

ORIGINAL ARTICLE

Polyphenol-enriched apple extract attenuates food allergy in mice

A. W. Zuercher, S. Holvoet, M. Weiss and A. Mercenier

Allergy Group, Department of Nutrition and Health, Nestle Research Center, Lausanne, Switzerland

Clinical & Experimental Allergy

Summary

Background The immune system may be modulated with nutrition to prevent the development or to treat the symptoms of allergy. Among other foods, consumption of apples has been linked to reduced incidence of atopic dermatitis and respiratory allergy.

Objective We evaluated the efficacy and mechanisms of a polyphenol-enriched apple extract in reducing symptoms of food allergy.

Methods In a model of food allergy to ovalbumin (OVA), BALB/c mice were fed with an apple extract either during sensitization or just before the challenge. After the challenge, allergic symptoms were scored, OVA-specific serum immunoglobulins were determined by ELISA, cytokine production by mesenteric lymph node (MLN) cells was measured by a multiplex assay and gene expression profiles in the intestine were addressed using quantitative real-time PCR.

Results Consumption of the apple extract reduced symptoms of food allergy upon challenge. This was paralleled by reduced levels of intestinal mast cell protease, diminished cytokine secretion by MLN cells and reduced local intestinal mRNA expression of various T-helper type-2 associated and pro-inflammatory genes. Mechanistic studies suggested decrease of mediator release by effector cells and reduction of allergenicity by protein–polyphenol interaction as potential mechanisms responsible for protection.

Conclusion Polyphenol-enriched apple extract can attenuate food allergy symptoms in sensitized mice via two distinct possible mechanisms.

Keywords apple extract, food allergy, mice, polyphenols

Submitted 5 August 2009; revised 6 November 2009; accepted 9 December 2009

Correspondence:

Dr Annick Mercenier, Allergy Group, Department of Nutrition and Health, Nestle Research Center, Vers-chez-les-Blanc, CH-1000 Lausanne 26, Switzerland.

E-mail:

annick.mercenier@rdls.nestle.com

Introduction

Nutritional intervention to prevent the development or to manage the symptoms of allergic diseases has been assessed in numerous pre-clinical and clinical settings. Typically, the protective effects of probiotic bacteria [1], prebiotics [2] or n-3 poly-unsaturated fatty acids (e.g. fish oil) [3] have been evaluated. Another group of ingredients that might have anti-allergic potential are dietary polyphenols. They are naturally occurring, reducing agents and thus fulfil an important role in preventing tissue damage due to oxidative stress. Several thousand polyphenols have been described in plants, but they can be grouped into some major classes, such as flavonoids, phenolic acids, stilbenes and lignans (reviewed in [4]). They are present in many fruits and vegetables and often the polyphenol profile is characteristic of a particular plant species. For example, apples have a unique polyphenol profile consisting of high levels of procyanidins,

catechins, flavonols (such as quercetin) and chlorogenic acid [5].

In vitro studies with primary human mast cells or with mast cell lines such as rat basophilic leukaemia (RBL)-2H3 showed that crude apple polyphenols or complex apple polyphenols [so-called apple condensed tannins (ACT)] inhibited the release of mediators, including histamine or leukotriene [6]. The effect seemed to be mediated by inhibition of FcεRI down-stream signalling and by interference with IgE–FcεRI interaction [7]. Similar results were obtained with purified quercetin [8, 9] or with purified procyanidin C1 [10]. In addition, one *in vitro* study established that quercetin stimulated IFN-γ and inhibited IL-4 secretion from human peripheral blood mononuclear cells (PBMCs) [11]. In animal models, it was shown that a quercetin-enriched diet attenuated respiratory symptoms in sensitized guinea-pigs [12]. Similarly, consumption of apple polyphenols reduced anaphylaxis and allergen-specific serum IgE antibody levels in

ovalbumin (OVA)-sensitized mice [13]. In pilot human intervention studies, it was shown that consumption of apple-condensed tannins over a period of 8 weeks reduced symptoms of atopic dermatitis/eczema [14]. Likewise, apple polyphenols attenuated symptoms of Japanese cedar pollinosis when consumed before and during the pollen season [15] as well as symptoms of perennial, house-dust mite-induced allergic rhinitis [16]. Finally, epidemiological and population-based case-control studies in Finland [17], the Netherlands [18] and the United Kingdom [19] have demonstrated a protective effect of quercetin/apple consumption on asthma incidence. In contrast to all the above findings, a potential role of apple polyphenols in the management of food allergy symptoms has not been studied to date.

Here, using a mouse model, we addressed whether apple-polyphenols could impact on symptoms of food allergy. Consumption of apple extract (1% w/w in powdered food) by sensitized mice attenuated clinical symptoms upon challenge. This was paralleled by decreased intestinal mediator release and reduced expression of T-helper type-2 (Th2)-associated genes in the ileum, including IL-5, IL-13 and CCL11. *In vitro* analyses suggested that inhibition of mast-cell degranulation and reduction of allergenicity of OVA through allergen-polyphenol interactions might be mechanisms conferring the observed effects.

Materials and methods

Materials

Apple extract Pomactiv HFV was purchased from Val de Vire Bioactives (Condé sur Vire, France). It is a water extract of cider apples, further enriched in polyphenols (> 40%), particularly flavonols (20–35% of total polyphenols) through a one-step alcohol extraction. The extract has a low protein content (batch used in this study: 2.3% protein) and contains fibres (<55%). Apple extract POMM9050ZZ was purchased from Diana Naturals (Antrain, France).

Ovalbumin food allergy model

The model used in this study was first described by Perrier et al. [20]. All studies were approved by a Nestec Internal Ethics Committee and the Service Vétérinaire of the Canton of Vaud, Switzerland (Authorization #1970). Briefly, 6-week-old conventional female BALB/c mice (10–15 animals/group) (Harlan Laboratories, Lyon, France) were sensitized via the oral route by a gavage at weekly intervals with 20 mg of OVA (Fluka; Sigma, Buchs, Switzerland)+10 µg/mouse of Cholera toxin (CT) as an adjuvant (List Biological Laboratories, Campbell, CA, USA) for 7 weeks. At the end of the sensitization period, an oral challenge with 100 mg of OVA was performed.

A nutritional intervention with apple extract (1% w/w) mixed with powdered mouse chow pellets (Kliba Nafga, Kaiseraugst, Switzerland) was performed either for primary prevention during the sensitization period (day 1–42) or for secondary prevention starting at the end of the sensitization phase (day 42–49) (schematic representation in Fig. 1). Starting 30 min after the challenge, mice were individually observed for 30 min. The allergic score was calculated as follows: 0: no symptoms, <4 episodes of scratching; 1: 4–10 episodes of scratching around the nose and head, no diarrhoea; 2: >10 episodes of scratching or bristled fur and immobility or soft stool; 3: diarrhoea or laboured respiration or cyanosis; 4: diarrhoea in combination with immobility after prodding, bristled fur, laboured respiration or cyanosis; and 5: anaphylaxis. Four hours after the challenge, mice were killed, and blood, mesenteric lymph nodes (MLN), the last centimetre of ileum and one Peyer's patch (PP) located in the same area were isolated.

Quantification of serum levels of mouse mast-cell protease 1

Mouse mast-cell protease 1 (MMCP-1) was quantified in mouse serum by ELISA, purchased from Moredun Scientific (Penicuik, Scotland) according to the manufacturer's instructions. The MMCP-1 concentration was obtained by converting OD values in pg/mL using a polynomial standard curve.

Quantification of serum levels of ovalbumin-specific immunoglobulin E

Specific IgE concentrations were detected by ELISA. Plates (NUNC Maxisorp; VWR, Nyon, Switzerland) were coated overnight at 4 °C with rat anti-mouse IgE (2 µg/mL; BD Pharmingen, Allschwil, Switzerland). After washing, wells were blocked with phosphate buffered saline (PBS)-1% BSA for 1 h at room temperature (RT). Serially diluted sera and standard (monoclonal mouse anti-OVA; ABD Serotec, Düsseldorf, Germany) were incubated for 2 h at 37 °C. Then biotinylated-OVA (1 µg/mL) was added to the plate for 1 h at 37 °C, followed by incubation with HRP-labelled streptavidin (1 : 1000; KPL; Socochim, Lausanne, Switzerland) for 30 min at 37 °C. Plates were developed with a tetramethylbenzidine substrate (KPL). The reaction was stopped with 1 M HCl (Merck, Darmstadt, Germany). Optical densities were measured at 450 nm. Concentrations were calculated by converting OD values in pg/mL using a polynomial standard curve.

Quantification of serum levels of ovalbumin-specific immunoglobulins G1 and G2a

Serum levels of anti-OVA IgG1 and IgG2a were determined by ELISA. Briefly, microtitre plates were coated

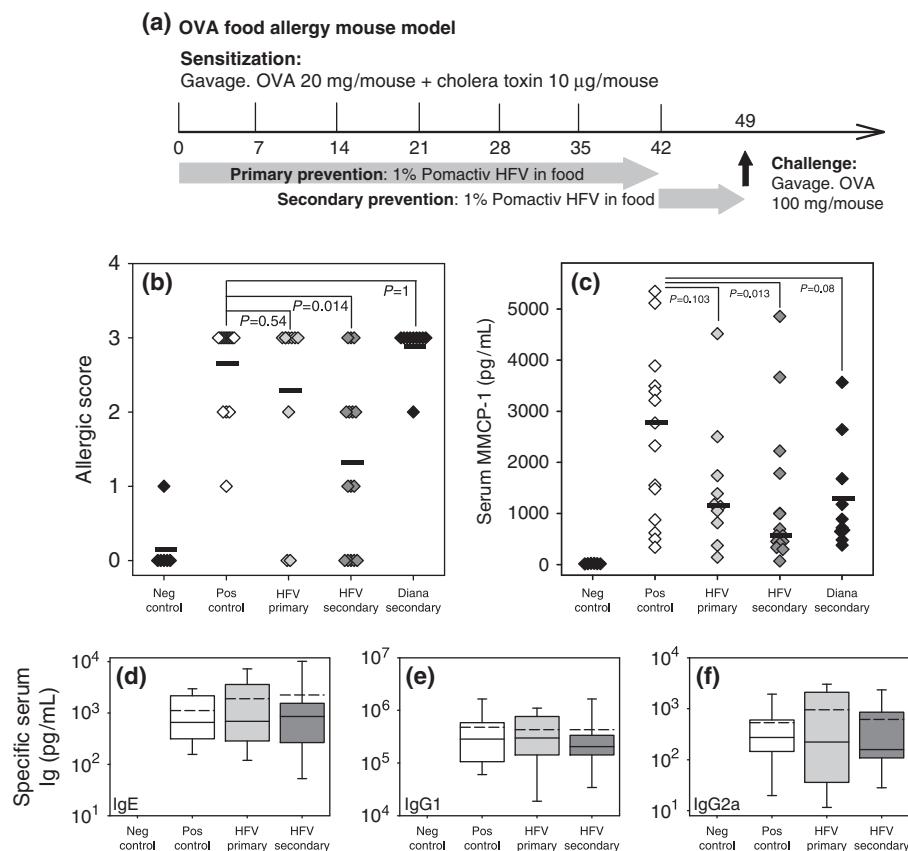


Fig. 1. Consumption of apple extract reduces allergic symptoms in sensitized mice upon challenge. Results from 1 representative out of three experiments are shown. (a) Schematic representation of the OVA Food Allergy mouse model. (b) Allergic scores after challenge. Results of individual mice (diamonds) and mean (horizontal bar). (c) Serum levels of Mouse Mast Cell Protease-1 (MMCP-1). Results of individual mice (diamonds) and median (horizontal bar). Serum levels of OVA-specific IgE (d), IgG1 (e) and IgG2a (f). Boxplots with a 25/75% interval (box), 5/95% interval (whiskers), median (solid line) and mean (dotted line).

with OVA (Sigma) (100 µg/mL) overnight at 4 °C. Wells were washed with PBS containing 0.05% Tween (Biorad, Reinach, Switzerland) and then blocked with PBS containing 1% BSA for 1 h at RT. Serially diluted standard (monoclonal mouse anti-OVA IgG1 and monoclonal mouse anti-OVA IgG2a from Antibody Shop; Lucerna-Chem, Lucerne, Switzerland) and serum samples were incubated for 2 h at 37 °C, followed by incubation for 2 h with an HRP-labelled goat anti-mouse IgG1 or IgG2a antibody (1 : 5000; Southern Biotech; Bioconcept, Allschwil, Switzerland). Plates were developed, read and analysed as described above.

Isolation and culture of mesenteric lymph node cells

MLN were homogenized using a syringe plunger in a cell strainer (BD Falcon; Milian, Meyrin, Switzerland). Cells were centrifuged and washed 2× in RPMI medium (Sigma) complemented with 10% fetal bovine serum (FBS; Bioconcept, Paris, France), 1% L-glutamine (Sigma), 1% Penicillin/Streptomycin (Sigma) and 0.1% Gentamycin (Sigma), 50 µM β-mercaptoethanol (Sigma). Cells

(3 × 10⁵ cells/well) were cultured in a 96-well flat-bottom plate (Corning, Milian, Meyrin, Switzerland) in the absence or presence of OVA (1000 µg/mL). After 72 h of culture, plates (including the supernatant and cells) were frozen at −20 °C.

Quantification of cytokine in the culture supernatant fluid

Mouse IL-4, IL-5, IL-10 and IFN-γ were measured using the mouse Th1/Th2 9-plex multiplex kit (Meso Scale Discovery®, Gaithersburg, MD, USA) according to the manufacturer's instructions.

Quantitative gene expression levels by real-time polymerase chain reaction (low-density array)

Total RNA from the ileum and PP were extracted according to the manufacturer's protocol using the SV Total RNA Isolation System kit (Promega, Dübendorf, Switzerland). RNA was quantified using the quant-IT Ribogreen Reagent kit purchased from Promega according to the manufacturer's protocol. Reverse transcription was performed on

1 µg of total RNA using the Multiscribe Reverse Transcriptase kit from Applied Biosystems (Foster City, CA, USA). Total RNA was mixed with 50 µM of random hexamers, 0.5 mM of dNTPs, 20 U of RNase inhibitor (Applied Biosystems), 62.5 U of Multiscribe reverse transcriptase, 1×RT buffer and 5.5 mM of MgCl₂ in a final volume of 50 µL. Reverse transcription was performed on a T3 thermocycler (Biomtra, Göttingen, Germany) with the following cycle program: 10 min at 25 °C, 30 min at 48 °C and 5 min at 95 °C to finish at 4 °C. Low-Density Arrays were designed online on the Applied Biosystems website. A complete list of genes analysed is shown in Table 1. The load and the run were performed according to the manufacturer's instructions on a quantitative ABI-Prism 7900HT (Applied Biosystems, Rotkreuz, Switzerland). The quantification was normalized with the mean of three house-keeping genes: β-actin, GAPDH and HPRT. The Cycle threshold (Ct) value for each gene was corrected by the Ct mean of these three house-keeping genes. Based on the Ct values obtained, a relative and normalized mRNA expression was determined for each gene using the ΔCt. The results were calculated as a relative expression using the formula $2^{-\Delta Ct} \times K$, where K is a 10⁶ factor. Fold increase result expression was normalized to expression levels in the negative (neg) control group.

In vitro characterization of apple polyphenols with rat basophilic leukaemia cells

RBL-2H3 cells (ATCC; LGC Standards Sarl, Molsheim, France) were grown in Eagle's minimal medium supplemented with 15% FCS. 4×10^5 cells/mL were cultured for 24 h in a 96-well microtitre plate in 0.1 mL/well. Fifty microlitre was replaced with H³-Serotonin (50 µL of 2 µCi/mL

in medium) and incubated for 3 h. IgE-mediated release: After discarding the supernatant and washing 3× with modified Hank's medium [0.1% bovine serum albumin (BSA)], cells were incubated for 2 h with 150 µL/well IgE-anti DNP (0.1 µg/mL). Cells were washed 2× with modified Hank's medium to eliminate free IgE-anti DNP and then incubated with 150 µL/well quercetin (10 mM) or Pomactiv HFV (0.5%) for 15 min. The supernatants were discarded and cells were washed 2× with modified Hank's medium and incubated for 30 min with 150 µL/well DNP-KLH (0.1 µg/mL). Alternatively, cells not sensitized with IgE were incubated with quercetin or Pomactiv HFV as described above and mediator release was induced by incubation with 150 µL/well compound 48/80 (300 µg/mL; Sigma) for 45 min. After centrifugation at 140 g for 8 min, 50 µL of the supernatant was transferred to an Optiwell 96-well plate and 200 µL of Microscint was added to each well. Serotonin release was measured in CPM using a TopCount Packard β-counter (Perkin Elmer, Schwerzenbach, Switzerland).

Antigenicity of polyphenol/ovalbumin complexes

ELISA plates were coated with polyclonal rabbit anti-chicken Egg Albumin (Sigma) overnight at RT in carbonate buffer. After washing with PBS Tween 1%, wells were blocked with PBS-5% milk for 1 h at 37 °C. Apple extract Pomactiv HFV and OVA were mixed at different ratios to allow the formation of macrocomplexes. After complex formation, samples were diluted to a theoretical final concentration of 100 ng/mL OVA in all the samples. Samples and, for comparison, non-complexed OVA, were serially diluted in PBS 0.5%-milk and incubated for 2 h at 37 °C. After washing, monoclonal mouse anti-OVA IgG1 (Antibody Shop) was added and incubated for 1 h at 37 °C. After washing, plates were incubated with HRP-labelled rabbit anti mouse-IgG1 for 1 h at 37 °C. Plates were developed, read and analysed as described above.

RBL-2H3 were cultured and loaded with H³-Serotonin as described above. After discarding the supernatant and washing 3× with modified Hank's medium (0.1% BSA), cells were incubated for 45 min with polyphenol/OVA complexes at different ratios as described above for 45 min. After centrifugation at 140 g for 8 min, 50 µL of the supernatant was transferred to an Optiwell 96-well plate and 200 µL of Microscint was added to each well. H³-Serotonin release was measured in CPM using a TopCount Packard β-counter.

Statistical analyses

Clinical scores were evaluated by Median or by Kruskal-Wallis tests, followed by the Wilcoxon test. All other outcomes were treated with the Kruskal-Wallis, followed by the Wilcoxon test. Corrections were applied following

Table 1. Gene expression levels in ileal Peyer's patches (mRNA)

Target gene	Relative gene expression (median±SEM) and fold change					
	Negative control		Positive control		Apple extract Pomactiv HFV	
	Relative*	Fold†	Relative	Fold	Relative	Fold
IL-4	112±31	1	333±91	+3.7	579±114	+5.2
IL-5	1±4	1	45±19	+85.3	1±21	+2.5
IL-13	1±4	1	175±50	+324.0	48±49	+88.9
CCL11	491±308	1	2388±1078	+4.9	974±1981	+2.0
CCL17	245±42	1	1994±687	+8.1	1288±366	+5.3
GATA3	3103±300	1	3622±462	+1.2	4549±615	+1.5
IL-10	197±34	1	913±108	+4.6	445±95	+2.3
IL-12a	1372±145	1	2163±605	+1.6	1974±327	+1.4
IFN-γ	74±28	1	220±102	+3.0	83±20	+1.1

*Relative gene expression levels were normalized to three house-keeping genes: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, hypoxanthine phosphoribosyltransferase (HPRT).

†Fold expression was normalized to expression levels in the Negative control group.

the Bonferroni–Sidak procedure. Results were considered as significant with a P -value ≤ 0.05 .

Results

Consumption of apple extract Pomactiv HFV reduces clinical symptoms and mediator release in a mouse model of food allergy to ovalbumin

We used a mouse model of food allergy to OVA [20] to test the anti-allergic effect of a polyphenol-enriched apple extract, termed Pomactiv HFV. Mice were sensitized via the oral route with the OVA+CT (Fig. 1a). Apple extract (1% w/w) was given mixed into powdered mouse chow pellets either during the sensitization phase (day 1–42) for the primary prevention of food allergy, or alternatively, sensitized mice were fed apple extract from days 42 to 49 of the experiment to assess whether symptoms would be attenuated upon challenge (secondary prevention) (Fig. 1a). Mice receiving only CT (neg control) did not develop symptoms after challenge with OVA, whereas sensitized, untreated mice [positive (pos) control] developed symptoms (Fig. 1b). Mice receiving apple extract during the sensitization phase (Pomactiv HFV primary) did not develop milder symptoms; in contrast, mice treated with apple extract in secondary prevention (Pomactiv HFV secondary) had a significantly reduced clinical score (Fig. 1b). Similarly, serum levels of MMCP-1 – a protease specifically released by activated intestinal mast cells – were significantly reduced in sensitized mice consuming apple extract for secondary prevention (Fig. 1c). These effects appeared to be most robust for apple extract Pomactiv HFV, because application of a different apple extract not enriched in polyphenol content (from Diana Naturals) and used for secondary prevention did not reduce symptoms (Fig. 1b) and only tended to reduce MMCP-1 serum levels (Fig. 1c). Apple extract consumption did not influence OVA-specific serum levels of IgE (Fig. 1d), IgG1 (Fig. 1e) and IgG2a (Fig. 1f).

Reduced cytokine secretion by mesenteric lymph node cells of ovalbumin-sensitized mice after consumption of apple extract Pomactiv HFV

To address the molecular bases of the observed clinical effects, we compared the cytokine profiles of MLN cells after restimulation with OVA. Oral sensitization and challenge led to induction of Th1 and Th2 cytokines (Fig. 2, neg control vs. pos control). Treatment with apple extract Pomactiv HFV led to a significant reduction of IL-10 secretion and a trend towards reduced production of IL-5 and IFN- γ . Similar results were obtained for secretion of IL-4 (Fig. 2). Statistics were performed for comparison of pos control vs. Pomactiv HFV secondary as data from individual mice were available only for these groups

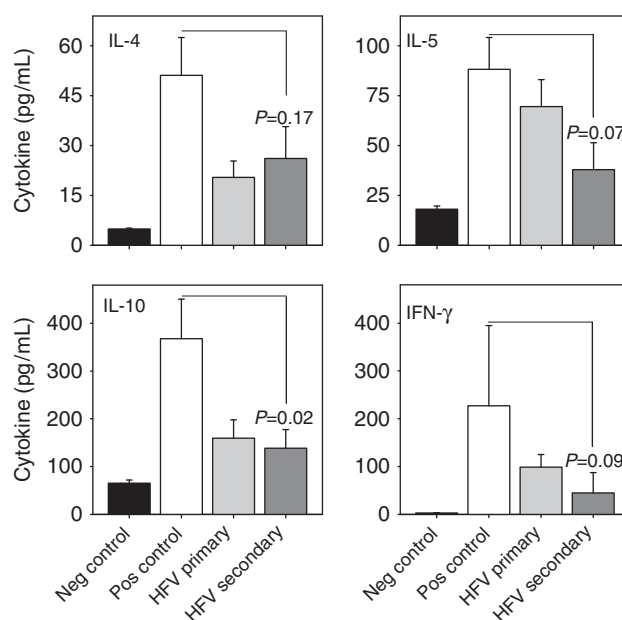


Fig. 2. Reduced cytokine secretion by mesenteric lymph nodes (MLN) cells of sensitized and challenged mice after consumption of apple extract. MLN of individual mice ($n = 15$ for neg control, pos control, HFV secondary) or four pools from 10 mice (two to three mice per pool) for HFV primary. Results (median and SEM) from one representative out of three experiments. Statistical analysis was performed between neg control and pos control (all values significant $P < 0.002$) and between pos control and HFV secondary.

($n = 15$ /group). Statistical significance was not achieved in all samples due to large individual variations within the groups.

Reduction of gene expression in ileal peyer's patches and ileum of ovalbumin-sensitized mice after consumption of apple extract Pomactiv HFV

To further evaluate the local events due to apple extract consumption, we determined mRNA expression levels of various genes in ileal PP and ileum by real-time PCR. Similar to the observations with MLN cells, apple extract led to a reduction of expression levels of multiple genes in the intestine. Genes showing more than a twofold reduction between the pos control and the apple extract Pomactiv HFV groups included IL5, IL13, CCL11, IL10 and IFNG in ileal PP (Table 1) and IL13, CCL11, GATA3, IL12 and IFNG in ileum (Table 2). A number of other genes were either not modulated by sensitization or by intervention with Pomactiv HFV (CCL20, CCL25, CCL5, CCR3, CCR4, CCR5, CCR6, CCR9, CD23, MUC2 and MUC4, data not shown). This demonstrates that consumption of apple extract Pomactiv HFV reduced the expression of some key genes induced by allergen challenge (4 h post-challenge) in ileal PP and the ileum of OVA-sensitized mice.

Table 2. Gene expression levels in ileum (mRNA)

Target gene	Relative gene expression (median±SEM) and fold change					
	Negative control		Positive control		Apple extract Pomactiv HFV	
	Relative*	Fold†	Relative	Fold	Relative	Fold
IL-4	6±1	1	16±84	+2.5	16±105	+2.5
IL-5	8±47	1	15±403	+1.8	16±114	+1.9
IL-13	6±1	1	648±360	+100.5	277±105	+43.0
CCL11	4703±3286	1	18 038±6797	+3.8	2118±1352	+0.5
CCL17	359±177	1	3754±888	+10.5	2203±543	+6.1
GATA3	2920±845	1	5827±1191	+2.0	2302±1059	+0.8
IL-10	306±163	1	2604±1540	+8.5	615±602	+2.0
IL-12a	100±148	1	830±438	+8.3	190±323	+1.9
IFN- γ	775±308	1	1747±738	+2.3	631±196	+0.8

*Relative gene expression levels were normalized to three house-keeping genes: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β -actin, hypoxanthine phosphoribosyltransferase (HPRT).

†Fold expression was normalized to expression levels in the Negative control group.

Apple extract Pomactiv HFV inhibits mediator release from rat basophilic leukaemia-2H3 cells

We hypothesized that one mechanism accounting for the results obtained might be inhibition of mediator release from mast cells by apple polyphenols. To formally prove this possibility, RBL-2H3 cells were sensitized with IgE-anti-DNP and incubated with pure quercetin or with apple extract Pomactiv HFV before inducing mediator release with DNP-KLH. Both quercetin and Pomactiv HFV inhibited the release of serotonin (Fig. 3a). Similarly, both ingredients inhibited IgE-independent mediator release induced by compound 48/80 (Fig. 3b), demonstrating a direct inhibitory effect of quercetin and Pomactiv HFV on the mast cells, independent of the IgE-Fc ϵ RI interaction. Similar results were obtained for secretion of β -hexosaminidase (data not shown). These data prove that Pomactiv HFV has the potential to reduce mediator release from (sensitized) mast cells.

Protein-polyphenol interactions between ovalbumin and apple extract Pomactiv HFV

As an alternative mechanism, we tested whether an interaction might occur between apple polyphenols in Pomactiv HFV and OVA and could thus reduce the allergenicity of OVA. Mixing of Pomactiv HFV and OVA at different ratios did indeed lead to the formation of macrocomplexes – as evidenced by ‘haze formation’ – and to protein precipitation (data not shown). Importantly, upon complexing of Pomactiv HFV and OVA, the reactivity of OVA with specific antibodies was strongly reduced (Fig. 4a). Moreover, RBL-2H3 sensitized with OVA-specific

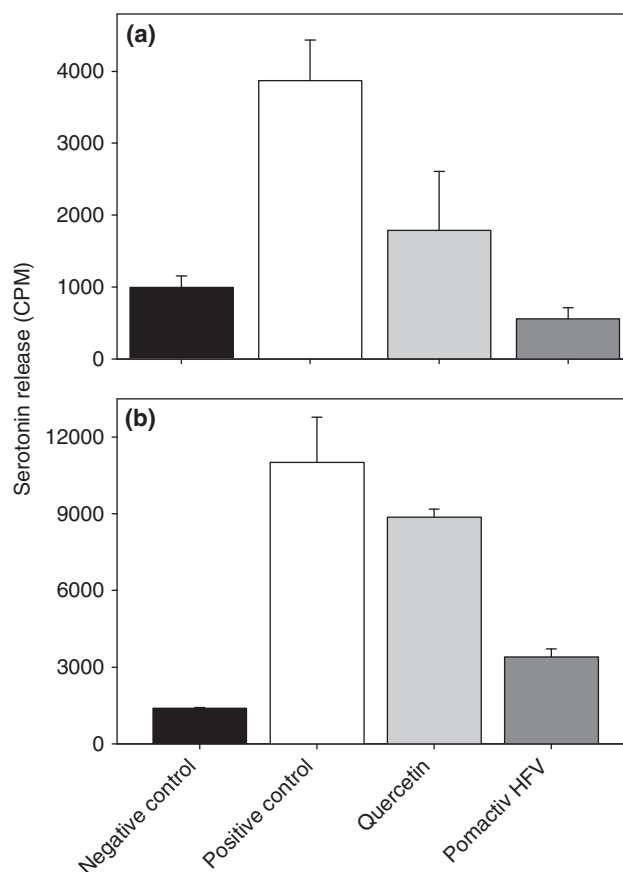


Fig. 3. Apple polyphenols reduce mediator release from rat basophilic leukaemia (RBL)-2H3 cells. RBL-2H3 cells were loaded with H^3 -serotonin. Inhibition of (a) IgE-dependent or (b) IgE-independent mediator release with quercetin or apple extract Pomactiv HFV. Mediator release was quantified by measurement of β -emission by H^3 -serotonin.

IgE showed diminished mediator release when triggered with polyphenol-complexed OVA compared with non-complexed OVA (Fig. 4b). These experiments establish that, in principle, the allergenicity of OVA might be reduced by interaction with apple polyphenols contained in Pomactiv HFV. Whether these interactions occur *in vivo* remains to be demonstrated.

Discussion

Our experiments using a mouse food allergy model showed that consumption of apple extract Pomactiv HFV reduced symptoms of food allergy as well as associated immune parameters such as MMCP-1 release by intestinal mast cells. These data are in line with a previous pre-clinical study by Akiyama et al. [13], where feeding of complex apple polyphenols (ACT) reduced systemic anaphylaxis induced by an intra-peritoneal allergen challenge. Our model includes a mucosal allergen challenge via the oral route at the end of the sensitization phase, and thus our data show that indeed local reactions such as

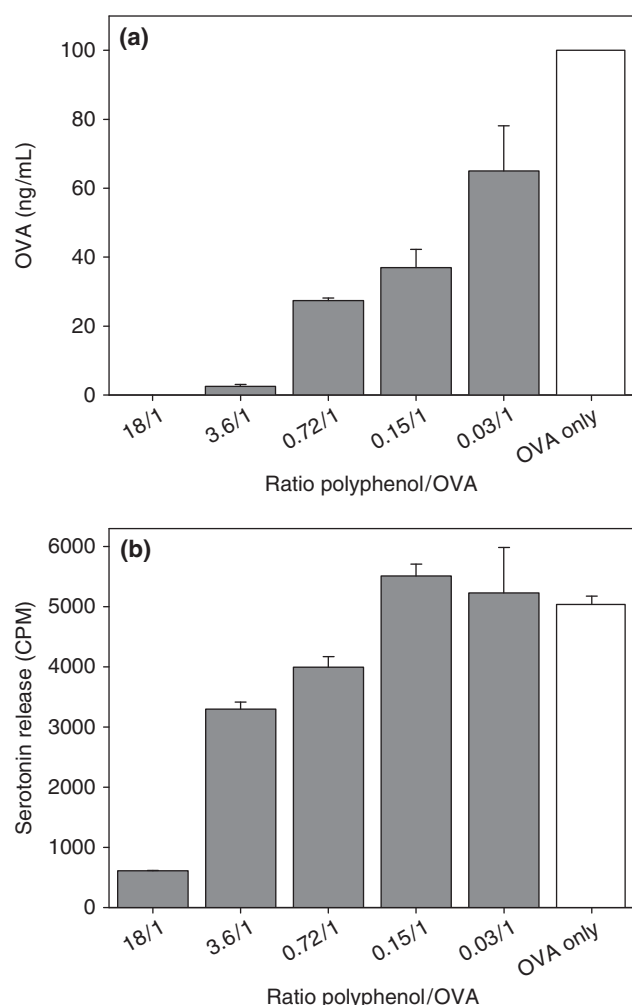


Fig. 4. Reduced antigenicity of ovalbumin (OVA) after complexing with apple extract Pomactiv HFV. Pomactiv HFV and OVA were mixed at different ratios to allow complex formation (a) Reduced binding of macro-complexes by anti-OVA antibodies determined by ELISA. (b) Mediator release from rat basophilic leukaemia-2H3 sensitized with anti-OVA IgE and loaded with H³-Serotonin after triggering with OVA only or with polyphenol: OVA complexes.

diarrhoea and mediator release from intestinal mast cells can be reduced by administration of apple polyphenols. Surprisingly, in the OVA food allergy model protection was only observed when apple extract was given for secondary prevention, i.e. after feeding to sensitized mice for 1 week before challenge (Fig. 1b and 1c). In contrast, feeding of apple extract during the sensitization phase did not confer any protection from subsequent challenge. In the study by Akiyama et al. [13], ACT were given *ad libitum* during the entire experiment. In line with this difference between the two studies, we did not observe significant modulation of OVA-specific serum Ig levels, whereas Akiyama and colleagues reported a strong reduction of OVA-specific serum antibodies. Other differences in the set-up of these two mouse models that might have

contributed to the diverging results include the mode of sensitization ('mechanical damage' through daily gavage as an adjuvant vs. CT as an adjuvant) and the route of application of apple polyphenols (1% v/v in drinking water vs. 1% w/w in food). Finally, differences in the exact polyphenolic composition of ACT vs. Pomactiv HFV may also have influenced the clinical outcome.

In the present study, reduction of symptoms was paralleled by a generalized attenuation of intestinal OVA-specific immune responses, e.g. reduction of both Th1 and Th2 cytokine secretion by OVA-restimulated MLN cells (Fig. 2.) and by decreased mRNA expression of Th2-associated genes in ileal PP and ileum (Tables 1 and 2). In contrast, *in vitro* experiments with human PBMC from healthy donors and purified apple polyphenols including quercetin showed an increase in IFN- γ production along with reduced IL-4 secretion [11]. Our own studies using Th2-skewed human PBMC yielded similar results, i.e. strong induction of IFN- γ production paralleled by profound inhibition of IL-5 secretion (data not shown). These findings suggest that, in principle, apple polyphenols might not only act at the level of effector cells but might be able to modulate cytokine profiles and thus influence adaptive immune responses. However, in the specific model we used, the effect appeared to be restricted to effector cells as no effect on specific serum immunoglobulin levels and no stimulation of Th1 (IFN- γ) or Th2 (IL-5) cytokines in MLN was observed. In keeping with this, all currently published human studies with apple polyphenols report a protective effect in reducing the symptoms of established atopic dermatitis [14] or allergic rhinitis [15, 16] in allergic individuals. In contrast, apart from the mouse studies by Akiyama et al. [13], no human studies have so far described the efficacy in the primary prevention of allergies. This indicates that the mouse model we used is, at least partly, predictive of the human situation. If further confirmed, this observation will facilitate selection of ingredients for human trials and will increase the chances of positive outcomes.

The levels of mRNA expression for various genes were reduced in ileum and ileal PP. The effects appeared strongest regarding the expression of typical Th2-associated genes such as IL-13 and IL-5, whereas the expression of another prototypic Th2 cytokine, namely IL-4, was not affected (Tables 1 and 2). This may be indicative of reduced levels of Th2-skewed T-cells present in the ileal lamina propria, even though no obvious explanation for the unaltered IL-4 mRNA levels can be provided. Moreover, mRNA transcription levels of CCL11 (eotaxin) and to a lesser extent of CCL17 (TARC) were down-regulated. Both of these chemokines are involved in the recruitment of effector cells such as eosinophils and Th2 cells in atopic dermatitis [21] and asthma [22]; likewise, the implication of CCL11 in eosinophil recruitment to the intestinal lamina propria has been documented [23]. Reduced recruitment of

these cell types may well have contributed to a decreased mediator release after challenge in our model. Again, these findings suggest an effect of apple polyphenols on the local immune status in the intestine of sensitized mice after challenge that goes beyond inhibition of mast-cell mediator release. Nevertheless, it is important to keep in mind that intestinal samples were isolated 4 h after allergen challenge. This time-point was considered to be optimal for the detection of serum MMCP-1. For analysis of gene expression levels, this may have been a relatively early time-point as the expression of many genes peaks only between 10 and 24 h after stimulation.

The diminished levels of MMCP-1 after challenge suggest inhibition of effector cells as an important mechanism responsible for symptom reduction. This was confirmed by *in vitro* studies using RBL-2H3 showing a blocking effect of Pomactiv HFV as well as pure quercetin on mediator release. Both IgE-dependent and IgE-independent mediator release was inhibited, strongly suggesting a direct inhibitory or stabilizing effect of polyphenols on mast cells, independent of allergen-IgE and IgE-FcεRI interactions. These data are in line with previous *in vitro* studies using different cell types and polyphenols [6–8]. Interestingly, our *in vitro* data seem to indicate a more profound inhibition by apple extract Pomactiv HFV than by pure quercetin. These findings imply that quercetin may be one but not the only active component of the apple extract contributing to mast cell stabilization. We speculate that other types of polyphenols or possibly even apple fibres (which constitute 50% of the extract) may have contributed to the inhibitory effect.

In addition, our *in vitro* experiments studying the interaction of Pomactiv HFV with OVA suggest a potential additional mechanism of action, namely reduction of allergenicity by epitope modification of the allergen. Gruber et al. [24] reported similar results with the major cherry allergen Pru av 1. They showed that interaction of Pru av 1 with various polyphenols, including epicatechin, gallic acid and quercetin, led to permanent modification of the tertiary structure of the allergen, which resulted in a strong reduction of the IgE-binding capacity of this allergen. Recently, similar findings were reported, demonstrating reduced allergenicity of peanuts after interaction with polyphenols [25]. Our experiments confirm these results and we show, in addition, that the reduced IgE binding of polyphenol-complexed OVA indeed has functional consequences, i.e. results in reduced mediator release by OVA-sensitized RBL-2H3 (Fig. 4b).

In addition to the two mechanisms proposed here, alternative effects of polyphenols such as reinforcement of intestinal barrier integrity [26] and induction of apoptosis through caspase activation [27] might contribute towards achievement of the anti-allergic effect.

Taken together, even if polyphenols clearly interact with the allergen *in vitro*, we speculate that mast cell

stabilization and consequently a reduced mediator release is the main effector mechanism by which apple polyphenols attenuate symptoms of food allergy in the present mouse model. It will now be important to study whether these mechanistic findings apply to humans too. To this end, a safety study in allergic children has been initiated.

Acknowledgements

We thank Aurore Deschamps, Christophe Maubert and Massimo Marchesini for assistance with animal studies and Mireille Moser for performing statistical analyses. We thank Philippe Sanoner (Val de Vire Bioactives) and Lionel Bovetto for their input on apple polyphenols. We appreciate the useful comments on the manuscript by Sonja Kierstein and Anurag Singh.

Conflict of interest statement: All co-authors are employees of Nestec S.A., who funded the work.

References

- 1 Osborn DA, Sinn JK. Probiotics in infants for prevention of allergic disease and food hypersensitivity. *Cochrane Database Syst Rev* 2007; CD006475.
- 2 Arslanoglu S, Moro GE, Schmitt J, Tandoi L, Rizzardi S, Boehm G. Early dietary intervention with a mixture of prebiotic oligosaccharides reduces the incidence of allergic manifestations and infections during the first two years of life. *J Nutr* 2008; 138:1091–5.
- 3 Dunstan JA, Mori TA, Barden A *et al.* Fish oil supplementation in pregnancy modifies neonatal allergen-specific immune responses and clinical outcomes in infants at high risk of atopy: a randomized, controlled trial. *J Allergy Clin Immunol* 2003; 112:1178–84.
- 4 Scalbert A, Williamson G. Dietary intake and bioavailability of polyphenols. *J Nutr* 2000; 130:2073S–85S.
- 5 Sanoner P, Guyot S, Marnet N, Molle D, Drilleau JP. Polyphenol profiles of French cider apple varieties (*Malus domestica* sp.). *J Agric Food Chem* 1999; 47:4847–53.
- 6 Kanda T, Akiyama H, Yanagida A *et al.* Inhibitory effects of apple polyphenol on induced histamine release from RBL-2H3 cells and rat mast cells. *Biosci Biotechnol Biochem* 1998; 62:1284–9.
- 7 Tokura T, Nakano N, Ito T *et al.* Inhibitory effect of polyphenol-enriched apple extracts on mast cell degranulation *in vitro* targeting the binding between IgE and FcεpsilonRI. *Biosci Biotechnol Biochem* 2005; 69:1974–7.
- 8 Kempuraj D, Madhappan B, Christodoulou S *et al.* Flavonols inhibit proinflammatory mediator release, intracellular calcium ion levels and protein kinase C theta phosphorylation in human mast cells. *Br J Pharmacol* 2005; 145:934–44.
- 9 Kimata M, Shichijo M, Miura T, Serizawa I, Inagaki N, Nagai H. Effects of luteolin, quercetin and baicalein on immunoglobulin E-mediated mediator release from human cultured mast cells. *Clin Exp Allergy* 2000; 30:501–8.
- 10 Nakano N, Nishiyama C, Tokura T *et al.* Procyanidin C1 from apple extracts inhibits Fc epsilon RI-mediated mast cell activation. *Int Arch Allergy Immunol* 2008; 147:213–21.

- 11 Nair MP, Kandaswami C, Mahajan S *et al.* The flavonoid, quercetin, differentially regulates Th-1 (IFN γ) and Th-2 (IL4) cytokine gene expression by normal peripheral blood mononuclear cells. *Biochim Biophys Acta* 2002; **1593**:29–36.
- 12 Dorsch W, Bittinger M, Kaas A, Muller A, Kreher B, Wagner H. Antiasthmatic effects of Galphimia glauca, gallic acid, and related compounds prevent allergen- and platelet-activating factor-induced bronchial obstruction as well as bronchial hyperreactivity in guinea pigs. *Int Arch Allergy Immunol* 1992; **97**:1–7.
- 13 Akiyama H, Sato Y, Watanabe T *et al.* Dietary unripe apple polyphenol inhibits the development of food allergies in murine models. *FEBS Lett* 2005; **579**:4485–91.
- 14 Kojima T, Akiyama H, Sasai M *et al.* Anti-allergic effect of apple polyphenol on patients with atopic dermatitis: a pilot study. *Allergol Int* 2000; **49**:69–73.
- 15 Kishi K, Saito M, Saito T *et al.* Clinical efficacy of apple polyphenol for treating cedar pollinosis. *Biosci Biotechnol Biochem* 2005; **69**:829–32.
- 16 Enomoto T, Nagasako-Akazome Y, Kanda T, Ikeda M, Dake Y. Clinical effects of apple polyphenols on persistent allergic rhinitis: a randomized double-blind placebo-controlled parallel arm study. *J Investig Allergol Clin Immunol* 2006; **16**:283–9.
- 17 Knekt P, Kumpulainen J, Jarvinen R *et al.* Flavonoid intake and risk of chronic diseases. *Am J Clin Nutr* 2002; **76**:560–8.
- 18 Willers SM, Devereux G, Craig LC *et al.* Maternal food consumption during pregnancy and asthma, respiratory and atopic symptoms in 5-year-old children. *Thorax* 2007; **62**:773–9.
- 19 Shaheen SO, Sterne JA, Thompson RL, Songhurst CE, Margetts BM, Burney PG. Dietary antioxidants and asthma in adults: population-based case-control study. *Am J Respir Crit Care Med* 2001; **164**:1823–8.
- 20 Perrier C, Thierry AC, Mercenier A, Corthésy B. Allergen-specific antibody and cytokine responses, mast cell reactivity and intestinal permeability upon oral challenge of sensitized and tolerized mice. *Clin Exp Allergy* 2009; **40**:153–162.
- 21 Pivarsci A, Homey B. Chemokine networks in atopic dermatitis: traffic signals of disease. *Curr Allergy Asthma Rep* 2005; **5**:284–90.
- 22 Garcia G, Godot V, Humbert M. New chemokine targets for asthma therapy. *Curr Allergy Asthma Rep* 2005; **5**:155–60.
- 23 Rothenberg ME, Mishra A, Brandt EB, Hogan SP. Gastrointestinal eosinophils. *Immunol Rev* 2001; **179**:139–55.
- 24 Gruber P, Vieths S, Wangorsch A, Nerkamp J, Hofmann T. Maillard reaction and enzymatic browning affect the allergenicity of Pru av 1, the major allergen from cherry (*Prunus avium*). *J Agric Food Chem* 2004; **52**:4002–7.
- 25 Si-Yin Chung, Elaine T. Champagne. Reducing the allergenic capacity of peanut extracts and liquid peanut butter by phenolic compounds. *Food Chem* 2009; **115**:1345–9.
- 26 Suzuki T, Hara H. Quercetin enhances intestinal barrier function through the assembly of zonula [corrected] occludens-2, occludin, and claudin-1 and the expression of claudin-4 in Caco-2 cells. *J Nutr* 2009; **139**:965–74.
- 27 Kern M, Pahlke G, Balavenkatraman KK, Bohmer FD, Marko D. Apple polyphenols affect protein kinase C activity and the onset of apoptosis in human colon carcinoma cells. *J Agric Food Chem* 2007; **55**:4999–5006.