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Human antibody response to N-glycans present on plant-made influenza virus-like particle (VLP) vaccines

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ABSTRACT

Background: Plant-made biotherapeutics are gathering momentum and some plant glycoproteins are allergens. Glycans with core β1-2xylose and α1,3fucose motifs and antennae terminated by mannose residues (*e.g.*: MMXF) are found on several plant allergens and can cross-react with glyco-epitopes from other sources. To date, reactivity to these cross-reactive determinants has not been associated with clinical symptoms.
Objective: We produced VLP vaccines bearing the hemagglutinin(HA) of H5(A/Indonesia/5/05) or H1(A/California/07/09) influenza viruses by transfection of *Nicotiana benthamiana*. Subjects enrolled in Phase I/II trials were followed for evidence of allergy/hypersensitivity and development of antibodies against plant glyco-epitopes.
Methods: A total of 280/349 subjects received either one (H1) or 2 doses (H5) of vaccine (5–45 μg of HA/dose) intramuscularly including 40 with pre-existing plant allergies. Subjects were monitored for 6 months. IgG and IgE to plant glyco-epitopes were measured by ELISA using corn-/egg-derived avidin and bromelain as target antigens.
Results: No subject developed allergic/hypersensitivity symptoms. Some (34%) developed transient IgG and, in some cases IgE, to plant glyco-epitopes but no subject mounted an IgE response to the MMXF motif. Antibodies returned to baseline by 6 months in most subjects.
Conclusion: VLP vaccines bearing influenza HA glycoproteins can elicit transient IgG and, in some cases, IgE responses that are not associated with either the development or worsening of allergic/hypersensitivity symptoms.

Capsule summary: Plant-made biotherapeutics and vaccines are rapidly approaching the market. In most cases, these products contain plant-specific glycans. Some plant glycan motifs (*e.g.*: MMFX, MUFX) have been associated with serious allergic reactions in humans. Allergic symptoms and humoral responses to plant glycans were monitored in 280 subjects enrolled in Phase I/II studies of plant-made, virus-like particle (VLP) vaccines targeting the hemagglutinin (HA) proteins of two influenza A viruses. Subjects received 1–2 doses intramuscularly of up to 5–45 μg of HA antigen ± alum (containing microgram quantities of plant-specific glycans). Among those who received these vaccines, 40 had declared allergies and 23 had allergies to plant materials. None of the 280 subjects either initiated or exacerbated allergic symptoms. Many subjects mounted weak and generally transient IgG and IgE responses to common plant glycans but no subject developed an IgE response to the MMFX or MUFX motifs associated with hypersensitivity responses.

1. Introduction

Plant-based production of recombinant proteins for human therapeutic and preventive uses is nearing an important milestone

Abbreviations: CCD, cross-reactive carbohydrate determinants; HA, hemagglutinin; IgG/E, immunoglobulin G or E; GlcNAc (Gn), *N*-acetylglucosamine; MMF^3, (Manα1-3)Manα1-6Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc; MMX, (Manα1-3)(Manα1-6Xylβ1-2)Manβ1-4GlcNAcβ1-4GlcNAc; $MMXF^3$, (Manα1-3)(Manα1-6Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc; $MUXF^3$, Manα1-6(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc; NA, neuraminidase; PBS, phosphate-buffered saline; VLP, virus-like particle.

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with several products in late-stage clinical development [1,2]. One product, a recombinant glucocerebrosidase produced in carrot cells for the treatment of Gaucher disease (Protalix™) received FDA approval in 2012.

Plant glycoproteins contain structural motifs not found on human glycoproteins (*e.g.*: core β1-2xylose and α1-3fucose). Since some of these motifs occur on known plant allergens, one theoretical risk of using plants for production of biotherapeutics is the induction of hypersensitivity. Many plant allergenic glyco-epitopes share biochemical and structural homology with epitopes found on glycoproteins from other sources (*e.g.*: insects, arthropods): so-called cross-reactive carbohydrate determinants (CCDs). Such CCDs can be involved in the binding of IgE and release of histamine by mast cells [3,4]. Roughly 20% of subjects with pollen and food allergies display *in vitro* CCD reactivity based on β1-2xylose or α1-3fucose. However, IgEs directed against CCDs appear to have little clinical significance, even in allergy-prone individuals [5–7].

Fortunately, only a small fraction of environmental proteins are allergenic and both experimental and computational studies suggest that allergen cross-reactivity is attributable primarily to protein sequence and structural similarities [8]. Although carbohydrates could theoretically contribute to such conformational epitopes, a recent study of CCD-reduced food allergens (*i.e.* less β1-2xylose and α1-3fucose) demonstrated that *in vivo* reactivity is largely driven by peptide- and not CCD-specific IgEs [9].

Despite the innocuous nature of most plant glycans, a small number of plant-specific glycans have been associated with IgE induction and clinical symptoms. Although there are exceptions [7], these allergens typically have complex glycans with core β1-2xylose and α1-3fucose and antennae terminated by mannose residues (*e.g.*: MMFX or MUFX). However, the presence of these motifs does not always lead to clinically-apparent allergic responses. For example, a recombinant human lactoferrin produced in transgenic rice plants containing these glycans [10] has recently been evaluated as a nutritional supplement. Almost all (82.7%) of the 29 subjects with pollen and food allergies had detectable IgE specific for rice-derived lactoferrin [11]. Although these IgEs had *in vitro* reactivity in a basophil histamine release assay, no subject challenged orally with 1 g of the rice-derived lactoferrin had an allergic reaction. Although antigen-specific IgE is generally accepted as a risk factor for type-I hypersensitivity, this study suggests a lower predictive value for IgE directed against plant glycans, for oral exposures at least. Indeed, orally-administered, plant-made vaccines have an excellent track record of safety [12]. The glycans found on most plant glycoproteins are typically more complex than the simple MUXF and MMXF motifs (Table 1).

In summary, most plant glycoprotein allergens have biochemical and structural characteristics associated with cross-reactivity that are not generally found on benign plant glycoproteins. Although some of these CCDs can lead to IgE-mediated histamine release *ex vivo* in patients with plant allergies, their clinical significance is uncertain. The allergenic nature of most of these plant glycoproteins is likely driven by the protein backbone rather than the glycan motifs.

In the current work, we measured IgG and IgE responses to plant glycans pre- and post-vaccination in two clinical trials, a Phase I of an H1 monovalent VLP vaccine and a Phase II of an H5 monovalent VLP vaccine.

2. Methods

2.1. Production of plant-made HA VLP influenza vaccines

The H1 and H5 VLPs were based on HA sequences of A/California/07/2009 H1N1 A/Indonesia/5/05 H5N1, respectively, using processes essentially as previously described [1]. Briefly, 6-days after infiltration of *Nicotiana benthamiana* with transgenic Agrobacteria bearing the target HA gene, leaves were harvested and VLPs were purified by a series of standard filtration and chromatographic steps [1]. Dosing was based on HA content. When an adjuvant was used, Alhydrogel™ (Cedarlane Laboratory, Burlington, ON: 0.5 mg aluminium/dose) was mixed with the vaccine immediately prior to administration.

2.2. N-glycan analysis

Site-specific N-glycan distribution on the extracellular region of H1 and H5 was analyzed by LC-ESI MS/MS before and after deglycosylation (NanoAcquity UPLC coupled to a QTOF micro: waters). Reduction (DTT) and alkylation (iodoacetamide) of cysteine residues were performed prior to overnight digestion with trypsin (37 °C) or endoproteinase Glu-c (25 °C). Deglycosylation was performed with peptide-N^4-(acetyl-β-glucosaminyl) asparagine amidase A (37 °C). Peptides and glycopeptides were chromatographically separated on a C18 column (New Objective, Woburn, MA) using a water-acetonitrile gradient and analyzed by MS/MS. Starting from the peptides identified after deglycosylation, Mascot (Matrix Science, Boston, MA) was used to generate a list of putative glycopeptides (*m/z*) based on the N-glycans typically found in plants. Detection of these theoretical *m/z* prior to deglycosylation indicated which glycans were attached at each site. Glycopeptide assignments were confirmed by the presence of reporter ions in the MS/MS spectra: *e.g.*: *m/z* 204, 366 and 512 indicating *n*-acetylglucosamine (GlcNAc), GlcNAc-Hexose (mannose or galactose) and GlcNAc-Hexose-Fucose, respectively. These three 'reporter ions' have previously been observed in the fragmentation patterns of N-linked glycopeptides [13].

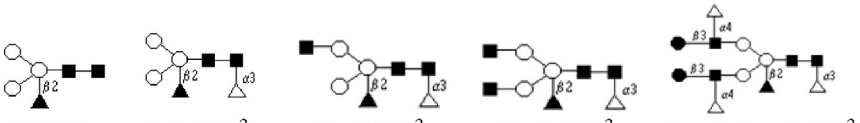
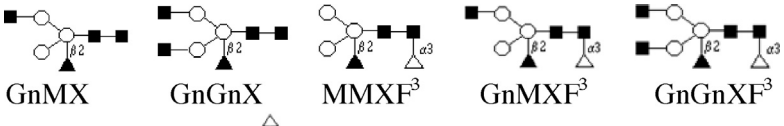
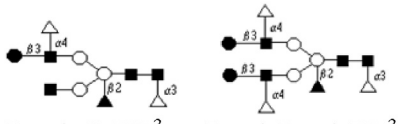
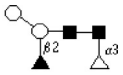
2.3. Clinical trials

The Phase 1 trial was a randomized, double-blind, placebo-controlled, dose-ranging study to evaluate a single non-adjuvanted dose of an H1 VLP influenza vaccine in healthy adults 18–49 years of age (NCT01302990 at ClinicalTrials.gov)[1]. Subjects ($n = 100$) received 5, 13 or 28 μg of H1 VLP vaccine, a licensed trivalent vaccine (Fluzone®) or placebo (PBS) by IM injection into the deltoid muscle (20/group). Serum samples were obtained at day 0 and at 21 and 201 after vaccination for hemagglutination-inhibition (HI) and detection of antibodies against plant glycans. The Phase 2 trial was a randomized, placebo-controlled, dose-ranging study to evaluate two doses of H5 VLP influenza vaccine ± Alhydrogel in healthy adults 18–60 years of age (NCT01244867 at ClinicalTrials.gov). The study included 255 subjects overall. In part A of the trial, 120 of whom received the alum-adjuvanted H5 VLP vaccine (30 at each of the 20, 30 or 45 μg/dose). Thirty received the 45 μg dose alone and 45 received placebo (PBS) (21 days between doses). Serum samples were obtained on day 0, 21 days after each dose and at 228 days after the 1st vaccination. Subjects with mild-moderate self-reported allergies, including sensitivity to plant allergens, were enrolled in both studies. No attempt was made to formally assess the severity of reported allergies.

2.4. Influenza-specific serologic responses

Standard hemagglutination inhibition (HI) testing was performed according to WHO recommendations [9] as previously described [1] on sera obtained on day 0 and 21 days after dosing (H1-VLP trial) and on days 0, 21 and 42 in the H5-VLP trial. Testing was also carried out on samples collected at 6 months but results are not presented here. The HI assays used whole inactivated A/California/7/09 H1N1 strain grown in MDCK cells and or the reassortant A/Indonesia/5/05 H5N1 strain grown in embryonic eggs.

Table 1
Structures of the principle glycans founds on the antigens used in the different antibody assays.

Antigen	Glycan structure[1]
Corn-derived avidin	 MMX MMXF^3 GnMXF^3 GnGnXF^3 LewisLewisXF^3
VLP[2]	 GnMX GnGnX MMXF^3 GnMXF^3 GnGnXF^3
	 LewisGnXF^3 LewisLewisXF^3
Bromelain	 MUXF^3

■ *N*-acetylglucosamine, △ Fucose, ○ Mannose, ▲ Xylose.
[1] Glycans of the HA found in the VLP vaccine were identified by LC–MS/MS as described in Section 2. Glycans present on the corn avidin and bromelain are as reported by the manufacturers.
[2] The same plant glycans were present on both H1- and H5-VLP vaccines.

Briefly, sera were pre-treated with receptor-destroying enzyme II (RDE II) (Denka Seiken Co., Tokyo, Japan) overnight at 37 °C and then PBS (Thermo Scientific, Rockford, IL) was added to create a 1:10 dilution. Sera were serially diluted 2-fold in V-bottom microtiter plates. Twenty five microliter of test virus (2–8 HAU/50 μL) were added to each well and plates were incubated at room temperature for 90 min prior addition of 0.5% chicken erythrocytes (Lampire Biologicals, Pipersville, PA). Plates were incubated at room temperature for 60 to 90 min and HI titers were defined as the reciprocal of the highest dilution causing complete inhibition of hemagglutination.

2.5. Analysis of the anti-plant glycan response

Antibodies to plant glycans were measured using two approaches. The first used paired ELISAs to measure serum IgG and IgE to plant specific glyco-epitopes of corn- *versus* egg-derived avidin (Table 1). Avidin is a 15.5 kDa protein with only one N-glycosylation site. When produced in transgenic corn, avidin contains plant-specific glycans (*e.g.*: core β1-2 xylose and α1-3 fucose [14]) that are not found on egg avidin. Thus, the only difference between the target antigens in the avidin assays run in parallel is the composition of the N-linked glycan. Results in these ELISAs are reported as the reciprocal of the last dilution yielding an OD of ≥0.1 over background (blank control) values. However, there are no reference standards for this test and the glycans on corn-derived avidin are unusual for plant glycoproteins, containing relatively few mannose moieties (termed 'paucimannose'). As a result, different reactivity in these paired assays must be interpreted with caution. As an additional control when using this approach to measure IgE titers, additional wells coated with the relevant VLP were included (all wells were coated overnight with 1 μg/mL protein in carbonate-bicarbonate buffer). Results in the VLP-based IgE ELISAs are also reported as the reciprocal of the last dilution yielding an OD of ≥0.1 over background (blank control) values. Only three of the five glycans known to be present on our VLP vaccines (GnGnXF^3, GnMXF^3 and Lewis) are represented in the corn avidin assay (Table 1).

In the second approach, IgE to MUXF3 was measured by ImmunoCAP (Pharmacia & Upjohn, Uppsala, Sweden). The target antigen in this ELISA (MUXF) is the only N-linked glycan of bromelain. IgE reactivity to MMXF/MUXF glycans has been correlated with clinically- relevant hypersensitivity [15]. Results of this assay are reported in arbitrary units (kUA/l) and graded for potential clinical relevance. Grade 0 is 'negative', grades 1 and 2 are 'low-' and 'moderate positive', respectively, while grades 3–6 define varying degrees of 'high' and 'very high' positive. Values >2 are thought to be predictive of clinically-relevant allergy. This assay was performed by Laboratoire Bio-Médic (Quebec, QC). Both MMXF and MMX are present on corn-derived avidin (Table 1).

2.6. Analysis of the anti-HA response

The response to HA of VLP was determined by a hemagglutination-inhibition (HI) assay as previously described [1] using either whole inactivated A/California/7/09 H1N1(X-179A strain) or reassortant A/Indonesia/5/05 H5N1 as the target antigens.

3. Results

3.1. Characterization of the H1 and H5 vaccines

The plant-made influenza vaccines used in these trials were ~135 nm diameter VLPs consisting of a lipid bi-layer studded with HA trimers as described in D'Aoust et al. [16]. HA accounted for more than 95% of total protein in the vaccine lots used (data not shown). Some plant membranes-derived proteins were present in miniscule amounts in both vaccines (*i.e.* ATPase, water channel protein).

No MUFX/MMXF/MMX glycans were found in either VLP formulation (Table 2). Six N-glycosylation sites were identified within the H1 and H5 extracellular regions and all sites were used. This proteomic analysis also identified 67% and 77% of the expected tryptic peptides of the H1 and H5 sequences, respectively, suggesting that

Table 2
Site distribution of N-linked glycans on H1 and H5. PNGase A: detection of deglycosylated peptides after treatment by PNGase A.

H1-VLP sites	PNGase A	N-linked glycans	H5-VLP sites	PNGase A	N-linked glycans
Asn11	+	$GnGnXF^3$	Asn11	+	$GnGnXF^3$
Asn23	+	$MMXF^3$ $GnMXF^3$	Asn23	+	$GnMXF^3$ $GnGnXF^3$ $LewisMXF^3$ $LewisLewisXF^3$
Asn87	+	GnMX GnGnX	Asn154	+	$GnMXF^3$ $GnGnXF^3$
Asn275	+	$GnGnXF^3$ $LewisGnXF^3$ $LewisLewisXF^3$	Asn165	+	$GnGnXF^3$ $LewisGnXF^3$
Asn287	+	GnMX $GnMXF^3$ $LewisMXF^3$	Asn286	+	$GnGnXF^3$ $LewisGnXF^3$ $LewisLewisXF^3$
Asn481	+	$GnGnXF^3$ $LewisGnXF^3$ $LewisLewisXF^3$	Asn484	+	$GnGnXF^3$ $LewisGnXF^3$ $LewisLewisXF^3$

the VLP vaccines do not have unusual post-translational modifications. All of the N-glycosylation sites of both HAs carried typical, complex plant N-glycans and glycan motifs differed only slightly between sites.

3.2. Clinical trials

The plant-derived HA VLP vaccines were well-tolerated and induced strong anti-HA responses in both trials. Among the 349 subjects enrolled in these trials, 280 received at least one dose of a plant-derived VLP vaccine. Five subjects for whom 6-month serum was not available (all in VLP groups) were excluded from analyses. Almost 13% of these subjects (36/280) reported at least one allergy at recruitment (*e.g.*: pollens, ragweed, penicillin, cats). No allergic symptoms were reported by any subject in the period immediately following immunization. No subject developed new allergies or the worsening of pre-existing allergies during the 6 month period. In both clinical trials, local and systemic reactions were comparable to those observed following the commercial trivalent vaccine included in the H1-VLP study (data not shown).

3.3. HAI responses

Most subjects responded well to either a single dose of the H1-VLP vaccine or to two doses of the H5-VLP vaccine over the range of doses administered (Tables 3 and 4). As expected, the antibody response was higher against the seasonal H1N1 strain compared to the pandemic H5N1 strain. A single dose of non-adjuvanted H1 VLP induced a four-fold increase in antibodies in 68.4–89.5% of subjects and in 94.4% of subjects who received Fluzone® (Table 3). Two doses of H5 VLP vaccine induced seroconversion rates of 58.6, 53.6 and 46.7% of subjects who received alum-adjuvanted doses of 20, 30 and 45 μg, respectively. The seroconversion rate for the non-adjuvanted dose of 45 μg (21.4%) was generally lower than that following adjuvanted vaccine doses but the differences did not reach statistical significant ($p = 0.170$, 0.289 and 0.548 *versus* alum-adjuvanted doses of 20, 30 and 45 μg, respectively).

3.4. Anti-glycan IgG response

Prior to immunization, 66/344 (19.2%) subjects had detectable IgG to corn avidin glycans. Among the 66 positive at screening, 35 had increased corn avidin IgG titers after vaccination (53%) and all were from VLP groups. Among the 278 who were negative at enrolment, 64 mounted IgG responses detectable in the corn avidin assay (23%). All but 5 of these were in VLP groups (92.2%). Of the 278 initially IgG-negative subjects, 22 mounted unequivocal responses to corn avidin glycans (IgG titers > 100). The remaining 55 subjects had weaker responses (titers 50–100). Overall, 94/99 (95%) of the subjects with increasing IgG in the corn avidin assay had received ≥1 dose of a VLP vaccine (see Table 5). Three subjects who received Fluzone® and 2 placebo recipients also had increased IgG titers in the corn avidin ELISA after vaccination. The proportion of VLP vaccine recipients with increasing IgG titers in the corn avidin assay was identical in those either seropositive or seronegative at enrolment (22/66 and 72/220, respectively: both 33%). Six months after the last dose of any vaccine, 98 of 344 subjects (29.3%) had detectable IgG to plant glycans; 9 had received only placebo or Fluzone® (9/65:14%) and 51 had been positive at screening (52%).

There was no obvious relationship between antibody responses to corn avidin glycans and the VLP-borne HA antigens: reported as geometric mean fold increases (GMFI) and HI titers, respectively. Spearman Rank correlation coefficients for the two-dose H5 study and the one-dose H1 study were 0.01 and 0.13 (data not shown). Responses to the viral HA antigens were more robust than the anti-glycan responses. Among those who mounted anti-corn avidin responses, GMFIs were highest in the 2 dose recipients. Neither the HA antigen dose nor alum had any significant effect on induction of anti-glycan IgG (Table 5).

3.5. IgE response to corn avidin glycans and VLPs

All subjects were seronegative for IgE to corn avidin glycans at enrolment (Table 6). Overall, only 12/344 (3.5%) had increased IgE levels at some point after vaccination and all had received at least 1 dose of a VLP vaccine (either H1 or H5) (Table 6). All the IgE-positive subjects also mounted IgG responses to corn-avidin glycans. The proportion with IgE responses was higher in two-dose (11/218 or 5%) than in one-dose (1/56 or 1.8%) recipients. Ten of the 11 two-dose subjects with IgE responses received the H5 VLP vaccine formulated with alum. IgE titers, when present, were invariably low (GMT ≤ 100). All but four of the 12 subjects who mounted apparent IgE responses following vaccination were seronegative at 6 months (1 with IgE to VLP, 3 with IgE to corn avidin glycans).

3.6. IgE responses in the bromelain assay

No subject had a response in the bromelain assay greater than grade 2 at any time. Only 10/374 (2.7%) subjects had positive IgE responses to bromelain at enrolment (grades 1 and 2) (Table 7). By chance, all ten had been randomized to VLP treatment groups. However, no subject in any group initiated an IgE response to the MUXF glycan after vaccination and none of the initial 10 bromelain-positive subjects increased their IgE levels in any of the assays at any time after vaccination. Eight of the 10 bromelain-positive subjects at enrolment were still positive 6 months later.

Table 3
Serum HI titers from Phase 1 clinical trial with monovalent H1 VLP vaccine (per protocol analysis).

	Placebo	5 μg H1 VLP vaccine	13 μg H1 VLP vaccine	28 μg H1 VLP vaccine	45 μg Fluzone®
Baseline	*n* = 19	*n* = 18	*n* = 19	*n* = 19	*n* = 18
Subjects with HI titer ≥ 1:40 (%(95%CI))	26.3 (9.1–51.2)	33.3 (13.3–59.0)	42.1 (20.3–66.5)	26.3 (9.1–51.2)	44.4 (21.5–69.2)
Geometric mean titer (95%CI)	19.9 (10.1–39.3)	17.5(7.9–38.5)	20.7 (10.1–42.6)	13.4 (7.2–25.0)	26.7 (12.8–55.6)
21 Days after vaccination	*n* = 19	*n* = 18	*n* = 19	*n* = 19	*n* = 18
Subjects with HI titer ≥ 1:40 (%(95%CI))	31.6 (12.6–56.6)	88.9 (65.3–98.6)	89.5 (66.9–98.7)	94.7 (74.0–99.9)	100 (81.5–100)
Subjects with seroconversion[a] (%(95%CI))	5.3 (0.1–26.0)	72.2 (46.5–90.3)	68.4 (43.3–87.4)	89.5 (66.9–98.7)	94.4 (72.7–99.9)
Geometric mean titer (95%CI)	21.5 (10.7–43.0)	290.4 (139.1–606.3)	218.3 (102.4–465.3)	543.6 (224.6–1315.9)	870.9 (540.9–4102.1)
Mean geometric increase	1.1	16.6	10.5	40.6	32.7
6 Months after vaccination	*n* = 17	*n* = 18	*n* = 16	*n* = 17	*n* = 17
Subjects with HI titer ≥ 1:40 (%(95%CI))	29.4 (10.3–56.0)	88.9 (65.3–98.6)	75.0 (47.6–92.6)	76.5 (50.1–93.2)	100 (80.5–100.0)
Subjects with seroconversion[a] (%(95%CI))	5.9 (0.1–28.7)	66.7 (41.0–86.7)	50.0 (24.7–75.3)	70.6 (44.0–89.7)	94.1 (71.3–99.9)
Geometric mean titer (95%CI)	20.4 (9.0–46.3)	148.2 (72.7–302.4)	99.2 (48.0–205.1)	230.5 (86.1–616.7)	361.5 (226.6–576.8)
Mean geometric increase	1.0	8.5	4.8	17.2	13.6

[a] Seroconversion was defined as a fourfold increase in HI titer or from baseline (≤10) to HI titers ≥40. Seroprotection was defined as the proportion of subjects with HI titer ≥40.

Table 4
Serum HI titers from Phase 2 clinical trial with H5 VLP vaccine (per protocol analysis).

	Placebo	20 μg + Al (H5 VLP vaccine)	30 μg + Al (H5 VLP vaccine)	45 μg + Al (H5 VLP vaccine)	45 μg (H5 VLP vaccine)
After first vaccination	*n* = 14	*n* = 30	*n* = 29	*n* = 30	*n* = 28
Subjects with HI titer ≥ 1:32 (%(95%CI))	0.0 (0.0–23.2)	6.7 (0.8–22.1)	0.0 (0.0–11.9)	10.0 (2.1–26.5)	10.7 (2.3–28.2)
Subjects with seroconversion[a] (%(95%CI))	0.0 (0.0–23.2)	6.7 (0.8–22.1)	0.0 (0.0–11.9)	10.0 (2.1–26.5)	10.7 (2.3–28.2)
Geometric mean titer (95%CI)	4.0 (4.0–4.0)	5.5 (4.2–7.3)	5.1 (4.3–6.1)	5.9 (4.1–8.5)	5.4 (3.7–7.7)
Mean geometric increase (95%CI)	1.0 (1.0–1.0)	1.4 (1.1–1.8)	1.3 (1.1–1.5)	1.5 (1.0–2.1)	1.4 (0.9–1.9)
After second vaccination	*n* = 13	*n* = 29	*n* = 28	*n* = 30	*n* = 28
Subjects with HI titer ≥ 1:32 (%(95%CI))	0.0 (0.0–24.7)	58.6 (38.9–76.5)	53.6 (33.9–72.5)	46.7 (28.3–65.7)	21.4 (8.3–41.0)
Subjects with seroconversion[a] (%(95%CI))	0.0 (0.0–24.7)	58.6 (38.9–76.5)	53.6 (33.9–72.5)	46.7 (28.3–65.7)	21.4 (8.3–41.0)
Geometric mean titer (95%CI)	4.0 (4.0–4.0)	24.3 (14.6–40.5)	22.4 (14.0–35.8)	19.3 (12.1–30.5)	11.9 (7.3–19.5)
Mean geometric increase (95%CI)	1.0 (1.0–1.0)	6.1 (3.7–10.1)	5.6 (3.5–9.0)	4.8 (3.0–7.6)	3.0 (1.8–4.9)
6 Months after vaccination	*n* = 13	*n* = 28	*n* = 28	*n* = 30	*n* = 27
Subjects with HI titer ≥ 1:32 (%(95%CI))	0.0 (0.0–24.7)	10.7 (2.3–28.2)	10.7 (2.3–28.2)	6.7 (0.8–22.1)	7.1 (0.923.5)
Subjects with seroconversion[a] (%(95%CI))	0.0 (0.0–24.7)	10.7 (2.3–28.2)	10.7 (2.3–28.2)	6.7 (0.8–22.1)	7.1 (0.9–23.5)
Geometric mean titer (95%CI)	4.9 (3.6v6.6)	9.4 (6.8–12.8)	7.7 (5.7–10.4)	8.2 (6.1–11.2)	6.3 (4.7–8.5)
Mean geometric increase (95%CI)	1.1 (1.0–1.3)	1.0 (0.8–1.1)	0.9 (0.8–1.0)	0.8 (0.7–1.0)	1.0 (0.9–1.1)

[a] Seroconversion is defined as a ≥4-fold from baseline or an HI titer ≥1:32 when there is no detectable titer at baseline.

Table 5
IgG responses to corn avidin glycans.

Clinical trial	Group	Number of subjects with detectable IgG[1] to corn avidin glycans at screening	Number of subjects with a corn avidin glycan IgG increase after vaccination	Number of subjects with detectable IgG to corn avidin glycans 6 months after vaccination
Phase 1 with H1 VLP (one dose)	Non-ad VLP (*n* = 58)	Positive: 13 (22%)	17%(10/58)	19%(10/52)
	Fluzone® (trivalent, *n* = 20)	Positive: 5 (25%)	15%(3/20)	33%(6/18)
	Placebo (*n* = 20)	Positive: 2 (10%)	10%(2/20)	6%(1/18)
Phase 2 with H5 VLP (two doses)	Ad VLP (*n* = 189)	Positive: 38 (20%)	38%(71/189)	38%(72/190)
	Non-ad VLP (*n* = 29)	Positive: 5 (17%)	45%(13/29)	25%(7/28)
	Placebo (*n* = 28)	Positive: 3 (11%)	0%(0/28)	7%(2/28)

[1] Positive ELISA responses based on OD readings >0.1 above background.

Table 6
IgE to corn avidin glycans or VLP.

Clinical trial	Group	Number of subjects with IgEs to VLP and corn avidin at screening	Number of subjects that showed an IgE increase one month after vaccination		Number of subjects that showed detectable IgEs six months after vaccination	
			VLP	Corn avidin	VLP	Corn avidin
Phase 1 with H1 VLP (one dose)	Non-ad VLP (*n* = 58)	0%(0/56)	1.8%(1/56)	1.8%(1/56)	1.9%(1/52)	0%(0/52)
	Fluzone® (trivalent, *n* = 20)	0%(0/18)	0%(0/18)	0%(0/18)	0%(0/18)	0%(0/18)
	Placebo (*n* = 20)	0%(0/19)	0%(0/19)	0%(0/19)	0%(0/18)	0%(0/18)
Phase 2 with H5 VLP (two doses)	Ad VLP (*n* = 192)	0.5%(1/189)	4.8%(9/189)	5.3%(10/189)	0%(0/189)	1.6%[1](3/189)
	Non-ad VLP (*n* = 29)	0%(0/29)	3.4%(1/29)	3.4%(1/29)	0%(0/28)	0%(0/28)
	Placebo (*n* = 27)	0%(0/28)	0%(0/28)	0%(0/28)	0%(0/28)	0%(0/28)

[1] Two of these subjects were negative after second dose and just above the limit of detection (LOD) at 6-months.

Table 7
IgE responses to bromelain glycans.

Clinical trial	Group	Number of subjects with IgEs ≥ grade 1 to bromelain at screening	Number of subjects that showed an IgE increase 1 month after vaccination	Number of subjects that showed detectable IgEs 6 months after vaccination
Phase 1 with H1 VLP (one dose)	Non-ad VLP (n = 58)	3.5% (2/57)	0% (0/57)	1.8% (1/56)
	Fluzone® (trivalent, n = 20)	0% (0/20)	0% (0/20)	0% (0/20)
	Placebo (n = 20)	0% (0/20)	0% (0/20)	0% (0/20)
Phase 2 with H5 VLP (two doses)	Ad VLP (n = 192)	3% (6/191)	0% (0/188)	3% (6/191)
	Non-ad VLP (n = 29)	7% (2/29)	0% (0/29)	4% (1/27)
	Placebo (n = 27)	0% (0/28)	0% (0/28)	0% (0/28)

Table 8
Phase 1 clinical trial with H1 VLP: Antibodies to plant glycans in subject reporting known allergies at screening.

Declared allergy	Vaccine treatment	n/N (%)	Subjects who experienced an increase in antibodies after vaccination to:			
			IgG to corn avidin glycans	IgE to corn avidin glycans	IgE to VLPs	IgE to bromelain glycans
Seasonal	VLP	8/280 (2.9%)	1	0	0	0
	Fluzone®	3/18 (16.7%)	1	0	0	0
	Placebo	4/51 (2.0%)	1	0	0	0
One or more of the following (antibiotics, hay fever, ragweed, pollen, allergic rhinitis, dust, cats, foods)	VLP	31/280 (11%)	15*	0	0	0
	Fluzone®	0/18 (0%)	0	0	0	0
	Placebo	0/51 (0%)	0	0	0	0

* Most common self-reported allergies were 'hay fever' (n = 6) and pollen (n = 3).

Table 9
Phase 1 clinical trial of H1 VLP: Antibody profiles of subjects who had a positive IgE response at any timepoint in at least one assay.

Treatment group	No. of subject	Declared allergies at screening	Timepoint	IgG corn avidin glycan	IgE corn avidin glycan	IgE VLP	IgE bromelain glycan
5 μg H1 VLP vaccine	02–048	None	Day 0	<LOD	<LOD	<LOD	Grade 2
			Day 21	50	<LOD	<LOD	Grade 2
			Day 201	100	<LOD	N/A	Grade 2
28 μg H1 VLP vaccine	m	None	Day 0	50	<LOD	<LOD	Negative
			Day 21	6400	100	50	Negative
			Day 201	400	<LOD	N/A	Negative
	02–068	None	Day 0	<LOD	<LOD	<LOD	Grade 1
			Day 21	<LOD	<LOD	<LOD	Grade 1
			Day 201	N/A	N/A	N/A	N/A

<LOD: below the limit of detection for IgG and IgE corn avidin glycan tests and the IgE VLP test. N/A: not available. Grade 1: Low Allergy. Grade 2: Moderately high allergy. In the H1 trial, two subjects (nos. 02–048 and 02–068) with no declared allergies were MUXF-positive at enrolment but did not increase these responses or mount an IgE response to VLPs or corn avidin glycans following VLP vaccination. The one for whom data was available was still bromelain-positive 6 months after vaccination. Subject no. 01–060 also had no declared allergies and was bromelain-negative at enrolment but had low IgG titers to corn avidin glycans. Following VLP vaccination, this subject mounted a strong IgG response to corn avidin and more modest and transient IgE responses to both corn avidin and VLPs. However, there was no increase in MUFX reactivity.

3.7. Incidence of IgE to plant glycans in subjects with declared allergies

Overall, 40/48 subjects with declared allergies had been assigned to VLP groups (Table 8). Allergies to suspected or known plant allergens (*e.g.*: seasonal allergies, hay fever, pollen, ragweed) accounted for 23 of the 48 (48%). None of these 'allergy-prone' subjects were positive in the bromelain assay at screening and none mounted an IgE response to the MUXF motif following vaccination. Some had increased IgG titers to corn avidin glycans following vaccination but similar responses were seen in non-allergic subjects (Table 2). Two of the 48 subjects with declared allergies (4%) mounted low-level IgE responses to corn avidin glycans and VLPs (both were in VLP-vaccinated groups).

3.8. Antibody profiles in subjects who mounted any IgE response

The individual antibody profiles of subjects who mounted detectable IgE responses at some point during these trials are shown in Tables 9 and 10.

3.9. No effect of VLP dose on glycan-specific serologic responses

In the Phase 1 H1 study, subjects received a single dose of the VLP vaccine at 5, 13, 28 μg/dose and, in the Phase 2, H5 study, subjects received two doses of VLP vaccine at 20, 30, 45 μg/dose. Neither the influenza-specific serologic responses (*e.g.*: HI, microneutralization: latter data not shown) nor the glycan-specific responses (*e.g.*: corn-avidin ELISA, bromelain assay) were significantly influenced by the dose of VLP received in either study (data not shown).

4. Discussion

From an early age, humans are exposed to a wide variety of plant products orally, by inhalation and on the skin. Given the frequency and intensity of such exposures, it would be maladaptive for the human immune system to over-react to plant-derived molecules. Indeed, relatively few people have historically suffered from life-threatening reactions to plant products. More recently, both hypersensitivity to plant products (*e.g.*: peanuts, latex) and seasonal allergies (*e.g.*: pollens) appear to be increasing. However, the large majority of such aberrant responses are directed against plant proteins rather than plant-specific glyco-epitopes [8]. Although hypersensitivity reactions to both mammalian (galactose alpha-1,3-galactose) and plant (MMXF/MUFX) carbohydrate motifs have been reported in rare individuals [17], most IgE responses to plant materials target so-called cross-reactive carbohydrate determinants (CCDs) and are not associated with clinical symptoms. In the current work, we have demonstrated that plant-made VLP

Table 10
Phase 2 clinical trial with H5 VLP: Antibody profiles of subjects who had a positive IgE response at any timepoint in at least one test.

Treatment Group	No. of subject	Declared allergies at screening	Timepoint	IgG glycan	IgE glycan	IgE VLP	IgE bromelain
20 μg H5 VLP Vaccine + Al	R15006	None	Day 0	<LOD	<LOD	<LOD	Negative
			Day 21	50	50	100	Negative
			Day 42	50	<LOD	50	Negative
			Day 228	<LOD	50	<LOD	Negative
	R25029	None	Day 0	100	<LOD	<LOD	Grade 1
			Day 21	200	<LOD	<LOD	Grade 1
			Day 42	400	<LOD	<LOD	Grade 1
			Day 228	100	<LOD	<LOD	Grade 1
	R45305	None	Day 0	<LOD	<LOD	<LOD	Negative
			Day 21	200	200	200	Negative
			Day 42	100	50	50	N/A
			Day 228	<LOD	<LOD	<LOD	Negative
	R45334	None	Day 0	<LOD	<LOD	<LOD	Negative
			Day 21	800	100	<LOD	Negative
			Day 42	800	100	50	Negative
			Day 228	100	<LOD	<LOD	Negative
	R45366	None	Day 0	<LOD	<LOD	<LOD	Grade 1
			Day 21	<LOD	<LOD	<LOD	Grade 1
			Day 42	50	<LOD	<LOD	Grade 1
			Day 228	<LOD	<LOD	<LOD	Grade 1
	R45367	None	Day 0	200	<LOD	<LOD	Negative
			Day 21	800	50	<LOD	Negative
			Day 42	1600	50	<LOD	Negative
			Day 228	400	<LOD	<LOD	Negative
	R45400	None	Day 0	<LOD	<LOD	<LOD	Negative
			Day 21	100	<LOD	<LOD	Negative
			Day 42	200	50	100	Negative
			Day 228	200	<LOD	<LOD	Negative
	R55425	Hay Fever	Day 0	100	<LOD	<LOD	Negative
			Day 21	200	100	100	Negative
			Day 42	400	100	200	Negative
			Day 228	200	N/A	<LOD	Negative
	R55517	Allergic Rhinitis	Day 0	100	<LOD	<LOD	Negative
			Day 21	100	100	50	Negative
			Day 42	200	50	50	Negative
			Day 228	400	N/A	<LOD	Negative
30 μg H5 VLP Vaccine + Al	R25020	None	Day 0	50	<LOD	<LOD	Negative
			Day 21	100	<LOD	<LOD	Negative
			Day 42	800	200	100	Negative
			Day 228	100	<LOD	<LOD	Negative
	R35121	None	Day 0	50	<LOD	<LOD	Grade 1
			Day 21	100	<LOD	<LOD	Grade 1
			Day 42	200	<LOD	<LOD	Grade 1
			Day 228	100	<LOD	<LOD	Grade 1
	R35125	None	Day 0	100	<LOD	<LOD	Grade 2
			Day 21	200	<LOD	<LOD	Grade 2
			Day 42	800	<LOD	<LOD	Grade 1
			Day 228	200	<LOD	<LOD	Grade 1
	R35152	None	Day 0	200	<LOD	<LOD	Grade 2
			Day 21	1600	<LOD	<LOD	Grade 2
			Day 42	1600	<LOD	<LOD	Grade 1
			Day 228	50	<LOD	<LOD	Negative
	R35161	None	Day 0	<LOD	<LOD	<LOD	Negative
			Day 21	100	<LOD	<LOD	Negative
			Day 42	1600	100	100	Negative
			Day 228	200	<LOD	<LOD	Negative
45 μg H5 VLP Vaccine + Al	R35116	None	Day 0	<LOD	<LOD	<LOD	Negative
			Day 21	<LOD	<LOD	<LOD	Grade 1
			Day 42	50	<LOD	<LOD	Negative
			Day 228	50	<LOD	<LOD	Grade 1
	R35127	None	Day 0	50	<LOD	<LOD	Grade 1
			Day 21	<LOD	<LOD	<LOD	Grade 1
			Day 42	100	<LOD	<LOD	Grade 1
			Day 228	100	<LOD	<LOD	Grade 1
	R35151	None	Day 0	200	<LOD	<LOD	Negative
			Day 21	400	<LOD	<LOD	Negative
			Day 42	800	100	50	Negative
			Day 228	50	<LOD	<LOD	Negative
	R35101	None	Day 0	100	<LOD	<LOD	Negative
			Day 21	6400	<LOD	50	Negative
			Day 42	12800	<LOD	50	Negative
			Day 228	1600	<LOD	<LOD	Negative

Table 10 (*Continued*)

Treatment Group	No. of subject	Declared allergies at screening	Timepoint	IgG glycan	IgE glycan	IgE VLP	IgE bromelain
	R35164	None	Day 0	<LOD	<LOD	<LOD	Grade 1
			Day 21	LOD	<LOD	<LOD	Grade 1
			Day 42	200	<LOD	<LOD	Grade 1
			Day 228	<LOD	<LOD	<LOD	N/A
	R35167	None	Day 0	LOD	<LOD	<LOD	Grade 1
			Day 21	100	<LOD	<LOD	Grade 1
			Day 42	200	<LOD	<LOD	Grade 1
			Day 228	<LOD	<LOD	<LOD	Grade 1

<LOD: below the limit of detection for IgG and IgE corn avidin glycan tests and the IgE VLP test. N/A: not available. Grade 1: low allergy. Grade 2: moderately high allergy. In the H5 VLP trial, none of the eight MUXF-positive subjects at enrolment mounted an IgE response to either VLP or corn avidin glycans following vaccination. Overall, no correlations were found between pre-existing IgE titers in any assay and reactivity to VLPs or between IgE responses to corn avidin and bromelain glycans. Of particular note, two of the 10 bromelain-positive subjects at reenrolment had lower IgE titers after vaccination with the plant-made VLP vaccine (subjects R35125 and R35152).

vaccines bearing HA molecules of different influenza A viruses can be given to humans without initiating or worsening allergic symptoms despite the development of transient IgG and IgE responses to plant glycans in some subjects.

The reception given to any new vaccine production platform is inevitably cautious. Indeed, IgE-mediated allergic reactions have been reported with almost all vaccines with an overall low incidence; ~1 per million doses [18,19]. With appropriate surveillance and investigation of cases, the risks associated with new platforms are typically clarified over time (*e.g.*: measles, influenza, yellow fever vaccines made in embryonated hens' eggs) [20–22]. After decades of cautious use, it is now clear that these vaccines, some of which contain micrograms of egg proteins, can be administered safely to the majority of egg-allergic subjects [22–24]. The situation with our plant-made vaccines is different from the outset. While classic egg allergens can be found in egg-based vaccines (*e.g.*: ovalbumin), no plant proteins with known allergenic potential are present even in trace amounts in the VLP vaccines. Furthermore, the plant-made proteins that are present do not contain glycan motifs typically associated with allergens. Assuming that all of the HA glycosylation sites are fully occupied by complex glycans in our VLP vaccines, each dose likely contains low microgram quantities of plant-specific glycans. Furthermore, both of the clinical trials administered VLP vaccines across a modest dose range (5, 13, 28 μg/dose for the H1 study and 20, 30, 45 μg/dose for each dose in the H5 study) and there was no clear effect of dose on any of the measured outcomes. Also, there was no significant effect of VLP dose or adjuvant on reported local or systemic adverse events following immunization and there was no correlation between influenza-specific responses and plant glycan-specific responses (Tables 3 and 4). Although the number of subjects studied to date is modest, our data do not suggest that these plant-made VLPs bearing influenza virus HAs and trace plant protein contaminants have important allergenic potential.

In addition to the dose of plant glycans contained in our VLP vaccines, their structure and organization also need to be considered. Since histamine release from mast cells and basophils requires IgE cross-linking, the target epitopes must be present in the form of an array. Given the number of glycosylation sites on the HA proteins on our VLPs (six for both the A/California/7/09 H1N1 and A/Indonesia/5/05) and the fact that each VLP has 400–500 trimeric HA 'spikes' (unpublished data), the opportunity for IgE cross-linking certainly exists with these vaccines. The structure of N-linked glycans also influences allergenic potential. The glycans found in most clinically-relevant allergens are MMXF, MMF or MMX structures devoid of terminal GlcNAc [7]. In contrast, our plant-made influenza VLP vaccines mostly contain complex glycans with terminal GlcNAc motifs. Theoretically, such glycans could even reduce the induction of IgE antibodies to fucose and xylose residues or the binding of pre-formed IgE to these motifs [7,25]. As shown in Table 6, 10/349 subjects enrolled in these studies were positive in the bromelain assay at enrolment, demonstrating that these potentially worrisome IgE antibodies occur naturally. Neither these subjects, all of whom were randomized to VLP vaccination groups, nor the subjects who were bromelain-negative at enrolment, mounted IgE responses to MUXF structures following vaccination. Only eight of the initially IgE-positive subjects were still positive at 6-months. These observations suggest that, even though plant-specific xylose and fucose motifs are found on the plant-origin proteins in our VLPs, these vaccines do not appear to induce IgE responses directed against MUFX motifs in healthy adults. The fact that none of the initially bromelain-positive subjects who received VLP vaccines ± alum increased their IgE titers is reassuring. Indeed, two of the MUFX-positive subjects at enrolment lost this reactivity during the course of these studies (one in each of the H1 and H5 VLP groups).

In contrast to the absence of MUFX responses, transient IgG and IgE responses to the complex glycans found on our VLPs were readily detectable by the corn avidin ELISA following VLP vaccination. However, none of the subjects with increased IgG/IgE reported allergic-like symptoms. Furthermore, even those with IgE responses to corn avidin glycans and/or VLPs failed to mount any response to the cross-reactive MUFX motif in the bromelain test. These results suggest that the relatively rare and transient glycan-specific IgE responses induced by our VLP vaccines are unlikely to be targeting CCD motifs.

Although some subjects in the placebo or comparator vaccine groups were IgG- and/or IgE-positive at baseline, all of the subjects who experienced increasing glycan-specific IgE titers following vaccination were in VLP treatment groups. Furthermore, both IgG and IgE responses tended to be most frequent in subjects receiving higher or multiple VLP doses. However, the incidence of glycan-specific IgE responses induced by VLP vaccination did not appear to be higher in the subjects with allergic histories, including those with reported plant allergies. Several groups have reported that IgEs to plant carbohydrates can cross-react with other allergens without triggering clinical symptoms [5,7,26]. In general, IgEs that recognize these CCDs appear to have weak biological activity in both skin tests and *in vitro* histamine release assays. Although the absence of clinical symptoms despite the presence of IgE directed against plant glycans is consistent with these observations, it is our intention to include limited *in vitro* testing in future studies.

The mechanism(s) that underlie the low biological activity of IgE directed against plant glycans is poorly understood. In the case of our plant-made VLPs, it seems likely that the absence of allergic symptoms is at least partly attributable to a parallel IgG response to the same carbohydrate epitopes. Approximately 20% of the subjects in our two studies (Table 2) already had detectable IgG titers to the MMXF, MMX, GnMXF, GnGnXF and Lewis glycans found on corn avidin at enrolment. This antibody 'profile' probably reflects

the typical response induced by natural exposures to plant glycans. After vaccination, 34% of the subjects who received a VLP vaccine mounted an IgG response to corn avidin glycans. Although small numbers of subjects in the comparator vaccine and placebo groups also mounted apparent IgG responses to plant glycans during the studies, 95% of the responders were in the VLP groups. It is possible that the induced IgG acts, at least in part, to 'block' potential IgE-mediated responses. Such blocking by IgG is thought to be an important mechanism contributing to allergen desensitization [27]. It is interesting that the two subjects in our studies who experienced a decrease in IgE levels in the bromelain test had simultaneous >8-fold increases in their IgG titers to plant glycans (Table 10).

To our knowledge, this work is the first systematic effort to evaluate the humoral response to the glycans on a plant-made biotherapeutic product. None of the 280 subjects exposed to plant-made VLPs bearing fully-glycosylated influenza H1 or H5 hemagglutinin proteins from influenza A virus experienced either new-onset allergic symptoms or worsening of pre-existing allergies. The absence of response to the known allergy-inducing MMXF/MUFX motifs despite readily detectable IgG and IgE responses to plant glycans following plant-made VLP vaccination was also reassuring. These observations support the continued development of plant-based platforms for the production of vaccines and other biotherapeutics.

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