Vaccine 34 (2016) 306–312

Contents lists available at ScienceDirect

Vaccine

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

Optimization of physiological properties of hydroxyapatite as a vaccine adjuvant

Masayuki Hayashi[a,b,c], Taiki Aoshi[a,b,1], Yasumichi Kogai[d], Daisuke Nomi[d], Yasunari Haseda[a,b,1], Etsushi Kuroda[a,b], Kouji Kobiyama[a,b,2], Ken J. Ishii[a,b,*]

[a] Laboratory of Adjuvant Innovation, National Institutes of Biomedical Innovation, Health and Nutrition (NIBIOHN), 7-6-8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan
[b] Laboratory of Vaccine Science, WPI Immunology Frontier Research Center (iFReC), Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan
[c] Vaccine Research Development Office, Biology Research Laboratories, Sohyaku, Innovative Research Division, Mitsubishi Tanabe Pharma Corporation, 1000 Kamoshida-cho, Aoba-ku, Yokohama 227-0033, Kanagawa, Japan
[d] Technical Development Center, SofSera Corporation, 2-7-1 Ayumino, Izumi, Osaka 594-1157, Japan

ARTICLE INFO

Article history:
Received 25 August 2015
Received in revised form
15 November 2015
Accepted 22 November 2015
Available online 5 December 2015

Keywords:
Adjuvant
Hydroxyapatite
Particles
Vaccine
Size
Calcium phosphate

ABSTRACT

Various particles such as Alum or silica are known to act as an adjuvant if co-administered with vaccine antigens. Several reports have demonstrated that the adjuvanticity is strongly affected by the physicochemical properties of particles such as the size, shape and surface charge, although the required properties and its relationship to the adjuvanticity are still controversial. Hydroxyapatite particle (HAp) composed of calcium phosphate has been shown to work as adjuvant in mice. However, the properties of HAp required for the adjuvanticity have not been fully characterized yet. In this study, we examined the role of size or shape of HAps in the antibody responses after immunization with antigen. HAps whose diameter ranging between 100 and 400 nm provided significantly higher antibody responses than smaller or larger ones. By comparison between sphere and rod shaped HAps, rod shaped HAps induced stronger inflammasome-dependent IL-1β production than the sphere shaped ones *in vitro*. However, sphere- and rod-shaped HAp elicited comparable antibody response in WT mice. Vice versa, *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$ or *Caspase1*$^{-/-}$ mice provided comparable level of antibody responses to HAp adjuvanted vaccination. Collectively, our results demonstrated that the size rather than shape is a more critical property, and IL-1β production *via* NLRP3 inflammasome is dispensable for the adjuvanticity of HAps in mice.

1. Introduction

Calcium phosphate is a natural constituent of hard tissue in human body such as bone and teeth, and hydroxyapatite (HA) composed of calcium phosphate is a biocompatible material and used for dental implant materials or bone generation [1,2]. HA implant can be resorbed either by a solution-mediated process (the solubility of implants in physiological solutions) [3] or a cell-mediated process. In particular, macrophages have the ability to degrade HA crystals [4], demonstrating HA is a biodegradable material.

Vaccine is the most effective agent to prevent us from infections, and vaccine adjuvants are used to improve the efficacy for some vaccines. Vaccine adjuvants are classified by different types of compounds such as microbial products, emulsions, particles or liposomes [5–7]. Among these adjuvants, aluminum salts (Alum) is widely used for human vaccines. However, recent studies have suggested some concerns on the safety of Alum, such as the severity of local tissue irritation, longer duration of the inflammatory reaction at the injection site and propensity to elicit undesirable IgE responses [8–10]. Therefore, there is a need for alternative adjuvants. Calcium phosphates (CPs) are one of successfully marketed vaccine adjuvant for human use and has established safety profile [9]. CPs have been shown to improve various vaccines efficacy

* Corresponding author at: Health and Nutrition (NIBIOHN) Innovation 7-6-8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan. Tel.: +81 72 641 8043; fax: +81 72 641 8079.
E-mail addresses: Hayashi.Masayuki@me.mt-pharma.co.jp (M. Hayashi), aoshi@biken.osaka-u.ac.jp (T. Aoshi), kogai@sofsera.co.jp (Y. Kogai), nomi@sofsera.co.jp (D. Nomi), y-sedaha@biken.osaka-u.ac.jp (Y. Haseda), kuroetu@ifrec.osaka-u.ac.jp (E. Kuroda), kobi@lji.org (K. Kobiyama), kenishii@biken.osaka-u.ac.jp, kenishii@nibiohn.go.jp (K.J. Ishii).
[1] Present address: Vaccine Dynamics Project, BIKEN Innovative Vaccine Research Alliance Laboratories, Research Institute for Microbial Diseases (RIMD), Osaka University, Osaka 565-0871, Japan.
[2] Present address: La Jolla Institute for Allergy and Immunology, La Jolla, CA, USA.

http://dx.doi.org/10.1016/j.vaccine.2015.11.059
0264-410X/

including diphtheria and tetanus toxoids [11,12]. In addition, CPs have some advantages over the other particle adjuvants. CPs are non-toxic, induce very little inflammation at injection site, have less local tissue irritation and high adjuvanticity without IgE induction [8,9].

The studies of several different types of particle adjuvant have demonstrated that particle size, surface morphology, surface charge and surface area are important parameters that influence on the adjuvanticity [13,14]. In addition, their proinflammatory activities have varied greatly with their physical properties. Rod- or needle-shaped hydroxyapatite (HA) crystals, which resembled the forms of HA aggregates found in the osteoarthritis (OA) synovium, induce higher IL-1β and IL-18 production *via* NLRP3 inflammasome activation in macrophages than sphere-shaped ones, suggesting ectopic deposition of HA crystals in joints induces inflammation closely associated with OA [15]. However the optimal size or shape for the adjuvanticity remains still unclear although nanoparticles of HA composed of CP have shown to have adjuvanticity in various studies [8,16].

In this study, we developed a unique method of synthesis of hydroxyapatite particle (HAp) that the size and shape are desirably controlled. By using several different sized and shaped HAps, we examined the required physicochemical properties and its relationship to the adjuvanticity of HAp in mice.

2. Materials and methods

2.1. Mice

Six-week-old female C57BL/6j mice were purchased from CLEA Japan. *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$ and *Caspase1*$^{-/-}$ mice have been described previously [17]. All animal experiments were performed in accordance with institutional guidelines for the National Institute of Biomedical Innovation, Health and Nutrition animal facility.

2.2. Antigens, antibodies, adjuvants and peptides

Ovalbumin (OVA) was purchased from Seikagaku-kogyo. SplitHA vaccine derived from A/California/7/2009 (H1N1) strain was provided from Institute of Microbial Chemistry (Japan, Osaka). Aluminum hydroxide (Alum) was purchased from Invivogen. Cytokine ELISA kits for IL-1β and TNFα were purchased from R&D systems.

2.3. Hydroxyapatite particles (HAps)

Preparation of HAps were described previously [18,19], with some modifications. Briefly, HAps synthesized by previous method were sterilized by dry heat treatment at 300 °C for 2 h in a pre-sterilized glass ample. After the ample was sealed, dry heat treatment was repeated again.

2.4. Scanning electron microscopy (SEM) analysis

The size and shape of HAps were observed by SEM (JSM-6301F; JEOL Ltd., Japan, Tokyo).

2.5. Immunization

C57BL/6 J mice were immunized twice i.d. (at the tailbase) with 2 weeks intervals (day 0 and 14). For antigen-specific ELISA, blood samples were taken on day 14 and 28. For During vaccination and bleeding, the mice were anesthetized with ketamine. Antigen-loaded Alum or HAp was rotated for more than 1 h before the immunization. Alum was used at 0.67 mg/mouse for immunization.

2.6. Ab titer

For ELISA, 96 well plates were coated with 1 μg/ml SplitHA in a carbonate buffer (pH9.6) for SplitHA-vaccinated groups or 10 μg/ml OVA for OVA-vaccinated groups. Wells were blocked with PBS containing 1% BSA and diluted sera from immunized mice were incubated on the antigen-coated plate. After washing, goat anti-mouse total IgG, IgG1 or IgG2c conjugated HRP (Southern Biotech) were added and incubated for 1 h at RT. After additional washing, the plates were incubated with TMB for 30 min and the reaction was stopped with 1 N H_2SO_4 and then the absorbance was measured. Ab titer was calculated. OD of 0.2 was set as the cut-off value for positive samples.

2.7. Virus neutralization titer

The sera from immunized mice were mixed with RDE II (Denka-Seiken) and incubated for overnight at 37 °C. After further incubation for 1 h at 56 °C, serially diluted sera and a final infecting titer of 100 $TCID_{50}$/ml of A/California/7/2009 (H1N1) influenza virus in MEM media containing 10 mM HEPES, 1% Penicillin-Streptomycin, 0.2% bovine albumin and 10 μg/ml trypsin were incubated for 30 min at 37 °C. After that, these were added to Madin–Darby Canine kidney (MDCK) cells. Four days after incubation at 37 °C with 5% CO_2, the MDCK cells were fixed with 10% formalin for 10 min at room temperature. The cells were stained with Naphtol Blue Black Solution (Naphtol Blue Black 0.5 g, Sodium Acetate 0.5 g, Acetic Acid 45 ml and distilled water 455 ml) for 30 min at room temperature. The stained cells were washed well with water and then dried. Subsequently, 0.1 M NaOH was added to the cells and the plates were read at 630 nm in a microplate reader. Virus neutralization titer was determined by maximum dilution ratio which gave higher absorbance than average of positive and negative controls.

2.8. In vitro *stimulation of macrophages*

For macrophages preparation, mice were i.p. injected with 3 ml of 4% (w/v) thioglycolate (SIGMA) solution. Four days later, macrophages were collected from peritoneal cavity and plated on 96 well plates. Macrophages were primed with 50 ng/ml LPS for 15 h, and stimulated with adjuvants for 8 h. IL-1β or TNFα in supernatants was measured by ELISA.

2.9. Statistical analysis

Statistical significance ($P < 0.05$) between groups was determined by Dunnet's Multiple comparison test or student *t*-test.

3. Results

3.1. Several hundred nanometer HAps have a better adjuvanticity than other larger or smaller ones

To investigate the relationship between particle size and adjuvanticity of HAp, we first prepared sphere-shaped HAps with sizes ranging from nanometer to micrometer including 40 nm (S40), 100 nm (S100), 170 nm (S170), 400 nm (S400), 1.8 μm (S1800) and 5 μm (S5000) on average (Table 1 and Fig. 1A–F). C57BL/6j mice were immunized twice with influenza splitHA vaccine (SV) plus three different doses (0.2, 1 or 5 mg) of these HAps, and the antigen-specific antibody response was examined. One hundred to 400 nm particles induced higher antibody response than smaller (40 nm) and larger (1.8 μm or 5 μm) sized particles (Fig. 2A–C). IgG subclass analysis also revealed that HAp induced more IgG1