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Detection of peanut allergen in human blood after consumption of peanuts is skewed by endogenous immunoglobulins

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ABSTRACT

Some studies have suggested that allergens may appear in the circulation after ingestion of allergenic food sources. The reported levels of allergen in serum, however, are low, and conclusions between studies differ. Here, we investigated factors that determine the detection of allergens in serum after consumption of peanuts.
Ten healthy volunteers ingested 100 g of light-roasted peanuts. Serum samples were taken at regular intervals for six hours. A double monoclonal sandwich ELISA was used to analyse the presence and quantity of the major peanut allergen Ara h 6 in serum.
In 4 out of 10 subjects, no Ara h 6 could be detected. Purified Ara h 6 that was digested *in vitro* was still reactive in the ELISA, rejecting the possibility that digestion leads to small peptides that could not be detected. Spiking of purified Ara h 6 in baseline serum showed that the pre-ingestion serum of these four subjects partially prevented Ara h 6 to react in the ELISA, with a reduction of reactivity of up to 3 orders of magnitude or more. Pre-ingestion serum of the other six subjects did not show such an effect. The reduction of reactivity of Ara h 6 coincided with high titres of IgG and IgG4, and removal of IgG from pre-ingestion serum abolished this effect completely, indicating that IgG and IgG4 inhibited the reactivity of Ara h6 in the ELISA.
We conclude that some individuals have IgG and IgG4 against food allergens in their blood, which interferes with detection of such food allergens in serum. Because this effect does not occur for each individual, the possibility of such interference should be taken into consideration when interpreting immunochemical studies on the absorption of food allergens in serum.

1. Introduction

Food allergies affect 2–3% of the population in Western countries, and peanut allergy is one of the most important food allergies because of 1) its persistent character, 2) the low amounts of peanut needed to trigger a reaction, and 3) peanut being a widely used food ingredient. Peanut conglutins Ara h 2 and Ara h 6 are the most potent allergens with regard to IgE-binding, skin prick test, and basophil test. IgE to these allergens has a high positive predictive value for diagnosing peanut allergy (Klemans et al., 2014).

A characteristic of conglutins (2S–albumins) is their relative stability to digestion (Astwood et al., 1996; Pfeifer et al., 2015). Digestion of Ara h 2 and Ara h 6 by pepsin and trypsin has been investigated in the past, showing that these are only partially hydrolysed resulting in relatively large peptides of around 4 to 5 kDa and 10 kDa in size (Sen et al., 2002; Koppelman et al., 2010; Apostolovic et al., 2016). Such digestion-resistant peptides are exposed to the intestinal immune system as large protein fragments, potentially explaining the potent allergenicity of these allergens with regard to their capacity to sensitize. It has also been shown that digestion-resistant peptides can be taken up in the circulation, leading to systemic reactions in peanut allergic individuals. Several studies investigated the uptake of digestion-stable food allergens such as peanut (Dirks et al., 2005; Baumert et al., 2009), egg (Husby et al., 1987) and wheat (Matsuo et al., 2005) in healthy individuals and animal models (Bernard et al., 2014). Ara h 2 and Ara h 6 have also been detected in breast milk of lactating women (Vadas et al., 2001; Schocker et al., 2016) and mice (Bernard et al., 2014). Moreover, a case report revealed that transfusion of a blood product into a peanut allergic recipient led to an anaphylactic reaction explained by residual peanut in

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the blood of one of the donors who consumed peanut prior to donation (Jacobs et al., 2011). Together these reports indicate that immunologically active fragments of peanut allergens can at least partially survive the gastro-intestinal tract, and may appear in the circulation to be transported to various compartments of the body. However, the reported values of peanut allergen taken up are low, and often close to the limit of detection. Also, large variability between studies and between individuals within studies is observed. Considering the digestion resistance of peanut conglutins and their fairly high abundance in peanut, one would expect more consistent and higher levels of such proteins in the circulation if these were indeed taken up by the intestine and transported into the blood.

The aim of this work was to identify possible confounding factors for the detection of peanut allergens in serum after consumption of peanut. We selected Ara h 6 as representative of the potent peanut conglutin allergens. Ara h 6 is biochemically well characterised and appears as a homogenous protein, with limited isoforms. We recruited a group of 10 healthy individuals, sampled blood before and after digestion of peanuts and analysed their serum for the presence of Ara h 6 at various time points. Moreover, we performed spiking experiments to investigate if detected levels corresponded to expected levels, and which sera-derived factors could be responsible for interference.

2. Materials and Methods

2.1. Healthy volunteers / subject

Ten healthy subjects were recruited via social media and email database of the university's human nutrition division (Wageningen University, the Netherlands). Exclusion criteria were having (suspected) allergic symptoms after peanut ingestion, smoking, use of illicit drugs, NSAIDs on a chronic basis, or use of any medication for gastric or intestinal complaints. None of the subjects donated blood during the last six weeks before the start of the study. Subjects were also instructed not to eat peanut-containing foods nor use alcohol for 2 days prior to the test day.

This study was approved by the medical ethical committee of Wageningen University (METC-WU 15/34; NTR5655), and conducted in accordance with the Declaration of Helsinki (revised version, October 2008, Seoul). All subjects gave their written informed consent after the nature and possible consequences of the study had been fully explained.

Each subject came to the university while having fasted overnight. Pre-ingestion blood was sampled after which they were asked to consume 100 g of light-roasted, unsalted peanuts obtained from a local supermarket (Albert Heijn; private label product) within 10 min. Blood was sampled in serum separator tubes 30, 60, 120, 240, and 360 min after finishing peanut consumption. Blood was left to clot in the dark for at least 20 min at room temperature, after which the tubes were centrifuged at 2000 g for 10 min at room temperature to obtain serum, which was directly aliquoted and stored at −80 °C until further analysis.

2.2. Peanut allergens

Virginia peanuts obtained from the US were used to purify the various Ara h 6 preparations. Ara h 6 was purified essentially as described earlier (Koppelman et al., 2005) with one additional hydrophobic interaction chromatography step to remove traces of impurities. Post-translationally proteolytically processed Ara h 6 (hereafter named naturally processed Ara h 6) was purified from side fractions of the Ara h 6 purification (Koppelman S.J., unpublished data), which contained disulfide-linked molecules consisting of a 5 and 10 kDa chain with estimated purify of >95%. Identity was proven by peptide mass finger printing (data not shown). Digestion-resistant peptides from peanut conglutin (equimolar mix of the heavy isoform of Ara h 2, the light isoform of Ara h 2, and Ara h 6 were prepared by incubation of peanut conglutin with immobilized trypsin (Apostolovic et al., 2016). The fraction of Ara h 6 in the equimolar mix was 0.30 based on mass. The protein concentration of the digestion-resistant peptide mix (hereafter named digested mix of Ara h 2 and Ara h 6) is expressed in mg of Ara h 6 per mL to simplify comparison with other Ara h 6 preparations.

2.3. ELISAs

The Ara h 6 ELISA was from Indoor Biotechnologies (EL-AH 6, Cardiff, UK), utilizing two monoclonal antibodies. The instructions of the manufacturer were followed, except that in some cases the reference material was replaced by other Ara h 6 preparations. By using extra dilutions of the standard, the lower limit of quantification was determined to be 0.024 ng/mL (defined as the value corresponding to an absorbance of the blank + 10 SD), and the lower limit of detection to be 0.012 (defined as the value corresponding to an absorbance of the blank + 3 SD). Samples were tested in duplicate and experiments were performed at least twice. To compare reactivity of different Ara h 6 forms, EC_{50} values, defined as the concentration that leads to half-maximal absorbance, were calculated using the Graphpad Prism software, version 6.07.

ELISAs for IgG and IgG4 were set-up as follows. Purified Ara h 6 was coated on ELISA plates (Nunc MaxiSorp® flat-bottom 96 well plate) at 3 μg/mL in PBS (pH 7.4; 136.89 mM NaCl, 1.47 mM KH_2PO_4, 8.10 mM Na_2HPO_4, 2.68 mM KCl), 100 μL/well, overnight at 4 °C. Plates were washed once with PBS and then blocked with PBS containing 2% (*W*/W) bovine serum albumin (BSA, A6003, Sigma Aldrich, Zwijndrecht, NL), for 2 h at 37 °C under shaking conditions (300 rpm). After washing (3 times with PBS containing 0.05% Tween 20 (PBS-T)), diluted serum samples were pipetted in the wells in duplicate and were allowed to incubate for 2 h at 37 °C under shaking conditions (300 rpm). Wells were washed again (3 times with PBS-T) and a 1:500 dilution of horseradish peroxidase conjugated anti-human IgG (05–4220, Invitrogen, Breda, NL) or 1:500 dilution of anti-human IgG4 (ab99817, Abcam, Cambridge, UK) in PBS-T with 1% BSA was applied on the plates for 1.5 h at 37 °C under shaking conditions (300 rpm). Plates were further developed using 100 μL of TMB substrate (T0440, Sigma Aldrich). Colour development was stopped after 10 min by adding 100 μL of Stop reagent (S5814, Sigma Aldrich). Absorbances with oversaturated values were imputed at 4.1, representing the highest absorbance that can be measured with the ELISA plate reader (Synergy HT, BioTek, Winoski, VT, USA).

2.4. Removal of IgG from serum

Two times diluted pre-digestion serum (in PBS) was applied to a Protein G Sepharose Column (6518, BioVision, ITK Diagnostics bv, Uithoorn, NL), equilibrated with PBS. The flow-through was collected and re-applied to the column four times. The final flow-through was collected as IgG-depleted serum. The column was washed and re-equilibrated according to the instructions of the manufacturer.

3. Results and Discussion

We applied an ELISA specific for Ara h 6 detection to study the presence of this allergen in serum of healthy individuals after consuming peanuts. Several hypotheses were tested to explain why the detected levels of Ara h 6 are low and variable.

3.1. Hypotheses formulation

An overview of some relevant subject characteristics of the study is provided in Table 1. Upon ingestion of peanuts, the serum samples at $t = 60$ min were tested for the presence and quantity of Ara h 6. In serum of four subjects (out of ten), Ara h 6 was undetectable, while in the serum of the other six subjects low levels of Ara h 6 of 0.30 ± 0.16 ng/mL could be detected. The total amount of peanuts consumed was 100 g. Using a protein content of 25% (Oerise et al., 1974), and