

Original article

Evaluation by double-blind placebo-controlled oral challenge of the clinical relevance of IgE antibodies against plant glycans

Background: The clinical relevance of immunoglobulin E (IgE) to plant glycans is a longstanding debate. We sought to evaluate their clinical reactivity using the human glycoprotein lactoferrin expressed in rice.

Methods: Allergic patients with IgE antibodies against plant glycans were analyzed for the presence of IgE against rice-produced lactoferrin. The potency of IgE to induce mediator release was assessed by basophil histamine release and skin prick tests (SPTs). Clinical relevance was evaluated by double-blind placebo-controlled oral challenge (DBPCOC).

Results: Twenty-four of 29 sera (82.7%) with IgE antibodies against plant glycans demonstrated IgE binding to transgenic lactoferrin. In three of five cases transgenic lactoferrin induced histamine release. Compared to a control major grass pollen allergen lactoferrin concentrations needed for biological activity of IgE were 5–6 orders of magnitude higher. Skin prick test and DBPCOC were negative in five patients with potential clinical reactivity that volunteered to undergo these *in vivo* challenges.

Conclusions: Poor or no biological activity and lack of clinical relevance of IgE-binding plant glycans (five out of five) was demonstrated using human lactoferrin expressed in rice as a model.

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Key words: basophil histamine release; cross-reactive carbohydrate determinants (CCD); double-blind placebo-controlled oral challenge; IgE; plant glycoproteins; transgenic plants.

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Plants are considered a promise as cost-effective and safe factories for the production of pharmaceutical proteins like monoclonal antibodies (1, 2). A potential problem linked to this approach lies in the difference between plant and mammalian glycosylation. Atypical glycosylation may result in immunogenicity of biopharmaceutical glycoproteins as well as changes in their functionality (2). For human use of genetically-modified crops, evaluation of potential allergenicity is an important element of the risk assessment process. It has been known for over two decades now that plant glycans can be recognized by immunoglobulin E (IgE) antibodies of mainly polysensitized pollen-allergic patients (3, 4). Production in plants of pharmaceutical glycoproteins of human origin will introduce such IgE-binding glycans on these proteins (2). Are they an allergenic risk? Since their discovery, there has been a debate on the clinical relevance of IgE antibodies against plant glycans (5, 6). The outcome of this discussion will of course have significant impact on the risk assessment of plant-derived biopharmaceuticals, but also on the validation of existing diagnostic tests for food allergy (6). The ubiquitous presence of plant glycans

in all plant foods makes it possible for specific IgE antibodies cross-react to a broad spectrum of foods. Perhaps the strongest argument against clinical relevance is the observation that pollen allergic patients with these cross-reactive antibodies do not suffer from food allergy, let alone to virtually all plant foods (4). Despite this, *in vitro* plant glycans can induce mediator release from effector cells of the allergic response like basophils. Effector-cell triggering is the first step towards a clinical reaction. Several reports, therefore have used such observations as support for potential clinical relevance of glycan-specific IgE (5, 7–9).

In this study we have investigated the *in vitro* and *in vivo* allergenicity of human lactoferrin (HLF) expressed in rice. HLF is present in human milk and other biological fluids (tears, saliva, synovial fluid, genital and nasal secretions) as well as in neutrophil granules (10). It has been shown to exhibit antibacterial, antiviral, antifungal and antiparasitic activities (11, 12). Recombinant HLF (rHLF) produced in transgenic rice plants was purified from rice seeds and is currently being evaluated for nutritional support of iron deficiency and acute

diarrhea (Ventria Bioscience, Sacramento, CA, USA) (13). Structurally and functionally this rHLF has been shown to be indistinguishable from natural HLF (14, 15), with the exception of its glycans that have typical plant characteristics, as reported by Fujiyama et al. (16). The authors described in detail the eight different glycan structures found on HLF expressed in rice. Two of three glycosylation sites on the molecules are used, and two glycan chains, showing the structure Man3FucXylGlcNAc2 or Man2FucXylGlcNAc2, represent about 83% of the eight different glycans, 93% carrying xylose or fucose. Fifty-five per cent of the glycan structures on rHLF are similar to those found in horse radish peroxidase (HRP), and 15% similar to bromelain glycans (16). These have been reported as the most common IgE-binding glycan structures among plant glycoproteins (6, 17). Assessment of potential allergenic risks of this plant glycosylation is also part of the evaluation process. The aim of this study was to evaluate the clinical relevance of IgE antibodies against plant glycans by double-blind placebo-controlled oral challenges (BDPCOCs) using HLF expressed in rice as a model glycoprotein. The outcome of this evaluation has great potential impact on the specificity of diagnostic tests and on the selection of patients' sera to be used in the safety evaluation of genetically-modified foods (18).

Methods

Purified and recombinant molecules

Recombinant HLF was produced in and purified from rice by Ventria Bioscience as described elsewhere (14, 15). Purified natural human (nHLF) lactoferrin was obtained from Sigma (St Louis, MO, USA). Recombinant major *Phleum pratense* grass pollen allergen Phl p 5 (rPhl p 5) was kindly provided by Biomay (Vienna, Austria) (19). A commercially available glycerinated *Phleum pratense* grass pollen extract with a known concentration of Phl p 5 (20 µg/ml), was used for skin prick test (SPT) (ALK-Abelló, Hørsholm, Denmark). Lactoferrins were used in basophil histamine release (BHR) test at concentrations ranging from 1 ng/ml to 1 mg/ml, rPhl p 5 at concentrations from 0.1 ng/ml to 0.1 µg/ml. A 500 µg/ml rHLF solution for SPT was prepared in 50% water : glycerine diluent. Ten-fold dilutions were obtained using the same diluent, with an antigen concentration down to 0.5 ng/ml. In DBPCOCs, rHLF was used at concentration ranging from 1 µg to 1 g.

Patients, skin testing and sera

The present study has been approved by the IDI-IRCCS institutional review board. Written informed consent was obtained for skin testing, blood sampling, and oral challenge from the patients and parents of patients younger than 18 years. From a cohort of 1243 patients with plant glycan-reactive IgE antibodies, as judged by IgE reactivity to two well-established marker plant glycoproteins, bromelain and HRP (specific IgE values greater than 0.35 kUA/l; ImmunoCAP system, Phadia, Uppsala, Sweden) (4), 29 subjects were recruited for the study (Fig. 1, panel A). Patients were symptomatic pollen and food allergic subjects with positive IgE to

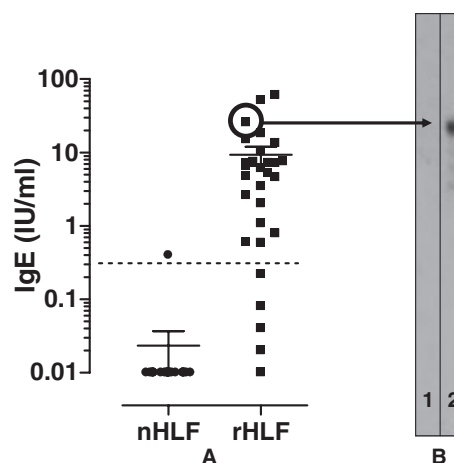


Figure 1. (Panel A) Specific IgE for rHLF and nHLF detected by RAST (values greater than 0.3 IU/ml are regarded as positive). Undetectable or negligible IgE against the natural lactoferrin in contrast to very significant binding upon expression in rice in 24 of 29 tested CCD-IgE tested subjects (82.7%). Circled serum was used for IgE immunoblotting. (Panel B) IgE immunoblotting using natural (nHLF, lane 1) and rice-produced recombinant human lactoferrin (rHLF, lane 2).

the major grass pollen allergen Phl p 5 (rPhl p 5) (20). Patients underwent SPT, with a standardized procedure and recording (21). Skin testing with increasing concentration of rHLF was performed every 15 min. A higher concentration was applied if a negative result was recorded. Sera were obtained from enrolled patients and stored at -20°C until required.

IgE detection and basophil histamine release test

IgE detection by radio allergosorbent test (RAST) was performed as described previously, using Sepharose-immobilized lactoferrin (1 µg/mg Sepharose) (22). Results were expressed in international units of IgE per ml (IU/ml) using a standard curve of a chimeric monoclonal IgE antibody against the major house dust mite allergen Der p 2 and sepharose-coupled recombinant Der p 2 (23). A RAST result greater than 0.3 IU/ml was regarded as positive. For the BHR assay, white blood cells were isolated from blood of a nonallergic donor by means of Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) centrifugation and stripped from IgE by using lactic acid treatment, as described elsewhere (24). Subsequently, cells were re-sensitized with the serum from five selected patients under study (Table 2). Released histamine was measured by using the fluorometric method, essentially as described by Siraganian (25). Stripped cells were used as a negative control and induced a histamine release of $<3\%$.

Double-blind placebo-controlled oral challenge

Double-blind placebo-controlled oral challenges were carried out following the guidelines of the European Academy of Allergy and Clinical Immunology on double-blind placebo control food challenges (26).

This includes testing of placebo and verum at two separate days. As rHLF was tasteless even at the highest concentration used, lyophilized rHLF was diluted in 100 ml natural drinking water

simply masked with 2 ml of concentrated commercial green-coloured mint syrup, free of plant extract. The same amount of mint syrup and water was used as placebo preparation. The preparations were made just before oral administration. Doses were administered every 30 min, starting from a lowest dose of 1 µg. The final cumulative dose was slightly over 1.11 g. A two hour observation period was included after the top dose had been reached. There was a 2-week interval between administration of verum and placebo.

Results

IgE recognition of rice-derived rHLF

Twenty-nine sera from grass pollen allergic patients with IgE antibodies against plant glycans were tested for IgE reactivity to rice-derived rHLF and nHLF (Fig. 1, panel A). Twenty-four (82.7%) showed significant binding to rHLF (ranging from 0.59 to 60.7 IU/ml) but showed negative or negligible specific IgE to nHLF. This demonstrated that introduction of plant glycans resulted in IgE binding to lactoferrin. For serum 4986-CV, this was visualized by immunoblot (Fig. 1, panel B).

Basophil histamine release assays

To investigate the biological activity of rHLF-reactive IgE antibodies, basophils from a nonatopic donor were sensitized with serum IgE antibodies of six patients, whose serological and clinical features are reported in Table 1, and incubated with rHLF and nHLF (except for GA23-LA). As a positive control, the major grass pollen allergen rPhl p 5 was used. In all six cases, rPhl p 5 induced significant histamine release (Fig. 2). The maximum release was between 20% and 50% and was reached at allergen concentrations around 1 ng/ml. None of the subjects showed significant histamine release upon incubation with nHLF. Two patients (4431-GEM and 2925-KSS) with lower levels of glycan-specific IgE (Fig. 1, panel A and Table 1), showed no histamine release with rHLF either. The other four subjects demonstrated low but significant histamine release with rHLF, reaching maximum release of 10–20% at concentrations between 0.1 and 1 mg. This is 5–6 orders of magnitude higher than the concentrations needed for rPhl p 5.

Skin test and DBPCOC

Three subjects showing BHR activity induced by rHLFs, and three additional patients having positive rHLF IgE, were invited to participate to the *in vivo* part of the study. Two of three subjects with positive BHR volunteered for SPT and DBPCOC with rHLFs and three of three of the second group accepted to undergo the *in vivo* testing. Skin prick test gave negative results with rHLF at concentrations up to 500 µg/ml in all five subjects (Table 1), whereas they all showed a positive SPT to the timothy grass pollen extract dilution containing 0.2 µg/ml Phl p 5. Five subjects underwent a DBPCOC with rHLF up to the top dose of 1 g without any subjective or objective adverse reactions (Table 1).

Discussion

In the present study it was demonstrated by DBPCOC with HLF expressed in rice, that IgE antibodies directed to plant glycans did not induce any clinical response in pollen allergic patients. This observation stresses the need to select patients' sera for evaluation of allergenicity of GMO's based on proven clinical reactivity and not merely on positive IgE serology. Ever since the discovery of plant glycan-specific IgE antibodies in serum of pollen-allergic patients, their clinical relevance has been a matter of debate (5, 6, 27). The most obvious support for their lack of clinical relevance comes from the simple observation that pollen-allergic patients with these type of cross-reactive IgE antibodies usually do not display food allergy to virtually all plant foods. If their pollen-food IgE cross-reactivity is exclusively based on anti-glycan IgE no food allergy is reported at all. This is also the case for the patients enrolled in this study. Where then lays the controversy? Several studies have shown that plant glycan-specific IgE can induce mediator release from effector cells of the allergic response (5, 7–9). In other words, glycan-specific IgE antibodies bound to high-affinity IgE receptors on basophils and mast cells can be cross-linked by glycoproteins carrying more than 1 glycan. This is also the case for the glycoprotein that served as a model in the present study, i.e., rHLF. Since

Table 1. Features of selected pollen allergic patients with IgE to plant glycans undergoing basophil histamine release test, SPT and DBPCOC

Code	Age	Gender	Clinically relevant IgE sensitization	Bromelain*	HRP*	rHLF	rPhl p 5*	SPT rHLF	DBPCOC rHLF
4431-GEM	60	F	Cypress, grass, mites	10.8	7.6	4.6	29.6	NEG	NEG
2925-KSS	46	M	Cypress, grass, parietaria	6.8	6.0	6.5	67.5	NEG	NEG
4969-GV	12	M	Cypress, grass, olive, parietaria, cow's milk	13.2	1.4	7.5	95.4	NEG	NEG
4837-MY	10	F	Cypress, grass, parietaria	30.1	2.3	13.5	84.8	NEG	NEG
4986-CV	14	M	Mites, cypress, grass, olive, parietaria	89.0	60.7	25.6	>100	NEG	NEG
GA23-LA	15	M	Cypress, peach, cat, parietaria, grass, mites	79.7	73.1	60.7	33.7	ND	ND

*IgE determined by ImmunoCAP system (Phadia, Uppsala, Sweden) and expressed in kUA/l.

SPT, skin prick test; DBPCOC, double-blind placebo-controlled oral challenge; rHLF, recombinant human lactoferrin; rPhl p 5, recombinant major *Phleum pratense* grass pollen allergen Phl p 5; ND, not done; NEG, negative.

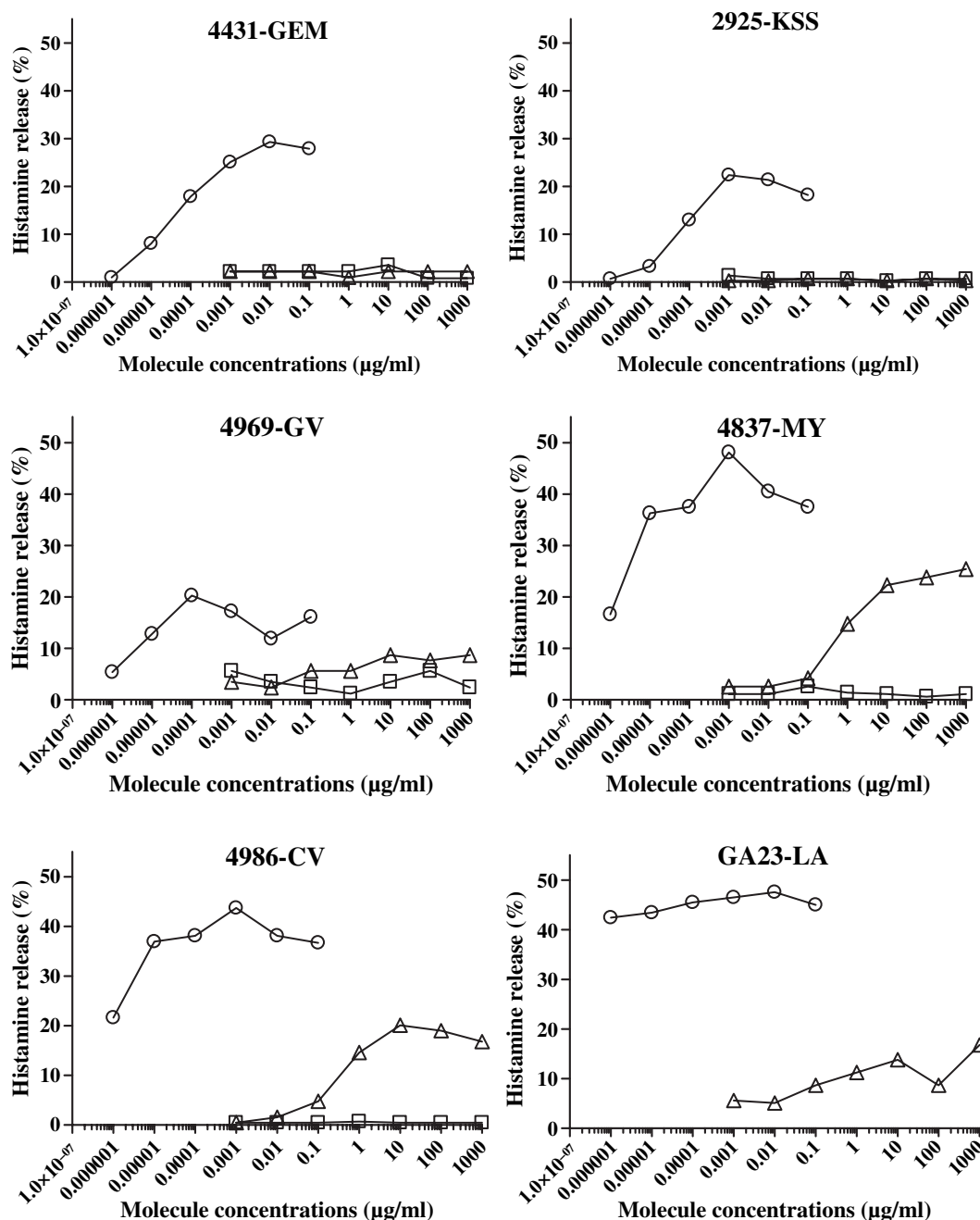


Figure 2. Basophil histamine release assays. Biological activity of rHLF (triangles) and nHLF (squares) (except for GA23-LA) is compared to that of the control major grass pollen allergen rPhl p 5 (circles).

biological activity of IgE is generally accepted as the starting point for immediate-type allergic reactions, the conclusion is easily drawn that glycan-specific IgE can induce such reactions. SPTs performed with rHLF are in line with earlier reports showing poor skin reactivity of another polyvalent plant glycoprotein HRP (4). Both glycoproteins were shown to share structurally identical glycans. In the present study it was now demonstrated for the first time by DBPCOC that significant *in vitro*

biological activity is not paralleled by clinical reactions, even with 1 g of rHLF. Recently, Bolhaar et al. reported for the first time a DBPCOC with a purified recombinant molecule, the major allergen from apple rMal d 1, in two apple allergic subjects (28). Concentrations causing initial local symptoms were in a range between 10 and 100 μg of the triggering allergen. One of the subjects presented quite severe systemic symptoms after ingesting 1 mg of rMal d 1. This amount is 6 orders of magnitude lower

than 1 g of rHLF given to and tolerated by our five candidates. How can we explain the discrepancy between *in vitro* biological activity and *in vivo* unresponsiveness? Histamine release was induced by rHLF at concentrations between 0.1 and 1 mg/ml. This is 6 orders of magnitude above the concentrations needed for the major grass pollen allergen rPhl p 5 (around 1 ng). The concentrations of specific IgE against rHLF are only about 1 order of magnitude lower than those against rPhl p 5. In other words, a similar amount of specific IgE against plant glycans requires orders of magnitude more allergen than specific IgE against a major grass pollen allergen. A possible explanation for this much lower biological activity could be a difference in avidity of the interaction between allergen and IgE. Recently, however, Altmann et al. reported that IgE antibodies against plant glycans are of high avidity (6, 29). To further address this issue, relative avidity of IgE against rPhl p 5 and rHLF was evaluated by homologous IgE inhibition assays. At similar specific IgE concentrations, 50% inhibition was attained 1.5 and 3.0 µg/ml, respectively (not shown). It is highly unlikely that this negligible difference can explain several orders of magnitude difference in biological

activity. Alternatively, the low number of epitopes available for cross-linking (two glycans) could be the explanation. The observation, however, that the subjects evaluated in the present study did not show *in vitro* histamine release with a glycoprotein with > 5 glycans (i.e. HRP) does not support this explanation (not shown). Therefore, alternative explanations for the poor biological activity of plant glycan-specific IgE need to be sought. In conclusion, this study has provided support for the safety of recombinant glycoproteins produced in plants for administration to allergic patients with plant glycan-specific IgE. Furthermore it has for the first time convincingly demonstrated the lack of clinical relevance of plant glycan-specific IgE antibodies that show significant but weak *in vitro* biological activity. This needs to be taken into account when interpreting positive *in vitro* diagnostic tests for food allergy.

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