune responses and sufficient protection, especially in elderly patients. In this study, chitosan nanospheres were employed as the drug delivery system. Influenza virus, CpG oligodeoxynucleotide (CpG ODN) and *Quillaja* saponins (QS) were incorporated in this nanospheric system. Three doses of dry powder nanosphere vaccine were nasally administered to rabbits on days 0, 45 and 60, followed by a final booster injection on day 75. Both humoral and cellular immune responses were investigated. Hemagglutination inhibition (HI) antibody titer was elevated in all groups compared to the control group at the end of vaccination in rabbits receiving nanospheres loaded with virus and CpG, CH(WV + CpG) ($P < 0.001$). Rabbit serum IgG raised significantly in all the vaccinated groups, with the highest responses in CH(WV + CpG) group. CH(WV + CpG) and CH(WV) induced significant sIgA titers ($P < 0.001$). CpG adjuvant also showed a prominent role in the stimulation and secretion of of IL-2 and IFN-γ cytokines (3 and 3.5 fold increase, respectively). Finally, as CH(WV + CpG) depicted to be effective in induction of humoral and cellular immune responses after nasal administration, this nanoparticulate adjuvant could be identified as an efficient adjuvant/delivery system for mucosal immunization against influenza virus.

1. Introduction

Influenza infection is an acute respiratory disease which easily circulates worldwide and causes epidemics and pandemics with high mortality and morbidity rates. In addition, this global threat imposes a considerable economical burden to countries. According to the WHO report, influenza epidemics inflict US $71–167 billion per year to the economy of the United States (WHO, 2003; Jefferson et al., 2012).

Influenza virus is phylogenetically classified into three different types, A, B and C. Due to the immunological memory against common strains, the yearly outbreak of type A usually turns into epidemics (Frank, 2002). To date, despite the available medications for the treatment and prophylaxis of influenza, vaccination against known pathogenic subtypes can be more cost-effective in the prevention of this disease (Amorij et al., 2008; Amour et al., 2012; Simonsen et al., 2011).

Since majority of the pathogenic microorganisms such as bacteria and viruses enter the body through mucosal surfaces, mucosal vaccine delivery is one of the most efficient vaccine delivery routes. Among all mucosal sites, nasal mucosa is the first site of contact with inhaled antigens and therefore, its use for vaccination especially against respiratory infections such as influenza, offers a promising prospect. Moreover, nasal vaccine delivery is a more convenient alternative to the classic parenteral vaccination, as it is able to enhance the systemic levels of specific immunoglobulin G and nasal secretory immunoglobulin A (Cox et al., 2004; Kadam et al., 1993; Ozsoy et al., 2009; Tafaghodi et al., 2008). Examples of the human efficacy of intranasal (IN) vaccines include those against influenza A

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http://dx.doi.org/10.1016/j.ijpharm.2014.08.032
0378-5173/

and B virus that have been successfully marketed and commercialized (Billich, 2000; Jin and Subbarao, 2014; Ozsoy et al., 2009), and others that are still in research and development such as proteosome-influenza (Treanor et al., 2006), adenovirus-vectored influenza (Park et al., 2009; Tutykhina et al., 2011), attenuated respiratory syncytial virus and parainfluenza viruses (Hu et al., 2005; Wyde et al., 2005).

Despite the aforementioned characteristics, nasal administration of free antigens generally induces weak mucosal and systemic immune responses and protection. Thereby, there is a crucial need for immunoadjuvants capable of inducing required immune response (Th1, Th2, antibodies, and CTLs). It is well known that especially in the viral infections both humoral and cellular immune responses are necessary to overcome the disease (Sun et al., 2009). However, extensive care required in choosing an adjuvant co-administered with the mucosal vaccines. Many experiments have shown that most of the adverse-reactions following nasal (or mucosal) vaccine administration are strongly associated with the adjuvants rather than the antigen itself (Spickler and Roth, 2003; Kulkarni, 2010). Thus, vaccine formulation should be designed in a way that provides a good immune response without causing any local irritation and other side effects (Bitter et al., 2011; Kulkarni, 2010).

One of the promising and successful steps toward amendment of nasal vaccines is incorporation of the antigen into mucoadhesive, biocompatible (nano- or micro) particles and antigen delivery in the form of dry powder. These particulate systems with encapsulating antigens can be tailor made by supplying with targeting ligands, adjuvants or endosomal escape mediators. This will form the desired vaccine and provides long lasting protective immunity (Slutter et al., 2008). On the other hand, the dry powder formulation offers physical and chemical stability for antigen and surmounts some problems of liquid vaccine formulations such as cold-chain and availability of medical personnel for administration (Kunda et al., 2013; Velasquez et al., 2011).

The dry powder nanospheres contain bioadhesive polymers, which usually absorb water on the surface of mucosa, swell, and become bulky and gel-like, so increase the residence time of the drug entrapped inside formulation on the mucus layer. Consequently, M-cells in the nasal associate lymphoid tissue (NALT) will rapidly uptake the nanoparticles, since they have a proven tendency to transport the small particles (particularly nanoparticles) from nasal cavity to lymphatic system and blood stream (Pandey et al., 2010; Pires et al., 2009). Various polymers have been used to prepare the particulate antigen delivery systems for nasal antigen delivery (Alpar et al., 2005; Bramwell and Perrie, 2005; Gordon et al., 2008; Tafaghodi et al., 2011; van der Lubben et al., 2001). It has been known that chitosan and its derivatives are biocompatible and biodegradable polymers used in encapsulation of antigens for nasal delivery. Many studies have used chitosan as carrier for different macromolecules. It can play the role of a carrier along with an adjuvant for one specific antigen in the form of aqueous dispersions, gels, sponges, and micro/nanoparticles (Arca et al., 2009; Gunbeyaz et al., 2010).

Selection of an animal model for in vivo evaluation of vaccines is highly dependent on the similarity of its immune system to human, the route of administration, and the vaccine dosage form. The studies on intranasal delivery of dry powder form of vaccines have frequently used rabbits as in vivo model, an animal with a nasal cavity structure/anatomy that closely resembles the human nasal cavity (Klas et al., 2008; Tafaghodi and Rastegar, 2010; Wang et al., 2012).

The objective of this research was to fabricate and characterize the chitosan nanospheres, loaded with influenza whole virus, and CpG oligodeoxynucleotide (CpG ODN) or *Quillaja* saponin (QS) adjuvants followed by evaluation of their immunogenicity by administration to rabbit's nostrils.

2. Material and methods

2.1. Materials

The influenza virus (A/New Caledonia/20/99H1N1) was replicated, harvested and UV-inactivated in the Influenza Research Lab, Pasteur Institute of Iran. Low-molecular-weight chitosan and purified QS were purchased from Sigma (France). The provided class B CpG ODN 2007 (5′-TCGTCGTTGTCGTTTTGTCGTT-3′) has a phosphorothioated backbone as indicated by the supplier (Micro-Synth, Switzerland) and has been successfully used in other studies on influenza vaccine (Lopez et al., 2006; Mallick et al., 2012). 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) and RNA extraction kit were obtained from Roche (Germany). New Zealand albino female rabbits were provided by Pasteur Institute of Iran. Goat anti-IgG and IgA antibodies were purchased from Thermo Scientific (USA). TMB (tetramethyl benzidine) solution as well as real time RT-PCR kit was obtained from Invitrogen (USA). All other chemicals used were of analytical grade. All the experiments were conducted according to the guidelines of ethics committee and were approved by a committee for animal experimentation.

2.2. Influenza virus suspension

The influenza whole virus (WV) propagated in Influenza Research Lab was inactivated using ultraviolet method as explained earlier (Deshmukh and Pomeroy, 1969). The virus inactivation was confirmed by three consecutive cell cultures in which no virus was detected by hemagglutination assay. Inactivated influenza virus was purified from the suspension by adapting the sucrose gradient method (Arora et al., 1985).

2.3. Preparation of chitosan nanospheres encapsulating antigen and adjuvants as powdered vaccines

The chitosan nanospheres were prepared by ionic gelation method (Xu and Du, 2003) as described in our previous study (Dehghan et al., 2013). Briefly, a 5% solution of tripolyphosphate (TPP) was emulsified in paraffin oil that contained Span 80 and Tween 80 (1:1 ratio), using a homogenizer (IKA, Germany). Chitosan (1% w/v) aqueous solution was also emulsified in the same oil phase. The latter emulsion was added dropwise to the first emulsion. The yielded nanospheres were dried under vacuum condition. WV, CpG and QS were added to the TPP solution before emulsification. The amount of virus and adjuvants were adjusted to achieve a concentration of 45 μg/dose of antigen and 10 μg/dose of each adjuvant.

2.4. Preparation of liquid influenza vaccines

The propagated influenza A/H1N1 virus was UV-inactivated and the harvested suspension was filter-sterilized using a 0.22 μm syringe filter. The amount of virus (based on its protein content) was adjusted to be 45 μg/dose.

2.5. Experimental groups and immunization schedule

Vaccination was accomplished using New Zealand albino female rabbits (1.5–2 kg, 6 weeks old). Seven groups of rabbits (6 animal/group) were immunized through the immunization schedule summarized in Table 1. Intranasal administration of vaccines requires booster immunizations to induce high immune responses (Haan et al., 2001; Singh et al., 2001). Six different formulations, three nanospheric and three liquid, were instilled into rabbits' nostrils (1 primer and 2 boosters) followed by a final