New strategies for simplifying influenza vaccination
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Chapter 4

Enhanced pulmonary immunization with aerosolized inactivated influenza vaccine containing delta inulin adjuvant

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Abstract

Vaccination is the primary intervention to contain influenza virus spread during seasonal and pandemic outbreaks. Pulmonary vaccination is gaining increasing attention for its ability to induce both local mucosal and systemic immune responses without the need for invasive injections. However, pulmonary administration of whole inactivated influenza virus (WIV) vaccine induces a Th2 dominant systemic immune response while a more balanced Th1/Th2 vaccine response may be preferred and only induces modest nasal immunity. This study evaluated immunity elicited by pulmonary versus intramuscular (i.m.) delivery of WIV, and tested whether the immune response could be improved by co-administration of delta (δ)-inulin, a novel carbohydrate-based particulate adjuvant. After pulmonary administration both unadjuvanted and δ-inulin adjuvanted WIV induced a potent systemic immune response, inducing higher serum anti-influenza IgG titers and nasal IgA titers than i.m. administration. Moreover, the addition of δ-inulin induced a more balanced Th1/Th2 response and induced higher nasal IgA titers versus pulmonary WIV alone. Pulmonary WIV alone or with δ-inulin induced hemagglutination inhibition (HI) titers > 40, titers which are considered protective against influenza virus. In conclusion, in this study we have shown that δ-inulin adjuvanted WIV induces a better immune response after pulmonary administration than vaccine alone.
1. Introduction

Influenza vaccines are important in controlling virus infection and spread during seasonal and pandemic outbreaks. Conventionally, inactivated influenza vaccines are administered by subcutaneous or intramuscular injection, with just live attenuated vaccines being administered by intranasal spray. However, non-invasive routes of inactivated influenza vaccine administration, including intranasal, sublingual, intradermal and pulmonary delivery have gained increasing attention [1–4]. The pulmonary route is an attractive route given its non-invasive nature, ease of administration and lack of need for trained health care workers. A further potential advantage of pulmonary immunization is the ability to induce local mucosal immune protection in the respiratory tract, e.g. via secretory IgA production [5]. However, there are also some short comings of pulmonary route of vaccination including a propensity to induce a dominant Th2 immune response [6,7]. A more balanced Th1/Th2 vaccine response is preferred as the combination of cellular with humoral immunity protects the host better against invading influenza viruses [8]. Furthermore, although pulmonary influenza vaccines elicit local immune responses in the respiratory tract, their ability to induce intranasal IgA antibody titers are only modest [6,7,9]. A strong mucosal response in the nose and lungs is desired to provide maximal protection at the port of entry for the influenza virus [10].

Addition of a suitable adjuvant to a pulmonary influenza vaccine could help improve vaccine immunogenicity and thereby its ability to protect against a viral infection. Carbohydrate-based adjuvants have gained recent attention with their ability to combine a high level of efficacy, tolerability and safety [11]. The lack of reactogenicity of carbohydrate-based adjuvants namely δ-inulin and δ-inulin, as demonstrated in both animal models and human vaccine trials [12], suggests their suitability for pulmonary use although to date they have predominantly been used by i.m. and s.c. routes [13]. δ-inulin, the most immunologically active inulin polymorph [14], was shown to have potent adjuvant activity when co-administered i.m. with seasonal or pandemic inactivated or recombinant influenza vaccines in the form of Advax™ adjuvant [12,15,16]. Therefore, we hypothesized that δ-inulin might similarly be able to enhance influenza vaccine immunogenicity when administered via the pulmonary route. This study therefore set out to compare mucosal and systemic WIV immunogenicity when administered by pulmonary versus i.m. routes with or without δ-inulin adjuvant.

2. Material and method

2.1 Vaccine

The WIV antigen (A/California/7/2009 (H1N1)) was produced and inactivated as previously reported [6]. After inactivation and dialysis, the protein content of the vaccine was determined by the micro Lowry assay [6]. The adjuvanted vaccine was prepared by mixing 200 µg of δ-inulin adjuvant (Advax™ adjuvant, 1 mg/ml,
Vaxine Pty Ltd, Adelaide, Australia) with WIV equivalent to 5 µg of HA (0.1 µg/µl) immediately prior to vaccination.

### 2.2 Immunization studies and sample analysis

The local animal welfare and use committee of the University of Groningen approved all animal experiment handling and work protocols. The study was carried out in 6-8 week old female BALB/c mice (Harlan, Zeist, The Netherlands). Mice were immunized twice at a two week interval (day 0 and 14) with WIV formulations containing 5 µg HA with or without 200 µg of δ-inulin adjuvant. For pulmonary immunization, mice were anesthetized by inhalation of isoflurane/O₂ then intubated in vertical position with a modified Autograde catheter (Becton Dickinson, Breda, The Netherlands). 50 µl of vaccine was delivered to the lungs of the mice using a IA-1C micro-sprayer attached to a FMJ-250 high-pressure syringe (Penn-Century Inc., Wyndmoor, USA). For i.m. immunization, 50 µl of vaccine was administered by dividing the dose equally between both hind limbs. Mice were then placed in a recovery incubator at a temperature of 25 °C for 2 hours, and then placed back in the housing facility.

Two weeks after the first dose, blood samples were withdrawn by facial vein technique for evaluation of the immune response. Two weeks after the second dose, the mice were sacrificed. After sacrifice, blood samples were withdrawn by heart puncture. Serum was stored at -20 °C until used. Nose wash was obtained using 1 ml PBS, pH 7.4, containing complete protease inhibitor cocktail tablets (Roche, Almere, The Netherlands). The collected samples were analyzed by ELISA to evaluate anti-influenza IgG, IgG1, IgG2a, and IgA antibody titers. Serum samples were also analyzed for anti-influenza HI titers, as previously described [7].

### 2.3 Statistical analysis

Immunoglobulin and HI titers are reported as geometric mean ± standard error mean. The differences in titers were analyzed by two-tailed Mann Whitney U-test at a confidence interval of 95 % (P≤0. 05). The significance level in figures is denoted by an increase in the number of “*” symbols: one symbol (P≤0.05); two symbols (P≤0.01) and three symbols (P≤0.001).

### 3. Results

#### 3.1 Anti-influenza IgG responses

The immune responses evoked in mice by i.m. or pulmonary immunization with or without δ-inulin adjuvant were first evaluated by measuring total serum anti-influenza IgG titers 14 days after the first and second immunization. Both i.m. and pulmonary immunization with or without δ-inulin adjuvant induced potent IgG responses even after the first immunization with further increases in anti-influenza
IgG titers seen after the second immunization (Figure 1). While after just a single immunization, anti-influenza IgG titers were significantly lower in the group receiving WIV alone by the pulmonary route when compared to WIV alone given by i.m injection, this reduced immunogenicity in the single dose pulmonary group was more than compensated for by the addition of δ-inulin adjuvant, with the single dose WIV+ δ-inulin pulmonary group achieving higher IgG titers than the equivalent single dose i.m. groups.

Furthermore, while after the second immunization in the i.m groups the increases in anti-influenza IgG were relatively modest (approximately 1 log), the increases in IgG titers after the second immunization in the pulmonary groups were much greater (approximately 3 logs for the WIV alone group and 1.5 logs for the WIV+ δ-inulin adjuvant group). Hence after two immunizations pulmonary immunization with or without δ-inulin adjuvant induced significantly higher IgG antibody titers than two doses of i.m. vaccine, either with or without adjuvant. Two weeks after the second vaccination, the mice that received i.m. vaccine with δ-inulin adjuvant achieved a significantly higher anti-influenza IgG response than mice that received i.m. WIV vaccine alone (P=0.0002), whereas two weeks after the second vaccination, there were no longer significant differences in anti-influenza IgG titers in mice that received pulmonary WIV vaccine with δ-inulin adjuvant compared to those that received pulmonary WIV vaccine alone.

**Figure 1.** WIV specific serum IgG antibody titers induced on day 14 (gray bars) and day 28 (black bars) after i.m. and pulmonary vaccination on day 0 and day 14.

**Figure 2.** WIV specific serum IgG subtypes, IgG1 (gray bars) and IgG2a (black bars) antibody titers induced on day 28 after i.m. and pulmonary vaccination on day 0 and day 14.
Phenotype of i.m versus pulmonary vaccine responses

Anti-influenza IgG subtypes were evaluated two weeks after the second vaccination (Figure 2). Pulmonary immunization with WIV alone induced primarily an IgG1 response, consistent with a Th2 dominant immune response, consistent with the results of previous studies [6,7]. This compared to the i.m groups with or without adjuvant where the titers of IgG1 and IgG2a were approximately equal (Figure 2), consistent with a more balanced Th1/Th2 response. The addition of δ-inulin adjuvant to the pulmonary immunization group significantly increased the IgG2a antibody titers when compared to WIV alone given pulmonary without compromising the IgG1 antibody titers, thereby resulting in a more balanced Th1/Th2 response. This was interesting as by contrast the addition of δ-inulin adjuvant to the i.m immunization group resulted in primarily in enhancement of the IgG1 rather than IgG2a response.

Hemagglutination inhibition titers after i.m. and pulmonary vaccination

Hemagglutination inhibition (HI) antibody titers reflect the subfraction of anti-influenza immunoglobulin that is able to block influenza virus attachment to mammalian sialic acid receptors and thereby more directly measures the amount of neutralizing antibodies induced by an influenza vaccine, with an HI titer of 40 considered to be protective [17]. After the second immunization, all groups whether

![Figure 3. WIV specific serum HI levels induced on day 14 (gray bars) and day 28 (black bars) after i.m. and pulmonary vaccination on day 0 and day 14. The HI titers were measured in triplicate. Since no differences in HI titers were found no error bars are shown](image)

![Figure 4. WIV specific nose IgA antibody levels induced by unadjuvanted (gray bars) and adjuvanted WIV (black bars) vaccine on day 28 after i.m. and pulmonary vaccination on day 0 and day 14.](image)
i.m and pulmonary, achieved an HI titer > 40, consistent with protection (Figure 3). There were no significant difference between the i.m WIV alone group and both pulmonary vaccine groups, with the only group having significantly higher HI titers than the i.m WIV alone group being the δ-inulin adjuvanted i.m. vaccine group (Figure 3).

Mucosal response after i.m. versus pulmonary immunization

The production of local secretory IgA at relevant mucosal respiratory surfaces was shown to be an important first line of defense against influenza virus attachment and infection. In general, mucosal IgA is not seen after intramuscular immunization. The mucosal immune response to i.m. or pulmonary immunization was therefore evaluated by measuring anti-influenza IgA levels in nasal washings of immunized mice (Figure 4). WIV vaccine given pulmonary with or without adjuvant induced significantly higher anti-influenza IgA titers in nasal washings than obtained with the i.m. vaccine. Furthermore, δ-inulin adjuvant significantly enhanced the anti-influenza IgA titers in nasal washings compared to WIV vaccine alone given pulmonary (P=0.02) (Figure 4).

4. Discussion and conclusions

To our knowledge, this is the first study to confirm that δ-inulin maintains its vaccine adjuvant activity when administered via the pulmonary route. Mice receiving δ-inulin adjuvanted influenza vaccine administered via the pulmonary route demonstrated a more balanced Th1/Th2 immune response whereas pulmonary administration of WIV alone demonstrated a marked Th2 bias in their antibody response. This Th1 enhancement of the antibody response to a pulmonary vaccine by δ-inulin is in contrast to the results seen when it was given i.m where it primarily enhanced IgG1 production consistent with a more Th2 action, and suggests that the immune pathways activated in the lung by δ-inulin may differ from the pathways it activates after i.m. injection. Another feature of δ-inulin when given pulmonary was its ability to enhance mucosal anti-influenza IgA titers. The most marked pulmonary effect of δ-inulin adjuvant on IgG titers was seen after a single immunization, when it enabled higher anti-influenza IgG titers to be achieved than obtained after even two i.m immunizations with WIV alone, or a single i.m immunization with WIV + δ-inulin. Previously, pulmonary or intranasal vaccines have generally required multiple booster vaccine doses to achieve satisfactory immunogenicity [6,18]. Based on these findings it may be possible to utilize δ-inulin adjuvant along with antigen dose optimization and delivery strategies to achieve single dose pulmonary vaccine protection, and this will be tested in future influenza challenge studies. While pulmonary delivery of WIV when compared to i.m. delivery induced a broader antibody isotype response than included IgA, and after two doses achieved HI titers considered protective, for as yet unexplained reasons there was a divergence such that whilst the anti-influenza IgG titers were substantially higher, the HI titers induced by pulmonary vaccination were
substantially lower than those induced by i.m. vaccination. Typically, WIV vaccines induce a Th1-biased immune response upon i.m. vaccination while Th2 dominant responses have usually been seen after pulmonary vaccination suggesting that the lung may have an innate Th2 bias [6,19]. The Th2 immune response helps to neutralize the virus whereas the Th1 immune response assists in clearing virally-infected cells from the host [8]. Hence, a balanced Th1/Th2 type of immune response should be optimal for influenza virus neutralization and clearance. Our results show that the δ-inulin adjuvanted pulmonary vaccine was capable of inducing a more balanced Th1/Th2 immune response than the WIV alone pulmonary vaccine. Furthermore, mucosal IgA is beneficial because it neutralizes the influenza virus at the port of entry and thus enhances protection [20,21]. Moreover, IgA antibodies are known for their broad spectrum protection, i.e. they are cross-protective against a range of drifted, heterologous influenza strains [22]. The results of this study show that significant anti-influenza IgA was only induced by pulmonary but not i.m. vaccination and was further improved by the δ-inulin adjuvant. Future studies will test the extent to which this enhanced mucosal IgA production might contribute to enhanced protection against influenza infection.

A wide variety of adjuvants including cholera toxins, squalene oil emulsions, saponins, CpG oligonucleotides, and ISCOMS have been tested for efficacy in animal models of intrapulmonary or intranasal immunization with variable results [23–28]. However, the single most important consideration for an adjuvant to be administered to the lung, nose or other mucosal surfaces is safety, given previous mucosal adjuvant misadventures most particularly the occurrence of facial nerve palsy in human trials of a Escherichia coli heat-labile toxin adjuvanted intranasal inactivated influenza vaccine [29], an event later shown to be directly attributable to the Escherichia coli heat-labile toxin adjuvant [30]. With these safety issues in mind, it is reassuring that δ-inulin has a strong safety and tolerability record when administered by the i.m. route in multiple preclinical and human clinical studies [12,15,16]. Of course, this i.m administration data does not guarantee against an unforeseen effect specific to lung administration and hence formal preclinical safety studies of δ-inulin’s intrapulmonary use will be required to exclude such a possibility, prior to any commencement of any human studies. However, no differences in animal well-being, e.g. weight loss, were observed between the pulmonary groups with or without adjuvant and the i.m groups (data not shown). The absence of any obvious pulmonary or systemic adverse events in mice in this study receiving δ-inulin adjuvant is reassuring and supports the idea that inulin particles might be safely administered to the lung as part of an intrapulmonary vaccine formulation.

In conclusion, our results clearly indicate that δ-inulin has adjuvant activity when administered through the pulmonary route with an inactivated influenza vaccine, inducing changes to the balance of IgG isotypes resulting in a more balanced Th1/Th2 response and promoting the production of secretory IgA in the nose. The extent to which this contributes to protective immunity will need to be clarified in future influenza virus challenge studies. These studies will also look at the effect of
changes in the dose of either the influenza antigen, the δ-inulin adjuvant or both on optimization of the anti-influenza immune response, with the ultimate goal of testing whether protection against influenza with a single dose of intrapulmonary vaccine is in fact achievable.

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Reference


Chapter 4


