High immunogenicity of plant-produced candidate influenza vaccine based on the M2e peptide fused to flagellin

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ABSTRACT

The ectodomain of the conserved influenza matrix protein M2 (M2e) is a promising target for the development of a universal influenza vaccines. Immunogenicity of M2e could be enhanced by its fusion to bacterial flagellin, the ligand for Toll-like receptor 5. Previously we reported the transient expression in plants of a recombinant protein Flg-4M comprising flagellin fused to 4 tandem copies of the M2e. The use of self-replicating recombinant vector based on the potato virus X allowed expression of Flg-4M in Nicotiana benthamiana leaves at a very high level, up to about 1 mg/g of fresh leaf tissue. Intranasal immunization of mice with Flg-4M induced M2e-specific serum antibodies and provided protection against lethal challenge with different strains of influenza A virus. Here we show that immunization with Flg-4M not only generates a strong immune response, but also redirects the response from the carrier flagellin toward the M2e epitopes. Significant IgG response to M2e was also developed in bronchoalveolar lavages of immunized mice. Protective activity of Flg-4M upon lethal influenza challenge correlated with a decrease of virus titers in lungs relative to the control. Overall these data show the potential for the development of a plant-produced M2e-flagellin universal influenza vaccine.

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Introduction

Influenza is a widely distributed viral infection of humans and other animals. The high variability of the influenza virus surface proteins, hemagglutinin and neuraminidase, results in the frequent appearance of new epidemic strains. In a promising solution to this problem is the development of recombinant vaccines that can be rapidly produced in standard expression systems. Moreover, the use of conserved virus proteins would allow the development of “universal” vaccines efficient against a wide range of influenza strains. M2e, the extracellular domain of the transmembrane protein M2 of influenza A virus is one of the most promising candidates since its sequence is virtually unchanged in all human isolates since 1933, and in strains of avian influenza A it differs in only a few amino acids, although these differences are important for the specificity of immune response. M2e is a short and poorly immunogenic peptide; however, when fused to an adjuvant or carrier virus-like particle it becomes highly immunogenic. In particular, bacterial flagellin, the ligand for Toll-like receptors (TLRs) which plays a critical role in controlling the adaptive immune response, significantly increases the immunogenicity and protective capacity of a linked antigen and is particularly active as mucosal adjuvant, opening the possibility of non-invasive intranasal administration of vaccines. It was previously demonstrated that immunization of mice with enterobacterial flagellin linked at its C terminus to 4 tandem copies of a human “consensus” M2e or a mix of 2 human and 2 avian (from strain A/Chicken/Kurgan/05/2005) M2e sequences, provides protection against lethal challenge with different influenza A strains.

Plants could became a promising biofactory for expression of recombinant proteins due to the low
final cost and inherent safety of products resulting from the absence of pathogens common to plants and animals.9,10 Recently, we reported11 the transient expression in plants of a recombinant protein Flg-4M comprising flagellin FljB of Salmonella typhimurium fused to 4 tandem copies of the M2e peptide, - 2 copies of human consensus M2e sequence (M2eh) and 2 copies of the M2e peptide of avian influenza virus strain A/Chicken/Kurgan/05/2005 (M2ek) arranged as Flg-M2eh-M2ek-M2eh-M2ek (Fig. 1). The use of a self-replicating recombinant viral vector based on the potato virus X allowed the expression of Flg-4M in Nicotiana benthaminana leaves at a very high level, about 1 mg/g of fresh leaf tissue. Intranasal immunization of mice with this candidate vaccine induced high levels of M2e-specific serum antibodies and provided protection against lethal challenge with different strains of influenza virus.11 Here we provide additional data on the characteristics of the immune response activated in mice upon intranasal immunisation with the plant-produced Flg-4M, indicating that immunisation not only generates a strong response against M2e, but also redirects this response from the carrier flagellin molecule toward the inserted M2e epitopes.

**Fusion of 4 copies of the M2e peptide to flagellin directs the immune response toward the inserted peptides**

Balb/c mice weighing 18–20 g were immunized intranasally thrice at 2 week intervals. The dose of plant-produced Flg-4M and Flg proteins was 10 μg/mouse and no additional adjuvant was used. The control group received PBS alone. The mouse sera and bronchoalveolar lavage (BAL) samples were analyzed 2 weeks after the third immunization.

Antibody titers were determined using sera and BAL from 5 individual mice. The serum and BAL samples were analyzed by ELISA to identify IgG antibodies directed against M2e using plates coated with synthetic peptides G-37 (SLLTEVETPIRNEWGCRCNDSSD) and G-50 (SLLTEVETPTRNEWECRCSDSSD), whose sequences corresponded to the human “consensus” M2e sequence and the M2e of influenza strain A/Chicken/Kurgan/05/2005, respectively, as described previously.12 Plates coated with purified empty flagellin (without M2e fusion) were similarly used to detect IgG antibodies directed against flagellin. The sera and BAL of non-immunized mice were used as a negative control for ELISA.

Serum IgG antibodies induced after the third immunisation efficiently bind to both synthetic M2e peptides G-50 and G-37, which are present in the candidate vaccine, although the immune response toward G-37, the human M2e sequence, was stronger (Fig. 2A). Antibodies against flagellin were also detected, but their titer was considerably lower than against M2e (Fig. 2A). Notably, immunisation with flagellin lacking M2e generates much higher titers of antibodies against flagellin, indicating that fusion of M2e epitopes at the C-terminus of flagellin not only confers immunogenicity to the epitopes, but also redirects the immune response toward M2e.

**M2e-specific antibody response in BAL**

To investigate the immune responses in mucosal secretions, M2eh- and M2ek-specific IgG titres were determined in the BALs 2 weeks after the third immunization in 5 mice from each group. As shown in Fig. 2B, intranasal immunisation with the Flg-4M fusion protein stimulated high levels of anti-M2e-specific IgG in BAL. Like in the serum samples, the titer of antibodies recognizing G-37, the human M2e sequence, was higher than the titer of M2ek-recognizing antibodies.

**Immunisation with Flg-4M fusion protein significantly reduced influenza virus load in lungs.**

Two weeks after the third immunization, mice from the test and control groups were infected intranasally with 5LD50 of A/PR/8/34 (H1N1) human influenza virus strain. Six days post-challenge, 5 mice from each group were sacrificed for the titration of residual lung virus.

The lungs were removed aseptically, homogenized in 2.7 ml PBS using a Tissue Lyser II homogenizer (Qiagen, USA) to achieve 10% (w/v) suspensions of

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**Figure 1.** Structure of recombinant protein Flg-4M. M2eh, human “consensus” M2e peptide; M2ek, M2e peptide of avian influenza strain A/Chicken/Kurgan/5/05 (H5N1). Sizes of boxes are not drawn to scale.
lung and centrifuged (15 min, 400g, 4°C) to remove cellular debris before storage at -20°C. In 96-well cell-culture plates, 10-fold serial dilutions of samples were added to monolayers of MDCK cells in quadruplicate and incubated as above for 72h. Viral cytopathic effect was observed daily and viral titer was determined by hemagglutination test with 0.5% chicken erythrocytes. The viral titer was calculated by the Reed and MENCH method and expressed as log 50% tissue culture infectious dose (TCID50).

We observed a significant decrease of virus reproduction in lungs following challenge of Flg-4M immunised mice with A/PR/8/34 compared to mice immunised with empty flagellin or PBS. Mice immunized with Flg-4M displayed log TCID50 of 2.0 in lungs, while virus titers reached 6.8 log TCID50 and 5.8 log TCID50 in lungs of mice immunized with flagellin and PBS, respectively.

**Discussion**

Huleatt et al. were the first to report the use of the TLR5 ligand flagellin of bacterium *Salmonella typhi-murium* as an adjuvant/carer for the influenza M2e peptide. A recombinant protein comprising flagellin fused to 4 tandem copies of the human consensus M2e was expressed in *Escherichia coli* and found to protect immunised mice from a lethal challenge with influenza A virus, and significantly reduced weight loss and clinical symptoms. Later, Stepanova et al. expressed a hybrid flagellin-M2e protein comprising 2 human and 2 avian (from strain A/Chicken/Kurgan/05/2005) M2e sequences in order to provide broader protection against infection with both human and avian influenza strains.

Immunisation with an adjuvant or carrier-linked epitope always induces not only the desired response to the target epitope, but also a carrier-specific immune response. The latter is undesirable since pre-existing immunity to the carrier peptide could reduce the efficiency of subsequent vaccination with the epitope-carrier complex. The relative efficiencies of immune responses to the carrier and the target epitopes depends on a number of factors, including the conformation of the complex. For example, insertion of the target epitope in the major immunodominant loop of the hepatitis B core antigen redirects the immune response toward the inserted epitope, while N-terminal insertions retain high HBc immunogenicity. In both the above mentioned studies on M2e-flagellin candidate influenza vaccine, comparative analysis of immune response toward M2e and flagellin was not reported.

Recently, evaluating an alternative expression system, we expressed Flg-4M in *Nicotiana benthamiana* plants at a very high level, and found that the plant-produced recombinant protein is immunogenic and provides protection against lethal influenza challenge. Here we show that immunization of mice with plant-produced Flg-4M
generates a strong immune response against M2e, while the titers of antibodies against the carrier flagellin are lower than in case of immunisation with empty flagellin without M2e. Such redirection of the immune response toward M2e is apparently beneficial. The position of the epitope is also important since the immune response against human M2eh, located at the C terminus of flagellin, is higher than against more distantly located M2ek. Similar differences between the levels of anti-M2eh and anti-M2ek antibodies were reported for a hybrid flagellin-M2e protein produced in E. coli. Studies examining the immunogenicity of flagellin fusion proteins offer conflicting findings regarding the immunogenicity of flagellin itself. Cuadros and colleagues (2004) reported that immunization with flagellin-green fluorescent protein fusion resulted in a low flagellin-specific response. In other studies anti-flagellin immune responses exceeding the response against the target epitope were observed.

Overall, we show that intranasal immunization of mice with a plant-produced flagellin-M2e fusion protein induces significant IgG response to M2e in both sera and BAL. Protective activity of Flg-4M upon lethal influenza challenge correlated with the decrease of virus titers in lungs relative to the control. These data show the potential for the development of plant-produced M2e-flagellin universal influenza vaccines. In addition to broad specificity provided by M2e, the use of flagellin opens the possibility of non-invasive intranasal administration of this candidate vaccine.

**Abbreviations**

BAL = bronchoalveolar lavage  
ELISA = enzyme-linked immunosorbent assay  
M2e = the extracellular domain of matrix protein 2 of influenza A virus  
PBS = phosphate buffered saline  
PVX = potato virus X  
TLR = Toll-like receptor

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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