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Al adjuvants can be tracked in viable cells by lumogallion staining

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ABSTRACT

The mechanism behind the adjuvant effect of aluminum salts is poorly understood notwithstanding that aluminum salts have been used for decades in clinical vaccines. In an aqueous environment and at a nearly neutral pH, the aluminum salts form particulate aggregates, and one plausible explanation of the lack of information regarding the mechanisms could be the absence of an efficient method of tracking phagocytosed aluminum adjuvants and thereby the intracellular location of the adjuvant.

In this paper, we want to report upon the use of lumogallion staining enabling the detection of phagocytosed aluminum adjuvants inside viable cells. Including micromolar concentrations of lumogallion in the culture medium resulted in a strong fluorescence signal from cells that had phagocytosed the aluminum adjuvant. The fluorescence appeared as spots in the cytoplasm and by confocal microscopy and co-staining with probes presenting fluorescence in the far-red region of the spectrum, aluminum adjuvants could to a certain extent be identified as localized in acidic vesicles, i.e., lysosomes.

Staining and detection of intracellular aluminum adjuvants was achieved not only by diffusion of lumogallion into the cytoplasm, thereby highlighting the presence of the adjuvant, but also by pre-staining the aluminum adjuvant prior to incubation with cells. Pre-staining of aluminum adjuvants resulted in bright fluorescent particulate aggregates that remained fluorescent for weeks and with only a minor reduction of fluorescence upon extensive washing or incubation with cells. Both aluminum oxyhydroxide and aluminum hydroxyphosphate, two of the most commonly used aluminum adjuvants in clinical vaccines, could be pre-stained with lumogallion and were easily tracked intracellularly after incubation with phagocytosing cells.

Staining of viable cells using lumogallion will be a useful method in investigations of the mechanisms behind aluminum adjuvants' differentiation of antigen-presenting cells into inflammatory cells. Information will be gained regarding the phagosomal pathways and the events inside the phagosomes, and thereby the ultimate fate of phagocytosed aluminum adjuvants could be resolved.

1. Introduction

Aluminum-based adjuvants in the form of aluminum salts have been used in pharmaceutical vaccine formulations for many decades. The aluminum adjuvants are dispersed in aqueous solvents at circumneutral pH where the aluminum salts form particles and aggregates in the nanometer to micrometer range. Despite their extensive use, a clear mechanistic understanding of how aluminum salts potentiate the induction of an immune response is still missing (Exley et al., 2010). The adsorption of antigens onto aggregates of the adjuvant and their subsequent release at the inoculation site has long been recognized as a possible *modus operandi* of the adjuvant (Lindblad, 2004). One alternative mechanism could be the induction of an inflammatory response by aluminum salts and indeed injection of aluminum adjuvants results in cell injury and a sterile inflammation (Rock et al., 2010). Cell injury at the injection site triggers the release of danger associated molecular patterns (DAMPs) and an infiltration of inflammatory cells together with increased levels of relevant chemokines has been shown at the inoculation site (Lu and HogenEsch, 2013). Furthermore, an inflammatory response may also be induced by the assembly and activation of the Nalp3 inflammasome by antigen-presenting cells (APCs) after phagocytosis of aluminum adjuvants, leading to the release of IL-1β and IL-18 (Hornung et al., 2008; Eisenbarth et al., 2008; Marrack et al., 2009). Considering the efficiency and continued use of aluminum adjuvants, experimental information concerning the mechanisms behind the induction of an immune response by aluminum adjuvants is surprisingly limited.

To be able to verify the distribution and action of aluminum salts after injection at immunization sites, a robust method allowing for

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their traceability is warranted. Lumogallion has been used as a histological stain to detect the presence of aluminum in both plant and mammalian tissues (Silva et al., 2000; Uchiumi et al., 1998), and it has recently been used to demonstrate the unequivocal presence of aluminum adjuvant in a monocytic cell line (Mold et al., 2014). In this paper, we intend to report upon the use of lumogallion in the staining of living cells after phagocytosis of aluminum adjuvants. For this purpose, we utilized a monocytic cell line with known phagocytizing capacity and a mouse glioma cell line. Staining of living cells by lumogallion will open up new possibilities of tracking intracellular aluminum adjuvants in viable cells thereby revealing the intracellular fate and pathway(s) of aluminum adjuvants after endocytosis.

2. Materials and methods

2.1. Cell lines

THP-1 (ATCC TIB-202) was obtained from LGC Standards, UK and the GL261 mouse glioma cells of C57BL/6 origin was kindly provided by Dr. G Safrany, Hungary.

2.2. Materials

The aluminum adjuvant preparations used herein were Alhydrogel AlO(OH) and Adju-Phos $AlPO_4$ purchased from Brenntag Biosector (Frederikssund, Denmark). Al_2O_3 60 nm nanoparticles coated with aluminic ester and aluminum hydroxide nanopowder/nanoparticles $Al(OH)_3$ 10–20 nm were obtained from US Research Nanomaterials (Houston, TX, USA).

Lumogallion (CAS 4386-25-8) came from TCI Europe N.V., Antwerp, Belgium, and morin hydrate (CAS 654055-01-3) was obtained as a powder from Sigma-Aldrich, St. Louis, MO, USA.

All other reagents were of analytical grade.

2.3. Cell culture

THP-1 and GL261 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum of EU grade (PAA laboratories, Linz, Austria) and 100 μg/ml of gentamicin (PAA laboratories, Linz, Austria). This medium will be referred to as R10. All cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 and the cells were maintained by sub-culturing once every third day.

2.4. Co-culture with aluminum adjuvants and staining of the cells

Triplicates of THP-1 cells 0.5×10^6 cells per ml were co-cultured in 96-well plates with Alhydrogel AlO(OH) adjuvant corresponding to final concentrations of aluminum content ranging from 0.8 to 50 μg/mL in a total volume of 200 μl R10 culture medium during 4 to 16 h (overnight) at 37 °C. Controls consisted of cells cultured in R10 medium in the absence of aluminum adjuvant. Lumogallion was then added from a stock solution in RPMI 1640 medium to final concentrations of 1.6 to 20 μM, and the cells were further incubated at 37 °C for various time intervals (30 min up to overnight). Controls consisted of cells cultured with and without any aluminum adjuvant and without any addition of lumogallion. Specified concentrations of aluminum, lumogallion and incubation periods of each experiment are described in the figure legends. After staining, the triplicates from the incubations were pooled and centrifuged at 1,000×*g* before the cells were re-suspended in 300 μl 1% (w/v) paraformaldehyde (PFA). Finally, the cells were analyzed by flow cytometry using an Accuri C6 flow cytometer and standard settings.

In experiments using morin, co-culture with aluminum adjuvant was performed as previously described before morin was added to the cells at a final concentration of 160 μM using a saturated solution of morin in RPMI 1640 medium (800 μM morin) as stock solution. Co-culture, washing, fixation and analysis by flow cytometry was performed as previously described.

2.5. Pre-staining of aluminum formulations

2.5.1. Staining of aluminum formulations with lumogallion

Various formulations of aluminum adjuvants corresponding to 500, 250, 125 or 62.5 μg aluminum/ml were incubated with 50 μM lumogallion in a total volume of 1 ml R10 medium. The suspensions were incubated at room temperature overnight on a rocking table, and the next day, the aluminum particles were collected by centrifugation for 10 min at 13,000×*g*. Finally, the particles were re-suspended in 1 ml R10 medium and the fluorescence of the suspensions was measured by spectrofluorometry using excitation 490 nm, emission 580 nm and a cut off filter of 530 nm.

2.5.2. Pre-staining of Alhydrogel AlO(OH) adjuvant

Alhydrogel AlO(OH) corresponding to 4 mg aluminum/ml was incubated overnight at room temperature on a rocking table with 50 μM lumogallion in a total volume of 1 ml R10 medium. The next day, the adjuvant was collected by centrifugation for 10 min at 13,000×*g*. The collected adjuvant was re-suspended in 1 ml R10 medium and stored in the refrigerator until further use.

2.5.3. Release of fluorescence from pre-stained Alhydrogel AlO(OH) upon washing

Alhydrogel AlO(OH) corresponding to 4 mg aluminum/ml was incubated overnight at room temperature on a rocking table with 50 μM lumogallion in a total volume of 1 ml R10 medium. The next day, the suspension was diluted 20 times with R10 medium, and 1 ml of the diluted suspension was withdrawn and centrifuged for 10 min at 13,000×*g*. The supernatant was collected, and the pellet was re-suspended in a new 1 ml portion of R10 medium. After 5 min on a rocking table, the sample was once again centrifuged for 10 min at 13,000×*g*. The supernatant was collected again and the pellet re-suspended in a new 1 ml portion of R10 medium and placed on a rocking table for 5 min before the sample was centrifuged, the supernatant collected and the pellet re-suspended in R10 medium. This procedure was repeated until 10 supernatants were collected. Finally, the fluorescence from all of the collected supernatants and a sample corresponding to the suspension obtained after the first re-suspension of stained aluminum adjuvant was measured by spectrofluorometry using excitation 490 nm, emission 580 nm and a cut off filter of 530 nm.

2.5.4. Pre-staining with morin

Alhydrogel AlO(OH) corresponding to 0.5 mg aluminum/ml was incubated overnight at room temperature on a rocking table with 250 μM morin in a total volume of 1 ml PBS. The next day, the adjuvant was collected by centrifugation for 10 min at 13,000×*g*. The collected adjuvant was re-suspended in 0.5 ml R10 medium and stored in the refrigerator until further use.

2.5.5. Zeta potential analysis

Zeta potential analysis was performed using a Zeta Potential/Particle Sizer NICOMP 380 ZLS, Particle Sizing System, Santa Barbara, CA, USA. Alhydrogel and pre-stained Alhydrogel were suspended in PBS at a concentration of 2 mg/ml and analyzed using ZPW388 Application Version 2.00 with the settings; temperature 20 °C, liquid visco: 1.002, liquid Index of Ref: 1.333, dielectric constant: 78.5 and electrode spacing: 0.4 cm.

2.6. Co-culture with pre-stained Alhydrogel AlO(OH)

Triplicates of THP-1 cells 0.5×10^6 cells per ml were co-cultured in 96-well plates with lumogallion or morin pre-stained Alhydrogel AlO(OH) adjuvant corresponding to final concentrations of aluminum