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Aluminum hydroxide nanoparticles show a stronger vaccine adjuvant activity than traditional aluminum hydroxide microparticles

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ABSTRACT

Aluminum hydroxide is used as a vaccine adjuvant in various human vaccines. Unfortunately, despite its favorable safety profile, aluminum hydroxide can only weakly or moderately potentiate antigen-specific antibody responses. When dispersed in an aqueous solution, aluminum hydroxide forms particulates of 1–20 μm. There is increasing evidence that nanoparticles around or less than 200 nm as vaccine or antigen carriers have a more potent adjuvant activity than large microparticles. In the present study, we synthesized aluminum hydroxide nanoparticles of 112 nm. Using ovalbumin and *Bacillus anthracis* protective antigen protein as model antigens, we showed that protein antigens adsorbed on the aluminum hydroxide nanoparticles induced a stronger antigen-specific antibody response than the same protein antigens adsorbed on the traditional aluminum hydroxide microparticles of around 9.3 μm. The potent adjuvant activity of the aluminum hydroxide nanoparticles was likely related to their ability to more effectively facilitate the uptake of the antigens adsorbed on them by antigen-presenting cells. Finally, the local inflammation induced by aluminum hydroxide nanoparticles in the injection sites was milder than that induced by microparticles. Simply reducing the particle size of the traditional aluminum hydroxide adjuvant into nanometers represents a novel and effective approach to improve its adjuvanticity.

1. Introduction

Many vaccines and antigens require an adjuvant to induce a strong immune response [1]. Aluminum-containing adjuvants are approved by the United States Food and Drug Administration for human use. There are two main aluminum-containing adjuvants, aluminum hydroxide and aluminum phosphate. Aluminum hydroxide adjuvant is composed of small primary fibers with an average calculated dimension of 4.5 × 2.2 × 10 nm, whereas the primary particles of aluminum phosphate adjuvant are around 50 nm [2]. In an aqueous solution, however, the size of the primary particles of both aluminum hydroxide and aluminum phosphate becomes 1–20 μm as a result of aggregation [3]. The mechanisms of immunopotentiation by aluminum-containing adjuvants have yet been fully elucidated. Originally, Glenny et al. (1931) proposed that aluminum-containing adjuvants could form an antigen depot in the injection site, from where the antigens are slowly released, and thereby the adsorption efficiency of antigens on aluminum-containing adjuvants is thought to be critical [1]. However, data from Hansen et al. showed that the tight binding of antigens onto aluminum-containing adjuvants may significantly reduce the amount of antigens that can elute from the aluminum salts, resulting in a weak antibody response [4]. Berthold et al. examined whether the full adsorption of antigens onto adjuvants is necessary by comparing the immune responses induced by two vaccine formulations: *Bacillus anthracis* recombinant protective antigen (PA) protein adsorbed onto aluminum hydroxide with a high binding efficiency, and PA admixed with aluminum phosphate with a negligible binding [5]. It was found that both formulations induced comparable anti-PA antibody responses, suggesting that the adjuvant activity of aluminum salts may not be entirely depended on the adsorption of the antigens onto the adjuvants [5]. Other mechanisms of immunopotentiation by aluminum-containing adjuvants have been proposed as well [2,6,7]. HogenEsch [6] summarized that aluminum-containing adjuvants may enhance immune responses by (i) direct or indirect stimulation of dendritic cells (DCs) [8]; (ii) activation of complements [9]; and (iii) induction of the release of chemokines [6,9]. More recently, aluminum-containing adjuvants have been shown to promote caspase-1 activation and IL-1β secretion through the NALP3 inflammasomes [10].

Due to their favorable safety profile, aluminum-containing adjuvants have been widely used in human vaccines for decades. Unfortunately, aluminum-containing adjuvants can only weakly or moderately potentiate antigen-specific antibody responses and are generally considered incapable of helping antigens to induce cellular immune responses [11]. As aforementioned, when dispersed in an aqueous solution, both aluminum hydroxide and aluminum phosphate form 1–20 μm particulates [3]. Recently, there had been extensive efforts in identifying the relationship between the size of particulate vaccine carriers and their adjuvant activities [12–14]. Although it remains controversial as to what particle size is associated with the most potent adjuvant activity, it is clear that the size of particulate vaccine carriers significantly affects their adjuvant activities, and there are data showing

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that particulate vaccine carriers of around 200 nm (or less) may be optimal. For examples, Fifis et al. reported that ovalbumin (OVA)-conjugated polystyrene particles of 230 nm induced stronger OVA-specific antibody and cellular immune responses than other larger OVA-conjugated polystyrene particles after intradermally injected into mice [13,15]. In a previous study, we also showed that small solid lipid nanoparticles of 200 nm have a more potent adjuvant activity than larger solid lipid nanoparticles of 700 nm, when OVA as an antigen is surface-conjugated on them [16]. The ability of the smaller nanoparticles to more effectively facilitate the uptake of antigens carried by them by antigen-presenting cells (APCs) and to up-regulate the expression of major histocompatibility complex and co-stimulatory molecules is likely related to their potent adjuvant activity [16]. Based on these findings, we proposed to improve the adjuvant activity of the traditional aluminum-containing adjuvants by reducing their particle size. We hypothesized that small aluminum hydroxide nanoparticles of ≤200 nm have a more potent vaccine adjuvant activity than the traditional aluminum hydroxide adjuvant with a particle size of 1–20 μm. To test this hypothesis, we synthesized aluminum hydroxide nanoparticles with a mean diameter of 112 nm and compared their adjuvant activity with that of the traditional aluminum hydroxide suspension with a mean diameter of 9.3 μm. OVA and *B. anthracis* PA protein were used as model antigens.

2. Materials and methods

2.1. Materials

Dried aluminum hydroxide gel was from Spectrum (Gardena, CA). Aluminum chloride hexahydrate, sodium hydroxide, OVA, horse serum, Laemmli sample buffer, fluorescein-5(6)-isothiocyanate (FITC), sodium bicarbonate, sodium carbonate, phosphate-buffered saline (PBS), and incomplete Freund's adjuvant (IFA) were from Sigma-Aldrich (St. Louis, MO). Goat anti-mouse immunoglobulins (IgG) were from Southern Biotechnology Associates, Inc. (Birmingham, AL). Carbon-coated 400-mesh grids were from Electron Microscopy Sciences (Hatfield, PA). Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) was from Vector Laboratories, Inc. (Burlingame, CA). *B. anthracis* PA protein was from List Biological Laboratories, Inc. (Campbell, CA). Bio-Safe™ Coomassie blue staining solution and Bio-Rad DC™ protein assay reagents were from Bio-Rad Laboratories (Hercules, CA). GM-CSF was from R&D Systems, Inc. (Minneapolis, MN). Tissue-Tek® O.C.T. compound medium was from Sakura Finetek USA, Inc. (Torrance, CA). Cell culture medium and fetal bovine serum (FBS) were from Invitrogen (Carlsbad, CA).

2.2. Mice and cell lines

Female BALB/c and C57BL/6 mice, 6–8 weeks of age, were from Charles River Laboratories, Inc. (Wilmington, MA). The OVA-expressing B16-OVA cell line was generously provided by Dr. Edith M. Lord and Dr. John Frelinger (University of Rochester Medical Center, Rochester, NY) [17] and cultured in RPMI1640 medium supplemented with 5% FBS and 400 μg/ml of geneticin (Sigma). Mouse J774A.1 macrophage cells (# TIB-67™) were from the American Type and Culture Collection (Manassas, VA) and grown in DMEM supplemented with 10% FBS, 100 U/ml of penicillin and 100 μg/ml of streptomycin, all from Invitrogen. DC2.4 cells (a mouse dendritic cell line) were originally created by Dr. Kenneth Rock (University of Massachusetts Medical School, Worcester, MA) [18] and grown in RPMI1640 medium supplemented with 10% FBS, 100 U/ml of penicillin and 100 μg/ml of streptomycin.

2.3. Preparation of aluminum hydroxide nanoparticles and microparticles

Aluminum hydroxide nanoparticles of less than 200 nm were synthesized by reacting aluminum chloride with sodium hydroxide in a solution. An equal volume of a 3.6 mg/ml $AlCl_3 \cdot 6H_2O$ solution and a 0.04 M NaOH solution were added into a glass vial, and a small volume of 0.01 M NaOH was added to adjust the pH to 7.0. After 20 min of stirring at room temperature, particle suspension was sonicated for 15 min to break down the particle size. A PD10 desalting column (Amersham Biosciences, Piscataway, NJ) was then used to remove sodium chloride in the suspension, and the eluted fractions were analyzed for nanoparticles by measuring the particle size using a Malvern Zetasizer Nano ZS (Westborough, MA), and for aluminum content using a Varian 710-ES Inductively Coupled Plasma Optical Emission Spectrometer in the Civil Architectural and Environmental Engineering Department at The University of Texas at Austin. The fourth fraction with the highest concentration of aluminum was used for further studies. Endotoxin was not detectable in the nanoparticle preparation with a ToxinSensor™ chromogenic limulus amebocyte lysate endotoxin assay kit from GenScript (Piscataway, NJ) [16]. Aluminum hydroxide microparticles were prepared by dispersing dried aluminum hydroxide gel into sterile water, followed by vigorous vortexing and 5 min of water-bath sonication, if needed. The size of the microparticles was determined using a Sympatec Helos laser diffraction instrument (Sympatec GmbH, Germany) equipped with a R3 lens.

2.4. Stability of aluminum hydroxide particles

The stability of aluminum hydroxide particles in suspension at 4 °C or room temperature was initially examined before adsorption with proteins. The particles in suspension were kept at 4 °C for 30 days, and their sizes were measured on days 0 and 30. In another study, the particles in suspension were kept at room temperature for 15 days, and their sizes were measured on days 0, 1, 7 and 15.

2.5. X-ray diffraction

The X-ray diffractograms of aluminum hydroxide particles were obtained with a Scintag X1 theta–theta powder diffractometer using Cu K-alpha radiation and a solid state Si(Li) detector in the Texas Materials Institute X-ray Facility in the Chemical Engineering Department at The University of Texas at Austin.

2.6. Adsorption of protein antigens on aluminum hydroxide particles

The adsorption of proteins (OVA or PA) on aluminum hydroxide particles was carried out by mixing the particles in suspension with the protein in solution. Briefly, a certain volume of the protein solution was added into a tube (10 μg OVA or 4 μg PA), followed by the addition of particles in suspension at a weight ratio of 1:5 to 1:1 (OVA *vs.* particles) or 1:5 (PA *vs.* particles). After 20 min of gentle stirring, the protein–particle mixtures were stored at 4 °C or freeze-dried, if needed, before further use.

The OVA-adsorbed aluminum hydroxide nanoparticles were lyophilized using a FreeZone plus 4.5 l cascade console freeze dry system (Labconco, Kansas City, MO). A proper cryoprotectant such as trehalose (2%, w/v) was needed to successfully freeze-dry the nanoparticles (Fig. S1A). In a short-term 28-day study and when stored as a lyophilized powder at 4 °C, the size of the lyophilized, OVA-adsorbed aluminum hydroxide nanoparticles did not significantly change (Fig. S1B), indicating that the antigen-adsorbed aluminum hydroxide nanoparticles may be stored long-term as a lyophilized powder.

2.7. Transmission electron microscopy (TEM)

The OVA-adsorbed aluminum hydroxide nanoparticles were examined using an FEI Tecnai Transmission Electron Microscope in the Institute for Cellular and Molecular Biology (ICMB) Microscopy and Imaging Facility at The University of Texas at Austin [19]. Carbon-coated 400-mesh grids were activated for 1–2 min. One drop of the OVA-

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