RESEARCH ARTICLE

A food matrix reduces digestion and absorption of food allergens in vivo

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Scope: Food allergy is caused by primary (class 1) food allergens, e.g. Bos d 5 (cow's milk) and Cor a 8 (hazelnut) or secondary (class 2) food allergens, e.g. Mal d 1 (apple). The latter cannot sensitize susceptible individuals but can cause allergy due to immunological cross-reactivity with homologous respiratory allergens. Here, we studied the effects of food matrix on gastrointestinal proteolysis, epithelial transport and in vivo absorption of class 1 and class 2 food allergens.

Methods and results: Mal d 1 lost its IgE-reactivity immediately after simulated gastric digestion whereas Bos d 5 and Cor a 8 did not. Only Cor a 8 maintained IgE-binding capacity after simulated intestinal proteolysis. The presence of hazelnut and peanut extracts, which served as protein-rich model food matrices, delayed gastrointestinal degradation and reduced epithelial transport rates of all allergens through CaCo-2 monolayers. Finally, IgE-reactive allergens were assessed at different time points in sera from rats fed with all three allergens with or without hazelnut extract. The levels of all allergens peaked 2 h after animals were fed without matrix and increased over 8 h after feeding.

Conclusions: A protein-rich food matrix delays gastrointestinal digestion and epithelial transport of food allergens and thereby may affect their sensitizing capacity and clinical symptoms.

Keywords:
Food allergens / Food allergy / Food matrix / Gastrointestinal digestion / IgE

1 Introduction

Food allergy is an important manifestation of Type 1 allergy and affects up to 6% of young children and 3–4% of adults [1, 2]. Food allergens have been classified into class 1 (true) food allergens and class 2 food allergens. This classification is mainly based on their capacity to induce allergic reactions either by a primary sensitization or as a consequence of IgE-cross-reactivity upon sensitization to homologous pollen allergens. In general, primary food allergens exhibit strong resistance to gastrointestinal degradation and are therefore believed to sensitize via the gut. Of note, recent studies have provided evidence that sensitization may also occur by cutaneous exposure [3]. An example of a class 1 food allergen is Bos d 5 (β-lactoglobulin) from cow's milk. Bos d 5 is the major whey protein recognized by IgE antibodies of 60–80% of cow's milk allergic patients [4]. Cow's milk allergy mainly affects children in the first 3 years of life [5] and is often outgrown by school age. In adults it is rare but still severe and Bos d 5 is one of the major milk allergens involved [6].

In Southern Europe, food allergy to Rosaceae fruits, legumes and other plant foods is most commonly associated with non-specific lipid transfer proteins (nsLTPs) [7–10]. Because nsLTPs often induce severe systemic reactions, their allergenic and immunological properties have been extensively studied. IgE and T-cell epitopes of Pru p 3, the nsLTP from peach, have been mapped [11–14] and the

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Abbreviations: nsLTP, non-specific lipid transfer protein; **TEER,** trans-epithelial electrical resistance

crystal structures of numerous nsLTPs have been solved [15–18]. Despite some controversy regarding their status as true food allergens because of reported respiratory sensitization to nsLTPs [19, 20], on account of their compact structure and strong resistance to gastrointestinal digestion, they are considered to be primary food allergens [21]. Furthermore, it was shown that T-cell clones specific for Pru p 3 express the gut homing factor integrin α4β7 [12], which strongly indicates that sensitization to this nsLTP has occurred in the gut.

Secondary food allergens are rapidly degraded by gastrointestinal enzymes. In intact form, they elicit symptoms due to cross-reactivity with homologous respiratory allergens to which the patient has originally been sensitized. Birch pollen-related food allergy is a very common and well-studied secondary food allergy [22, 23]. Here, sensitization occurs to Bet v 1, the major birch pollen allergen, and immunological cross-reactivity with Bet v 1-related allergens from various foods causes allergic reactions [24, 25]. In Northern Europe, fruits belonging to the Rosaceae family, especially apples, are the main cause for birch pollen-related food allergy [26–28]. Mal d 1, the Bet v 1 homolog in apple is a well-characterized secondary food allergen [29, 30] that immediately loses its IgE-binding capacity during gastrointestinal digestion [31].

Proteolytic degradation and absorption of food allergens in the gastrointestinal tract determine their allergenicity. The composition of foods may affect these processes, e.g. by preventing gastrointestinal proteolysis of allergens [32]. However, in most studies performed so far, pure allergens have been subjected to enzymatic digestion assays excluding a potential impact of a food matrix. In this study, we investigated the gastrointestinal digestion and absorption of the model food allergens Bos d 5 from cow's milk, Cor a 8 the nsLTP from hazelnut, and Mal d 1 from apple in vitro and in vivo in the presence or absence of food matrix.

2 Materials and methods

2.1 Allergens and food extracts

Recombinant Mal d 1 was produced as described previously [33]. Briefly, Mal d 1 was expressed in BL21 *E. coli* cells and purified from the soluble fraction by anion exchange chromatography using a HiTrap Q-Sepharose column (GE Healthcare, Vienna, Austria). Purified natural Bos d 5 (β-lactoglobulin A) was purchased from Sigma-Aldrich, Steinheim, Germany. Cor a 8 was expressed in yeast and purified as described previously [34].

Non-roasted hazelnuts and peanuts were purchased in a local food store and homogenized at −80°C by means of a mechanical blender. The ground hazelnuts or peanuts were stirred in double-distilled water for 1 h at room temperature. Each extract was then centrifuged for 30 min at 20 000 *xg*. The lipid content forming the top layer was collected and stored at 4°C until use. The recovered aqueous supernatant was vacuum-filtered and its protein content was determined using a BCA protein assay kit (Thermo Scientific, Rockford, USA). Subsequently, the extract was lyophilized and stored at −20°C until further use.

2.2 Simulated gastrointestinal digestion

Digestion of allergens was performed as previously described with slight modifications [35]. Briefly, gastric digestion was simulated using Enzynorm® forte, (Pharma.select Handels, Wien, Austria). One Enzynorm® tablet was dissolved in 100 mL of 0.15 M NaCl (2.5 mg/mL pepsin). To simulate intestinal digestion Pankreoflat® (Solvay Pharmaceuticals, Hannover, Germany) was used. One tablet consists of 170 mg pankreatin (400 Fédération Internationale Pharmaceutique (FIP) units proteases including trypsin, chymotrypsin, elastases, endopeptidases, exopeptidases and carboxypeptidases A and B; amylases, 5500 FIP units; and lipases, 6500 FIP units) and was dissolved in 100 mL of 0.15 M NaCl (1.7 mg/mL pancreatin). Twenty microliters of each allergen (1 mg/mL) were mixed with 10 μL of gastric solution (2.5 mg/mL pepsin; allergen:pepsin ratio 1:1.25) and the reaction mix was made up to a final volume of 300 μL with 0.15 M NaCl. The pH was set to 1.5 and digestion was carried out for 2 h at 37°C under gentle agitation. To simulate intestinal digestion, 20 μL of each allergen (1 mg/mL) were incubated with 60 μL of pancreatic solution (1.6 mg/mL pancreatin; allergen: pancreatin ratio 1:5), at pH 7 for 45 min at 37°C and gentle agitation. Vieths et al. have used an allergen:pepsin ratio of 1:1.25 and an allergen:pancreatin ratio of 1.5:1 [35]. Aqueous hazelnut or peanut extract (1980 or 380 μg protein) was added to make up 99 or 95% of total protein, respectively. To assess the effect of lipids, 60% v/v hazelnut or peanut oil harvested during extract production were added and incubated with the allergens overnight at room temperature under heavy agitation prior to the addition of enzymes. Samples were taken after 5, 15, 30, 60 and 120 min for simulated gastric digestion and after 5, 10, 15, 30 and 45 min for simulated intestinal digestion and immediately mixed with non-reducing SDS-containing sample buffer and heated to 95°C to stop the enzymatic reaction.

2.3 IgE immunoblot

Samples were run on a 15% SDS-PAGE gel and blotted onto a nitrocellulose membrane. IgE-binding of allergens was analyzed by incubation of blots with patients' sera containing allergen-specific IgE. For each allergen, sera from two different patients with clinical reactions, positive skin prick tests and ImmunoCAP to the respective food as well as positive IgE levels to the food allergens as determined by ELISA were used. Mal d 1^+ patients were negative for Cor a

1, the homolog in hazelnut. Incubation of blots with patients' sera was followed by incubation with I^{125}-goat anti-human IgE (Demeditec, Kiel, Germany) and autoradiography.

2.4 Allergen transport through a CaCo-2 monolayer

Bos d 5 and Cor a 8 were labeled with FITC and Mal d 1 with Rhodamine B (Sigma-Aldrich) to allow the analysis of concomitant transport. Briefly, 1 mL of each allergen (each at 2 mg/mL in sodiumcarbonate buffer, pH 9) was mixed with 0.5 mL of either FITC or Rhodamine B (1 mg/mL in DMSO) and incubated in the dark for 8 h at 4°C. Unbound dye was removed by dialysis.

CaCo-2 cells were grown on permeable polycarbonate filter trans-well inserts (3 μm pore size, Becton-Dickinson, Erembodagam, Belgium). Each filter chamber was placed inside a 24-well plate (Becton-Dickinson). Cells were seeded at a density of 5×10^5 cm^2 and provided with fresh medium every 48 h. After 14–20 days of culture, integrity and functionality of the monolayer were monitored by trans-epithelial electrical resistance (TEER) evaluation and phenol-red absorption [36]. TEER was measured using EndOhm-6 tissue resistance measurement chambers (WPI, Berlin, Germany). Transport studies were performed in duplicates when TEER measurements were $> 300\,\Omega \cdot \text{cm}^2$. Culture medium was aspirated from apical and basolateral chambers and both cell surfaces were washed three times with PBS. Phenol-red free cell medium (0.9 mL) was added to the basolateral chamber and fluorescent-labeled allergens (25 μg/well found to be optimal in preliminary experiments) with or without hazelnut extract (2475 μg protein/well) were added to the apical chamber. Medium alone served as negative control. At indicated time points, basolateral samples were collected and stored at −20°C until further use. Thereafter, the integrity of the monolayer was again tested by TEER measurements and phenol-red absorption to exclude that proteases in the food matrix had effects on the cell layer [36]. The concentration of FITC-Bos d 5, FITC-Cor a 8 and Rhodamine-Mal d 1 was determined by measuring fluorescence intensity with a spectral fluorometer (Infinite 200 Pro, Tecan, Switzerland) at the wavelengths 490 nm (excitation) and 530 nm (emission) for FITC and at 530 and 590 nm for Rhodamine B, respectively. A standard curve was generated to quantify the amounts of allergens transported through the monolayer. All readings were done in duplicate. IgE-reactivity of translocalized allergens was confirmed by inhibition ELISA (data not shown).

2.5 In vivo absorption of food allergens

Twelve male 12-wk old Sprague-Dawely rats (250–300 g) were obtained from Besondere Einrichtungen für Biomedizinische Forschung, Himberg, Austria and kept in a temperature-regulated environment under a controlled 12 h light/dark cycle with free access to water and without food for 24 h. The animals were divided into four animals per group. Group A was fed with 2 mL of 0.15 M NaCl containing a mixture of Mal d 1 (10 mg), Bos d 5 (10 mg) and Cor a 8 (4 mg) administered by gavage. Group B received 2 mL of 0.15 M NaCl containing a mixture of Mal d 1 (10 mg), Bos d 5 (10 mg) and soluble hazelnut extract (1 g). Group C was fed with 2 mL of 0.15 M NaCl as control. Animals were sacrificed at indicated time points (2, 4, 6 and 8 h). Three microliters of stomach content were spotted onto pH indicator paper (Acilit pH 0.5-5.0, pH-Box, Merck, Darmstadt, Germany) to determine gastric pH values. Sera were obtained and stored at −20°C until further use. All experiments were approved by the Animal Experimentation Ethics Committee of the Medical University of Vienna and the Ministry of Science and Research (GZ 66.009/0150-II/10b/2008).

2.6 Inhibition ELISA

Microtiter plates (Maxisorp, Nunc, Denmark) were coated with allergens (1 μg/mL) in carbonate buffer (pH 9.6) overnight at 4°C. Patient sera (10 μL) were pre-incubated with rat sera (90 μL) overnight at 4°C. Allergen-coated plates were washed twice and saturated with 1% BSA in PBS/0.05% Tween 20 for 2 h at room temperature. Subsequently, pre-absorbed patient sera were transferred and incubated overnight at 4°C. After five washing steps, bound IgE was detected using an alkaline-phosphatase-conjugated mouse anti-human IgE (BD Biosciences Pharmingen, San Diego, CA). Furthermore, 4-nitrophenyl phosphate salt hexahydrate was used as substrate (Sigma-Aldrich). For quantification, a mixture of Bos d 5, Mal d 1 and Cor a 8 was spiked into control rat serum (Group C) at known concentrations to generate a standard curve.

3 Results

3.1 Simulated gastrointestinal digestion in the presence of food matrix

Bos d 5, Cor a 8 and Mal d 1 were incubated with gastric or pancreatic enzymes, in the absence or presence of either aqueous hazelnut or peanut extract and their IgE-reactivity was determined by means of immunoblotting. Bos d 5 remained intact to gastric enzymes for 120 min (Fig. 1A). After 60 min, an additional IgE-binding fragment of approximately 16 kDa was detected. This fragment was not visible when 95 and 99% hazelnut extract had been added to the allergen. After 10 min of simulated intestinal digestion, Bos d 5 was degraded beyond level of detection (Fig. 1B). However, 95 and 99% hazelnut extract prolonged its detection by IgE antibodies to 120 min. Cor a 8 remained intact throughout the tested time period of simulated gastric or intestinal digestion (Fig. 1A). Of note, an IgE-binding

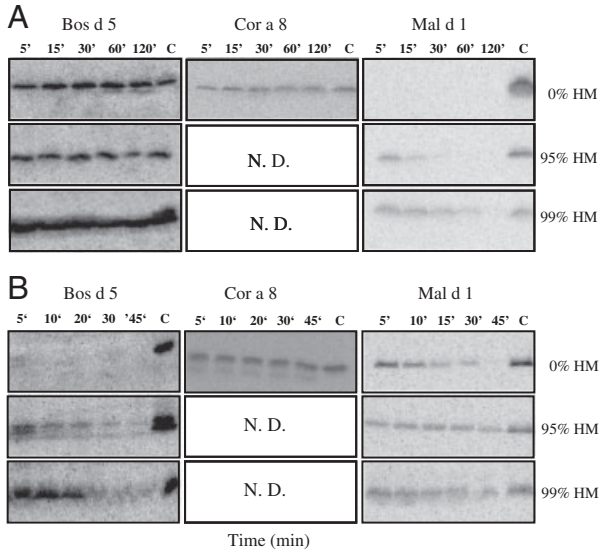

Figure 1. IgE-reactivity of allergens after simulated gastrointestinal digestion in the presence or absence of a food matrix. Bos d 5, Cor a 8 and Mal d 1 were incubated with (A) gastric enzymes for 5–120 min and (B) intestinal enzymes for 5–45 min and IgE-binding was determined by immunoblot. (C) Undigested control; HM; hazelnut matrix; N.D; not determined. One representative experiment is shown.

fragment of about 6 kDa appeared after 5 min of intestinal proteolysis and remained detectable for up to 45 min (Fig. 1B). Since Cor a 8 was resistant to gastrointestinal proteolysis and is contained in hazelnut extract, no digestion experiments in the presence of a hazelnut matrix were performed. Mal d 1 was immediately degraded under gastric conditions but remained intact for up to 30 min under intestinal conditions (Fig. 1A). Addition of 95% hazelnut extract prolonged its detection to 15 min and the presence of 99% hazelnut extract for up to 60 min. Simulated gastric and intestinal digestion of Bos d 5 and Mal d 1 in the presence of 95 and 99% peanut extract, respectively, revealed a similar delay of enzymatic proteolysis as observed with hazelnut extract (data not shown). IgE antibodies from patients sensitized to Bos d 5 and Mal d 1 did not show any reactivity to hazelnut or peanut extract alone (data not shown). The addition of 60% hazelnut or peanut lipids, respectively, did not affect the degradation of Bos d 5 and Mal d 1 (data not shown).

3.2 Epithelial transport of allergens in the presence of food matrix

FITC-labeled Bos d 5 and Cor a 8 and Rhodamine-labeled Mal d 1 with or without 99% aqueous hazelnut extract were loaded onto a CaCo-2 cell monolayer. After 2, 4, 6 and 8 h, the basolateral side was checked for the presence of transported allergens. A continuous increase of all three allergens was detected (Fig. 2). In the absence of hazelnut extract, 2.9 μg of Bos d 5 (11.6% of the loaded amount), 1.5 μg of Cor a 8 (6.3%) and 2.0 μg of Mal d 1 (8%) were detected in the basolateral chamber after 8 h. In the presence of 99% hazelnut extract 1.7 μg of Bos d 5 (6.8%), 0.56 μg of Cor a 8 (2.3%) and 0.92 μg of Mal d 1 (3.7%) had passed through the cell layer after 8 h. Summarizing the transport data of all three allergens revealed statistically significant differences between the absence and presence of food matrix at each time point (Wilcoxon Signed Ranks test, $p < 0.05$). Thus, the presence of food matrix significantly hampered the transepithelial transport of all allergens in vitro.

3.3 Gastric pH after in vivo digestion

Rats were fed with a cocktail of all allergens with or without 99% aqueous hazelnut extract by means of a stomach tube. After 2, 4, 6 and 8 h, animals were sacrificed and the gastric pH was assessed. Two hours after food administration, the pH within the stomach was raised to a value of 5 (Fig. 3). Thereafter, the pH started to decline over time. However, a gastric pH of 1.5 was not reached until 6 h after feeding. No difference in pH values between the presence or absence of a food matrix was found.

3.4 Detection of in vivo absorbed IgE-reactive allergens

IgE-binding allergen and allergen fragments in rat sera collected 2, 4, 6 and 8 h after allergen feeding with or without 99% hazelnut matrix were assessed by their capacity to inhibit binding of patients' IgE to Bos d 5, Cor a 8 and Mal d 1. In sera from animals fed without-food matrix levels of all three model allergens peaked 2 h after feeding. Thereafter, detectable allergens decreased (Fig. 4). The serum levels of allergens were different in animals fed with food matrix. Two hours after feeding, only low amounts of all three allergens were found. Thereafter, the levels of Bos d 5 and Cor a 8 remained relatively stable whereas Mal d 1 levels continuously increased over the investigated time period.

4 Discussion

We sought to study the in vivo absorption of primary and secondary animal and plant food allergens in the absence or presence of a food matrix. Three model allergens with different susceptibilities to digestion enzymes were employed (Fig. 1): Mal d 1, which is immediately degraded in the simulated gastric fluid but shows more stable properties in the simulated intestinal environment [31]; Bos d 5, which is resistant to simulated gastric digestion but is readily degraded by intestinal enzymes [37] and Cor a 8,

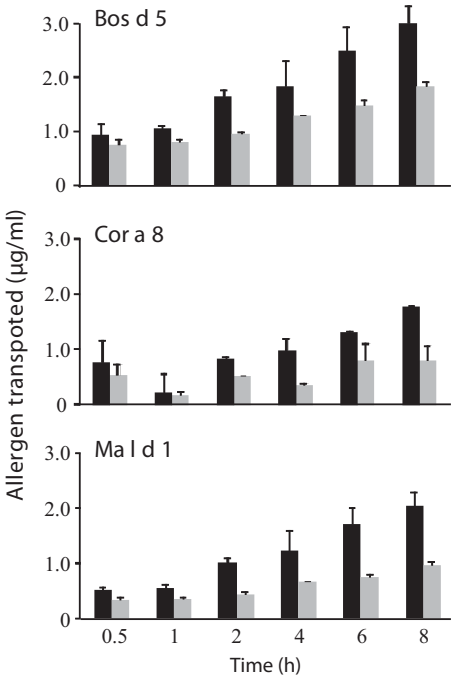

Figure 2. Epithelial transport of allergens. The amount of FITC-Bos d 5, FITC-Cor a 8 and Rhodamine-Mal d 1 with (grey bars) and without (black bars) hazelnut extract transported through CaCo-2 cell monolayers was fluorometrically assessed. Mean values of duplicates and standard deviations are shown. One of two experiments is shown.

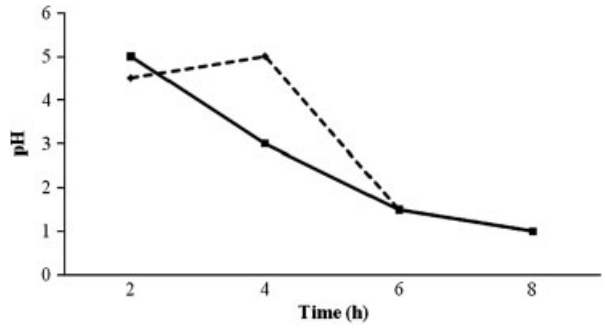

Figure 3. pH values in stomach after food ingestion. Rats were fed with allergens with (solid line) and without (dotted line) 99% hazelnut extract, sacrificed and the pH was determined at indicated time points.

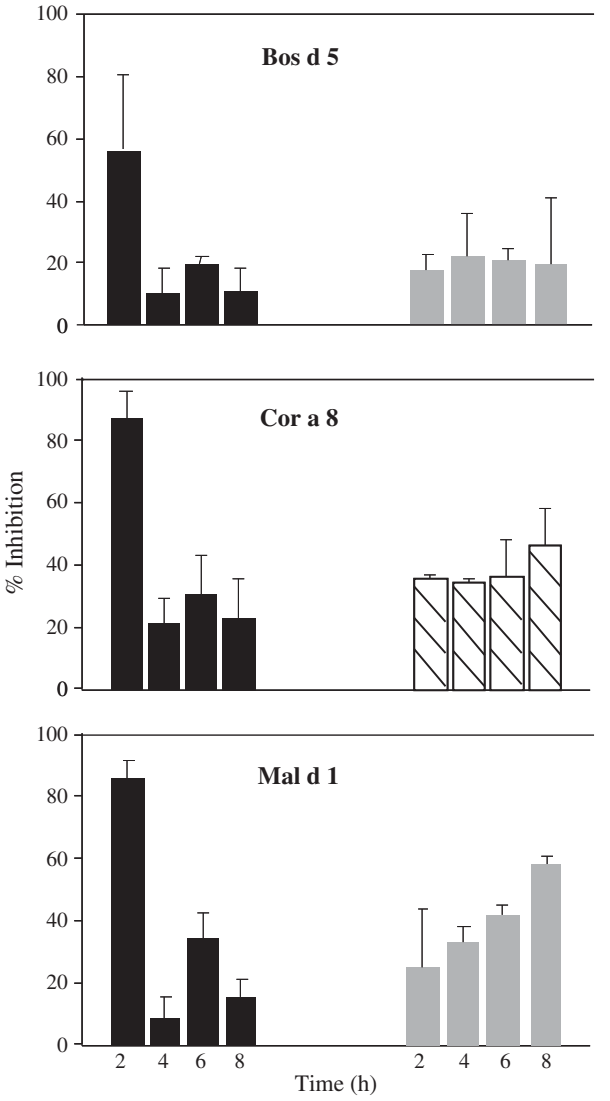

Figure 4. In vivo absorption of IgE-reactive food allergens. Sera from allergic donors containing allergen-specific IgE were pre-incubated with sera from rats fed recombinant allergens with (grey bars) or without (black bars) food matrix. Animals fed with food matrix were not administered recombinant but natural Cor a 8 present in the hazelnut extract (hatched bars). Inhibition of IgE binding to Bos d 5, Cor a 8 and Mal d 1 was assessed. Mean values and standard deviations of sera from two different patients are shown.

which we found to be resistant to gastric and intestinal enzymes as described previously for other nsLTPs [21, 38]. We chose hazelnuts and peanuts as model matrices because their nutritional composition is rich in protein (15 and 25%, respectively), lipids (60 and 48%, respectively) and carbohydrates (16.5 and 15%, respectively) [39]. Furthermore, both foods contain clinically relevant food allergens. Employing these model constituents, we found a strong influence of a food matrix on the in vivo absorption of food allergens. In sera from rats fed allergens without hazelnut extract, maximum concentrations of all three allergens were detected 2 h after feeding (Fig. 4) corresponding to 0.56 µg of Bos d 5, 0.4 µg of Cor a 8 and 0.57 µg of Mal d 1/mL. These levels declined over time. In contrast, in animals fed allergens with hazelnut extract, the serum levels of allergens were low after 2 h but increased continuously, reaching 0.2 µg/mL of Bos d 5, 0.15 µg/mL of Cor a 8 and 0.45 µg/mL of Mal d 1 after 8 h. Estimating the blood volume of a rat to be 20 mL, approximately 0.04–0.09% of food allergens that had been administered in a matrix were absorbed after 8 h.

In vitro experiments revealed that a matrix affects both, gastric and intestinal proteolysis as well as epithelial transport of food allergens. The presence of a food matrix derived from hazelnut extract prevented simulated gastric and intestinal digestion of Mal d 1 and Bos d 5 (Fig. 1). Based on the observations that a high protein content of the food matrix enhanced the stability against simulated gastrointestinal degradation, we consider carbohydrates and proteins to be mainly responsible for the protection of food allergens. Polysaccharides were shown to hamper pepsin activity [40–42]. In addition, we speculate that large amounts of proteins in a matrix create a competitive environment for enzyme cleavage, thereby delaying gastrointestinal proteolysis of food allergens. In a similar way, food proteins may compete with allergens for active epithelial transport as reflected by the strongly reduced transport rates of all three model allergens through CaCo-2 cell monolayers in the presence of 99% hazelnut extract (Fig. 2).

Like Mal d 1, the Bet v 1-related allergen in hazelnut, Cor a 1, is considered to be a secondary food allergen because in pure form it is rapidly degraded by gastric enzymes and looses its IgE-binding ability within a few seconds [31]. However, we previously found evidence for a potential sensitizing capacity of Cor a 1 based on the identification of T-cell clones that did not cross-react with Bet v 1 [43]. Moreover, it has been reported that young children display IgE antibodies specific for Cor a 1 without prior respiratory sensitization to the major birch pollen allergen [44]. These findings tempted us to investigate whether the natural matrix embedding Cor a 1 may contribute to its sensitizing capacity. We determined that hazelnut extract contains less than 1% of Cor a 1 (data not shown). Feeding a similar ratio of hazelnut extract and the Cor a 1-homolog Mal d 1 resulted in continuously increasing serum levels of the apple allergen (Fig. 4) suggesting its protection from gastric proteolysis and consecutive intestinal absorption of intact protein over a time period of 8 h in vivo. Similar to Mal d 1 (Fig. 1), Cor a 1 is relatively resistant to degradation by trypsin [31]. Therefore, we conclude that a food matrix rich in carbohydrates and proteins together with the naturally high pH in the stomach of children [44] contributes to a sensitizing capacity of the major hazelnut allergen independently from Bet v 1.

The finding of relatively high in vivo levels of IgE-reactive Mal d 1 detected 2 h after feeding without food matrix was unexpected (Fig. 4). We refer this finding to the protection of Mal d 1 from gastric digestion due to the increased pH (Fig. 3) rendering pepsin inactive [45] combined with the lack of a matrix preventing its intestinal absorption. Consequently, Mal d 1, generally considered to induce only mild, local symptoms such as oral allergy syndrome [26], could trigger more severe reactions. Indeed, systemic reactions of birch pollen-allergic patients after consuming fruit and vegetable smoothies freshly prepared from apple and/or carrot occur occasionally (C. Ebner, personal communication). Usually, such smoothies are drunk very rapidly at so-called bio-bars. The combination of an empty stomach, the rapid rise of the gastric pH and the low protein content in both foods (0.3 and 1.0% of protein in apple and carrot, respectively [39]) may enable the absorption of large amounts of IgE-reactive Bet v 1-related food allergens causing systemic anaphylaxis. This cascade of events may also explain the severe anaphylactic reactions observed in birch pollen-allergic patients after ingestion of soy-based foods, in particular soy milk, containing the Bet v 1-homolog Gly m 4 [46, 47].

In summary, a food matrix has a strong impact on gastrointestinal degradation and uptake of food allergens and thereby may have negative and positive consequences for susceptible individuals. We conclude that matrices rich in proteins and carbohydrates may under certain circumstances endow secondary food allergens with sensitizing capacity. On the other hand, the presence of such matrices may occasionally also attenuate the severity of food-induced allergic reactions. Thus, the respective food matrix should be considered when the allergenicity of food allergens is determined.

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