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## **Immunological Assessment of Plant-derived Avian Flu H5/HA1 Variants**

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Abbreviations: *hemagglutinin, **HA**; plant-derived HA1 antigen, **pHA**; pHA1 fused with mouse or human heavy chain IgG Fc fragment, **pHA1-mFc** or **pHA1-hFc**;*

Key words: *Plant biotechnology/ avian flu H5N1/recombinant subunit vaccine/*

## **ABSTRACT**

Polypeptide variants of the HA1 antigenic domain of the H5N1 avian influenza virus hemagglutinin (HA) molecule were produced in plants using transient and stable expression systems and fused with His/c-myc tags or with mouse or human Fc antibody fragments. The resulting peptides were purified and used for intramuscular immunization of mice. While the recombinant HA1 variants induced a significant serum humoral immune response in the mice, none of the HA1 preparations induced virus-neutralizing antibodies. Fusion with the Fc fragment improved overall yield of the constructs and allowed purification requiring only a single step, but led to no detectable fusion-related enhancement of immunogenicity or quality of immune response.

## 1. INTRODUCTION

The development of cost-effective avian flu vaccines for both humans and domestic poultry is an urgent priority to prevent pandemic flu outbreaks [1]. Recombinant subunit vaccines that incorporate antigenic viral membrane glycoproteins, such as hemagglutinin (HA), are particularly attractive candidates, since these molecules can induce virus-neutralizing antibodies [2]. The HA1 antigenic domain of HA has been shown to induce an immune response equal to that of the full-size protein [3, 4]. Here, we describe the successful production of recombinant HA1 antigenic variants using transient and stable plant expression systems, a promising alternative to microbial and/or animal pharmaceutical protein production systems [5].

## 2. MATERIALS AND METHODS

### *2.1 Expression of HA1 variants in plants*

The cDNA template of the HA from A/Viet Nam/1203/2004 (H5N1), obtained from CDC (Atlanta, GA), was used for PCR-based cloning of the following HA constructs (**Fig. 1A**): the full-size mature HA without viral leader peptide (aa 1-549); 37 kDa HA1 polypeptide containing major HA antigenic domains (aa 1-330); and two shorter versions, 34 kDa -lacking C-terminal fragment containing cleavage site and sequences with potential poor solubility (aa 1-277) and 27 kDa fragment lacking N-terminal part (aa 68-277). A set of appropriate PCR primers carrying restriction site adapters *NcoI* (forward) and *NotI* (reverse) were used to clone each variant of the HA gene in-frame with C-terminal His- and c-myc tags of the Impact Vectors system used for stable plant transformation (Plant Research International, Wageningen, Netherlands). For some of the variants, compatible restriction sites of transient MagnIcon pro-vector pICH11599 (IconGenetics, Halle, Germany) were used to expedite expression [6]. To generate HA1-Fc fusions (*HA1-Fc*), cDNA clones encoding the heavy chain of human anti-rabies monoclonal antibody SO57 [7] and the heavy chain of murine antibody CO17-1A [8] were used as PCR templates. HA1 and Fc fragments were joined by a flexible bridge of seven amino acids {GGGSGNS}. Both human (*HA1-hFc*) and mouse (*HA1-mFc*) fusions were arranged using the Impact Vector system (tagged vectors # 1.1 and 1.3). The resulting cassettes carrying two

affinity-purification tags (c-myc and 6xHis) at the C-terminus were subcloned into the Impact binary vector *AscI-PacI* restriction sites and used for *Agrobacterium*-mediated stable transformation of tobacco plants as described [9]. The plant signal peptide for apoplast secretion and the HDEL C-terminal ER retention signal were used for transient (*Nicotiana benthamiana*) and stable (*Nicotiana tabacum*) expression, respectively.

## 2.2 Isolation and purification of recombinant proteins

HA-based polypeptide variants were isolated from plant tissues and purified by a 2-step procedure using His- and c-myc tags exactly as described [6]. HA-Fc fusion proteins were purified in a single-step protocol using protein A beads as described [7]. *In vitro* characterization and quantification of protein expression were done by ELISA and Western blot analysis as described [6, 7]. Full-size baculovirus-expressed H5 protein from BEI Resources (Manassas, VA) was used as positive control.

## 2.3 Immunological assessment of HAI variants in mice

Six- to 8-week-old female BALB/c mice (5 per group) were immunized intramuscularly with recombinant peptides 2 times (two weeks interval) at a dose of 10 µg per immunization using alum-CpG adjuvant essentially as described [6]. Ten days after the last immunization, mice were sacrificed and sera from individual mice were analyzed by ELISA and Western blotting as described [6], followed by virus micro-neutralization and hemagglutination inhibition assays [10]. Results are expressed as the mean ±SD for groups of five mice. All animal experiments were conducted in accordance with the institutional guidelines for animal welfare.

## 3. RESULTS AND DISCUSSION

Initial attempts to express the entire H5 protein (~65 kDa) or its *HAI* domain (~37 kDa) in plants yielded minor quantities of the recombinant material by stable and/or transient transformation procedures (data not shown). Further testing revealed that the major part of *HAI* containing the antigenic region can be efficiently expressed either as a truncated 34- or 27-kDa protein by transient system (Fig. 1A, B) or as a 37-kDa polypeptide fused at the C-terminus with a 26-kDa human or mouse Fc fragment (Fig. 1C) by stable transformation.

All recombinant plant-produced proteins pHA1 and pHA1-Fc were present in the soluble protein fraction and showed the expected molecular weight of the resulting products. Magnicon-based expression of 34 kDa HA1 yielded up to 1 mg/kg of fresh tissue. Stably transformed tobacco plants (>15 independent transgenic lines) confirmed high-level expression of recombinant Fc-fusion proteins, i.e., up to 4 mg/kg of fresh tissue, as determined by Western blot analysis using c-myc- (Fig. 1C) or HA1-specific antibodies (not shown). Intense secondary bands resolved from the Fc fusion constructs suggested a propensity for cleavage/degradation, especially in older leaf material (Fig. 1C, lanes 1b-3b).

The pHA1 fragments produced by the MagnICON system were purified using a 2-step Ni<sup>++</sup> and c-myc chromatography procedure, while pHA1-Fc constructs were purified using single-step protein A chromatography (Fig. 1D, E). The purification procedure increased degradation of both human and mouse Fc-fused polypeptides. In Western blots the c-myc-detectable degradation product of approximately 40kDa is the result of the HA1/HA2 cleavage site left 97 aa upstream of the Fc. Addition of protease inhibitor cocktail (Sigma) during purification improved stability only marginally. Nevertheless, purification of HA1-Fc constructs by simple single-step protein-A chromatography yielded around 2 mg of HA1-Fc/kg of fresh plant tissue (50% yield).

### ***3.1 Immunogenicity of HA1 protein in mice***

The purified peptides from the 34kDa HA1 and HA1-Fc, HA1-mFc fusions were used to immunize mice at similar doses (~10 µg per immunization). Dosage for HA1-Fc constructs was calculated based on Western blot band intensity of commercial proteins. ELISA analysis revealed a strong H5-specific serum IgG response to all three HA1 constructs (Fig. 2A), and Western blot analysis confirmed the specificity of the serum immune response against baculovirus-expressed full-size H5 and plant-derived HA1 (Fig. 2B). High titers of mouse anti-human IgG were also detected in sera of mice immunized with pHA1-hFc (Fig. 2C).

Several recent reports demonstrated that HA and HA1 fragment containing the majority of antigenic determinants are responsible for generation of virus-neutralizing antibodies [4, 11-15]. A transient expression system was successfully used to produce in

plants HA from A/Wyoming/03/03 (H3N2) virus that generated neutralizing antibodies in mice [11]. However, in our plant-based system, despite the high serum titers of H5-specific IgG in mice immunized with all three HA1 variants, none of the sera demonstrated neutralizing activities in a virus micro-neutralization assay (all titers less than 20) or hemagglutination inhibition assay (all titers less than 10). Moreover, although fusion of antigen with the IgG Fc domain may increase expression levels, improve stability and, in some cases, increase immunogenicity of antigens [16], we did not detect improvement of immunogenicity and with mouse Fc fusion observed a slight decrease of HA1 immunogenicity. The ease of purification procedure as a function of this fusion was confirmed. At present time we can not pinpoint the reason for the lack of neutralizing activity in our case. The other research differs either in selection of virus strain(s) and/or the area of HA polypeptides expressed. There are also differences to the delivery systems, such as subunit vaccines, DNA vaccines and viral vectors; choice of adjuvants, etc. The question also remains to whether lack of neutralizing antibodies is due to the dosage, peptide folding, purity etc. [12,17,18]. All these may have an impact on the ability of HA to generate a protective antibody response. Lower immunogenicity of H5 may be resolved by using better adjuvant(s) and/or different methodology of administration [19]. Further research is needed to optimize production of H5-derived antigens in plants.

Conclusions: both the Magnicon transient system and Fc-fusion served to improve yield of viral glycoproteins otherwise difficult to express in plants. Fusion of HA1 fragments with the Fc region of human or mouse IgG heavy chains allowed simple protein A-based purification of the final product but did not have a significant impact on immunogenicity or quality of the immune response against H5.

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## **FIGURE LEGENDS**

### **Figure 1. Expression and purification of HA1, HA1-hFc and HA1-mFc polypeptides.**

(A) HA1 glycoprotein cloning strategy. (I) Schematic diagram of the full-length H5 protein comprised of a HA1, HA2 domains and cleavage site (shaded square). Putative glycosylation sites are indicated with asterisks; 37-kDa HA1 peptide (aa 1-330); 34-kDa peptide (aa 1-277); and 27-kDa peptide (aa 68-277). (II) 37-kDa HA1 peptide (aa 1-330) linked to the heavy chain Fc-fragment of either mouse (IgG-mFc) or human (IgG-hFc) immunoglobulin. (B) Western blot analysis of plant-derived (transient transformation) 34-

and 27-kDa HA1 peptides. Equal amounts of total soluble protein (TSP) extracted from two independent transfection samples were loaded onto the SDS-polyacrylamide gel and analyzed by Western blotting using c-Myc Mab. (C) Western blot analysis of plant-derived HA1-hFc after stable transformation, detected in TSP extracts of three independent transgenic lines by c-Myc Mab. Bands of the expected molecular weight are seen in young (a) leaves (arrowhead) and some degradation products are seen in old (b) leaves (double arrowheads). (D) Western blot analysis of two representative plant-derived purified samples HA1-hFc (pHA1-hFc) and HA1-mFc (pHA1-mFc) using c-myc antibodies and HA1-specific antibodies (data not shown). Full-size H5 (+) containing c-myc tag was used as positive control. Products of expected size are marked with arrowhead. Sub-products (double arrowheads) were detected in both HA1-Fc and commercial H5. (E) Coomassie-stained gel showing the resolved purified batches of pHA1-hFc as compared to commercial IgG (heavy and light chain) titration.

**Figure 2. Serum antibody response against pHA1-based polypeptides in mice.**

(A) H5-specific IgG titers after second immunization of mice with 34 kDa pHA1, pHA1-mFc and pHA1-hFc as determined in ELISA against the full-size H5 from BEI Resources (Manassas, VA). (B) Western blot analysis of pooled mouse sera (pHA1-mFc, pHA1-hFc or 34 kDa pHA1, see panel A) using the commercial full-size H5 preparation and the plant-derived HA1-hFc. Right panel shows detection of pHA1-hFc with c-Myc Mab. (C) ELISA titers, determined using mouse anti-human IgG, obtained after second immunization with pHA1-hFc.