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Brief report

Effect of particulate adjuvant on the anthrax protective antigen dose required for effective nasal vaccination

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ABSTRACT

Successful vaccine development is dependent on the development of effective adjuvants since the poor immunogenicity of modern subunit vaccines typically requires the use of potent adjuvants and high antigen doses. In recent years, adjuvant formulations combining both immunopotentiators and delivery systems have emerged as a promising strategy to develop effective and improved vaccines. In this study we investigate if the association of the mast cell activating adjuvant compound 48/80 (C48/80) with chitosan nanoparticles would promote an antigen dose sparing effect when administered intranasally. Even though the induction of strong mucosal immunity required higher antigen doses, incorporation of C48/80 into nanoparticles provided significant dose sparing when compared to antigen and C48/80 in solution with no significant effect on serum neutralizing antibodies titers. These results suggest the potential of this novel adjuvant combination to improve the immunogenicity of a vaccine and decrease the antigen dose required for vaccination.

1. Introduction

Strategies to reduce the antigen dose in vaccines are highly desirable since they could reduce the vaccine manufacturing cost, improve its availability and therefore increase the supply of vaccines worldwide. This is particularly relevant for mucosally-administered vaccines that usually require high antigen doses due to enzymatic degradation on the mucosae. The use of potent vaccine adjuvants is one of the strategies able to provide antigen sparing. More recently there has been a growing recognition of the potential of adjuvant combinations in vaccine development [1–4]. However, the advantage, in terms of antigen dose-sparing, of having an immunopotentiator co-delivered by nanoparticles instead of its soluble form has not yet been thoroughly evaluated. The aim of this study was to investigate if having an effective immunopotentiator incorporated within nanoparticles would increase adjuvant potency resulting in an antigen dose sparing effect. To test this hypothesis, compound 48/80 (C48/80), a mast cell activator with well established immunopotentiator properties [5–10], was associated with mucoadhesive chitosan nanoparticles. Mice were intranasally vaccinated with different doses of protective antigen of anthrax (PA) plus compound 48/80 (C48/80), in solution or incorporated in nanoparticles, and the respective immune response evaluated. To the best of our knowledge, this is the first report that shows the effect of combining C48/80 and the antigen in chitosan nanoparticles on the antigen dose required for induction of the desired immune response.

2. Methods and materials

2.1. Materials

Low molecular weight chitosan (deacetylation degree 95%), was purchased from Primex BioChemials AS (Avaldsnes, Norway) and used after a purification process adapted from [11]. Compound 48/80 was purchased from Sigma–Aldrich (St. Louis, MO, USA). Recombinant protective antigen of anthrax (PA) and recombinant lethal factor (LF) were purchased from List Biologicals (Campbell, CA, USA).

2.2. Nanoparticle preparation and characterization

C48/80 loaded chitosan nanoparticles (Chi-C48/80 NP) were prepared by adding dropwise 3 mL of an alkaline solution (5 mM

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NaOH) of C48/80 and Na_2SO_4 (0.3 mg/mL and 2.03 mg/mL, respectively) to 3 mL of a chitosan solution (1 mg/mL in 0.1% acetic acid) under high speed vortexing. The nanoparticles were formed after further maturation for 60 min under magnetic stirring and PA was loaded by physical adsorption after incubation for 30 min with Chi-C48/80 NP. Particle size and zeta potential were measured in a Zetasizer Nano ZS (Malvern). Loading efficacy of C48/80 and PA was determined according to a method validated by our group [12] and Pierce BCA protein assay, respectively. Endotoxin content of materials and nanoparticles was evaluated using the PYROGENT™ Gel Clot LAL assay.

2.3. Nasal immunization

Six- to eight-week-old C57BL/6NCr female mice were purchased from Charles River (National Cancer Institute, Frederick, MD). Mice (5 per group) were intranasally immunized on days 0, 7 and 21 with 0.4 μg, 1 μg or 2.5 μg of PA adjuvanted with 15 μg of C48/80 in solution or C48/80 incorporated in Chi NP. Controls included a naïve group that received saline and an antigen alone group vaccinated with 2.5 μg of PA. Each mouse received 15 μL of vaccine formulation, 7.5 μL per nostril, under isoflurane anesthesia. Serum was collected on days 14, 21 and 42. Nasal washes, fecal material and vaginal lavage were collected on day 42 and processed as previously described [13]. All animal procedures were approved by Duke University Division of Laboratory Animal Resources and Duke University Institutional Animal Care and Use Committee (IACUC).

2.4. ELISA

Titers of rPA-specific IgG, IgG isotypes and IgA antibodies were determined by ELISA as previously described [14]. The log 2 endpoint titers were used for statistical analysis. Samples with undetectable titers were assigned a titer of one less than the first dilution tested.

2.5. LeTx neutralization assay

A macrophage toxicity assay using J774A.1 mouse macrophages (ATCC, Manassas, VA) was used to determine the ability of serum anti-PA antibodies to neutralize lethal toxin (LeTx). The assay was performed as previously described [14]. LeTx neutralizing titers for each mouse were calculated as 50% neutralization titers (NT50), *i.e.*, the serum dilution needed to neutralize 50% of LeTx. Samples with an NT50 less than 1:128 were below our tested range and were assigned a value of 1:64 for graphical representation and statistical evaluation.

2.6. Statistical analysis

Statistical analysis was performed with GraphPad Prism v 5.03 (GraphPad Software Inc., La Jolla, CA, USA). Student's *t*-test and one-way ANOVA followed by Tukey's post-test were used for two samples or multiple comparisons, respectively. A P value < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Anthrax protective antigen was efficiently adsorbed on the surface of Chi-C48/80 particles

Previous work in our lab demonstrated that nasal immunization with Chi-C48/80 NP as adjuvant resulted in high levels of protective antibodies and significantly higher nasal IgA titers than those induced by C48/80 in solution or Chi NP. Fecal and vaginal IgA levels were also significantly greater in mice immunized with Chi-C48/80 NP when compared to the responses induced by Chi NP [15]. Having demonstrated the potent adjuvant activity of Chi-C48/80 NP, the focus of this study was to assess if the incorporation of C48/80 in Chi NP had the further advantage of providing an antigen dose sparing effect when compared with C48/80 in solution.

Chi-C48/80 NP were prepared by a method optimized in our laboratory. The loading efficacy of C48/80 on NP was $18.7 \pm 3.0\%$ which corresponded to a loading capacity of 35.3 μg of C48/80 per 1 mg of nanoparticles. These nanoparticles were thereafter loaded with different amounts of PA by physical adsorption to obtain vaccine formulations with the desired antigen doses. The vaccine formulations displayed a unimodal size distribution (Fig. 1A) with an average size in the range of 550 nm to 680 nm and were positively charged (Fig. 1B). All formulations had more than 70% of the PA adsorbed to the particles and the remaining antigen was free on the nanoparticle suspension (Fig. 1C). The loading of different quantities of antigen on nanoparticle surface did not significantly affect the size and charge of the formulations ($P > 0.05$). To rule out the possibility of endotoxin contamination of the nanoparticles, endotoxin in the final formulation were evaluated and found to be below the level of detection (0.125 EU/mL) of the assay.

3.2. Incorporation of C48/80 in nanoparticles lowers the antigen dose required for the induction of serum Lethal Toxin neutralizing antibody responses

To evaluate if the incorporation of C48/80 in nanoparticles would improve its adjuvanticity leading to an antigen sparing effect, mice were intranasally immunized with 0.4 μg, 1 μg or 2.5 μg of PA adjuvanted with 15 μg of C48/80 in solution or incorporated in Chi NP (Chi-C48/80 NP). After the first boost (day 14), all adjuvants induced serum anti-PA IgG titers significantly greater than those induced by immunization with 2.5 μg PA alone (Fig. 2A). Chi-C48/80 NP plus 2.5 μg or 1 μg of PA induced anti-PA serum IgG titers significantly higher than all the other vaccine formulations while 0.4 μg of PA plus C48/80 in solution induced the lowest levels of PA-specific IgG. The beneficial effect of the encapsulation of the C48/80 was also observed by the direct comparison of C48/80 in solution with Chi-C48/80 NP at each antigen dose. Chi-C48/80 NP was more effective at inducing serum anti-PA IgG than C48/80 at all antigen doses tested. After the second boost (at day 42), 0.4 μg of PA plus C48/80 was again the group with lower anti-PA IgG when compared with all other adjuvanted groups (Fig. 2B). On the other hand, high levels of PA-specific IgG were detected in the serum of mice vaccinated with Chi-C48/80 NP even with only 0.4 μg of antigen. The increase in serum anti-PA IgG titers after the second boost was particularly evident in the group immunized with the lowest dose of PA adjuvanted with Chi-C48/80 NP. In fact, 0.4 μg of PA plus Chi-C48/80 NP induced PA-specific IgG titers significantly higher than the IgG responses induced by any dose of PA adjuvanted with C48/80 in solution.

The use of PA allowed us to evaluate the functionality of the immune response using an *in vitro* macrophage toxicity assay that assesses the ability of the induced anti-PA serum antibodies to neutralize LeTx. While vaccination with 0.4 μg of PA plus free C48/80 failed to induce LeTx neutralizing titers significantly higher than those observed in mice immunized with antigen alone, the administration of C48/80 incorporated into nanoparticles resulted in strong LeTx-neutralizing activity even when using a lower dose of antigen (Fig. 2C). Interestingly, 1 μg of PA co-administered with Chi-C48/80 NP induced LeTx neutralizing titers significantly higher than those induced by any dose of PA adjuvanted with C48/80 in solution. The influence of antigen dose on the quality of the immune response was assessed by monitoring anti-PA IgG1 and IgG2c titers at day 42. All adjuvant formulations induced levels of anti-PA IgG1 significantly higher than IgG1 titers in mice

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