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A novel vaccinological evaluation of intranasal vaccine and adjuvant safety for preclinical tests

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ABSTRACT

Vaccines are administered to healthy humans, including infants, so the safety and efficacy must be very high. Therefore, evaluating vaccine safety in preclinical and clinical studies, according to World Health Organization guidelines, is crucial for vaccine development and clinical use. A change in the route of administration is considered to alter a vaccine's immunogenicity. Several adjuvants have also been developed and approved for use in vaccines. However, the addition of adjuvants to vaccines may cause unwanted immune responses, including facial nerve paralysis and narcolepsy. Therefore, a more accurate and comprehensive strategy must be used to develope next-generation vaccines for ensuring vaccine safety. Previously, we have developed a system with which to evaluate vaccine safety in rats using a systematic vaccinological approach and 20 marker genes. In this study, we developed a safety evaluation system for nasally administered influenza vaccines and adjuvanted influenza vaccines using these marker genes. Expression of these genes increased dose-dependent manner when mice were intranasally administered the toxicity reference vaccine. When the adjuvant CpG K3 or a CpG-K3-combined influenza vaccine was administered intranasally, marker gene expression increased in a CpG-K3-dose-dependent way. A histopathological analysis indicated that marker gene expression correlated with vaccine- or adjuvant-induced phenotypic changes in the lung and nasal mucosa. We believe that the marker genes expression analyses will be useful in preclinical testing, adjuvant development, and selecting the appropriate dose of adjuvant in nasal administration vaccines.

1. Introduction

Vaccination is one of the most effective methods of preventing various infectious diseases, and vaccination against influenza is the most effective way to prevent seasonal and pandemic influenza infections. Influenza viruses are negative-stranded RNA viruses of the family *Orthomyxoviridae*. Individuals infected with influenza virus generally display symptoms such as fever, headache, muscle pain, fatigue, rhinitis, and coughing. The immediate availability of influenza vaccines to the world's populations is a critical factor in ensuring effective coverage against seasonal influenza. Current quadrivalent seasonal influenza vaccines contain inactivated influenza antigens from two of influenza A strains and influenza B strains, respectively. This quadrivalent vaccine is classified as a subvirion influenza vaccine (HAv), which induces adequate immunity with a low frequency of adverse effects compared with whole-particle influenza virus vaccines (WPVs). WPVs were the first used in widespread annual influenza vaccination programs. Although WPVs vaccination induces a strong immune response and is still

Abbreviations: ATT, abnormal toxicity test; DPT, diphtheria–tetanus–pertussis; HA, hemagglutinin; HE, hematoxylin and eosin; IFN, interferon; JEV, Japanese encephalitis virus; LTT, leukopenic toxicity test; MPO, myeloperoxidase; NALT, nasal-associated lymphoid tissue; SA, physiological saline; QGP, QuantiGene Plex; HAv, subvirion influenza vaccine; RE, toxicity reference vaccine; TLR, Toll-like receptor; WBC, white blood cells; WPV, whole-particle influenza virus vaccine.

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licensed for use against pandemic influenza viruses, it causes local and systemic adverse effects more often than HAv [1]. Therefore, in Japan, most recent seasonal influenza vaccines manufactured since the 1970s have been HAv.

Intranasal mucosal immunization has received much attention in recent years because it has several advantages, including needle-free delivery and enhanced mucosal immune responses to infections such as influenza, and it can be enhanced by the coadministration of effective mucosal adjuvants, such as the mutant heat labile toxin of *Escherichia coli* [2]. However, this immunization approach raised concerns when a strong epidemiological association was reported between facial nerve paralysis (Bell's palsy) and the intranasally administered inactivated virosomal influenza vaccine "NasalFlu" [3], containing an enzymatically active mutant labile toxin adjuvant [4]. Therefore, concern about the safety of intranasal vaccines has been increasing. Since 2004, a live attenuated influenza vaccine delivered with an intranasal spray ("FluMist") was licensed in the United States [5]. This vaccine induces an immune response that more closely resembles natural immunity than does the response elicited by its subcutaneous administration or intramuscular injection [6]. The nasal vaccine induces significantly higher local immunoglobulin A (IgA) antibodies in the nasal mucosa and local immune-cell-mediated immunity, which might improve its efficiency [7,8]. It is fitting that the safety of vaccines be evaluated with the same administration route proposed for human use [9]. Therefore, the development of a method to evaluate the safety of a specific injection route (intranasal or intramuscular) is critical for the development of influenza vaccines.

WPVs are less safe but potentially more immunogenic than subvirion formulations [10]. The goal of vaccination is the generation of a strong immune response to the influenza antigen that can afford long-term protection against influenza infection without a high frequency of adverse reactions. To achieve this, several adjuvants have been approved for use with HAv [11]. The most commonly used vaccine adjuvant, aluminum salt, is currently used in pandemic influenza vaccines. Oil-in-water (o/w) emulsions have also proved suitable adjuvants for influenza vaccines. MF59 was the first of these adjuvants approved for use with influenza vaccines in 1997 in the United States [11]. Like MF59, AS03 is also an o/w emulsion based on squalene. The development of suitable adjuvants for influenza vaccines is imperative. However, besides increasing the immunity of the antigens, the addition of adjuvants to influenza vaccines can induce unwanted immune responses [12]. Therefore, evaluating the safety of adjuvanted vaccines is important, and might be even more warranted than the evaluation of nonadjuvanted vaccines [13]. The benefits of adjuvant incorporation into any vaccine must be balanced against the risk of adverse reactions [14,15], which can be both systemic and local, and include pain, local inflammation, swelling, fever, eosinophilia, and immunotoxicity (i.e., autoimmune disease) [9,16]. Unfortunately, potent adjuvant activity often correlates with an increased frequency of adverse reactions [13]. Therefore, a major challenge in adjuvant research is to achieve a potent adjuvant effect with minimal toxicity, which makes the development of new effective adjuvants challenging.

Vaccines are administered to healthy humans, including infants, so the demands for safety and efficacy are very high. Therefore, evaluating the safety of a vaccine in preclinical and clinical studies, according to the World Health Organization guidelines, is crucial for vaccine development and clinical use, as well as in lot release testing and protocol reviews by national control laboratories. In Japan, the abnormal toxicity test (ATT) was introduced into the guidelines for lot release testing, and it is mandatory that the safety evaluations of all inactivated vaccines and toxoids include ATT and other specific toxicity tests [17,18]. In addition to ATT, the mouse leukopenic toxicity test (LTT) is also used to assess the immunotoxicity of influenza vaccines [18]. Although these animal tests play a critical role in the quality control of influenza vaccines, several aspects must be improved for the next generation of influenza vaccines. We have previously developed a vaccine safety evaluation system using a systematic vaccinological approach and 20 marker genes expressed in the lung with which to evaluate the batch-to-batch consistency and safety of the H5N1 vaccine [19]. As previously reported, the expression of the 20 marker genes increased when the WPV was intraperitoneally injected into rats, but not when HAv was administered in the same way [19]. Therefore, we consider that the expression of these 20 marker genes partly reflects the biological effects of the vaccine, which are also related to the adverse reactions to WPV. We previously also showed the potential utility of these marker genes in evaluating the safety of influenza vaccines and their possible replacement of ATT [20,21].

In this study, we investigated whether these 20 marker genes can be used to evaluate the safety of a nasally administered adjuvanted influenza vaccine. It is appropriate that a safety evaluation be conducted with the same administration route as proposed for human use [9]. Therefore, in this study, we tested whether the intranasally administered vaccine could be evaluated with marker gene expression. We also assessed the potential utility of these biomarkers in assessing the safety of adjuvanted influenza vaccines. We selected CpG K3 as the adjuvant and dosing amount of CpG K3 was set at maximum 10 μg/mouse in reference to the previous study [22]. Our results confirm the utility of the biomarkers in assessing the safety of the adjuvant itself and that of the adjuvanted influenza vaccines.

2. Methods

2.1. Animals and ethics statement

Female 6–7-week-old BALB/c mice (16–20 g) were obtained from SLC (Tokyo, Japan). All mice were housed in rooms maintained at 23 ± 1 °C and 50 ± 10% relative humidity, under a 12 h light/dark cycle. The mice were acclimated for at least 2 days before the experiments. All animal experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee of the National Institute of Infectious Diseases (NIID), Tokyo, Japan. The study was approved by the Institutional Animal Care and Use Committee of NIID.

2.2. Vaccines and adjuvant

The national reference influenza vaccine (RE) is a toxicity reference issued by NIID (Japan). RE is a lyophilized whole-virion preparation of an inactivated influenza virus, and is consisted from the three different type of inactivated whole-virion: A/Newcaledonia/20/99 (H1N1), A/Hiroshima/52/2005 (H3N2), and B/Malaysia/2506/2004. RE is used as the toxicity reference in the LTT in Japan [17]. It was reconstituted in the appropriate volume of physiological saline (SA). Serial dilutions with SA were performed to prepare solutions of approximately 2.5, 5.0, and 10 μg each strain of hemagglutinin (HA)/30 μl. The influenza A virus (A/New Caledonia/20/99; H1N1)) split-product vaccine (HAv) was kindly provided by Dr. Takeshi Tanimoto (The Research Foundation for Microbial Diseases of Osaka University, Kan-on-ji, Kagawa, Japan). Like RE, HAv was reconstituted in the appropriate volume of SA and serially diluted with SA to prepare solutions of approximately 2.5, 5, and 10 μg HA/30 μl. CpG K3 (underline indicates phosphorothioate bonds: (5′-ATC GAC TCT CGA GCG TTC TC-3′)) was synthesized by GeneDesign (Osaka, Japan) and was reconstituted in

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