

Clinical Allergy to Hazelnut and Peanut: Identification of T Cell Cross-Reactive Allergens

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Key Words

Allergens • Cross-reactivity • Hazelnut • Peanut • T cell

Abstract

Background: Peanut and tree nut allergies are life-threatening conditions for many affected individuals worldwide. Currently there is no cure. While co-allergy to peanut and tree nuts is a common clinical observation, and IgE cross-reactivity between peanut and tree nuts is reported, T cell cross-reactivity is poorly defined. **Methods:** Hazelnut-specific T cell lines were established using peripheral blood mononuclear cells from 5 subjects with co-allergy to hazelnut and peanut. These lines were stimulated with hazelnut and peanut extracts and purified major peanut allergens, Ara h 1 and Ara h 2. Proliferation was determined by ³H-thymidine incorporation and secretion of key Th1 (IFN- γ) and Th2 (IL-5) cytokines analysed by ELISA. **Results:** Hazelnut-specific T cell lines from all 5 subjects proliferated upon stimulation with both hazelnut and peanut extracts and for 4 subjects, to Ara h 1 and/or Ara h 2. Proliferating cells were mainly CD4+ T cells and produced both IL-5 and IFN- γ in response to hazelnut and peanut stimulation. Mitogenicity of extracts and allergens was excluded by their lack of stimulation of house dust mite-specific T cells. **Conclusion:** Our finding that hazelnut and peanut co-allergy is associated with cross-reactive T cell responses, driven partly by cross-reactivity to the major pea-

nut allergens Ara h 1 and Ara h 2, points to future development of allergen immunotherapy by targeting cross-reactive T cells.

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Introduction

Peanut allergy is a major health care problem worldwide. It affects up to 2% of the population and is the leading cause of fatal food-induced anaphylaxis [1–5]. Tree nut allergy is also an important clinical problem with a reported prevalence of 0.4% [2]. Studies report 20–40% subjects presenting to allergy clinics have peanut and tree nut co-allergy [2, 6, 7], but in our adult allergy clinic up to 60% of peanut-allergic patients are allergic to one or more tree nuts, with the most common reactivity, 49%, to hazelnut [unpublished data]. There is no cure for peanut or tree nut allergy, which typically persist lifelong [8].

Correlation between serum levels of peanut-specific IgE and hazelnut-, Brazil nut- as well as almond-specific IgE in peanut-allergic children suggests that cross-reactive immune responses underlie co-allergy to peanut and tree nuts [9]. IgE antibody cross-reactivity between peanut and tree nuts is reported [10–13], and we demonstrated biological relevance of this cross-reactivity by tree nut-induced activation of basophils sensitized with purified

Table 1. Clinical characteristics of hazelnut- and peanut-allergic subjects

	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5
Gender	F	F	F	M	M
Age, years	24	27	50	30	33
Allergic disease	asthma, rhinitis, eczema	asthma, rhinitis, eczema	nil	asthma	asthma, rhinitis
Known nut sensitivities	peanut, hazelnut, almond, walnut, cashew	peanut, hazelnut, walnut	peanut, hazelnut	peanut, hazelnut	peanut, hazelnut, almond, walnut
Clinical features on peanut and tree nut exposure	laryngeal oedema, facial angioedema, asthma	laryngeal oedema, generalized urticaria, facial angioedema	laryngeal oedema, generalized urticaria, facial angioedema	laryngeal oedema, facial angioedema, asthma, GIT upset	laryngeal oedema, facial angioedema, asthma
Age at first reaction, years	1	10	18	12	3
Time since last reaction, months	24	2	21	18	36
Peanut CAP-FEIA score, kU _A /l	5 (63.5)	2 (2.07)	3 (6.87)	1 (0.51)	3 (13.9)
Hazelnut CAP-FEIA score, kU _A /l	2 (2.31)	4 (17.5)	1 (0.4)	1 (0.56)	3 (13.52)
Positive skin prick test	GP, HDM, C, D, M	GP, HDM, C, D, M	GP, HDM, C	HDM	HDM

GIT = Gastrointestinal; GP = grass pollen; C = cat; D = dog; M = mould.

peanut-specific IgE antibodies [14]. In contrast, reports of human T cell cross-reactivity between peanut and tree nuts are limited to our early pilot study, showing proliferation of cloned peanut-specific T cells to both hazelnut and peanut, but induction of Th2 cytokine secretion only for the clinically relevant nut [15].

Although taxonomically peanut is distantly related to tree nuts, both are edible seeds with conserved protein families and similar functions in plant development [16]. A number of allergens have been identified officially in both peanut and tree nuts as listed by the Allergen Nomenclature Sub-committee (<http://www.allergen.org>) of the International Union of Immunological Societies. Ara h 1 and Ara h 2 are designated major allergens [17], and each shows considerable sequence homology with various tree nut allergens (<http://www.allergen.org>) [16, 17]. IgE-binding epitopes have been mapped for each of these peanut allergens with subsequent reports of shared IgE-binding epitopes in tree nut allergens. In particular, Ara h 1 shares IgE-binding epitopes with walnut (Jug r 2), hazelnut (Cor a 11) and cashew nut (Ana o 1) [18], and Ara h 2 with almond and Brazil nut allergens [19].

In this study, proliferative and cytokine responses of hazelnut-specific T cell lines demonstrated T cell cross-reactivity between hazelnut and peanut in subjects sensitized and clinically allergic to both foods. This cross-reactivity was partly attributable to the major peanut allergens, Ara h 1 and Ara h 2.

Subjects and Methods

Subjects

Five subjects with both hazelnut and peanut allergy were recruited from the Alfred Hospital Allergy Clinic (table 1). Hazelnut and peanut allergy were defined by a history of clinical symptoms on allergen exposure and detection of specific-IgE by immunoassay (score >1, Pharmacia CAP System™; Pharmacia Diagnostics, Uppsala, Sweden). In addition, two non-peanut-allergic controls were recruited, one of whom was also non-atopic (as defined by the lack of symptoms of allergic disease and negative skin prick testing to common environmental aeroallergens). The study was approved by the Alfred Hospital Ethics Committee (No. 35/99) and informed written consent was obtained from each subject.

Preparation of Allergen Extracts

Aqueous extracts were prepared from commercially available hazelnuts and peanuts, and from house dust mite (HDM; kindly provided by Allergy Therapeutics Ltd., Worthing, UK) as described previously [13, 20]. For use in cell culture, the extracts were filter sterilized using a 0.2-µm filter and stored at -20°C.

Purification of Ara h 1

Peanut extract was re-equilibrated in 10 mM Na₂HPO₄ buffer, pH 6.7 and loaded onto a Pharmacia Mono S 2-ml cation exchange column (GE Healthcare, Little Chalfont, UK) attached to a Bio-Rad Biologic Duoflow HPLC system (Bio-Rad Laboratories, Hercules, Calif., USA). The column was washed with 10 mM Na₂HPO₄ buffer, pH 6.7, with the run-off collected for subsequent Ara h 2 purification. The bound proteins were eluted using a 26-ml three-step NaCl gradient from 23 to 40% using 10 mM Na₂HPO₄ buffer, pH 6.7 as buffer A and 1 M NaCl in 10 mM Tris HCl pH 6.7 as buffer B. Protein-containing fractions were analysed by 14% SDS-PAGE and those containing Ara h 1 (based on molecular

mass) were pooled and concentrated by centrifugation at 3,000 g using a 50-kDa Sartorius Vivaspin 6 ml filtration unit (Sartorius, Göttingen, Germany). The concentrate was dialysed against PBS and filter sterilized using a 0.2- μ m filter before storage at -20°C .

Purification of Ara h 2

Flow-through collected from the cation exchange column during the purification of Ara h 1 was found to be enriched for Ara h 2 and consequently used for the purification of Ara h 2 by anion exchange chromatography. The pooled flow-through was re-equilibrated in 10 mM Tris HCl pH 7.6 and loaded onto a Bio-Scale Q 2 ml column (Bio-Rad Laboratories) attached to a Bio-Rad Biologic Duoflow HPLC system (Bio-Rad Laboratories). The bound proteins were eluted using a 20-ml linear NaCl gradient from 0 to 50% of 10 mM Tris HCl pH 7.6 as buffer A and 1 M NaCl in 10 mM Tris HCl pH 7.6 as buffer B. Protein-containing fractions were analysed by 14% SDS-PAGE and those containing Ara h 2 (based on molecular mass) pooled, dialysed against PBS, sterilized using a 0.2- μ m filter and stored at -20°C . For N-terminal sequencing, proteins separated by 14% SDS-PAGE were transferred onto PVDF membranes, stained with Coomassie Brilliant Blue (CBB) and individual bands cut out and sent to the Australian Proteome Analysis Facility, University of New South Wales, Sydney, for sequencing by Edman degradation.

Testing Allergen Preparations for Mitogenicity, Toxicity and Endotoxin

Mitogenicity and toxicity of hazelnut and peanut extracts as well as purified Ara h 1 and Ara h 2 was excluded by culturing with oligoclonal HDM-reactive CD4⁺ T cells in the presence of irradiated peripheral blood mononuclear cells (PBMC) and co-culture of antigens with HDM-reactive T cells in the presence of IL-2, respectively (data not shown). Endotoxin estimation using a limulus amoebocyte lysate assay (Bio-Whittaker, Walkersville, Md., USA) revealed negligible endotoxin in our peanut extract and purified Ara h 1 and Ara h 2 preparations (0.0079, 0.0024 and 0.0011 EU/ μ g of protein, respectively).

SDS-PAGE and Immunoblotting

Hazelnut and peanut extracts and purified Ara h 1 and Ara h 2 were resolved by 12 or 14% SDS-PAGE under reducing conditions and proteins stained with CBB (Sigma, St. Louis, Mo., USA) as described [11]. For immunoblotting, hazelnut and peanut proteins separated by 14% SDS-PAGE were transferred onto nitrocellulose membranes (0.45 μ m; Schleicher and Schuell Inc., Dassel, Germany) as described [11]. Membranes were blocked with 10% skim milk powder/PBS, washed and incubated with peanut/tree nut-allergic donor sera and control sera diluted 1:5 with 0.05% Tween-20/PBS containing 1% skim milk powder, followed by rabbit polyclonal anti-human IgE antibody (1:200; DAKO, Carpinteria, Calif., USA) and horseradish peroxidase-labelled goat anti-rabbit IgG antibody (1:2,000; Promega, Madison, Wisc., USA). IgE binding was detected using the substrate 4-chloro-1-naphthol (Sigma) in the presence of hydrogen peroxide.

PBMC Proliferation Assays

PBMC separated from heparinized blood using Ficoll Paque gradient (Amersham Biosciences, Uppsala, Sweden) were cultured in 96-well U-bottom plates (1×10^5 PBMC/well; Linbro ICN Biomedical, Costa Mesa, Calif., USA) in complete medium

[RPMI 1640 supplemented with 2 mM L-glutamine, 100 IU/ml penicillin-streptomycin (Invitrogen Life Technologies, Carlsbad, Calif., USA) and 5% heat-inactivated human AB serum (Sigma)] and different concentrations of hazelnut, peanut and HDM extracts (0–400 μ g/ml) or as positive controls, tetanus toxoid (0.35 LFU/ml; Calbiochem, Merck KGaA, Darmstadt, Germany) and phytohaemagglutinin (PHA; 2 μ g/ml; Wellcome Diagnostics, Dartford, England) for 7 days at 37°C , 5% CO_2 . Cells were pulsed with ^3H -thymidine (1 μCi /well; Amersham Biosciences) for the last 16 h of culture and ^3H -thymidine incorporation was measured by liquid scintillation spectroscopy. Results are expressed as means $\pm$ SD (cpm) for triplicate cultures or as stimulation indices (SI; cpm stimulated cultures/cpm unstimulated cultures) with SI values >2.5 defined as significant.

Generation of Short-Term Hazelnut-Specific Oligoclonal T Cell Lines

Purified PBMC were cultured in 24-well plates (Greiner Bio-One, Frickenhausen, Germany) at 2.5×10^6 cells per well in complete medium with hazelnut extract (at the concentration that induced maximal proliferation in the polyclonal PBMC assay) for 7 days at 37°C , 5% CO_2 . On day 7, cells were washed once, resuspended at $1\text{--}1.5 \times 10^6$ /ml and mixed together with 1×10^6 /ml irradiated autologous PBMC (3,000 rad; Gammacell 1000 Elite, Nordion International Inc., Kanata, Ont., Canada) and restimulated with hazelnut extract. On days 2 and 4 following restimulation, 25 U/ml of recombinant human IL-2 (rIL-2; Cetus, Emeryville, Calif., USA) was added. Due to low cell numbers at 2 weeks in some experiments, 3-week T cell lines were generated by restimulation with hazelnut extract and rIL-2 as above for a further week.

Oligoclonal T Cell Proliferation Assays

Oligoclonal T cell blasts (5×10^4 /well) from hazelnut-specific T cell lines were stimulated in triplicate with different concentrations of hazelnut, peanut, HDM extracts, purified Ara h 1 and Ara h 2 (0–400 μ g/ml) or medium alone, and rIL-2 as negative and positive controls, respectively, in the presence of autologous irradiated PBMC (3,000 rad; 5×10^4 /well) for 3 days. For the last 16 h of culture, cells were pulsed with ^3H -thymidine (1 μCi /well) and ^3H -thymidine incorporation was measured as before.

Analysis of IFN- γ and IL-5 Production by Hazelnut-Specific Oligoclonal T Cells

Following stimulation of hazelnut-specific T cell lines with hazelnut, peanut, Ara h 1 and Ara h 2 for 48 h, production of IFN- γ and IL-5 in culture supernatants was measured by sandwich ELISA using mouse anti-human IFN- γ (Endogen, Woburn, Mass., USA) and mouse anti-human IL-5 (PharMingen, San Diego, Calif., USA) capture monoclonal antibodies and biotinylated rat-anti-human IFN- γ (Endogen) and biotinylated rat-anti-human IL-5 (PharMingen) detection monoclonal antibodies [21]. Standard curve construction and determination of cytokine levels were performed using Packard I-Smart software (Packard Instrument Company, Meriden, Conn., USA). The lower limits of detection for IFN- γ and IL-5 were 4 and 2 pg/ml, respectively.

Surface Marker Phenotype of Oligoclonal Hazelnut-Specific T Cells by Flow Cytometry

Oligoclonal hazelnut-specific T cells (0.5×10^6 /test) were stained with mouse anti-human CD4-FITC (BD Biosciences, San

Jose, Calif., USA), mouse anti-human CD8-PE (BD Biosciences), mouse anti-human CD19-PE (BD Biosciences), mouse anti-human CD14-PE (BD Biosciences) or the relevant isotype controls, according to the manufacturer's instructions. The percentage of stained cells was determined from 100,000 events using a FACS-Calibur flow cytometer (BD Biosciences) and Cell Quest software (BD Biosciences).

Results

Subject Characterization

The clinical characteristics of the 5 hazelnut- and peanut-allergic subjects are summarized in table 1. While the entire group described clinical features of anaphylaxis to both hazelnut and peanut, subjects 1, 2 and 5 also described reactions to other tree nuts (almond, walnut and/or cashew). Each subject suffered reactions in the previous 3 years, reflecting the difficulty in avoiding peanut and tree nut exposure over a lifetime. All subjects were skin prick test positive to HDM, allowing the use of this antigen as a control allergen extract for T cell assays.

Purification of Ara h 1 and Ara h 2

Two protein-rich fractions of peanut extract separated using cation and anion exchange chromatography were analysed by 12 or 14% SDS-PAGE (fig. 1). The first fraction contained a prominent protein band of about 63 kDa which is in agreement with the published molecular mass of Ara h 1 [22]. Also present in that fraction was a minor protein component of about 30 kDa. The second fraction comprised two closely migrating bands with a mean molecular mass of approximately 17 kDa which is similar to that found for Ara h 2 in previous studies [23, 24]. N-terminal sequencing of each band gave the same result for the first 5 amino acids (R-Q-Q-X-E), that is, consistent with that published for Ara h 2 [24]. The purity of the Ara h 1 and Ara h 2 preparations was greater than 95% as determined by SDS-PAGE and CBB staining. Immunoblotting using a panel of peanut-allergic sera confirmed the strong IgE reactivity of these purified preparations of Ara h 1 and Ara h 2 (data not shown).

Analysis of Serum IgE Reactivity to Hazelnut and Peanut Proteins by Immunoblotting

Hazelnut and peanut extracts were resolved by 14% SDS-PAGE and either stained with CBB or electrophoretically transferred onto nitrocellulose membranes for analysis of reactivity with serum IgE from hazelnut- and peanut-allergic subjects. As shown in figure 2a, hazelnut extract proteins had molecular masses ranging from ap-

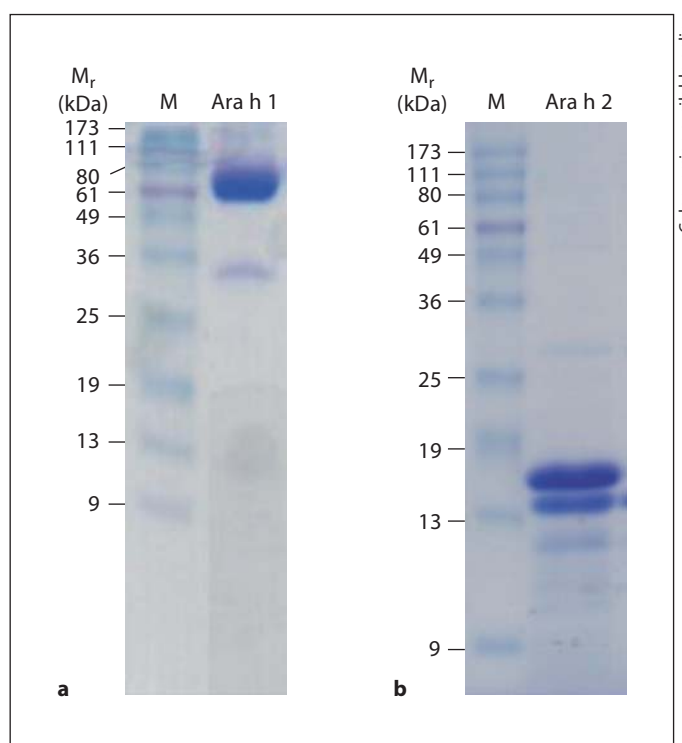
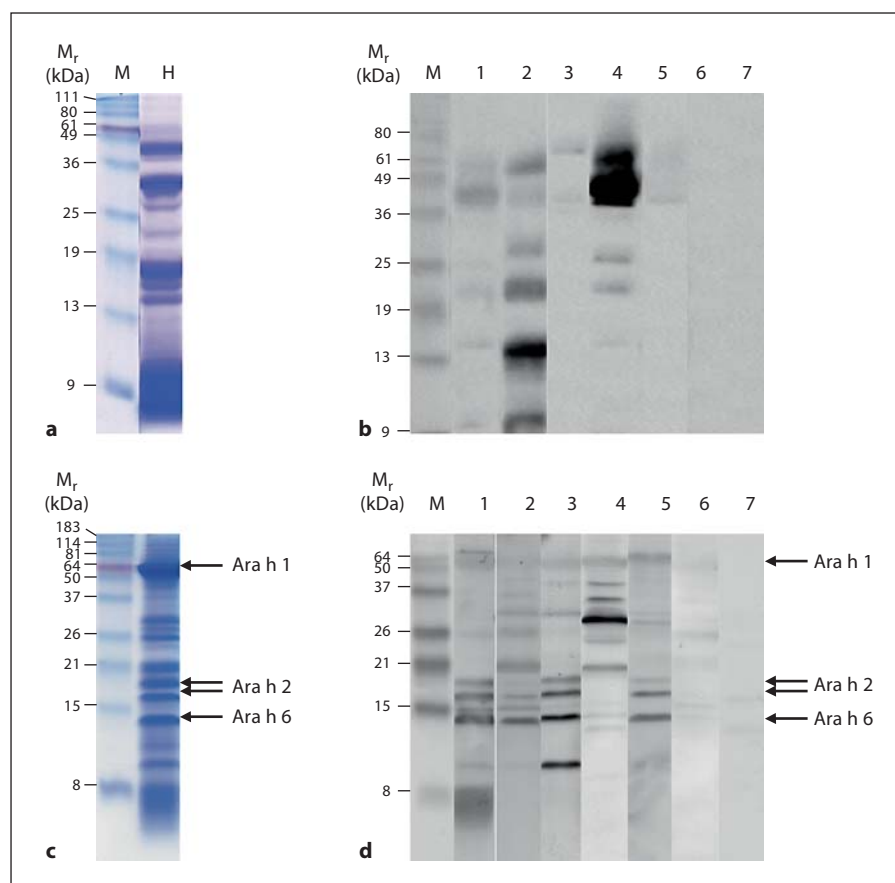


Fig. 1. SDS-PAGE of purified Ara h 1 (a) and Ara h 2 (b). Cation exchange and ultrafiltration purified Ara h 1 as well as cation and anion exchange purified Ara h 2 were resolved by 14% (Ara h 1) or 12% (Ara h 2) SDS-PAGE under reducing conditions and stained with CBB. M indicates position of molecular mass markers.

proximately 8 to 61 kDa, with the protein of about 8 kDa being the most abundant protein. Numerous serum IgE-reactive hazelnut protein bands were detected (fig. 2b), ranging in molecular mass from approximately 10 to 61 kDa. Four of the five hazelnut- and peanut-allergic subjects in this study showed serum IgE reactivity with a band of about 40 kDa, one very strongly. Proteins of about 55, 22 and 15 kDa were the next most frequently associated with hazelnut allergy, being IgE reactive in 3 of the 5 subjects. Other proteins binding serum IgE antibodies from 2 of the 5 subjects were about 27 and 9 kDa in molecular mass. Serum from an atopic, non-peanut/tree nut-allergic control subject did not show IgE reactivity to any of the hazelnut proteins.

SDS-PAGE of peanut extract revealed numerous proteins (fig. 2c), some of which have been classified as major peanut allergens, namely Ara h 1 (63 kDa) and Ara h 2 (17–19 kDa double band). Immunoblotting showed that both Ara h 1 and Ara h 2 were recognized by serum IgE from four subjects, along with the minor allergen

Fig. 2. 14% SDS-PAGE and immunoblotting of hazelnut and peanut extract. Hazelnut (**a, b**) and peanut (**c, d**) extract were resolved by 14% SDS-PAGE under reducing conditions. Proteins were either stained with CBB (**a, c**) or electroblotted onto nitrocellulose membranes (**b, d**) and probed with serum IgE from 5 subjects with both hazelnut and peanut allergy (lanes 1–5) and 1 atopic, non-hazelnut/peanut-allergic subject (lane 6). Lane 7 shows a membrane incubated in secondary and tertiary antibody only.



Ara h 6 which shares extensive homology with Ara h 2 [25] (fig. 2d). For two subjects (lanes 2 and 4), serum IgE bound to a series of bands between approximately 13 and 45 kDa consistent with proteolytically cleaved Ara h 3 [26]. Serum IgE reactivity was also detected for a number of other peanut proteins, but these have not been identified or extensively characterized. Minimal IgE reactivity to the peanut proteins was observed with the atopic, non-peanut/tree nut-allergic negative control serum (fig. 2d).

Polyclonal T Cell Proliferative Responses to Hazelnut, Peanut and HDM Extracts

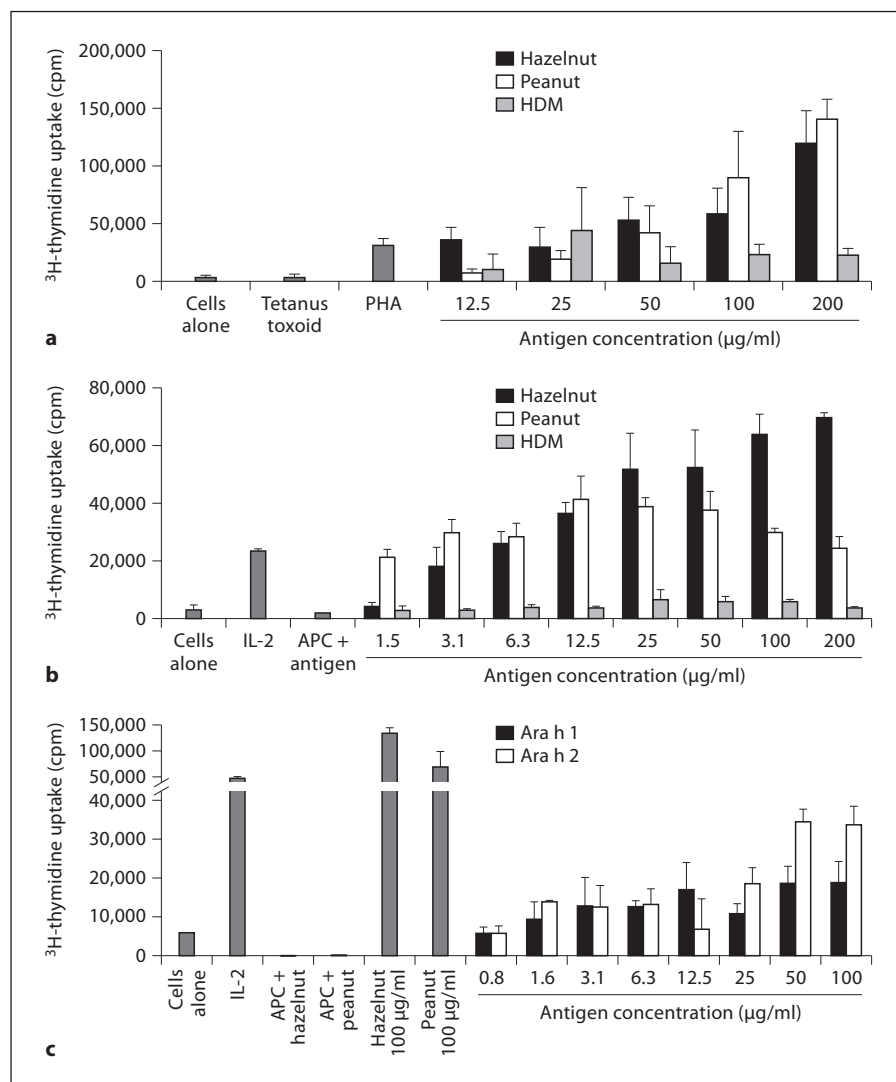
T cell reactivity of the subjects in this study to hazelnut, peanut and HDM was first established by assessing PBMC proliferative responses to the allergen extracts. Figure 3a shows representative data for a hazelnut- and peanut-allergic subject, demonstrating a dose-dependent PBMC proliferative response to hazelnut, peanut and HDM. The maximum polyclonal (PBMC) T cell proliferative responses to hazelnut, peanut and HDM for

each subject are summarized in table 2. Maximum proliferative responses ranged from 3.7×10^4 to 1.5×10^5 cpm for hazelnut, 4.0×10^4 to 1.4×10^5 cpm for peanut and 2.9×10^4 to 9.2×10^4 cpm for HDM. SI values for hazelnut, peanut and HDM in all subjects were greater than 2.5, indicating significant proliferation to these allergens.

Cross-Reactivity of T Cell Proliferative Responses to Hazelnut and Peanut

To determine whether T cell proliferative responses to hazelnut and peanut were due to the expansion of T cell populations specific to cross-reactive antigens present in hazelnut and peanut, hazelnut-specific oligoclonal T cell lines were generated for each subject and stimulated with hazelnut and peanut extracts as well as HDM extract as a negative control. A representative proliferation assay is shown in figure 3b whereby a dose-dependent proliferative response to both hazelnut and peanut was observed for a hazelnut-specific oligoclonal T cell line. In contrast, minimal proliferation was detected with the negative

Fig. 3. Representative proliferation assays for a hazelnut- and peanut-allergic subject (subject 1 from table 1). **a** Proliferation of PBMC to hazelnut extract, peanut extract and HDM-negative control extract. PBMC were cultured with antigens over a concentration range for seven days. Positive control antigens included tetanus toxoid and phytohaemagglutinin (PHA). Hazelnut-specific T cell lines were generated from PBMC and stimulated with hazelnut extract, peanut extract and HDM-negative control extract (**b**) or purified Ara h 1 and Ara h 2 (**c**) over a concentration range for 3 days. Proliferation was assessed by ^3H -thymidine incorporation and expressed as mean cpm of triplicate responses.



control HDM extract. Flow-cytometric analysis of the surface markers of T cells expanded in a hazelnut-specific T cell line confirmed that CD4+ T cells were the predominant proliferating cell type (77% CD4+, 10% CD8+, <1% CD14+ and <1% CD19+), consistent with our previous experience for allergen-specific T cell lines generated using this methodology.

The maximum proliferative responses and SI for all subjects are summarized in table 3. For the hazelnut-positive control stimulation, the maximum proliferative responses ranged from 2.6×10^4 to 8.1×10^4 cpm, with all SI values greater than 2.5. Similarly, SI values greater than 2.5 were obtained following stimulation of all hazelnut-specific oligoclonal T cell lines with peanut extract, with maximum proliferative responses ranging from $1.4 \times$

10^4 to 5.2×10^4 cpm. In contrast, SI values obtained with HDM extract were all less than 2.5, with maximum proliferative responses ranging from 2.6×10^3 to 1.4×10^4 cpm.

To examine whether cross-reactive T cell responses between hazelnut and peanut could be attributed to the major peanut allergens Ara h 1 and Ara h 2, hazelnut-specific oligoclonal T cell lines were cultured with each peanut allergen at different concentrations and cell proliferation was measured after 3 days. Representative data are shown in figure 3c, indicating proliferation to Ara h 1 and Ara h 2 in a hazelnut-specific T cell line from a hazelnut- and peanut-allergic subject. The maximum proliferative responses and SI to Ara h 1 and Ara h 2 for hazelnut-specific oligoclonal T cell lines from all 5 sub-

Table 2. PBMC proliferative responses to crude hazelnut, peanut and HDM allergen extracts

Subject	Hazelnut	Peanut	HDM	Tetanus toxoid	PHA
<i>Maximum proliferation, cpm ± SD</i>					
1	120,241 ± 28,422 (33.4)	140,667 ± 16,893 (71.0)	44,043 ± 37,389 (12.2)	2,553 ± 650 (0.7)	30,762 ± 6,552 (8.5)
2	149,132 ± 41,232 (8.5)	72,464 ± 33,111 (88.3)	91,851 ± 49,016 (5.2)	170,022 ± 49,264 (9.7)	13,388 ± 1,311 (0.76)
3	49,466 ± 41,320 (16.7)	40,280 ± 12,793 (45.9)	66,759 ± 46,257 (22.5)	2,603 ± 955 (0.9)	12,346 ± 2,194 (4.2)
4	37,375 ± 31,297 (6.1)	61,510 ± 9,016 (12.9)	29,763 ± 6,544 (4.9)	42,598 ± 6,967 (7.0)	88,596 ± 8,885 (14.5)
5	127,627 ± 90,636 (22.0)	115,277 ± 7,778 (34.4)	68,256 ± 2,5686 (11.8)	149,223 ± 7,873 (25.7)	19,852 ± 1,501 (3.4)

Figures in parentheses show stimulation index scores. Maximum proliferation of 7-day PBMC cultures over a concentration range (0–400 µg/ml allergen extract). Control cultures stimulated with tetanus toxoid (0.35 LFU/ml) or PHA (2 µg/ml). Results expressed as means ± SD for triplicate cultures. Responses where the stimulation index (cpm stimulated cultures/cpm unstimulated cultures) is greater than 2.5 are bolded.

Table 3. Hazelnut-specific TCL proliferative responses to crude peanut, hazelnut and HDM extracts

T cell line	Hazelnut	Peanut	HDM	IL-2
<i>Maximum proliferation, cpm ± SD</i>				
1.3	78,871 ± 2,876 (27.2)	41,471 ± 8,195 (14.3)	6,475 ± 3,415 (2.2)	23,459 ± 1,404 (8.1)
2.3	72,704 ± 5,836 (30.2)	25,801 ± 1,695 (10.7)	2,658 ± 890 (1.1)	38,452 ± 1,889 (16.0)
3.3	47,951 ± 2,953 (13.5)	14,462 ± 1,122 (4.1)	4,104 ± 726 (1.2)	38,156 ± 1,107 (10.7)
4.3	81,768 ± 7,070 (29.2)	52,384 ± 5,293 (18.7)	7,012 ± 2,004 (2.5)	35,464 ± 4,435 (12.7)
5.3	26,363 ± 2,273 (3.9)	17,440 ± 816 (2.6)	14,024 ± 3,377 (2.1)	55,299 ± 3,213 (8.2)

Figures in parentheses show stimulation index scores. Maximum proliferation of 3-day TCL cultures over a concentration range (0–400 µg/ml allergen extract). Control cultures stimulated with rIL-2 (50 U/ml). Results expressed as means ± SD for triplicate cultures. Responses where the stimulation index is greater than 2.5 are bolded.

jects are summarized in table 4. Maximal proliferative responses to Ara h 1 ranged from 2.0×10^3 to 1.9×10^4 cpm but only 2 of 5 T cell lines demonstrated significant proliferation to Ara h 1, with SI > 2.5. For Ara h 2, maximal proliferative responses ranged from 7.6×10^3 to 3.4×10^4 cpm with significant proliferation for 3 of the 5 T cell lines. In summary, 4 of the T cell lines responded to either one or both of Ara h 1 and Ara h 2, but only 1 of these lines demonstrated significant proliferation to both Ara h 1 and Ara h 2 (T cell line 1.2; table 4).

Cytokine Profile of Cross-Reactive Hazelnut-Specific T Cells

Functional phenotype was determined by measurement of IL-5 and IFN-γ levels using ELISA in 48 h supernatants of hazelnut-specific oligoclonal T cell lines, stimulated with hazelnut or peanut (table 5). Moderate to high levels of IL-5 and IFN-γ were detected in supernatants for

all hazelnut- and peanut-stimulated T cell lines with low to negligible amounts of these cytokines in HDM-stimulated culture supernatants, in agreement with the proliferative responses of the T cell lines.

Discussion

Co-sensitivity to the groundnut peanut and tree nuts is a common clinical observation and may be attributed to immunological cross-reactivity. Previous studies demonstrated IgE cross-reactivity between peanut and various tree nuts, suggesting the existence of shared IgE-binding epitopes [11, 13, 27]. This study reports T cell cross-reactivity between hazelnut and peanut using oligoclonal hazelnut-specific T cell lines derived from PBMC from 5 subjects with clinical sensitivity to both hazelnut and peanut. This observed cross-reactivity is

Table 4. Hazelnut-specific TCL proliferative responses to purified Ara h 1 and Ara h 2

T cell line	Ara h 1	Ara h 2	Peanut	Hazelnut
<i>Maximum proliferation, cpm ± SD</i>				
1.2	18,880 ± 5,309 (3.1)	34,245 ± 3,385 (5.7)	69,645 ± 30,648 (11.6)	136,399 ± 10,615 (22.6)
2.2	10,175 ± 5,104 (2.9)	5,078 ± 4,121 (1.5)	29,529 ± 20,122 (8.5)	81,051 ± 7,213 (23.4)
3.2	8,415 ± 2,368 (2.4)	19,645 ± 2,611 (5.5)	19,264 ± 5,511 (5.4)	131,811 ± 59,466 (37.0)
4.2	2,085 ± 1,001 (1.0)	18,862 ± 4,848 (9.4)	46,176 ± 19,852 (22.9)	82,203 ± 26,178 (40.8)
5.2	11,492 ± 1,905 (2.0)	7,615 ± 2,982 (1.3)	12,647 ± 798 (2.2)	19,951 ± 6,568 (3.4)

Figures in parentheses show stimulation index scores. Maximum proliferation of 3-day TCL cultures over a concentration range (0–100 µg/ml purified allergen). Control cultures stimulated with peanut (100 µg/ml) or hazelnut (100 µg/ml) extract. Results expressed as means ± SD for triplicate cultures. Responses where the stimulation index is greater than 2.5 are bolded.

Table 5. Maximum IL-5 and IFN-γ concentrations in supernatants from hazelnut-specific TCL following stimulation with hazelnut, peanut and HDM extracts

T cell line	Hazelnut		Peanut		HDM	
	IL-5	IFN-γ	IL-5	IFN-γ	IL-5	IFN-γ
<i>Concentration, pg/ml</i>						
1.3	932	1,730	54	513	0	114
2.3	1,957	1,289	305	286	14	63
3.3	1,144	368	57	107	2	0
4.3	2,549	1,180	1,162	822	2	7
5.3	79	326	70	237	0	140

partly attributable to the major peanut allergens, Ara h 1 and Ara h 2, which induced proliferation in 4 of the 5 hazelnut-specific T cell lines. The specificity of the hazelnut-specific T cell responses to peanut allergens was confirmed by the lack of stimulation of HDM-specific T cell lines by the peanut allergens.

In this study, stimulation with peanut extract induced significant proliferation in hazelnut-specific T cell lines from each of the 5 subjects tested, suggesting that a substantial frequency of hazelnut-specific T cells recognize shared T cell epitopes between hazelnut and peanut proteins. Thus, T cell as well as IgE cross-reactivity between peanut and hazelnut [13] may contribute to the high incidence of co-sensitization to hazelnut commonly observed in peanut-allergic subjects. Given that the proliferative responses of hazelnut-specific T cell lines to peanut stimulation were lower than those induced by hazelnut, it is likely that only a proportion of the T cells in these lines were cross-reactive to peanut. It could also be that the cross-reactive T cells were more efficiently

stimulated by specific epitopes within hazelnut than the shared epitopes within peanut.

The sequence homology of allergens may contribute to multiple food sensitivities through cross-reactive T cells. T cell cross-reactivity has been observed between the major allergens of birch pollen (Bet v 1) and apple (Mal d 1) [28]. Bet v 1 and Mal d 1 share approximately 64.5% sequence identity, with the cross-reacting T cell-reactive peptide fragments sharing 50% sequence identity. However, other studies have not demonstrated T cell cross-reactivity between allergens with a similar degree of sequence identity, as was the case for the major latex allergens, Hev b 1 and Hev b 3, despite 47% sequence identity [29]. In light of these studies, and given that single amino acid substitutions can lead to loss of T cell reactivity [30–32], it is likely that shared key amino acid motifs are more important for T cell cross-reactivity than whole epitope sequences.

In this study, T cell cross-reactivity between hazelnut and peanut was partly attributed to major peanut allergens, Ara h 1 and Ara h 2. As some hazelnut allergens belong to the same protein families as these peanut allergens, sequence homology between these proteins are likely. Indeed, the major hazelnut allergens Cor a 11 and Ara h 1 are both 7S vicilin-like proteins with conserved sequences and shared specific IgE-binding epitopes [18]. Ara h 2, a 2S albumin protein [33], could potentially be a homologue of the 32-kDa 2S albumin protein identified as allergenic in hazelnut [34], but the latter has not been sequenced and there are no reports of shared epitopes. Interestingly, not all of the hazelnut-specific T cell lines responded to Ara h 1 and Ara h 2 stimulation, despite all lines demonstrating a significant proliferative response to peanut. Furthermore, for two hazelnut-specific T cell lines combined responses to Ara h 1 and Ara h 2 were

substantially lower than the response to whole peanut extract. This suggests that other peanut allergens could contribute to T cell cross-reactivity between peanut and hazelnut, also supported by the immunoblotting data demonstrating serum IgE reactivity to other peanut proteins. Candidate cross-reactive allergens include Cor a 9 and Ara h 3, members of the legumin or 11S globulin family of seed storage proteins [35–37], which according to our Genbank sequence comparisons using the BLAST program (www.ncbi.nlm.nih.gov/blast/) share 44% identity and 60% homology. Similarly, Cor a 1.04 and Ara h 8 are both members of the pathogenesis-related protein family 10 [38, 39] and are lipid transfer proteins, allergenic in both hazelnut and peanut [40–42]. Cross-reactive carbohydrate determinants can contribute to IgE cross-reactivity between peanut and rye grass pollen allergens [43], and T cell cross-reactivity between non-allergen antigens may occur via carbohydrate side chains [44]. Ara h 1 and Ara h 2 are glycoproteins, but whether or not carbohydrate side chains of hazelnut- and peanut-allergenic glycoproteins contribute to T cell cross-reactivity remains to be elucidated.

This study confirms and extends our earlier preliminary observation of T cell cross-reactivity between ha-

zelnut and peanut [15], and provides further characterization of cross-reactive T cells in co-allergic subjects. Proliferating cross-reactive oligoclonal T cells were predominantly CD4+ with substantial production of the Th2-type cytokine IL-5, suggesting their potential role in the pathogenesis of shared peanut and hazelnut allergy. The additional detection of the Th1 cytokine IFN- γ as part of this response is not surprising, having also been described in T cell responses to other food allergens, including milk and egg [45, 46]. In conclusion, our findings provide clear evidence for a mechanistic role of cross-reactive T cell responses in the development of co-sensitization to hazelnut and peanut. Future studies are required to define the fine specificity of the cross-reactive T cell epitopes.

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