

The Effect of the Food Matrix on *In Vivo* Immune Responses to Purified Peanut Allergens

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There is little knowledge about the factors that determine the allergenicity of food proteins. One aspect that remains to be elucidated is the effect of the food matrix on immune responses to food proteins. To study the intrinsic immunogenicity of allergens and the influence of the food matrix, purified peanut allergens (*Ara h 1*, *Ara h 2*, *Ara h 3*, or *Ara h 6*) and a whole peanut extract (PE) were tested in the popliteal lymph node assay (PLNA) and in an oral model of peanut hypersensitivity. In the PLNA, peanut proteins were injected into the hind footpad of BALB/c mice; in the oral exposure experiments C3H/HeOuJ mice were gavaged weekly with PE or allergens in the presence of cholera toxin (CT). Upon footpad injection, none of the allergens induced significant immune activation. In contrast, PE induced an increase in cell number, cytokine production, and activation of antigen-presenting cells. Furthermore, the presence of a food matrix enhanced the immune response to the individual allergens. Oral exposure to the purified allergens in the presence of CT induced specific IgE responses, irrespective of the presence of a food matrix. These results suggest that purified peanut allergens possess little intrinsic immune-stimulating capacity in contrast to a whole PE. Moreover, the data indicate that the food matrix can influence responses to individual proteins and, therefore, the food matrix must be taken into account when developing models for allergenic potential assessment.

Key Words: peanut allergens; food matrix; mouse model; immunogenicity.

INTRODUCTION

Food allergy has emerged as a major health problem in westernized countries, and its prevalence is increasing (Sicherer *et al.*, 2003). Type I food allergy is characterized by the production of protein-specific serum IgE antibodies that can reside on mast cells. Upon re-exposure to the offending food, cross-linking of the IgE antibodies provokes degranula-

tion of mast cells and release of mediators, leading to a variety of symptoms.

In the past decade, considerable research has been conducted to characterize the food proteins that are responsible for allergic reactions. Knowledge of the general properties of proteins in relationship to their allergenicity is needed to assess the allergic potential of novel (including genetically modified) foods and foods produced by new processing techniques (Ladics *et al.*, 2003; Maleki, 2004; Metcalfe, 2003).

Foods are composed of proteins, carbohydrates, lipids, and micronutrients. Major food allergens, in general, are glycoproteins ranging from 10 to 70 kDa that are abundant in the food and that possess multiple IgE binding epitopes (Bannon, 2004). To be able to induce allergic sensitization, allergens have to be absorbed through the gut epithelial barrier in an immunologically intact form (Taylor *et al.*, 1987). Accordingly, food allergens are generally thought to be resistant to food-processing methods and digestion (Astwood *et al.*, 1996). However, several food allergens have been identified that are sensitive to these processes; in addition, many non-allergenic proteins are as resistant to degradation as allergenic proteins (Fu, 2002). At present, the sum of properties that renders a food protein allergenic is unknown.

One aspect of food allergens that remains to be elucidated is the influence of the food matrix on the immune responses to food proteins. It has been hypothesized that the food body, consisting of fats, carbohydrates, and other proteins, may affect allergenic potential of proteins (Teuber, 2002). For example, high allergenic foods such as tree nuts and peanuts have medium protein concentrations but high levels of fat (Lehrer *et al.*, 2002). The fats may protect proteins during the digestion process, or they may influence the activation of immune cells, which may result in enhanced allergenicity of a protein. These considerations prompted us to investigate the contribution of the food matrix to immune responses against peanut allergens.

Peanut allergy accounts for the majority of severe food allergic reactions; it tends to be persistent, and trace amounts of peanut can induce allergic responses (Al-Muhsen *et al.*, 2003).

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Several peanut proteins belonging to different families of seed storage proteins have been identified by immunoblotting as major or minor allergens, and seven of them have been characterized and officially termed *Ara h 1–7* (Kleber-Janke *et al.*, 1999). We have recently shown that allergen-specific antibody and cytokine responses can be determined after oral or ip exposure to a whole peanut extract (PE) (van Wijk *et al.*, 2004).

To study the intrinsic immunogenicity and adjuvanticity of purified peanut allergens versus a whole peanut protein extract, and to reveal the effect of the food matrix, two different peanut protein extracts (with or without fat), several purified major peanut allergens (*Ara h 1*, *Ara h 2*, and *Ara h 3*), and one minor allergen (*Ara h 6*) were used in the current experiments.

To determine the intrinsic immune-stimulating capacity of the purified allergens compared to the whole peanut extract, the popliteal lymph node assay (PLNA), an established model to test immune stimulation was used. After injection of 1 mg PE or purified allergen, PLN cell numbers, cytokine production, and expression of costimulatory molecules (CD80, CD86, and CD54), a measure for antigen-presenting cell (APC) activation, were determined. Data derived from this study show an important role of the PE food matrix (irrespective of the presence of fat) in activating immune cells, thereby driving immune responses to peanut proteins.

The effect of the food matrix on IgE responses against peanut allergens was also examined, because the elicitation of specific IgE responses is one of the absolute requirements for food allergens to induce immediate hypersensitivity responses. This effect was studied in an established mouse model of peanut hypersensitivity, using the relevant oral route of exposure in combination with a mucosal adjuvant, cholera toxin (CT). All purified peanut allergens induced IgE-specific serum antibody responses with only little additional effect of the food matrix. From these studies we concluded that purified peanut allergens possess little intrinsic adjuvanticity in contrast to whole PE. Furthermore, the results suggest that the food matrix can influence responses to individual proteins.

MATERIALS AND METHODS

Mice. Female, specific pathogen-free BALB/c mice (6 weeks of age) and C3H/HeOuJ Ico mice (4 weeks of age), bred on a SM R/M-Z CRL VRF1 diet (ssniff Spezialdiäten GmbH, Germany) were purchased from Charles River (Lyon, France). Mice were maintained under barrier conditions in filter-topped Macrolon cages with wood chip bedding, at mean temperature of $23^{\circ} \pm 2^{\circ}\text{C}$, relative humidity of 50–55%, and a 12-h light/dark cycle. Drinking water and standard laboratory food pellets (rat and mouse breeder and grower, Special Diet Services, Essex, England) were provided *ad libitum*. No peanut proteins were present in the diets. The experiments were approved by the animal experiments committee of the faculty of veterinary medicine, Utrecht University.

Chemicals and reagents. Chemicals were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands) unless stated otherwise. For cytokine measurements, IL-2, IL-4, and IFN- γ capture and detecting antibodies, and an IL-10

ELISA kit were obtained from BD Pharmingen (Hamburg, Germany); and streptavidin–horseradish peroxidase (HRP) was from Sanquin (Amsterdam, The Netherlands). BD Pharmingen was the supplier of rat anti-mouse IgE, and the Digoxigenin (DIG)-coupling kit was obtained from Boehringer Mannheim (Mannheim, Germany); anti-DIG-peroxidase Fab fragments were from Roche Diagnostics (Mannheim, Germany). Alkaline phosphatase (AP)-conjugated goat anti-mouse, human adsorbed IgG1 and IgG2a were purchased from Southern Biotechnology Associates (Birmingham, AL). Campina Melkuni (Eindhoven, The Netherlands) was the supplier of milk powder used in blocking buffer. Medium used for the *in vitro* restimulation was complete RPMI 1640 with Glutamax-I (Life Technologies, Paisley, Scotland) supplemented with 10% fetal calf serum (ICN Pharmaceuticals, Costa Mesa, CA) and 2% penicillin-streptomycin.

Preparation of peanut extract and purification of allergens. Peanuts from the Runner cultivar (Cargill, Dawson, GA) were kindly provided by Imko Nut Products (Doetinchem, The Netherlands). The PE was prepared as described by Koppelman *et al.* (2003). Briefly, peanuts were ground and the protein was extracted by mixing 25 g ground peanut with 200 ml 20 mM Tris buffer (pH 7.2). After this mixture was stirred for 2 h at room temperature, the aqueous fraction was collected by centrifugation ($3000 \times g$ for 30 min) and subsequently centrifuged at $10,000 g$ for 30 min to remove residual traces of fat and insoluble particles. The extract consisted of 4.3% protein, 0.2% fat, 0.5% carbohydrates, 0.2% ash, and 94.8% water, as determined by Kjeldahl analysis and acid hydrolysis. For the preparation of fatty PE (PE fat), fat was not removed after centrifugation, resulting in a PE–oil emulsion consisting of 3.6% protein, 3.5% fat, 0.4% carbohydrates, 0.2% ash, and 92.3% water. Protein concentration (PE 30 mg/ml and PE fat 25 mg/ml) was determined with a BCA protein assay kit (Pierce, Rockford, IL). Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of PE and PE fat confirmed the presence of proteins migrating at the same molecular weights as the purified allergens *Ara h 1* (14%), *Ara h 2* (6.5%), *Ara h 3* (50%), and *Ara h 6* (4.5%), and the two extracts showed a similar protein pattern (data not shown). Their relative amounts (expressed as percentage of total protein) were quantified by densitometry as previously described (Koppelman *et al.*, 2001). Purification of the allergens *Ara h 1*, *Ara h 2*, and *Ara h 3* was performed according to previously described methods (de Jong *et al.*, 1998; Koppelman *et al.*, 2003), and *Ara h 6* was similarly purified from a side-fraction. Endogenous endotoxin content was measured by Limulus Amebocyte Lysate assay (Bio Whittaker, Walkersville, MD) and was $<0.02 \mu\text{g LPS/mg protein}$ ($\leq 200 \text{ EU/mg protein}$) for all purified allergen and PE solutions.

PLNA protocol. For the PLNA, a standard protocol was used as previously described (Blokma *et al.*, 1995). Pilot studies were performed to determine the optimal concentration of PE that also served as a positive control. Different doses of purified allergens (0.1 mg, 0.5 mg, and 1 mg) were tested, and no differences were found between the different dosing groups for all parameters measured. The findings for the 1-mg dose group are presented in the *Results*. Accordingly, BALB/c mice ($n = 4$) were subcutaneously injected into the hind footpad with 1 mg of protein (PE, PE fat, *Ara h 1*, *Ara h 2*, or *Ara h 6*) dissolved in 50 μl sterile NaCl, and control animals were injected with 50 μl vehicle. *Ara h 3* could not be dissolved at this concentration and was therefore not included in the PLNA. Seven days after protein injection, mice were killed by cervical dislocation, and the draining popliteal lymph nodes (PLN) were excised. Popliteal lymph node single-cell suspensions were prepared under aseptic conditions in complete RPMI 1640 and adjusted to 2.5×10^6 cells/ml.

Oral treatment protocol. C3H/HeJ mice ($n = 6$) were orally exposed to the purified allergens *Ara h 1*, *Ara h 2*, *Ara h 3*, *Ara h 6* (0.1 and 1 mg), PE (1 and 6 mg), or PE fat (6 mg) in the presence of the mucosal adjuvant CT. Oral exposure was performed by intragastric dosing of protein on 3 consecutive days, followed by weekly dosing (4 weeks). Cholera toxin (10 μg) was coadministered on days 1, 2, 3, 8, 15, and 21. At day 30, mice received a double dose of protein. Mice were killed by cervical dislocation 31 days after the onset of exposure.

Cell culture and cytokine measurement. Popliteal lymph node single-cell suspensions ($150\ \mu\text{l}$ of 2.5×10^6 cells/ml in complete RPMI 1640) were incubated in the presence of PE (200 $\mu\text{g/ml}$), *Ara h 1* (200 $\mu\text{g/ml}$), *Ara h 2* (200 $\mu\text{g/ml}$), *Ara h 6* (200 $\mu\text{g/ml}$), Con A (5 $\mu\text{g/ml}$), or medium alone in 96-well plates (Costar, Cambridge, MA) for 96 h (Con A 24 h) at 37°C , 5% CO_2 . After centrifugation for 10 min at $150 \times g$, supernatant was collected and stored at -20°C until analysis.

In the culture supernatants, IL-2, IFN- γ , IL-4, and IL-10 levels were determined by sandwich ELISA. Plates (highbond 3590) were coated overnight with 1 $\mu\text{g/ml}$ rat anti-mouse IL-2, IL-4, or IFN- γ , and the following day plates were blocked with PBS-Tween/3% milk powder for 4 h at room temperature. Samples and cytokine standards were added in several dilutions and incubated overnight at 4°C . Plates were incubated with 0.25 $\mu\text{g/ml}$ rat anti-mouse IL-2, IL-4, or IFN- γ conjugate for 1 h at room temperature, followed by streptavidin-HRP incubation for 45 min. Finally, tetramethylbenzidine (TMB)-substrate (0.1 mg/ml) was added, and the color reaction was stopped with 2 M H_2SO_4 . Absorbance was measured at 450 nm. The IL-10 ELISA was performed in accordance with the manufacturer's instructions.

Flow cytometry. For flow cytometric analysis, 2×10^5 PLN cells were incubated with 50 μl of predetermined dilutions of fluorescein isothiocyanate (FITC)-, CyChrome (CY)-, and biotin-conjugated monoclonal antibodies in 96-well plates (30 min darkness at 4°C). Cells were characterized based on the following monoclonal antibodies: CD54 FITC (3E2), CD80 FITC (16-10A1), CD86 FITC (GL1), isotype controls for CD80 (hamster IgG2 FITC), and CD86 (Rat IgG2a FITC) and MHC-II biotin (NIMR-4), all obtained from BD Pharmingen. After incubation with biotin-conjugated monoclonal antibodies, cells were washed and incubated with streptavidin-CY (BD Pharmingen) in the same way. Finally, cells were washed, stored in formalin (0.1%), and analyzed within 18 h on a FACScan with standard FACSflow using CellQuest software (BD Biosciences, Franklin Lakes, NJ). Cells expressing CD80, CD86, or CD54 were checked for MHC-II expression to assure antigen-presenting capability.

Measurement of serum IgE antibodies. Blood of orally exposed mice was collected at weekly intervals, and levels of PE and *Ara h*-specific IgE were measured by sandwich ELISA. Plates (highbond 3590; Costar, Cambridge, MA) were coated overnight with 1.5 $\mu\text{g/ml}$ purified rat anti-mouse IgE in carbonate buffer (pH 9.6), followed by 1 h of blocking (37°C) with PBS-Tween/3% milk powder. Each test serum was titrated starting at 1:8 dilution and incubated for 2 h (37°C). A pre-sera pool was used as reference value (dilution 1:4). Subsequently a PE- or *Ara h*-DIG conjugate solution was added (1 h at 37°C). The coupling of DIG to PE or the *Ara h*'s at an optimal labeling efficiency was performed according to the manufacturer's instructions. Briefly, the coupled proteins were separated on a Sephadex G-25 column, and the concentration of coupled protein was determined spectrophotometrically at 280 nm. After incubation (1 h at 37°C) with peroxidase-conjugated anti-DIG fragments, a TMB-substrate (0.1 mg/ml) solution was used, and the color reaction was stopped with 2 M H_2SO_4 . Absorbance was measured at 450 nm using an ELISA reader ELX800 (BIOTEK Instruments, Winooski, VT). The reciprocal of the furthest test serum dilution resulting in an extinction higher than the reference value was read as a titer. Background values were <0.100 (OD).

Measurement of serum IgG1 and IgG2a antibodies. Blood was collected at weekly intervals, and levels of PE-specific antibodies were measured by ELISA. Plates (highbond 3590; Costar, Cambridge, MA) were coated overnight with 20 $\mu\text{g/ml}$ PE in carbonate buffer (pH 9.6), followed by 1 h of blocking (37°C) with PBS-Tween/3% milk powder. Each test serum was titrated starting at 1:8 dilution and incubated for 1 h (37°C). A pre-sera pool was used as reference value (dilution 1:4). After incubation, AP-conjugated antibodies were added (1 h at 37°C). Subsequently 1 mg/ml p-nitrophenylphosphate in diethanolamine buffer was used for the color reaction, which was stopped with a 10% EDTA solution, after which absorbance was measured at 405 nm using an ELISA reader ELX800 (BIOTEK Instruments, Winooski, VT). Background values were <0.100 (OD).

Statistics. Multiple comparisons of group means were performed using one-way analyses of variance (ANOVA) with Bonferroni as a *post hoc* test. For PLN cell numbers, cytokine levels, and serum antibody levels, statistical analysis was performed following logarithmic transformation (to achieve normal distribution), whereas the levels of cell surface molecules were untransformed. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Peanut Extract, But Not Purified Allergen, Induces PLN Cell Proliferation

To examine the intrinsic immune stimulating capacity and adjuvant activity of purified peanut allergens versus whole PE, the PLNA was used. Mice were injected with 1 mg of protein in the hind footpad, and at day 7 total cell numbers of the draining PLN were determined (Fig. 1). After injection with PE fat or PE, total lymph node cell numbers increased significantly (fourfold), and no difference was found between the PE fat- and PE-treated groups. In contrast, no cell proliferation was observed after injection with the purified peanut allergens *Ara h 1*, *Ara h 2*, or *Ara h 6*.

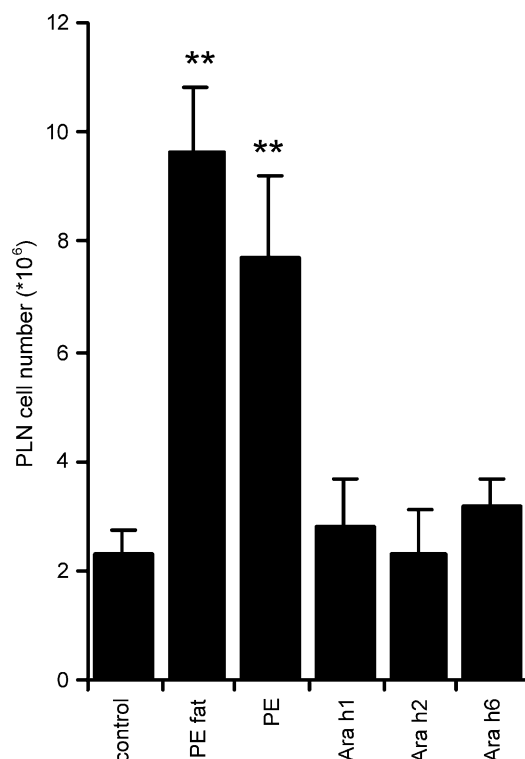


FIG. 1. Total cell numbers in the PLN. Mice were injected in the hind footpad with vehicle alone (control) or with 1 mg of protein (PE fat, PE, *Ara h 1*, *Ara h 2*, or *Ara h 6*) dissolved in NaCl. At day 7, the draining lymph nodes were isolated and cells were counted. Levels are expressed as group means $\pm$ SD of four mice per group. **Significantly different ($p < 0.01$) from the control group.

Purified Allergen Treatment Fails to Induce Cytokine Production in Con A Restimulated PLN Cell Cultures

To reflect the general activation of T cells, PLN cells were cultured for 24 h in the presence of concanavalin A (Con A), and the concentrations of the Th2-associated cytokines IL-4 and IL-10, and of the Th1-associated cytokine IFN- γ , were measured in the supernatant (Fig. 2). Popliteal lymph node cells derived from PE- and PE fat-treated mice secreted similar high amounts of IL-4, IL-10, and IFN- γ compared to cells derived from control mice. No Th2 or Th1 bias was observed in either of the PE treatment groups.

With respect to the purified allergens, no significant IL-10 or IFN- γ cytokine production was found in PLN cell cultures of *Ara h 1*-, *Ara h 2*-, and *Ara h 6*-treated groups, and only *Ara h 6*-injection resulted in significant IL-4 production upon restimulation with Con A.

Cytokine Responses Induced by Restimulation with Purified Allergen Are Enhanced by the Presence of a Food Matrix at the Time of Injection

To examine antigen-specific T cell responses, PLN cells were obtained 7 days after the initial injection with 1 mg of PE, PE fat, or purified allergen and cultured for 96 h in the presence of PE, *Ara h 1*, *Ara h 2*, *Ara h 6*, or medium alone. Th1-associated (IL-2 and IFN- γ) and Th2-associated (IL-4 and IL-10) cytokine production was measured in the supernatant (Table 1).

Popliteal lymph node LN cell cultures derived from PE- and PE fat-treated mice restimulated with PE induced high levels of IL-2, IL-4, IL-10, and IFN- γ . No significant differences in cytokine concentration were found between the two groups, and a mixed Th1/Th2 cytokine response was observed.

Regarding the purified allergens, only *Ara h 6* treatment resulted in a low but significantly increased cytokine production (IL-2, IL-4, IL-10, but not IFN- γ), whereas *Ara h 1*- and *Ara h 2*-injection failed to induce considerable cytokine

production in PLN cell cultures when restimulated with the corresponding purified allergen.

Interestingly, cells derived from PE-treated or PE-fat-treated groups secreted higher levels of cytokines than cells derived from *Ara h 1*- and *Ara h 6*-treated animals when restimulated with *Ara h 1* or *Ara h 6*. Restimulation with *Ara h 2* only induced low IL-2 production, and levels of IL-2 were significantly higher in cell cultures of PE-treated and PE-fat-treated mice than in those of *Ara h 2*-treated mice. Popliteal lymph node cells cultured in the presence of medium alone showed no production of any of the cytokines examined (data not shown). These results indicate that T cell responses to purified allergens can be influenced by the food matrix.

Peanut Extracts But Not Purified Allergen Induces Expression of Co-stimulatory Molecules on APC

Activation of antigen-presenting cells (APC) is considered one of the requirements for initiating active immune responses. Therefore, the expressions of the activation markers CD80, CD86, and CD54 were determined on PLN APC, 7 days after protein injection (Fig. 3). Both PE fat and PE injection significantly augmented the percentages of CD80-, CD86-, and CD54-expressing cells in the PLN, whereas no increase was found after injection with *Ara h 1*, *Ara h 2*, or *Ara h 6*.

Together, these data indicate that purified peanut allergens, in contrast to the whole extract, lack intrinsic immune-stimulating capacity and thereby fail to induce significant immune responses in the PLNA.

Purified Allergens Induce Specific IgE Responses upon Oral Exposure, Irrespective of the Food Matrix

To further explore the role of the food matrix in immune responses to peanut proteins, an established oral model of peanut hypersensitivity was used. In this model, PE- and allergen-specific IgE responses can be determined using the

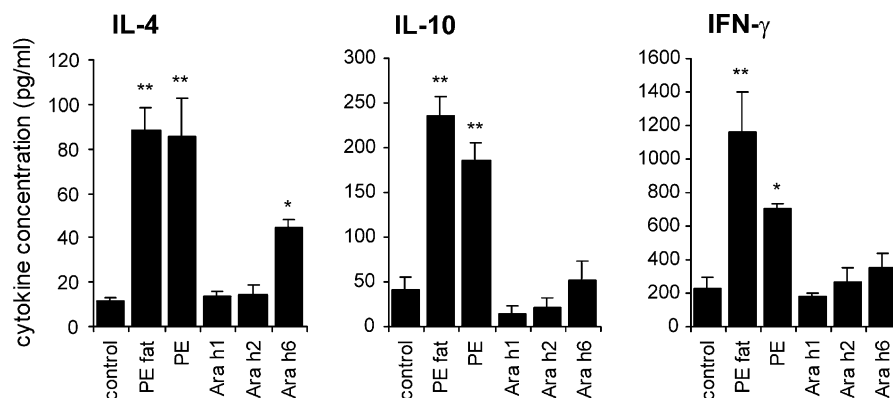


FIG. 2. Cytokine production after *in vitro* restimulation of PLN cells with Con A. Mice were injected in the hind footpad with vehicle alone (control) or with 1 mg of protein (PE fat, PE, *Ara h 1*, *Ara h 2*, or *Ara h 6*) dissolved in NaCl. At day 7, the draining lymph nodes were isolated and cultured for 24 h in the presence of Con A (5 μ g/ml). Culture supernatants were collected and cytokine levels of IL-4, IL-10, and IFN- γ were determined by ELISA. Data are presented as mean cytokine levels $\pm$ SEM of four mice per group. *Significantly different ($p < 0.05$), ** $p < 0.01$) from the control group.

TABLE 1
Ex Vivo Cytokine Production by PLN Cells upon Restimulation (96 h) with Antigen

Treatment group	Restimulation	IL-2 (pg/ml) ^a	IL-4 (pg/ml) ^a	IFN- γ (pg/ml) ^a	IL-10 (pg/ml) ^a
Control		4.33 $\pm$ 0.63	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
PE fat	PE	220.9 $\pm$ 44.8 **	391.2 $\pm$ 27.8 **	650.6 $\pm$ 318.1 **	4785 $\pm$ 1696 **
PE		225.9 $\pm$ 64.2 **	311.1 $\pm$ 62.4 **	511.1 $\pm$ 183.3 **	4315 $\pm$ 1881 **
Control	Ara h 1	6.9 $\pm$ 5.6	0.7 $\pm$ 0.67	1.5 $\pm$ 1.5	0 $\pm$ 0
PE fat		27.5 $\pm$ 4.2 *	43.4 $\pm$ 11.8 **	77.1 $\pm$ 22.1 *	428.1 $\pm$ 142.9 **
PE		21.7 $\pm$ 3.4 *	36.9 $\pm$ 8.2 **	38.0 $\pm$ 14.1	245.7 $\pm$ 101.8 **
Ara h 1		8.9 $\pm$ 0.7	2.7 $\pm$ 0.97 ¶ §	2.1 $\pm$ 0.7 †‡	27.9 $\pm$ 6.7†‡
Control	Ara h 2	6.4 $\pm$ 1.3	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
PE fat		12.1 $\pm$ 1.3 **	1.47 $\pm$ 1.4	0 $\pm$ 0	0 $\pm$ 0
PE		10.8 $\pm$ 0.2 *	0.37 $\pm$ 0.4	0 $\pm$ 0	0 $\pm$ 0
Ara h 2		3.8 $\pm$ 0.6 †‡	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
Control	Ara h 6	3.8 $\pm$ 1.1	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
PE fat		41.4 $\pm$ 10.9 **	59.9 $\pm$ 14.4 **	79.8 $\pm$ 46.2 **	508.2 $\pm$ 96.1 **
PE		40.2 $\pm$ 11.2 **	87.8 $\pm$ 44.1 **	28.1 $\pm$ 15.7 ** †	745.8 $\pm$ 281.9 **
Ara h 6		15.1 $\pm$ 1.3 *	34.2 $\pm$ 2.5 **	0 $\pm$ 0 †‡	221.3 $\pm$ 23.2 **

^aLevels are expressed as group means $\pm$ SEM of four mice per group.

*Significantly different ($p < 0.05$; ** $p < 0.01$) from control.

†Significantly different ($p < 0.05$) from PE fat.

‡Significantly different ($p < 0.05$) from PE.

relevant route of exposure to food proteins (van Wijk *et al.*, 2004). Mice were dosed by gavage for 4 weeks with PE (1 and 6 mg), Ara h 1, Ara h 2, Ara h 3, or Ara h 6 (0.1 and 1 mg) in the presence of the mucosal adjuvant CT.

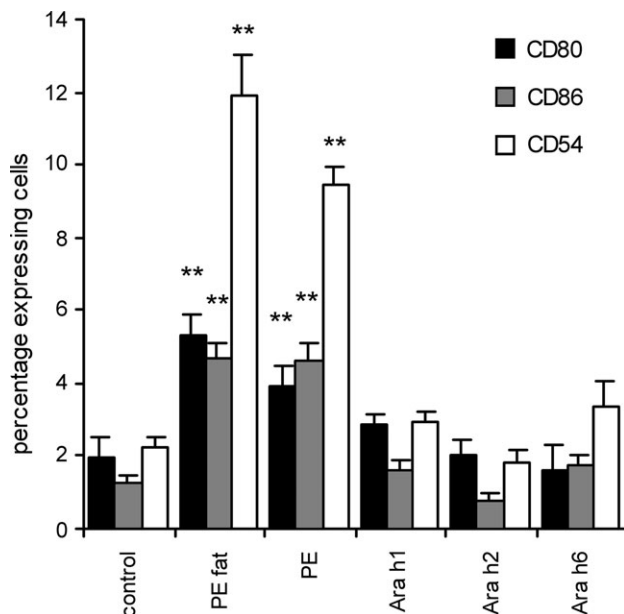


FIG. 3. Expression of the costimulatory molecules on APCs. Mice were injected in the hind footpad with vehicle alone (control) or with 1 mg of protein (PE fat, PE, Ara h 1, Ara h 2, or Ara h 6) dissolved in NaCl. At day 7, the draining lymph nodes were isolated and the expressions of CD80, CD86, and CD54 on MHCII-positive cells were determined by flow cytometric analysis. Data are presented as percentages $\pm$ SD of four mice per group. **Significantly different ($p < 0.01$) from the control group.

All purified allergens were able to induce specific IgE responses, but only for Ara h 1 treatment was a significant difference found between the 0.1 and 1 mg dosing groups (Fig. 4). Exposure to PE (1 mg or 6 mg) also resulted in Ara h-specific IgE responses, with levels comparable to those observed after purified allergen exposure. In the control group (oral exposure to PBS + CT) no Ara h-specific IgE responses were found (data not shown).

No Difference in Antibody Responses upon Oral Exposure to PE or PE Fat

To examine the effect of fat content on orally induced antibody responses to PE, mice were orally exposed to PE fat or PE in the presence or absence of the mucosal adjuvant CT. After 4 weeks of oral exposure, both Th1-associated (IgG2a) and Th2-associated (IgG1 and IgE) PE-specific antibodies were determined in the serum (Fig. 5). In the absence of CT, only low levels of IgG1 and IgG2a, and no IgE, were found, with no differences between the PE fat- and PE-treated group. In the presence of CT, similar high levels of IgG2a, IgG1, and IgE were found in both PE treatment groups. In line with the results found in the PLN, the presence of fat does not affect orally induced immune responses to PE.

DISCUSSION

No definitive characteristics of food allergens have been identified other than that they need to reach and stimulate

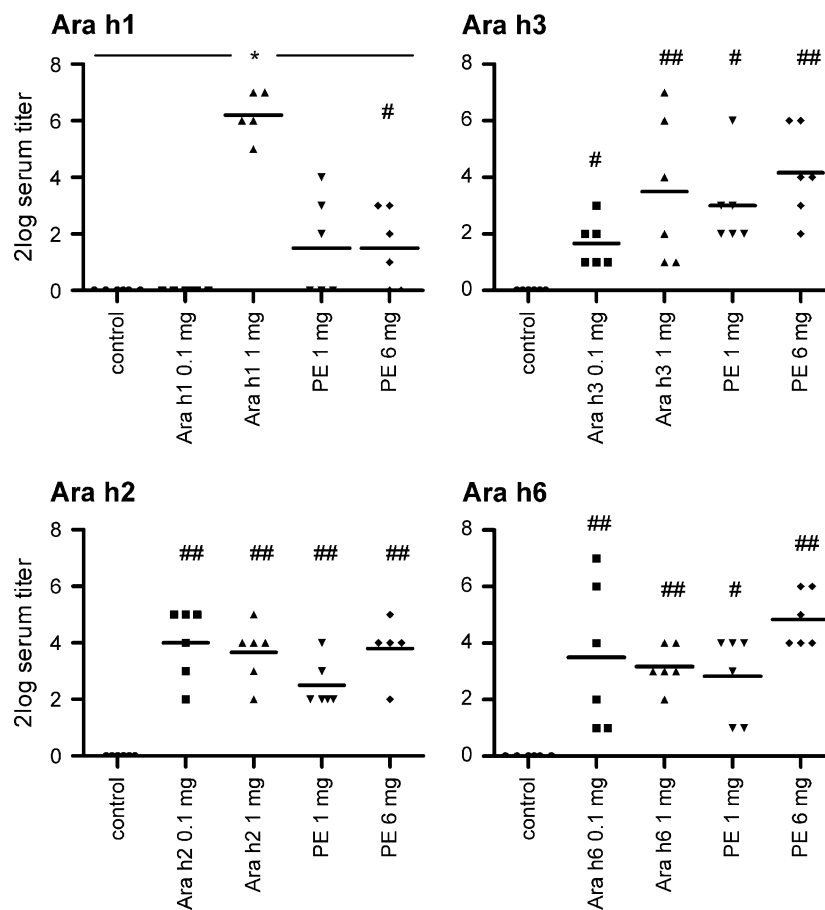


FIG. 4. Ara h-specific IgE serum levels after 4 weeks of oral treatment. Ara h-specific IgE serum levels were determined after 4 weeks of oral exposure to the purified allergens *Ara h 1*, *Ara h 2*, *Ara h 3*, and *Ara h 6* (0.1 and 1 mg) or PE (1 mg and 6 mg) in the presence of CT. The data are presented as the mean $^2\log$ IgE titer of six mice per group (symbols indicate individual animals). #Significantly different from the control group (# $p < 0.05$, ## $p < 0.01$). *Significantly different ($p < 0.05$) from indicated groups.

immune cells in order to induce sensitization. It has been suggested that both properties may be influenced by the food matrix (Teuber, 2002). In the present study we have shown that purified peanut allergens, unlike a whole PE, possess little

intrinsic immune-stimulating capacity and that the immune response to these allergens can be adjuvated by the presence of a food matrix. Furthermore, it was demonstrated that the purified allergens are stable enough to get into contact with the immune system after oral exposure, both in the presence and in the absence of a food matrix.

Although *Ara h 1* and *Ara h 2* are recognized as major peanut allergens, they were unable to induce PLN cell proliferation or cytokine production, suggesting that they possess little intrinsic immune-stimulating capacity. In contrast, PE induced a strong PLNA response (without causing inflammation in the paw) and was also able to adjuvate specific T cell responses against the allergens present in the extract. In line with these results, *Ara h 1* and *Ara h 2* appear to be poor IgG and IgE inducers in mice when injected ip (G. Ladics, Dupont, personal communication), whereas ip PE injection results in considerable *Ara h 1*-specific and *Ara h 2*-specific IgG and IgE responses (van Wijk *et al.*, 2004).

The actual amount of individual allergens in PE and PE fat was always lower than the 1 mg of allergen injected in a purified form. This may raise the issue that the discrepancy

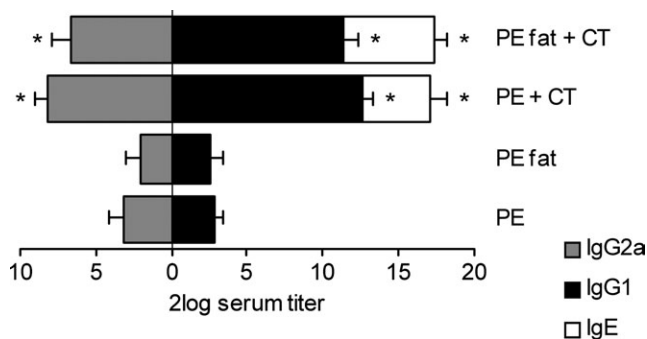


FIG. 5. PE-specific serum antibody levels. PE-specific Th1-associated (IgG2a) and Th2-associated (IgG1 and IgE) serum Ab levels were determined after 4 weeks of oral exposure to PE or PE fat (both 6 mg) in the presence or absence of CT. The data are presented as the mean $^2\log$ antibody titer $\pm$ SEM of six mice per group. *Significantly different from corresponding non-CT-treated group ($p < 0.05$).

in dose has affected the outcome of the immune response and that the high dose injection (1 mg) has induced non-responsiveness. However, injection of 0.1 and 0.5 mg of purified allergen also failed to induce an immune response in the PLNA (data not shown).

The difference in immune-stimulating capacity between the purified allergens and the extract may be explained by selective activation of APC. Generally considered, APC activation and the accompanying upregulation of costimulatory molecules is required for the induction of an active immune response. For contact allergens, which possess high intrinsic adjuvanticity due to their reactive groups (Kapsenberg, 1996), it has been shown that they selectively upregulate costimulatory molecules (e.g., CD54 and CD86) on dendritic cells *in vitro* (Rougier *et al.*, 2000; Tuschl *et al.*, 2000). Furthermore, drugs associated with immune-mediated hypersensitivity reactions were demonstrated to upregulate the expression of costimulatory molecules on APC in the PLNA (Nierkens *et al.*, 2002; Popovic *et al.*, 2004). Interestingly, none of the purified peanut allergens tested was able to increase expressions of costimulatory molecules on APC, whereas PE injection resulted in an upregulation of CD80, CD86, and CD54. Hence, in contrast to sensitizing chemicals, soluble peanut allergens do not possess intrinsic adjuvanticity, and they therefore need an accompanying adjuvant (in the case of PE provided by the food matrix) to be able to activate APC and to induce subsequent immune stimulation.

Peanut consists of proteins, carbohydrates and fatty acids, and all these different components—and their interactions—may be responsible for the adjuvant effect. For instance, it has been described that peanut oils can have an adjuvant effect in a vaccine model (Eghafona, 1996). Remarkably, in the PLNA, the presence of fat did not seem to play a major role in the induction of the immune response (PLN cell numbers, cytokine production, and APC activation). In addition to nutrients, non-nutritive compounds such as lectins (Watzl *et al.*, 2001) and food contaminants such as LPS (Brix *et al.*, 2004) and aflatoxins (Kocabas and Sekerel, 2003) may affect immune responses to food proteins. For future experiments, it would be interesting to test different PE components separately in order to elucidate their immune-modulating properties.

Concerning the relevant route of exposure, the food matrix has been suggested to affect the allergenic properties of orally administered proteins by providing adjuvant stimuli to the specialized gut mucosal immune system or by protecting them from digestion (Lehrer *et al.*, 2002; Teuber, 2002), but to our knowledge no *in vivo* data are available. In general, oral administration (by feeding or gavage) of soluble food proteins to mice results in peripheral hyporesponsiveness (reviewed by Mayer *et al.*, 2001; Nagler-Anderson *et al.*, 2001). Administration of proteins with adjuvants induces a productive immune response to this normally tolerogenic form of antigen (Nagler-Anderson *et al.*, 2001). We have previously shown that, in the present oral model, coadministration of the mucosal adjuvant

CT is indispensable to the induction of antigen-specific IgE responses. Clearly, the intrinsic adjuvant activity of PE (*i.e.*, without fat) is not sufficient to induce sensitization via the oral route (van Wijk *et al.*, 2005). Consequently, it might be impossible to determine the adjuvant activity of the food matrix itself when CT is coadministered. Yet, in the current study, the presence of fat did not affect the magnitude or polarization (type 1 versus type 2) of the antibody responses against PE, irrespective of the presence of CT (Fig. 5). Fatty acids have been described as immune modulators following high-dose oral supplementation (Harbige and Fisher, 2001; Wu 2004), covalent coupling to a protein or peptide (Oliveira *et al.*, 2002; BenMohamed *et al.*, 2002), or the use of liposome-entrapped proteins (Sehra *et al.*, 1998), but it remains to be elucidated whether fats present in a normal diet have similar immune-modulating properties.

Regarding the aspect of digestion, the present results indicate that peanut allergens are at least stable enough to reach the gut-associated lymphoid tissue, even without the presence of a protecting food matrix. Although it has been shown *in vitro* that polysaccharide–protein and protein–protein interactions may influence allergen digestion (Fremont *et al.*, 2004; Maleki *et al.*, 2003), these interactions seemed not to affect the immune response to allergens in the present *in vivo* model. Our data are in accordance with *in vitro* data showing that *Ara h 1* and *Ara h 2* are highly resistant to enzymatic digestion (Maleki *et al.*, 2000; Sen *et al.*, 2002). Furthermore, treatment of purified *Ara h 1* and PE with pepsin resulted in the production of nearly identical sets of digestion products, suggesting an intrinsic stability of *Ara h 1* (Kopper *et al.*, 2004). The current results demonstrate that the major peanut allergens *Ara h 1*, *Ara h 2*, *Ara h 3*, and the minor allergen *Ara h 6* are stable enough *in vivo* to elicit specific IgE responses.

In recent years there has been considerable interest in the development of experimental animal models to predict the allergenicity of proteins. Our data suggest that assays designed to measure immune stimulation, such as the PLNA, are clearly not suitable to identify allergenic proteins when injected in a purified form, because they do not appear to possess high intrinsic immune-stimulating capacity. Because PE injection did result in immune stimulation, further studies may reveal the feasibility of subcutaneous injection with whole food extract as a tool for identifying the adjuvating capacity of compounds within a food matrix.

After oral exposure, all purified peanut allergens were shown to be IgE inducers, which confirms that they possess IgE epitopes, and that they are able to reach the immune system of the gut in a relatively intact form, two qualities required for the induction of allergic sensitization. However, for the future use of this model as a screening tool, other allergens and especially non-allergens need to be tested. Recently, Gaudry *et al.* (2004) have examined several food allergens and non-allergens in a similar oral model and have shown that mice and humans respond similarly to major allergens and non-allergens.

Although a broader panel of (non)allergens should be tested and the use of an adjuvant remains a topic of debate, the measurement of IgE antibody responses following oral exposure to proteins seems to be a promising tool for predicting allergenicity of these proteins. Furthermore, the oral route represents the most adequate route of exposure to study possible *in vivo* digestion and food matrix effects.

In summary, our results indicate that purified peanut allergens possess little intrinsic immune-stimulating capacity and that they probably need co-localization of an adjuvant compound to induce sensitization. Moreover, this study shows that the food matrix may affect immune responses to individual allergens; as a consequence, a possible influence of the food matrix has to be considered in the development of animal tests to predict allergenic potential.

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