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A new adjuvanted nanoparticle-based H1N1 influenza vaccine induced antigen-specific local mucosal and systemic immune responses after administration into the lung

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ABSTRACT

Annually influenza virus infections are responsible for hospitalization and mortality, especially in high risk groups. Constant antigenic changes in seasonal influenza viruses resulted from antigenic shifts and antigenic drifts, enable emerging of novel virus subtypes that may reduce current vaccine efficacy and impose the continuous revision of vaccine component. Currently available vaccines are usually limited by their production processes in terms of rapid adaptation to new circulating subtypes in high quantities meeting the global demand. Thus, new approaches to rapidly manufacture high yields of influenza vaccines are required. New technologies to reach maximal protection with minimal vaccine doses also need to be developed.

In this study, we evaluated the systemic and local immunogenicity of a new double-adjuvanted influenza vaccine administered at the site of infection, the respiratory tract. This vaccine combines a plant-produced H1N1 influenza hemagglutinin antigen (HAC1), a silica nanoparticle-based (SiO_2) drug delivery system and the mucosal adjuvant candidate bis-(3′,5′)-cyclic dimeric guanosine monophosphate (c-di-GMP). Mice were vaccinated by intratracheal route with HAC1/SiO_2 or HAC1/c-di-GMP (single-adjuvanted vaccine) or HAC1/SiO_2/c-di-GMP (double-adjuvanted vaccine) and evaluated for target-specific immune responses, such as hemagglutination inhibition and hemagglutinin-specific IgG titers, as well as local antibody (IgG and IgA) titers in the bronchoalveolar lavage (BAL). Furthermore, the HAC1-specific T-cell re-stimulation potential was assessed using precision-cut lung slices (PCLS) of vaccinated mice.

The double-adjuvanted vaccine induced high systemic antibody responses comparable to the systemic vaccination control. In addition, it induced local IgG and IgA responses in the BAL. Furthermore, HAC1 induced a local T-cell response demonstrated by elevated IL-2 and IFN-γ levels in PCLS of c-di-GMP-vaccinated mice upon re-stimulation.

Overall, the present study showed the potential of the double-adjuvanted vaccine to induce systemic humoral immune responses in intratracheally vaccinated mice. Furthermore, it induced a strong mucosal immune response, with evidence of antigen-primed T-cells in the lung.

1. Introduction

Annual influenza-associated cases of hospitalization and up to 500,000 deaths during frequent virus outbreaks and sporadic pandemics illustrate the serious health burden of influenza virus infections [1]. The high mutational rate of the virus and frequency of interspecies transmission and/or zoonosis leading to new

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virus subtypes makes influenza infections highly unpredictable [2,3]. Therefore, there is a need of developing novel and effective influenza vaccines.

Traditionally, only systemic administration of inactivated influenza vaccines, mostly intramuscularly, has been used. In 2003 Flumist®, the first nasal influenza vaccine with live attenuated influenza viruses, has been approved in the US [4], which protects locally at the site of virus entry and infection. An advantage of delivering vaccines via the respiratory route is, besides the inductions of local immune responses at virus settlement, the non-invasive application which is likely to increase public compliance. However, it has been described that intranasal antigen administration induces poor immune responses when applied without an appropriate mucosal adjuvant [5]. Thus, many new effective mucosal adjuvants are in preclinical development (s. review [6]). In 2007, bis-(3′,5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) was introduced as a mucosal adjuvant with promising activity [7]. Madhun et al. showed that c-di-GMP improved the immunogenicity of an intranasally delivered subunit influenza vaccine, compared to antigen only, by inducing strong mucosal and systemic immune responses [8]. Additionally, the authors showed that intranasal administration of the c-di-GMP adjuvanted antigen induced protective antibody titers and cellular immune responses that far exceeded the responses induced by intramuscular administration of the same vaccine [8]. Moreover, Svindland et al. tested vaccination with c-di-GMP combined with a second adjuvant, Chitosan, and showed that vaccination with the combination of these molecules can further improve the humoral and cellular immune responses against target antigens [9]. Besides its adjuvantive effects, Chitosan is used as an intranasal delivery system. Other drug delivery systems such as silica nanoparticle (NP) have also been previously shown to have adjuvant properties [10,11]. Recently, we have shown that a combination of a plant-produced recombinant hemagglutinin (HA) antigen from the H1N1 influenza virus A/California/04/09 (HAC1) with silica-NP (SiO_2) was able to recall a previously established immune response in human lung tissue [12]. Manufacturing of recombinant proteins in plants for influenza vaccine development evolved as an alternative to the conventional egg-based vaccine production to overcome the limitations in quantity and time consumption [13]. This bottleneck of egg-produced vaccines can have serious consequences during influenza pandemics, when the production of sufficient amounts of vaccine in an adequate time frame to serve the global market could be difficult.

Regarding the need of rapidly produced vaccines in times of pandemics and the time consuming limitation of the egg-based vaccines, the here presented study tested the recombinant antigen of a highly immunogenic H1N1 strain responsible for the 2009/2010 pandemic. Furthermore, the study extends the published work with HAC1 and SiO_2 and evaluates the immunogenicity of this vaccine formulation when combined with c-di-GMP and administered at the site of virus entry. Overall, it showed the potential of the c-di-GMP/SiO_2 double-adjuvanted vaccine to induce systemic humoral and strong mucosal immune responses, with IgA in the airways. Furthermore, it presented evidence of antigen-primed T-cells in the lung in intratracheally vaccinated mice.

2. Materials and methods

2.1. Animals

Female wild-type BALB/c mice aged 6–8 weeks (Charles River, Sulzfeld, Germany) were kept at an animal facility under conventional housing conditions (22 °C, 55% humidity, 12-h day/night cycle) with food and tap water ad libitum. The randomized study was approved by a local agency (Application-No. 33.9-42502-04-11/0465) and conducted according to the German Animal Protection law.

2.2. Media

Reagents were, if not stated otherwise, purchased from Sigma–Aldrich (Munich, Germany). Phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+}, pH 7.4, Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 HAM (DMEM) with L-glutamine, 15 mM HEPES and 7.5% w/v sodium bicarbonate without phenol red, pH 7.2–7.4, RPMI 1640 and Earle's Balanced Salt Solution (EBSS) were obtained from Gibco (Darmstadt, Germany). Cell/tissue cultivation medium was supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin.

2.3. Vaccine and adjuvants

HAC1 was produced as previously described [14]. Briefly, the HA nucleotide sequence, encompassing amino acids 18–530 of the A/California/04/09 influenza strain (H1N1, NCBI accession number ACQ76318.1) were optimized for expression in plants and synthesized. The optimized HA sequence contains a 6× His affinity purification tag and the ER retention signal KDEL at the C-terminus. This gene was inserted into the pGRD4 launch vector and transformed into *Agrobacterium tumefaciens*. The transformed bacterium was introduced into hydroponically grown *Nicotiana benthamiana* by vacuum infiltration and leaf tissues were harvested, homogenized, extracted, filtered and chromatographically purified after a one-week growing period [14]. Aliquots of purified HAC1 were kept in PBS at −80 °C until usage. For silica-NP, DMEM and SiO_2 nanopowder (HDK 200, Wacker Chemie, Germany) were mixed and dispersed by ultrasonic sonotrode. The production of c-di-GMP has been described before [15,16]. Lyophilized c-di-GMP was stored at −20 °C. Immediately prior to immunization, HAC1 was admixed with the adjuvant and/or silica-NP and swirled ≥ 10 min on an overhead shaker to ensure complete mixing.

2.4. Vaccination and sample collection

Mice were immunized on days 0 and 21 with either 5 μg antigen (HAC1), single- or double-adjuvanted vaccine (5 μg HAC1/10 μg SiO_2; 5 μg HAC1/7.5 μg c-di-GMP; 5 μg HAC1/10 μg SiO_2/7.5 μg c-di-GMP) by intratracheal route (50 μl). For intratracheal immunization mice were tilted (∼45°) and the vaccine administered into the deep lung with subsequent insufflation with an air bolus. A systemic control group to ensure the effectiveness of the vaccination protocol, received 1 μg HAC1 adsorbed on aluminum hydroxide (Alum) intraperitoneally (200 μl). Blood was obtained by retrobulbar sampling and sera were collected on days 0, 21, 35, and 49 to determine HA-specific antibody response by hemagglutination inhibition (HAI) and enzyme-linked immunosorbent (ELISA) assays. On day 49, mice were sacrificed with an intraperitoneal overdosing of pentobarbital-Na (Merial, Hallbergmoos, Germany) and cutting the *Vena cava inferior*. BAL fluids, agarose-filled lungs, and spleens were sampled and used for immunoglobulin (Ig) measurements and re-stimulation assays.

2.5. HAI assay

Collected sera were treated with a receptor-destroying enzyme (Denka Seiken, Japan). HAI assay was performed using 0.75% turkey erythrocytes and A/California/07/09 × 179A virus (CDC #2009713114) with an initial serum dilution of 1:20 as described previously [14]. HAI endpoint titers were determined as reciprocal

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