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Isolated IgE reactivity to native walnut vicilin-like protein (nJug r 2) on ISAC™ microarray is due to cross-reactive carbohydrate epitopes

Abstract

Background: The last version of the microarray-based testing ImmunoCAP ISAC 112™ includes the native walnut (*Juglans regia*) molecules 2S albumin (nJug r 1), vicilin (nJug r 2) and lipid transfer protein (nJug r 3). In view of the many unexpected cases of isolated positivity to nJug r 2 occurring in daily practice, we evaluated the association of these reactivities with clinical symptoms, as well as the relationship between sIgE and nJug r 2 and cross-reactive carbohydrate determinants (CCDs).

Methods: Sera from 320 consecutive allergic outpatients tested by ImmunoCAP ISAC™ 112 were considered. The medical records of all nJug r 2 positive patients were reviewed to assess clinical symptoms related to walnut allergy. A linear regression analysis was performed to evaluate the correlation between nJug r 2 and CCDs (nMUXF3) sIgE values, and a CAP inhibition assay was carried out to confirm the possible cross-reactivity between CCDs and nJug r 2.

Results: Thirty-seven out of 320 sera tested (11.6%) were positive to nJug r 2. Among them three (8.1%) and eight (21.6%) scored positive for nJug r 1 and nJug r 3 as well, respectively. Twenty-seven (73%) sera showed isolated nJug r 2 positivity. Only nJug r 1 reactors had symptoms referred to walnut allergy. Twenty-five/37 nJug r 2-positive sera (67.6%) showed a simultaneous positivity to nMUXF3 and a significant correlation ($p < 0.0001$) between the IgE levels to nJug r 2 and nMUXF3 ($r^2 = 0.787$). After incubation with nMUXF3 a complete inhibition of sIgE reactivity to both nMUXF3 and nJug r 2 was shown.

Conclusions: The unexpected isolated sIgE reactivity to nJug r 2 found by ImmunoCAP ISAC™ 112 is frequently related to reactivity to cross-reactive carbohydrate epitopes and it is lacking clinical significance.

Keywords: cross-reactive carbohydrate determinants (CCDs); ImmunoCAP ISAC™; microarray; native walnut vicilin (nJug r 2) nMUXF3.

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Introduction

English walnut (*Juglans regia*) is one of the most common allergenic foods. The importance of allergy to walnut is related not only to its prevalence in the general population (around 0.2%–0.7%) [1], but also to the severity of allergic reactions. The register of food anaphylaxis-related fatalities in the US reported that three out of 32 cases between 1994 and 1999 were caused by walnut [2]; as well in the UK five out of 37 food-induced fatalities between 1992 and 1998 were due to walnut allergy [3, 4].

The characterization of the major walnut allergens may allow a component resolved diagnosis (CRD) and an improvement of the in vitro diagnosis of walnut allergy. The walnut allergens identified so far include Jug r 1 [5], Jug r 2 [6], Jug r 3 [7], and Jug r 4 [8], corresponding to walnut 2S albumin, vicilin-like protein, lipid transfer protein (LTP), and 11S globulin, respectively.

In the last version of the ImmunoCAP ISAC 112™, a microarray-based test able to detect simultaneously IgE (sIgE) specific for 112 different allergens, the native (n) molecules of Jug r 1, Jug r 2 and Jug r 3 are present. During the first year of the routine use of the ImmunoCAP ISAC™ 112 we observed many unexpected isolated IgE reactivities against nJug r 2. In the present study we investigated how many patients showing isolated sIgE positivity to nJug r 2 display symptoms associated with walnut allergy. In addition, since vicilin-like proteins are post-translationally N-glycosylated glycoproteins, we evaluated the possible

correlation between sIgE positivity to nJug r 2 and sIgE to cross-reactive carbohydrate determinants (CCDs), which are often found in the sera from pollen allergic patients and may interfere in sIgE assays for latex, hymenoptera venoms and plant foods [9].

Materials and methods

The study was carried out on 320 consecutive sera from allergic outpatients seen from November 2011 to December 2012 at the Allergy Unit of Pordenone and tested for sIgE with the microarray-based ImmunoCAP ISAC™ 112 (Thermo Fisher Scientific, Uppsala, Sweden). During the visit all the patients were thoroughly interviewed in order to ascertain respiratory and/or food allergy symptoms, and underwent skin prick testing (SPT) with a large panel of inhalant and/or food allergens based on clinical history.

Specific IgE were detected by ImmunoCAP ISAC™ 112 following manufacturer's instructions. Briefly, reaction sites were incubated with 30 µL of patients' serum for 2 h. After rinsing, washing, and drying, allergen-specific IgE complexes were stained with fluorescence-labeled anti-human IgE for 30 min. After further washing, a laser scanner took fluorescence readings and results were expressed as ISAC standardized units (ISU)/L. Levels higher than 0.3 ISU/L were regarded as positive.

The medical records of all nJug r 2-positive patients were reviewed to ascertain the presence or absence of clinical symptoms related to walnut ingestion. The reported symptoms were classified as oral allergy syndrome (OAS), defined as the occurrence of oral itching with or without angioedema of the lips and/or tongue a few minutes after the food ingestion, urticaria, asthma and anaphylaxis.

In the same patients reactivity to the other walnut allergens (Jug r 1 and Jug r 3), profilin (Bet v 2 and homologous proteins), pathogenesis-related protein 10 (PR-10) (Bet v 1 and homologous proteins) and CCDs (nMUXF3) was evaluated.

CAP-inhibition assay

An inhibition assay was carried out to evaluate whether nJug r 2 reactivity was associated with sIgE to CCDs. Three randomly selected sera among those reacting to both nJug r 2 and nMUXF3, showing sIgE levels of 6.0, 2.6 and 8.4 ISU/L, and 9.6, 3.2 and 8.6 ISU/L, respectively, was used to this end. Forty microliters of serum were incubated for 30 min at 37°C in a CAP coated with nMUXF3 (Thermo Fisher Scientific; code o214) and in a CAP coated with Bet v 1 (Thermo Fisher Scientific, code t215) as control. Subsequently, sera were recovered by centrifugation of the CAPs at 800 g for 10 min, and re-tested in two separate ImmunoCAP ISAC™.

As all in vivo and in vitro tests were performed within routine clinical activity no Ethical Committee approval was needed for the study.

Statistical analysis

The correlation between sIgE levels was assessed by linear regression analysis. The non-parametric Mann-Whitney U-test was used to

compare nJug r 2 sIgE levels in nMUXF3-positive and -negative proteins. Probability values below 0.05 were considered significant.

MedCal for Windows, version 7.4.1.0 (MedCal Software, Mariakerke, Belgium) was used for the statistical analysis.

Results

Thirty-seven out of 320 sera tested (11.6%) showed sIgE to nJug r 2. Three of them (8.1%) reacted to walnut 2S albumin (nJug r 1) and 8 (21.6%) to walnut LTP (nJug r 3) as well. Twenty-seven (73%) sera showed isolated nJug r 2 positivity.

The evaluation of the medical records demonstrated that all three patients with both nJug r 2 and nJug r 1 reactivity had a clinical history of symptoms clearly related to walnut ingestion (anaphylaxis, urticaria, and urticaria plus asthma, respectively). Three out of eight nJug r 3-positive patients reported OAS, but only with fruits of the Rosaceae family, while walnut was tolerated. Twenty-three out of the 27 (85%) patients with isolated nJug r 2 positivity tolerated all the foods, while the remaining four reported OAS after ingestion of raw fruits of the Rosaceae family. However, two of the latter patients were PR-10 (Bet v 1 and homologous) reactors and two profilins (Bet v 2 and homologous) reactors.

Twenty-five out of the 37 nJug r 2 positive sera (67.6%) showed a simultaneous positivity of sIgE to the CCDs (nMUXF3). Further, nJug r 2-positive but nMUXF3 negative patients had sIgE levels to nJug r 2 significantly lower than patients positive for both allergens (0.95 ± 0.212 ISU/L vs. 4.65 ± 4.48 ISU/L; $p=0.007$) (Figure 1). A significant correlation ($p<0.0001$) was found between sIgE to nJug r 2 and nMUXF3 ($r^2=0.787$; slope= 1.020 ± 0.1107 ; Y-intercept= 0.2773 ± 0.5302 ; X-intercept= -0.2719 ; $1/\text{slope } 0.9806$) (Figure 2).

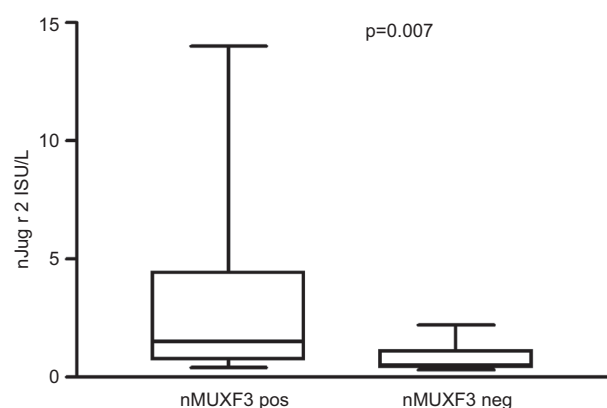


Figure 1 Levels of sIgE to walnut vicilin (nJug r 2) in MUXF3-positive and negative sera.

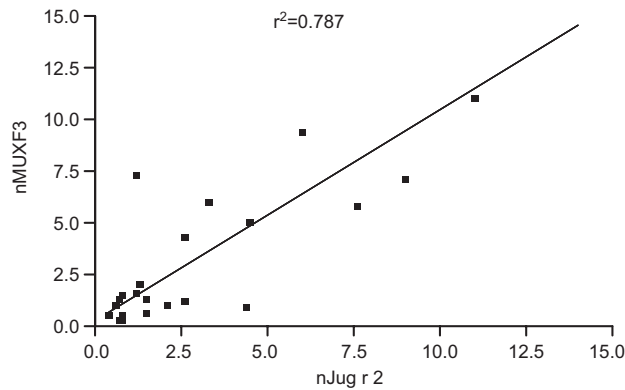


Figure 2 Correlation between sIgE sera levels (expressed in ISU/L) to nJug r 2 and nMUXF3.

The CAP inhibition assay showed a complete inhibition of IgE reactivity to both nMUXF3 and nJug r 2 following incubation of the three nJug r 2 positive sera with nMUXF3, whereas any change in IgE reactivity was observed after incubation with Bet v 1 (Figure 3).

Discussion

Advances in knowledge of specific allergenic components present in allergenic sources and availability of recombinant allergens led to the development of the new concept of CRD in allergology, allowing the identification of potential disease-eliciting molecules. By CRD it is possible to distinguish between species-specific allergen components, which are unique markers for a certain allergen source, and cross-reactivity markers, which share similar epitope structures and IgE binding properties in many different allergen sources. In food allergy CRD is essential to detect the sensitizing allergens and to evaluate the risk of reaction on exposure to different sources and the potential severity of the reactions [10].

Using the microarray-based ImmunoCAP ISAC™ 112 it is possible to determine the IgE reactivity profile of the single patient and to assess the clinical sensitization pattern. However, the presence of a predefined large number of allergen components in the panel may bear

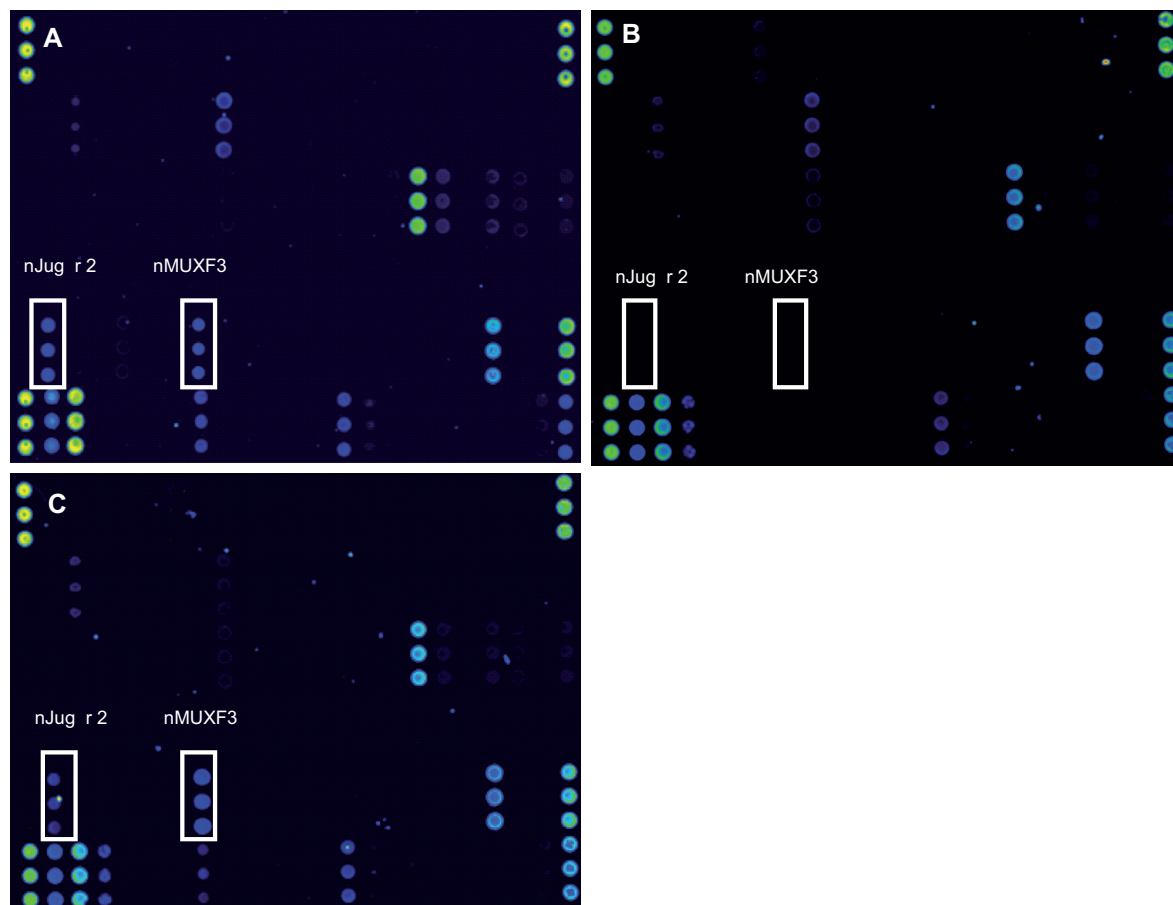


Figure 3 CAP inhibition assay with a serum showing sIgE levels to nJug r 2 and to MUXF3 of 6.0 and 9.6 ISU/L, respectively. (A) sIgE to nJug r 2 and nMUXF3 before the inhibition assay. (B) sIgE to nJug r 2 and nMUXF3 after pre-absorption with CCDs (nMUXF3). (C) sIgE to nJug r 2 and nMUXF3 after pre-absorption with the control allergen (Bet v 1).

the risk of unexpected positivities that must be correctly interpreted.

During the routine use of the ImmunoCAP ISAC™ 112 we observed several unexpected isolated positivity of sIgE to nJug r 2, a marker of primary walnut sensitization. However, the present study shows that only patients co-sensitized to Jug r 1, a 2S albumin, have a history of walnut allergy, whereas patients with isolated nJug r 2 reactivity or co-sensitized nJug r 3 do not. We found a strong association between reactivity to nJug r 2 and to CCDs components (nMUXF3), and the pre-adsorption of a nJug r 2 positive sera with CCDs completely abolished the reactivity to walnut. This suggests that most of the unexpected anti-nJug r 2 positivities found on ImmunoCAP ISAC™ microarray are due to sIgE against CCDs. These glyco-epitopes can share significant structural homologies beyond the limits of protein families and are thus responsible for extensive cross-reactivity between allergenic or non-allergenic glycoproteins containing a carbohydrate chain [11]. However, the presence of IgE against CCDs are clinically irrelevant.

Vicilins of peanut and tree nuts (walnut, hazelnut and cashew nut) are quantitatively important seed storage glycoproteins and represent major allergens of these allergenic sources. Surface-exposed IgE binding epitopes have been characterized on the vicilins of peanut, walnut, hazelnut and cashew nut, and correspond to linear stretches of conserved amino acid sequences mainly located along the C-terminus of the polypeptide chains [12]. These epitopes seem to be associated with tree nut allergy and cross-allergenicity. However, vicilins are post-translationally N-glycosylated during their processing along the endoplasmic reticulum and walnut vicilin has one potential N-linked glycosylation site at amino acid position 230 [13]. The N-glycans protrude from the

molecular surface of the vicilin becoming more exposed than the corresponding peptide IgE-binding epitopes. sIgE against these glyco-epitopes (CCDs) may, on one hand, exert some masking effect on the peptide-epitopes and, on the other hand, determine an in vitro cross-reactivity with homologous N-glycans contained in glycoprotein from different sources (pollens, plant foods, latex, hymenoptera venom) as shown in our study.

In conclusion, the finding of an isolated, unexpected nJug r 2 reactivity in the in vitro microarray-based sIgE detection is generally due to reactivity to cross-reactive carbohydrate epitopes and is clinically irrelevant. Since Jug r 2 is a major allergen involved in walnut allergy, a possible solution is to use a recombinant molecule (rJug r 2) without or with limited glycosylation. Moreover, the lesson from this study is that unexpected results, unrelated to the initial clinical question, can be frequently found using extended, not disease-oriented microarray-based multiplexed testing for sIgE detection. As a consequence, the results obtained by such diagnostic approach need to be carefully interpreted as allergen sensitization does not necessarily imply a clinical reaction.

Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.

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