RESEARCH ARTICLE

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Digestion and Transport across the Intestinal Epithelium Affects the Allergenicity of Ara h 1 and 3 but Not of Ara h 2 and 6

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Scope: No accepted and validated methods are currently available which can accurately predict protein allergenicity. In this study, the role of digestion and transport on protein allergenicity is investigated.
Methods and results: Peanut allergens (Ara h 1, 2, 3, and 6) and a milk allergen (β-lactoglobulin) are transported across pig intestinal epithelium using the InTESTine model and afterward basophil activation is measured to assess the (remaining) functional properties. Additionally, allergens are digested by pepsin prior to epithelial transport and their allergenicity is assessed in a human mast cell activation assay. Remarkably, transported Ara h 1 and 3 are not able to activate basophils, in contrast to Ara h 2 and 6. Digestion prior to transport results in a significant increase in mast cell activation of Ara h 1 and 3 dependent on the length of digestion time. Activation of mast cells by Ara h 2 and 6 is unaffected by digestion prior to transport.
Conclusions: Digestion and transport influences the allergenicity of Ara h 1 and 3, but not of Ara h 2 and 6. The influence of digestion and transport on protein allergenicity may explain why current in vitro assays are not predictive for allergenicity.

1. Introduction

The introduction of novel foods should not add to the burden of food allergy and accurate allergenicity assessment is therefore needed. However, there are currently no regulatory accepted and validated methods available which can accurately evaluate and predict protein allergenicity.[1] A possible reason for this could be the use of purified or recombinant proteins which are not subjected to physiological processes (e.g., processing, digestion, and epithelial transport) to which food normally is exposed to upon consumption. This can hamper the interpretation and development of a predictive in vitro assay for allergenicity assessment. Current method development could therefore potentially be improved by adapting to more physiologically relevant conditions.[2,3]

An European COST action (ImpARAS) (www.imparas.eu) was established to evaluate and improve current allergenicity risk assessment strategies.[4] The ImpARAS group developed an adverse outcome pathway (AOP) for food sensitization (http://www.saaop.org/).[5] This AOP describes the molecular initiating events (MIE) and key events (KE) that are associated with the development of food-allergic sensitization, which are interesting starting points for the development or improvement of predictive in vitro assays for allergenicity assessment of novel food proteins. It was advised that a first evaluation project should focus on methods addressing

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MIE and KE involving intestinal uptake and epithelial cell activation.

In a healthy individual, proteins are mainly transported by means of endocytosis via the enterocytes and microfold (M) cells. Transport via the M cells leaves a larger part of the proteins intact, while transport via enterocytes exposes 90% of the internalized protein to intracellular degradation in lysosomes.[6] M cells contain fewer lysosomes, and have a thinner glycocalyx layer which results in less protein degradation.[7] After intracellular degradation, the resulting fragments can still be allergenic if their size is at least 3.5 kDa.[8] Besides lysosomal degradation, proteins are also subjected to gastro-intestinal degradation. The effects of digestion on protein stability, structure, and allergenicity are well-characterized and reviewed in recent publications by the European Food Safety Authority.[9,10] However, the combined role of digestion and protein transport across the intestinal wall has not been investigated. Diagnostic tests and in vitro allergy models might be improved by more knowledge on this role, because current in vitro tests mainly use purified, native, or recombinant proteins for allergenicity assessment and allergenicity prediction. The effect of digestion and protein transport could be an explanation for the current situation in which in vitro methods fail to accurately predict the allergenicity of proteins because the physiological conditions in which the allergen is encountered are ignored.[11,12]

In this study, the effect of digestion and transport across pig intestinal epithelium of peanut allergens (Ara h 1, 2, 3, and 6) on allergenicity was investigated. The different peanut allergens were chosen because of their diverse physicochemical properties such as size, structure, and stability in a pepsin resistance test. Allergenicity was assessed in basophil and mast cell activation assays using serum from peanut-allergic patients.

2. Experimental Section

2.1. Protein Isolation and Radioactive Labeling Using [^{14}C]-Formaldehyde

Isolation of Ara h 1, 2, 3, and 6 has been described previously.[13,14] β-lactoglobulin was isolated by dissolving 40 g whey protein isolate (Bipro, Davisco) in 4 L 20 mM Tris-HCl, pH 8.0, and loaded on a 3.6 L Source Q column. β-lactoglobulin containing fractions were pooled and desalted in Milli-Q water using a 7200 mL Sephadex G25 column. Proteins were labeled with [^{14}C]-formaldehyde (ARC USA) in a 1:20:20 protein:sodium cyanoborohydride:[^{14}C]-formaldehyde ratio according to the reductive methylation method as described in Habeeb et al.[15]. Habeeb et al. showed that radiolabeling by reductive methylation did not influence the molecular weight, net charge, and enzymatic activity of lysozyme. Furthermore, Wallace has shown that ^{14}C-labeled bovine serum albumin and casein proteins have similar rates of digestion as their native counterparts.[16] Labeling by the reductive methylation method was therefore chosen. Ara h 2, 3, 6 and β-lactoglobulin (10 mg mL^{-1}) were prepared in 0.16 M phosphate buffer (pH 8.0); Ara h 1 was prepared in 0.1 M phosphate buffer. The protein solutions (0.2 µmol) were pipetted into glass tubes and sodium cyanoborohydride (4.0 µmol) and [^{14}C]-formaldehyde (4.0 µmol) was added and incubated for 30 min at room temperature. One milliliter of 0.05 M borate buffer (pH 8.0) was added and to stop the reaction. The solutions were transferred to a dialysis membrane (Spectra/Por dialysis membrane MWCO 3500). The radiolabeled proteins were dialyzed against ice-cold 0.05 M borate buffer containing 0.02% sodium azide (pH 7.0) for 2 days until less than 1% of the radioactivity (% of dose) was present in the dialysis fluid. The purity of the protein solutions was checked by HPLC analysis using a Vydac 218TP54 protein and peptide column coupled to a β-RAM detector and UV-detector. The organic solvent was evaporated in an evaporator (TurboVap LV) and the remaining aqueous solution was analyzed for radioactivity by liquid scintillation counting (LSC).

2.2. Intestinal Tissue Isolation and Mounting of Tissue in the InTESTine System

Residual porcine intestinal tissue was made available by Gemeenschappelijk Dierenlaboratorium, part of the Utrecht University. For the studies described here, pigs of 4–7 months old and between 55 and 146 kg were used. Preparation and mounting of the intestine tissue segments has been described previously.[17,18] The protocol for this study was approved by the Animal Ethics Committee Utrecht (Ethics Committee Permit Number 2013.III.01.005, Utrecht, The Netherlands).

2.3. Tissue Integrity and Functionality Markers

FITC Dextran 4 (50 µM FD4, Sigma Aldrich) was used as a marker for tissue integrity. The fluorescence of FD4 was measured using a multi-mode microplate reader (Synergy HT, Biotek using Gen 5 software) (excitation: 490 nm, emission: 520 nm). The functionality of the intestinal tissue was assessed by determining the caffeine/mannitol apparent permeability coefficient (P_{app}) ratio, which was markers for paracellular and transcellular transport, respectively. 10 µM was added to each incubation for both. The P_{app} caffeine/P_{app} mannitol ratio (>3) and the FD4 leakage (≤0.5%) results validated the InTESTine experiments and demonstrated an intact and functional intestinal barrier.

2.4. InTESTine Incubations

Incubation with Ara h 1, 2, 3, 6, and β-lactoglobulin (1–3 mg mL^{-1}) was conducted on a shaker platform (≈60 RPM) in a humidified incubator at 37 °C with 90% O_2 and 5% CO_2. Incubations of Ara h 1, 2, 3, 6, and β-lactoglobulin were performed in triplicate and later pooled to generate one sample per protein condition for evaluation in the activation assays. For radioactive analysis, aliquots from both the apical (1 mL) and basolateral compartments (7.5 mL) were taken after 45 and 105 min of incubation in scintillation vials and analyzed by LSC. At t = 105 min, the remaining apical (dosing) solution and 4 mL of the basolateral solution was transferred to collection tubes which contained 1X protease inhibitor (Halt Protease Inhibitor Cocktail 100X, Thermo Fisher Scientific). The protein transport was expressed as percentage of dose and calculated by dividing the radioactivity of the basolateral solution by the apical solution (dose), multiplied by 100%.

Table 1. Clinical characteristics of the food challenge confirmed peanut-allergic patients.

Patient number	Sex	Age	SPT peanut [mm]	CAP [kU/L]				
				Total	Peanut	Ara h 1	Ara h 2	Ara h 3	Ara h 6
1	F	39	14.0	502	>100	69.1	70.5	52.5	31.8
2	M	33	12.0	641	>100	99.2	>100	22.3	51.6
3	F	37	8.5	3592	47.4	0.1	32.6	0.1	24.1
4	M	49	10.0	2749	>100	>100	>100	78.5	>100

2.5. Radioactivity Measurements by Liquid Scintillation Counting

In order to determine the amount of radioactivity present in the samples, 10 mL (per sample) of liquid scintillant (Ultima Gold, PerkinElmer) was added to all samples followed by vigorous mixing. Radioactivity was determined by LSC on a Tri-Carb 3100TR liquid scintillation counter using QuantaSmart software in which all counts were converted to disintegrations per minute using transformed Spectral Index of external standards coupled to Automatic Efficiency Correction.

2.6. Patient Selection

Serum derived from food challenge confirmed peanut-allergic patients ($n = 4$) who visited the Allergology Outpatient Clinic at the University Medical Center Utrecht was used in the immunoglobulin E (IgE) immunoblotting, indirect basophil activation test (in-BAT), and in the human mast cell (hMC) activation assay. Peanut specific IgE was measured by ImmunoCAP (ThermoFisher, Uppsala, Sweden). Patients also underwent skin prick testing with peanut extract. The clinical characteristics per patient are reported in **Table 1**. The study was approved by the local ethics committee (18-428).

2.7. Indirect Basophil Activation Test

The in-BAT was performed as described by Koppelman et al.[13]. In brief, IgE antibodies were stripped from the basophil membrane by incubation with lactic acid (pH 3.9) for 2 min 15% v/v serum from peanut-allergic subjects supplemented with 10 µg mL^{-1} heparin and 4 mM EDTA was added to the peripheral blood mononuclear cells and incubated for 60 min. Cells were stimulated for 30 min with the pooled samples of Ara h 1, 2, 3, 6, and β-lactoglobulin retrieved from the apical and basolateral compartments from the InTESTine studies. A fourfold serial dilution which ranged from 1:10^3 to 1:10^6 for the apical samples and a fivefold serial dilution which ranged from 1:3 to 1:10^3 for the basolateral samples was used to stimulate the cells. A single sample per dilution of the dilution series was incubated. Degranulation was quantified by flow cytometry and was expressed as percentage CD63$^+$ cells using a BD FACS Canto II (BD Biosciences) equipped with a High Throughput Sampler. The BD FACSDIVA software was used for data acquisition and the data analysis was performed with FLOWJO, LLC.

2.8. Digestion of Proteins by Pepsin

18 mg of protein was dissolved in 7.5 mL 20 mM Tris-HCl and 150 mM NaCl (pH 7.2). 1.5 mL of saliva (pool) was added and the solution was incubated in a water bath at 37 °C for 5 min. Subsequently, 6 mL of digestion buffer (46 mM citric acid and 0.76 mM Na_2HCO_3, pH 2.5) was added and incubated for another 5 min at 37 °C. 20 µL of pepsin (pepsin from porcine gastric mucosa, ≥250 units per mg, Sigma-Aldrich) was added to obtain a concentration of 90 000 U mL^{-1}. The ratio of pepsin:substrate was based on prior research.[19,20] After 0, 15, and 30 s and after 1, 5, 10, 30, and 60 min of digestion, a 6.5 mL aliquot was transferred to a tube containing 1.44 mL of stopping buffer (1 M Tris-HCl, pH 11.0). Reactivity of selected digests was evaluated using the aforementioned in-BAT technique.

2.9. SDS-PAGE Analysis and IgE Immunoblotting

The digested peanut proteins were evaluated by SDS-PAGE using a 15% acrylamide/Tris-HCl gel (Criterion, Biorad, Germany). The samples were diluted with 5X sample buffer containing 250 mM Tris-HCl pH 6.8, 10% SDS, 50% glycerol, 10% β-mercaptoethanol. Digested samples (15 µL) were loaded onto the gel and the proteins in the gel were visualized using InstantBlue (Coomassie) gel staining (Expedion, UK) or the gels were used for IgE immunoblotting. The proteins were transferred from the gel to a polyvinylidene difluoride membrane (immune-Blot PVDF membrane sandwiches, Bio-Rad). The membrane was blocked with 4% w/v Protifar (Nutricia, Cuijk, The Netherlands) and 0.1% Tween 20 in PBS for 60 min. Peanut-allergic serum was diluted 400-fold in PBS containing 1% Protifar and 0.1% Tween 20, and the blots were incubated for 1 h at room temperature. After washing three times with wash buffer (0.1% Tween 20 in PBS), the membrane was incubated with peroxidase-conjugated goat-anti-human IgE (1 mg mL^{-1}, 1:30 000 diluted, KPL, Maryland, USA) in PBS containing 1% protifar and 0.1% Tween 20 for 1 h at room temperature. After washing, the blots were developed using a chemiluminescent peroxidase substrate kit and the data was collected using a Chemidoc XRS+ image scanner with Imagelab software (Bio-Rad).

2.10. Human Mast Cell Generation from Peripheral Blood

CD34$^+$ progenitor stem cells were isolated from buffy coats (Sanquin Blood Bank, The Netherlands) to generate hMCs which

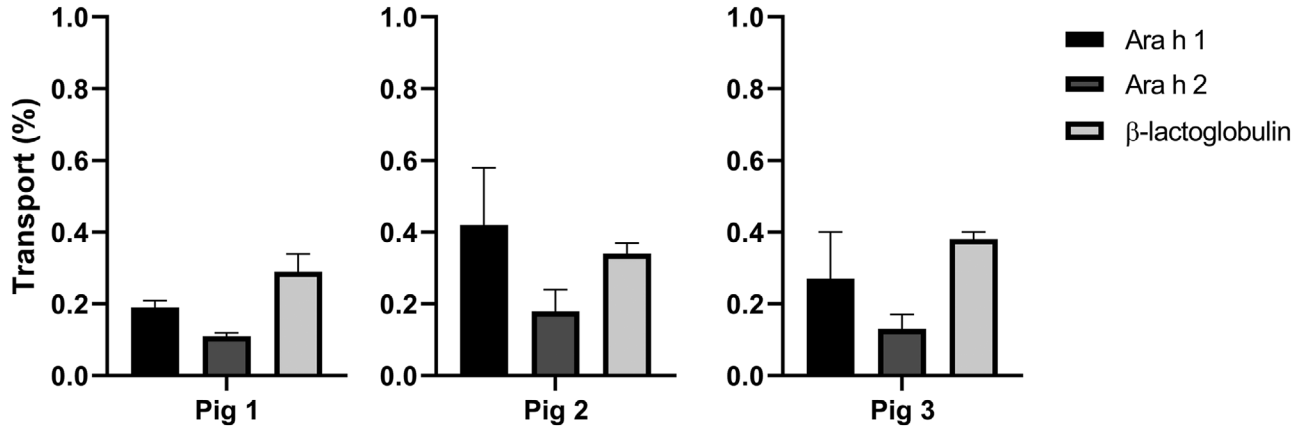

Figure 1. Percentage of Ara h 1, Ara h 2, and β-lactoglobulin transport across the intestinal epithelium of three pigs. Per allergen the data is presented as mean percentage ($n = 3$) of transport ± SD.

has been described previously.[21,22] Stem cells were isolated according to the manufacturer's protocol (EasySep, Stemcell technologies). Stem cells were cultured in StemSpan medium (StemCell technologies) supplemented with 10 µg mL^{-1} ciprofloxacin (Sigma-Aldrich, St Louis, MO), human IL-6 (50 ng mL^{-1}), human IL-3 (10 ng mL^{-1}), and human stem cell factor (100 ng mL^{-1}) (Peprotech, Rocky Hill, NJ) in a humidified incubator (37 °C, 5% CO_2). After 30 days, the cells were progressively transferred to culture medium consisting of Iscove's modified Dulbeccos medium with GlutaMAX-I, 50 µM 2-mercaptoethanol (Life Technologies, Carlsbad, CA), 0.5% AlbuMAX (Gibco), 1% insulin-transferrin-selenium (Life Technologies, Carlsbad, CA), 10 µg mL^{-1} ciprofloxacin, 50 ng mL^{-1} human IL-6, and 3% supernatant of Chinese hamster ovary transfectants secreting murine stem cell factor. Maturation of hMCs was confirmed by the presence of CD117 and FcεRIa on the cell membrane.

2.11. hMC Activation Assay

For the hMC activation assay, digested samples from the following time points were used: 0 and 30 s, and 60 min for Ara h 1 and 3 and 0, 5, and 60 min for Ara h 2 and 6. hMCs were sensitized overnight with 5% v/v serum from peanut-allergic patients in a humidified incubator. The cells were washed twice with RPMI 1640 medium supplemented with 2% fetal bovine serum (HyClone, GE Healthcare) and centrifuged for 5 min at 350 x g. After that, cells were incubated in a humidified incubator for 90 min with the digested and transported peanut allergens (Ara h 1, 2, 3, and 6) pooled from three intestinal segments per pig at a dilution of 1:1, 1:2.5, 1:5, 1:10, 1:10^2, and 1:10^3 in duplicate. Rabbit anti-human IgE (6 µg mL^{-1}; DAKO) and purified Ara h 1, 2, 3, and 6 were used as positive controls. After incubation, 5 µL 10% Triton X-100 was added and cells were centrifuged for 5 min at 400 x g. 50 µL of the supernatant was transferred to a 96-wells plate and 50 µL of β-hexosaminidase substrate (100 µL of 10 mM 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide diluted in 5 mL 0.1 M citric acid [pH 4.5]) was added and incubated for 1 h in a humidified incubator. The reaction was stopped with 100 µL 0.1 M glycine buffer (pH 10.7). Fluorescence of the 4-MUG substrate cleavage by β-hexosaminidase was measured (excitation 360 nm, emission 452 nm) using a Fluoroskan Ascent FL (Labsystems) fluorometer. Degranulation was reported as percentage release compared to anti-IgE corrected for the medium control.

2.12. Data Analysis and Statistics

Descriptive statistics were performed to report the mean values and standard deviations of the percentage of transport using radiolabeled proteins, the percentage CD63$^+$, and the percentage β-hexosaminidase release. The mean percentage of transport of Ara h 1 and 2, and β-lactoglobulin in three pigs was investigated by the Kruskal–Wallis test followed by Dunn's multiple comparison test. The mean of the dilution series in the in-BAT and the hMC activation assays were calculated based on the five (in-BAT) or six (hMC activation assay) dilution points for each unique combination of patient serum and pig used. Differences between in the reactivity of the digestion times in the hMC activation assay were then investigated by the Kruskal–Wallis test followed by Dunn's multiple comparison test. Differences in the CD63 expression between apical and basolateral stimulation with Ara h 1, 2, 3, and 6 were investigated using the Mann–Whitney *U* test. A *p*-value of <0.05 was considered statistically significant. Statistical analysis was performed and graphs were drawn using GraphPad Prism 8.3 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Transport of Ara h 1, Ara h 2, and β-Lactoglobulin across the Intestinal Epithelium Is Comparable

Transport of Ara h 1, Ara h 2, and β-lactoglobulin (control) across the intestinal epithelium of pigs was quantified using radioactive labeled proteins and the InTESTine model. The results are expressed as percentage transport and presented in **Figure 1**. Approximately 0.1–0.4% of the protein dose applied on the apical side was transported without significant differences between the mean amount of transported radioactively labeled Ara h 1, Ara h 2, and β-lactoglobulin or fragments thereof ($p > 0.05$). The results from the three pigs were comparable.

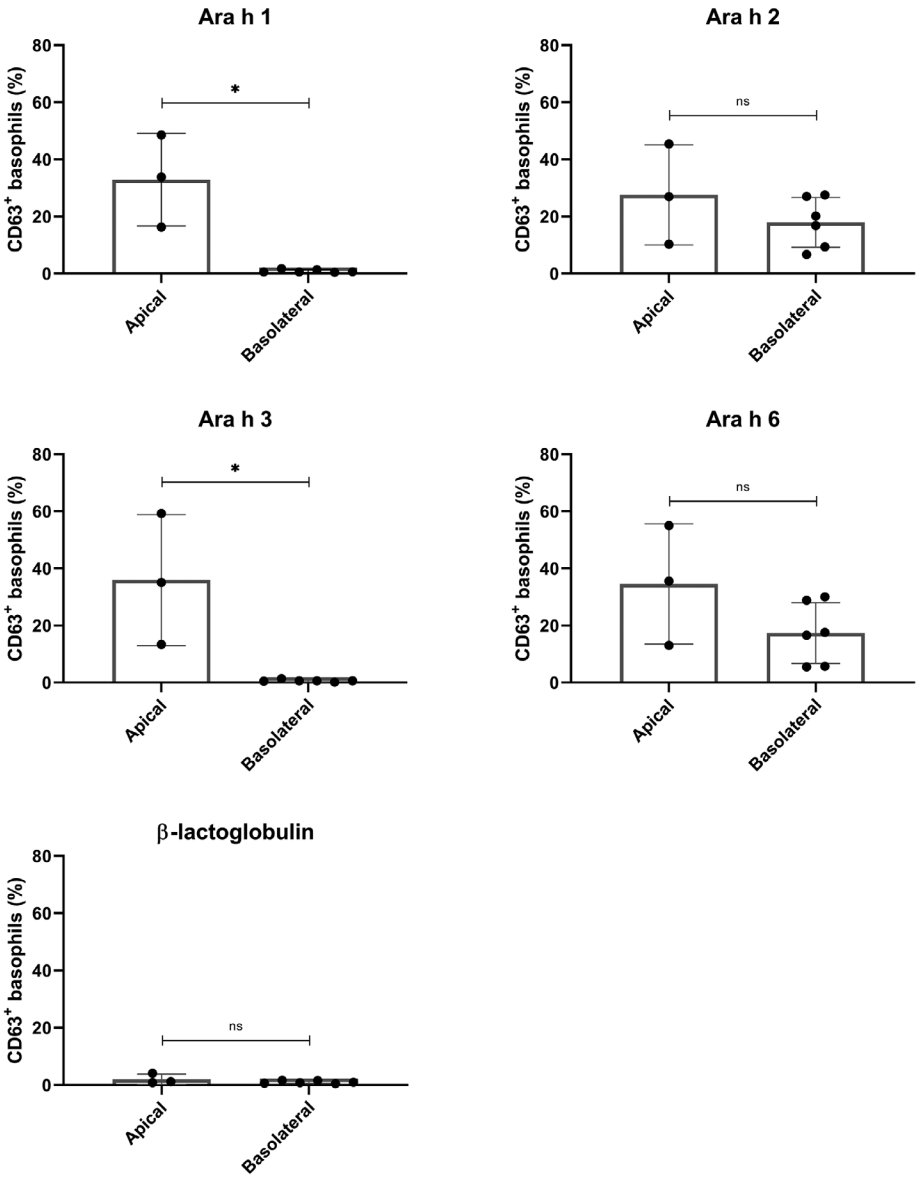

Figure 2. Basophil activation after stimulation with a serial dilution of peanut allergens Ara h 1, Ara h 2, Ara h 3, Ara h 6, and β-lactoglobulin retrieved from the apical ($1{:}10^3$, $1{:}10^4$, $1{:}10^5$, $1{:}10^6$) compartment and from the basolateral (1:3, 1:10, 1:50, $1{:}10^2$: $1{:}10^3$) compartment after transport across the intestinal epithelium of two pigs (pig 4 and pig 5) in the InTESTine model. Serum of three peanut-allergic patients was used to reload the basophils. The data is presented as the percentage of $CD63^+ \pm$ SD of the means of the serial dilutions (2 pigs and 3 patients sera, $n = 6$). The specific reactivity for each dilution is given in Figures S1 and S2, Supporting Information. Reactivity of the basolateral samples compared to the apical samples was evaluated by a Mann–Whitney *U* test. The number of symbols indicates the level of significance between the apical and basolateral CD63 expression: $*p < 0.05$.

3.2. Diminished Activation of Ara h 1 and 3 after Transport in an in-BAT

The effect of transport on the immune response of Ara h 1 and 2 was evaluated. To confirm the Ara h 1 and 2 results, Ara h 3 and 6 were also included in the following transport studies. Ara h 3 and 6 have comparable physicochemical properties (size, structure, and stability) with respect to Ara h 1 and 2. **Figure 2** shows the result of basophil activation by the apical and basolateral Ara h 1, 2, 3, and 6, and β-lactoglobulin. All peanut allergens retrieved from the apical compartment were able to activate the basophils as shown by the increased CD63 expression. This indicates that the basophils sensitized with serum from peanut-allergic patients were able to react to all peanut allergens before transport across the intestinal epithelium. Inter-donor differences between peanut-allergic donors were seen which was expected due to differences in peanut specific IgE values. No reaction was found for β-lactoglobulin (negative control). Ara h 1, 2, 3, and 6, and β-lactoglobulin were transported over the intestinal epithelium of two pigs and an in-BAT was performed with the basolateral samples. Ara h 1 and 3 were not able to activate basophils after epithelial transport. The means of the serial dilutions of the

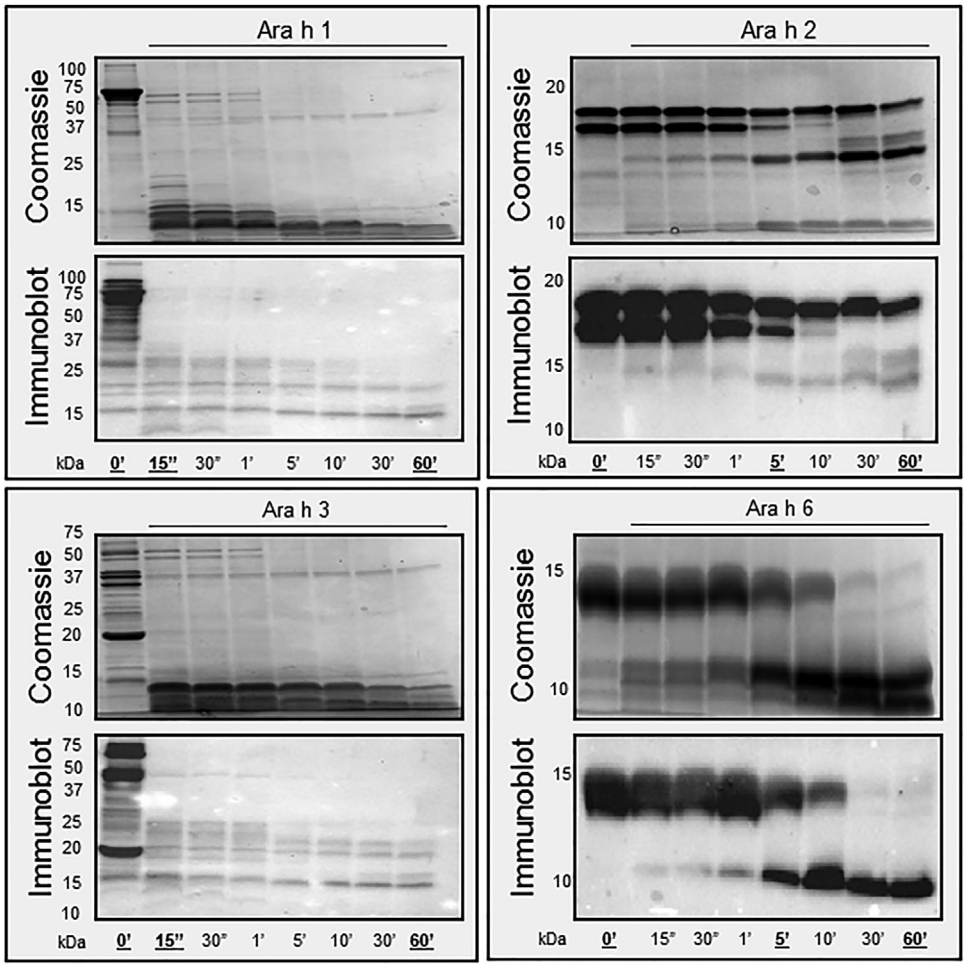

Figure 3. Digestion kinetics of Ara h 1, 2, 3, and 6 visualized using Coomassie stained 15% SDS-PAGE gels and IgE immunoblots incubated with the serum from peanut-allergic patient 4. The samples were collected after 0, 15 and 30 s and 1, 5, 10, 30 and 60 min of digestion, respectively.

patients were significantly lower after stimulation with basolateral Ara h 1 (0.9 ± 0.6) compared to apical Ara h 1 (32.9 ± 16.2). This difference was also found for Ara h 3 (apical: 35.9 ± 23.0 vs basolateral: 0.7 ± 0.4). No significant difference was found before and after transport of Ara h 2 (apical: 27.6 ± 17.5 vs basolateral: 17.9 ± 8.7) and Ara h 6 (apical: 34.6 ± 21.0 vs basolateral: 17.4 ± 10.7), respectively. The results were consistent for the sera from three peanut-allergic donors. The serial dilutions can be found in Figures S1 and S2, Supporting Information. It must be noted that, although no difference between apical and basolateral samples was found for Ara h 2 and 6, the apical samples were ≈100 times more diluted for the in-BAT with respect to the basolateral samples. The lower amount of protein in the basolateral samples can be attributed to the limited transport rate of the intestine samples within the InTESTine model.

3.3. Ara h 1 and 3 Are Less Stable in a Pepsin Digestion Test than Ara h 2 and 6

The fragmentation of Ara h 1, 2, 3, and 6 after pepsin digestion for multiple time points was evaluated using Coomassie staining and IgE immunoblotting (**Figure 3**). The typical major bands of Ara h 1 (64 kDa) and Ara h 3 (45, 42, 23 kDa) were rapidly digested within 15 s. IgE immunoblotting with peanut-specific IgE showed IgE binding to the major band of Ara h 1 at 64 kDa and to the peptide fragments. This pattern was also seen for the major bands of Ara h 3 and the resulting peptide mixture. Ara h 2 shows two protein bands on the gel belonging to the two Ara h 2 isomers. The protein digestion pattern of Ara h 6 (15 kDa) showed similarities with that of Ara h 2. Time points 0, 30 s, and 60 min were chosen as turning points in the digestion for Ara h 1 and 3 and were evaluated for their immunoreactive potential in the in-BAT. For Ara h 2 and 6, 0, 5, and 60 min of digestion were chosen. Reactivity of all Ara h 1, 2, 3, and 6 digests was confirmed using the sera of patient 1, 2, and 4 in an in-BAT (**Figure 4**).

3.4. Digested Ara h 1 and 3 Are Able to Activate Mast Cells after Transport

Figure 5 shows that when Ara h 1 and 3 are digested for a longer time period prior to epithelial transport, a higher percentage

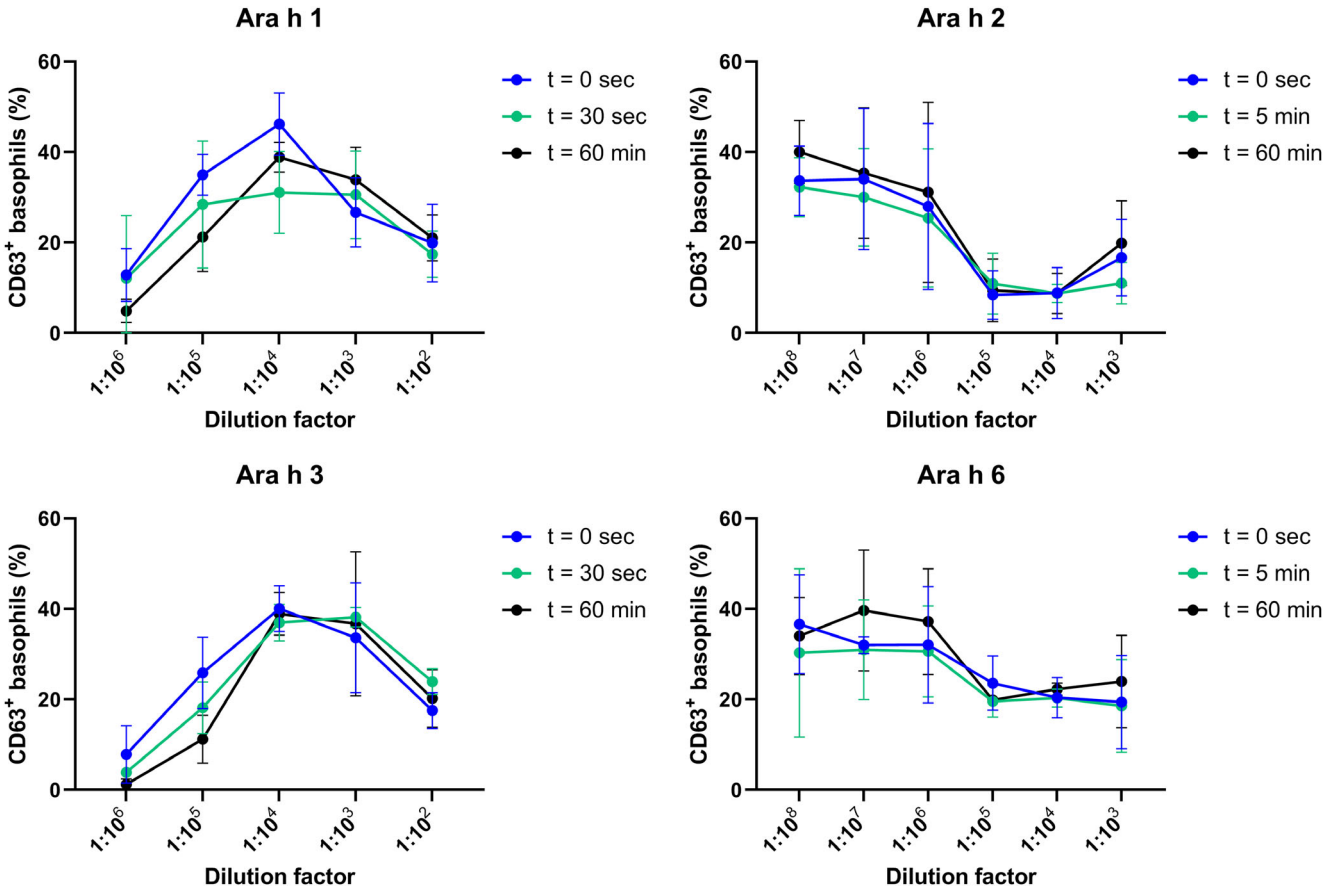

Figure 4. Percentage CD63$^+$ basophils after stimulation with a serial dilution of digests from peanut allergens Ara h 1, 2, 3, and 6. Serum of three peanut-allergic patients (1, 2, and 4) was used to reload the basophils. The data is presented as the mean percentage of CD63$^+$ ± SD and all peanut allergen digests showed reactivity.

β-hexosaminidase release from mast cells sensitized with serum from peanut-allergic patients was found. The means of the serial dilutions of the three patients and three pigs (nine in total) were evaluated for significant differences. Ara h 1 which was digested for 60 min (23.7 ± 9.7) was significantly more reactive after epithelial transport compared to Ara h 1 that was digested for 0 s (7.5 ± 5.8). There was no significant difference between 60 min and 30 s of digestion. Comparable results were found for Ara h 3. A digestion time of 60 min (21.8 ± 8.7) resulted in a significant higher activation compared to 30 s (7.1 ± 4.0) and 0 s (3.7 ± 2.9). No significantly different release was found for β-lactoglobulin compared to the medium control (data not shown). No significant difference was found in the percentage β-hexosaminidase release when hMCs were stimulated with digests of Ara h 2 (60 min vs 0 and 5 min). The mean release elicited by Ara h 2 that was digested for 60 min (50.6 ± 17.2) was not significantly different compared to 0 s (56.9 ± 12.7) and 5 min (43.9 ± 13.9). For Ara h 6, similar results were seen with no significant difference between 60 min (44.1 ± 10.7) and 0 s (54.1 ± 10.4) and 5 min (46.8 ± 11.1) of digestion. Subsequently, it can be concluded that the digestion time does not significantly influence activation by Ara h 2 and 6. Activation by Ara h 2 and 6 was seen after digestion and epithelial transport in every pig and for every digestion time point. The serial dilutions of Ara h 1, 2, 3, and 6 can be found in Figures S3 and S4, Supporting Information.

4. Discussion

In this study, we showed that Ara h 1 was unable to activate basophils after transport across the pig intestinal epithelium while Ara h 2 could, even though an equal amount of radiolabeled Ara h 1 and 2 or fragments thereof were transported across the intestinal epithelium. The percentage of transport, however, does not evaluate important characteristics such as protein state and bioactivity. This difference in basophil activation was also shown for two physiologically comparable peanut allergens Ara h 3 and 6. In contrast, when Ara h 1 and 3 were digested with pepsin, the resulting peptide fragments were able to activate hMCs after transport. Ara h 2 and 6 were not significantly affected in their ability to activate basophils by transport and digestion. Protein fragmentation and aggregation could have occurred during digestion and transport which are processes that influence the capacity to elicit degranulation of the investigated allergens. However, no reliable information regarding protein state and form could be gathered using multiple techniques. In summary, the functional properties of Ara h 1 and 3 are clearly affected by trans-

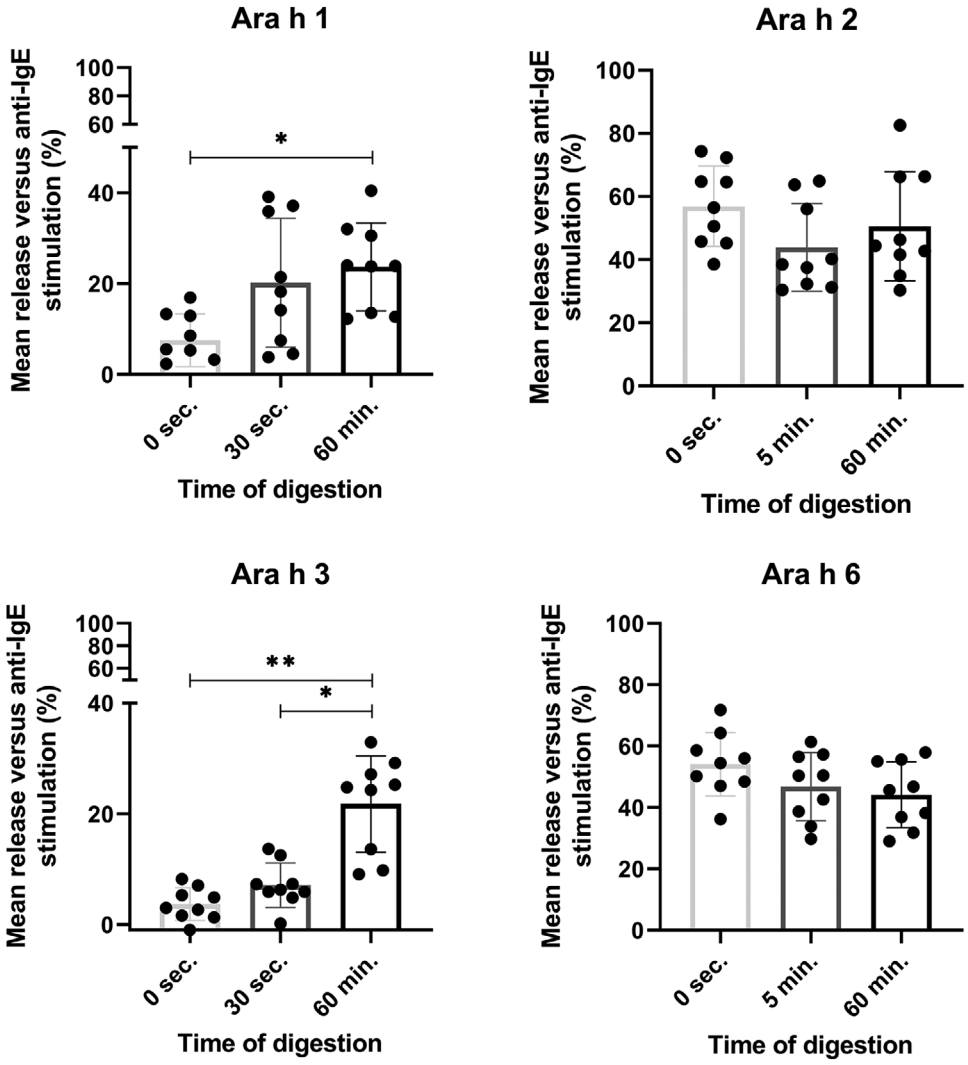

Figure 5. Mast cell activation after stimulation with digested ($t = 0$, 30 s, or 5 min, and 60 min) and transported Ara h 1, 2, 3, and 6 across the intestinal epithelium of three different pigs (pig 6, 7, and 8). Data is presented as the percentage β-hexosaminidase release ± SD with respect to the release induced by incubation with anti-IgE. The means of the serial dilutions (1:1, 1:2.5, 1:5, 1:10, $1{:}10^2$, $1{:}10^3$) are displayed and the β-hexosaminidase release of the specific dilution points can be found in Figures S3 and S4, Supporting Information. The reactivity between the different digestion times was evaluated by a Kruskal–Wallis test followed by Dunn's multiple comparison test. The number of symbols indicates the level of significance between the digestion times: * $p < 0.05$, ** $p < 0.005$.

port and by the combination of digestion and transport. This is an important finding regarding the interpretation and development of a predictive in vitro assay for allergenicity assessment.

The difference in activation of Ara h 1 and 3 compared to Ara h 2 and 6 after transport is remarkable. The difference in reactivity between Ara h 1 and 3 and Ara h 2 and 6 can have various causes. Proteins are transported over the intestinal epithelium via different routes. Soluble proteins are mainly transported across the intestinal epithelium by enterocytes, and 90% of the internalized protein is subjected to intracellular degradation in lysosomes.[7] All peanut proteins (Ara h 1, 2, 3, and 6) are water-soluble proteins.[23] It can be hypothesized that Ara h 1 and 3 are subjected to degradation by lysosomal proteases, which results in the degradation of IgE binding epitopes and subsequently in reduced IgE binding, whereas Ara h 2 and 6 are less degraded by lysosomal proteases. This hypothesis is supported by Smit et al. who investigated the processing of peanut allergens in dendritic cells.[24] Peanut allergens were coupled to latex beads and intracellular phago-lysosomal degradation in dendritic cells was quantified by flow cytometry. Intracellular protein degradation was found to be higher for Ara h 1 and 3 than for Ara h 2 and 6. Additionally, Mattison et al. showed that certain fragments of Ara h 1 were also cleaved by endolysosomal proteases.[25] More information is therefore needed regarding the effect of lysosomal degradation and the resulting effect on allergenicity to evaluate if this is a possible point of interest to accurately predict the allergenicity of novel proteins using in vitro cell-based assays.

The difference in reactivity of the nondigested and transported Ara h 1 and 3 between the six pigs is interesting. In four out of the six pigs, we clearly see no activation of effector cells by the nondi-

gested Ara h 1 and 3, while in two pigs a small effect is seen at the highest concentrations. This difference can be attributed to inter-pig differences. The age of the pigs can also be an important factor in relation to intestinal integrity, as we also see with humans.[26]. In our study, 4–7 month old pigs were used. Gut permeability plays an important role in transport and allergy as reviewed by Perrier et al.[27]. Differences in intestinal integrity can be expected between pigs and might be the reason for the differences seen between the six pigs.

The resistance to pepsin digestion is currently one of the main pillars for assessing the allergenic potential of (novel) proteins.[28]. Pepsin digestion of the peanut allergens in our study was similar to the results found by others.[19] Koppelman et al. also showed that Ara h 2 and 6 are very stable, even when subjected to harsh conditions while Ara h 1 and 3 are not. Our study shows that both intact and fragments of all tested peanut allergens were able to bind to IgE of peanut-allergic patients. However, after transport of Ara h 1 and 3, no activation of effector cells was seen, in contrast to Ara h 1 and 3 fragments obtained after digestion. Ours and other studies have shown that after digestion the fragments of Ara h 1, 2, and 6 exhibit immunoreactivity.[29–31] As previously hypothesized, it is possible that endolysosomal enzymes have cleaved nondigested Ara h 1 and 3 during transport resulting in the degradation of IgE-binding epitopes. However, the increase in activation seen for Ara h 1 and 3 when these allergens are subjected to pepsin digestion prior to epithelial transport may be associated with a different route of transport. Besides transcellular transport where proteins are affected by endolysosomal enzymes, immunoreactive fragments could potentially be transported via paracellular transport. Small proteins with a maximal radius of 15 Å (±3.5 kDa) can cross the barrier via paracellular transport.[32,33] According to Maleki et al., pepsin digestion of Ara h 1 resulted in fragments smaller than 5 kDa.[34] Proteins which are transported via the paracellular route are not exposed to lysosomes and therefore not degraded further. It is possible that the route of transport across the intestinal epithelium is responsible for the difference in immunological response between intact and fragmented Ara h 1 and 3. Differences in immunological response caused by differences in transport route was also reported by Roth-Walter et al.[35]. Roth-Walter et al. showed in mice that sensitization to milk proteins (α-lactalbumin, β-lactoglobulin and casein) mainly occurred when the aggregated proteins were transported by M cells and elicitation to milk proteins was observed after intracellular transport of non-aggregated milk proteins by enterocytes. The difference in immunological response could be caused by differences in exposure to lysosomal degradation and the possible destruction of IgE epitopes. This might be the reason why intact Ara h 1 and 3 are not able to activate effector cells after transport, whereas digested Ara h 1 and 3 are able to activate effector cells after transport. Aggregation of Ara h 1 and 3 fragments might also play a role. Khan et al., reported that after digestion of Ara h 1 at pH 2 (stomach), the resulting fragments formed aggregates when transferred to a basic environment (intestine).[36] This can result in potentially new immune reactive epitopes that can induce an allergic response and might be the reason as to why digested and subsequently aggregated Ara h 1 and 3 induced a response while Ara h 2 and 6 did not.[36] It would be interesting to gain more knowledge regarding the protein state and fragmentation status after transport. However, we were not able to get this information due to the low amount of transported proteins and fragments thereof and the contamination of the samples with pig intestinal proteins.

It is important to realize that the immune cells in the human body do not solely encounter digested peptide fragments or intact proteins. Due to the way the gastro-intestinal tract functions, continuous gastric emptying results in the passage of protein–peptide mixtures containing intact allergens and fragments in various stages of breakdown.[37,38] These mixtures of proteins and peptides will come into contact with the epithelial cells even before further digestion in the intestine takes place. In our study, three different digestion time points were chosen to mimic some of the variability of this situation. We indeed found that these different peptide/protein mixtures induce different immunological effects after transport across the intestinal epithelium. The next step would be to include intestinal digestion preferably using a dynamic digestion model (such as the TIM model) to mimic the real life situation even better.

Currently, in vitro methods used in allergenicity assessment cannot predict the allergenicity of proteins accurately.[39,40] As concluded by Remington et al., processing is generally not performed but is important when preparing material for testing in in vitro studies. The methods which can be used to investigate transcellular transport are discussed in an ImpARAS publication.[41] The importance to study transport in relation to allergenicity is highlighted in this publication. However, only a few isolated studies using a limited number of allergens have been conducted. Therefore, we advise to evaluate more food proteins using these existing techniques in combination with an effector cell assay. Multiple factors (food processing, food matrix, stability, digestion, and transport) influence the allergenicity of proteins and ideally these should be included in an allergenicity assessment assay to improve their predictability. However, we realize that including these factors might be very challenging and will hamper the development of fast and cheap screening assays for allergenicity prediction.

In this study, we showed that digestion and epithelial transport influenced the immune response of Ara h 1 and 3, but not for Ara h 2 and 6. The influence of digestion and transport on protein allergenicity might explain why current in vitro assays are not predictive for allergenicity. Ideally, factors such as processing, digestion, and transport should be included in an allergenicity assessment assay; however, the feasibility and practicality of including these factors should be taken into account.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

All authors contributed to the interpretation and review of the manuscript. I.N. and K.V. contributed to the conception and design of the experiments. A.J. purified the proteins, M.S. and I.N. performed the experiments, and I.N., K.V., and M.S. analyzed the data. M.S. drafted the manuscript. All authors reviewed the manuscript and approved its final version.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

allergenicity prediction, basophil and mast cell activation, food allergy, intestinal protein transport, protein digestion

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