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Toward understanding the mechanism underlying the strong adjuvant activity of aluminum salt nanoparticles

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ABSTRACT

Aluminum salts such as aluminum oxyhydroxide and aluminum hydroxyphosphate are commonly used human vaccine adjuvants. In an effort to improve the adjuvant activity of aluminum salts, we previously showed that the adjuvant activity of aluminum oxyhydroxide nanoparticles is significantly more potent than that of aluminum oxyhydroxide microparticles. The present study was designed to (i) understand the mechanism underlying the potent adjuvant activity of aluminum oxyhydroxide nanoparticles, relative to microparticles, and (ii) to test whether aluminum hydroxyphosphate nanoparticles have a more potent adjuvant activity than aluminum hydroxyphosphate microparticles as well. In human THP-1 myeloid cells, wild-type and NLRP3-deficient, both aluminum oxyhydroxide nanoparticles and microparticles stimulate the secretion of proinflammatory cytokine IL-1β by activating NLRP3 inflammasome, although aluminum oxyhydroxide nanoparticles are more potent than microparticles, likely related to the higher uptake of the nanoparticles by the THP-1 cells than the microparticles. Aluminum hydroxyphosphate nanoparticles also have a more potent adjuvant activity than microparticles in helping a model antigen lysozyme to stimulate specific antibody response, again likely related to their stronger ability to activate the NLRP3 inflammasome.

1. Introduction

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Vaccines have been an indispensable tool in the fight against infectious diseases. For most modern vaccines, an adjuvant is often needed to help induce strong pathogen-specific humoral and cellular immune responses. Aluminum salts are the most commonly used human vaccine adjuvants in the U.S., although their adjuvant activity is relatively weak. Two types of aluminum salts, namely aluminum oxyhydroxide (AlO(OH), or AH, also known as aluminum hydroxide) and aluminum hydroxyphosphate ($Al(OH)_x(PO)_y$, or AP, also known as aluminum phosphate), are commonly used. Both aluminum oxyhydroxide and aluminum hydroxyphosphate are composed of small nanometer-scale primary particles, which if not stabilized, aggregate in an aqueous solution to form larger microparticles of 1–20 μm [1,2].

In an effort to improve the adjuvant activity of aluminum salts, previously we reported that the adjuvant activity of aluminum oxyhydroxide nanoparticles of ~110 nm is significantly stronger than that of aluminum oxyhydroxide microparticles of ~9 μm [3]. The present study was designed to understand the mechanism underlying the stronger adjuvant activity of aluminum oxyhydroxide nanoparticles, and to test whether aluminum hydroxyphosphate nanoparticles also have a stronger adjuvant activity than microparticles.

The exact mechanisms underlying the adjuvant activity of aluminum salts have yet been fully elucidated [4,5]. Proposed mechanisms of immunopotentiation by aluminum-containing adjuvants include formation of antigen depot [6,7], stimulation of dendritic cells [8], complement activation [9], and stimulation of chemokine release [4,9]. However, recent evidence points out that aluminum salt-based adjuvants activate an intracellular pathogen pattern recognition receptor signaling pathway involving the NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome [10,11]. The activation of NLRP3 inflammasome controls the maturation and secretion of cytokines such as IL-1β and IL-18 whose potent proinflammatory activity directs host responses to infection and injury [12]. Therefore, in the present

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study, we focused on examining whether the adjuvant activity of aluminum oxyhydroxide nanoparticles (AH-NPs) is dependent on NLRP3 inflammasome activation, and whether AH-NPs are more potent than aluminum oxyhydroxide microparticles (AH-MPs) in activating the NLRP3 inflammasome. In addition, we prepared aluminum hydroxyphosphate nanoparticles (AP-NPs) and microparticles (AP-MPs) and compared their abilities in activating NLPR3 inflammasome in cell culture and in helping enhance antigen-specific immune responses in a mouse model.

2. Materials and methods

2.1. Materials

Aluminum Hydroxide Nanopowder/Nanoparticles (high purity, 99.9%, 10–20 nm, hydrophilic, # US3026) were from US Research Nanomaterials, Inc. (Houston TX). Aluminum chloride hexahydrate, sodium hydroxide, polyvinylpyrrolidone, Laemmli sample buffer, sodium bicarbonate, sodium carbonate, phosphate-buffered saline (PBS), CA-074-Me, beta-mercaptoethanol, and N-acetyl-L-cysteine (NAC) were from Sigma-Aldrich (St. Louis, MO). Aluminum hydroxide Alhydrogel® liquid suspension (Alum), lipopolysaccharide (LPS), phorbol 12-myristate 13-acetate (PMA), monosodium urate (MSU) crystal, HygroGold, and normocin were from InvivoGen (San Diego, CA). The human IL-1β ELISA kit was from R&D Systems (Minneapolis, MN). Goat anti-mouse immunoglobulins (IgG) were from Southern Biotechnology Associates, Inc. (Birmingham, AL). Bio-safe™ Coomassie blue staining solution and Bio-Rad DC™ protein assay reagents were from Bio-Rad Laboratories (Hercules, CA). Cell culture medium, antibiotics, and fetal bovine serum (FBS) were from Invitrogen (Carlsbad, CA).

2.2. Preparation and characterization of aluminum oxyhydroxide particles

Previously, we synthesized aluminum oxyhydroxide nanoparticles by reacting aluminum chloride with sodium hydroxide, whereas the aluminum oxyhydroxide microparticles were prepared by suspending commercially available aluminum hydroxide dried gel in aqueous medium [3]. In the present study, to ensure that the aluminum oxyhydroxide nanoparticles and microparticles are prepared from the same material, the Aluminum Hydroxide Nanopowder/Nanoparticles powder from the US Research Nanomaterials was used. The powder was dispersed in water (5 mg/ml) and separated into AH-NPs and AH-MPs by centrifugation. Briefly, the Aluminum Hydroxide Nanopowder/Nanoparticles powder was slowly added into warm water while stirring. The suspension was probe-sonicated and spun at 900 rpm for 10 min. The supernatant was probe-sonicated repeatedly and spun down again at 900 rpm for 10 min. The resultant supernatant suspension was stabilized by adding polyvinylpyrrolidone (1%, w/v) and used as nanoparticles (AH-NPs). The sediment was re-suspended and used as microparticles (AH-MPs) in subsequent studies.

The size and size distribution of the AH-MPs in suspension were determined using a Sympatec HELOS laser diffraction instrument equipped with a R3 lens (Sympatec GmbH, Germany). The particle size and size distribution of the AH-NPs were determined using a Malvern Zeta Sizer Nano ZS (Westborough, MA). The AH-NPs and AH-MPs were also examined using an FEI Tecnai Transmission Electron Microscope (TEM) in the Institute for Cellular and Molecular Biology (ICMB) Microscopy and Imaging Facility at The University of Texas at Austin. Carbon-coated 400-mesh grids were activated for 1–2 min. One drop of the particle suspension was deposited on the grids and incubated for 2 min at room temperature. The grids were washed with water and dried for 1 min. Extra water was removed using filter paper and allowed to dry for 15 min before observation [3]. The aluminum contents in the AH-NPs and AH-MPs preparations were determined using a Varian 710-ES Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) in the Civil Architectural and Environmental Engineering Department at the University of Texas at Austin.

2.3. Synthesis and characterization aluminum hydroxyphosphate particles

To prepare aluminum hydroxyphosphate particles, an aqueous solution of $AlCl_3$ (180 mM) was slowly added under magnetic stirring to an aqueous solution of dibasic hydrogen phosphate (108 mM). The resultant suspension was probe-sonicated and centrifuged at 900 rpm, and the supernatant was collected. The process was repeated twice with the collected supernatant. Particles in the supernatant are considered as nanoparticles (*i.e.*, AP-NPs), while the sediment was re-suspended and considered as microparticles (*i.e.*, AP-MPs). The particle size and size distribution and the aluminum contents in samples were determined as mentioned above.

2.4. Cell culture

Human THP-1 cells from the American Type Culture Collection (ATCC, Manassas, VA) were grown at 37 °C with 5% CO_2 in RPMI-1640 medium supplemented with 10% FBS (v/v), 100 U/ml-100 μg/ml penicillin-streptomycin, and 50 μM β-mercaptoethanol. The NLRP3-deficient THP-1 cells (THP1-defNLRP3, InvivoGen) were grown at 37 °C with 5% CO_2 following InvivoGen's instruction in RPMI-1640 medium supplemented with 10% FBS (v/v), 200 μg/ml HygroGold, and 100 μg/ml normocin. HEK-Blue IL-1β cells that contain an IL-1β-sensitive reporter were from InvivoGen and grown following InvivoGen's instruction at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FBS (v/v), 4.5 g/l glucose, 2 mM GlutaMAX medium, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml zeocin, 200 μg/ml hygromycin, and 100 μg/ml normocin (all from Life Technologies or InvivoGen).The level of secreted embryonic alkaline phosphatase (SEAP) protein, a truncated form of human placental alkaline phosphatase released into the culture medium, was used as a measurement of NF-κB activation through IL-1β stimulation with the QUANTI-Blue™ detection reagent.

2.5. IL-1β production after THP-1 cells are stimulated with aluminum salt particles

THP-1 cells, wild type or defNLRP3, in 100 μl culture medium were plated at the density of 3×10^4 per well in 96-well plates in the presence of 1 μg/ml PMA for 16 h to differentiate the monocytes into macrophages. The medium was replaced with fresh medium, and cells were treated with AH-NPs, AH-MPs, AP-NPs, or AP-MPs (50 μg of aluminum/ml) in the presence of LPS (10 ng/ml) for 6 h. Inflammasome activation requires two signals for the production of mature IL-1β, and thus the differentiated THP-1 cells were cultured in the presence of LPS [13,14]. Our own data also showed that aluminum particles alone (*e.g.*, Alhydrogel®, AH-NPs, AP-NPs) are weak in inducing THP-1 cells to produce IL-1β, but treating the cells with LPS significantly increases the aluminum particles' ability to induce the production of IL-1β (data not shown). The supernatant of the activated cells was collected to determine IL-1β content using an ELISA kit (R&D Systems) or the InvivoGen's Quanti-Blue® SEAP reporter assay following the manufacturers' instructions.

To examine the effect of specific inhibitors on IL-β release, THP-1 cells were pre-treated with NAC (a radical scavenger, antioxidant, and glutathione precursor, 25 mM) or CA-074-Me (a protease cathepsin B inhibitor that also inhibits lysosomal rupture [15],

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