

Modulation of Allergic Reactions to Apple by Pesticide Residues

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The question whether pesticides can modulate allergy relevant reactions is a controversial issue. In the allergist's office many food-allergic patients believe themselves to react particularly to 'chemically treated' food, especially fruits. Investigations on the influence of pesticides upon apple allergy should bring the problem closer to scientific understanding. Apples of the variety 'Golden Delicious' from 'biologic-dynamic' cultivation and the pesticides chlorpropham and thiram were studied in the experiments. Self-prepared apple protein extracts as well as the pesticides were examined in the in vitro mediator release test from human basophil granulocytes either alone or in combination (spiking experiments). The mediators histamine and leukotriene C₄ were measured by high-performance liquid chromatography (HPLC). The possible binding of chlorpropham to the apple protein was examined using the enzyme allergosorbent test and immunoblotting with chlorpropham spiked apple allergen extracts. Chlorpropham but not thiram acted as a direct histamine liberator. Leukotriene C₄ secretion was not induced by any pesticide. In the spiking experiments, when chlorpropham was added to apple extract, it showed a concentration dependent inhibition of histamine release compared to thiram, which had no effect. In an HPLC examination a binding of chlorpropham to apple protein up to 13% could be observed. In the enzyme allergosorbent test chlorpropham caused a reduced specific IgE antibody binding to the apple protein. In the immunoblot there was weakening by chlorpropham of the 18 kDa major allergen band (Mal d I). It is assumed that chlorpropham modulates the apple allergens leading to reduced antibody binding thereby decreasing allergenic potency.

Keywords: Apple-allergy, pesticide, carbamate, chlorpropham, thiram, basophils, mediator release, histamine, leukotriene C₄, modulation

INTRODUCTION

The role of xenobiotic food components in the induction, elicitation and maintenance of hypersensitivity reactions to foods is of increasing interest. A possible connection between the increase in allergy prevalence and environmental pollution is frequently discussed (Ring & Vieluf, 1991; Ring *et al.*, 1991; Schlipkoeter, 1991). Recently it could be shown that polychlorinated biphenyls, heavy metals (cadmium, lead, mercury) and some pesticides (*p,p'*-DDT, hexachlorobenzene, dieldrin, lindane, carbaryl) are able to influence immune reactions (Behrendt *et al.*, 1988; Behrendt & Ring, 1997). There are no studies on the effects of these chemicals on allergic diseases from foods. The opinion of many food-allergic patients that they react particularly to 'chemically treated' foods is common knowledge. Moreover in placebo controlled clinical studies using oral provocation tests substantiating this phenomena are lacking (Ring *et al.*, 1991; Schlipkoeter, 1991).

The aim of this examination was to improve the scientific understanding of this problem. A possible influence of pesticide residues on allergic reactions to apple was examined in the mediator release test from human basophil granulocytes.

The pesticides chlorpropham (carbamate) and thiram (thiuram), belong to chemical compound classes which have been found to be present in commercially available foods in pesticide residue monitoring were tested both alone and in combination with self-prepared apple allergen extracts (Beil *et al.*, 1997). The hypothesis was that the selected compounds could modulate the 'natural' allergenicity of apple. A possible chlorpropham-apple protein binding should be examined by high-performance liquid chromatography (HPLC), followed by an enzyme allergosorbent test (EAST) and immunoblotting using sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE).

MATERIAL AND METHODS

Patients

For the examination serum and blood samples from 25 persons, 12 apple-allergic patients (positive history of immediate type allergy to apple plus CAP-RAST ≥ 3) and 13 control persons (CAP-RAST = 0), was used.

Chemicals and Reagents

Histamine release. Tris buffer (3.0 g tris(hydroxymethyl)-aminomethane (Tris) + 7.0 g NaCl + 3.0 g KCl + 0.0147 g CaCl₂ + 0.0246 g MgCl₂, with dist. water ad 1000 ml, with 4 mol l⁻¹ HCl ad pH 7.4, with NaCl ad 300 mosm); anti-IgE stock solution (78 µg rabbit immunoglobulin directed to human IgE [Dako, Copenhagen, Denmark] in 1 ml 0.1 mol l⁻¹ NaCl); apple protein extract stock solution (260 µg ml⁻¹ in phosphate-buffered saline buffer [Sigma, Deisenhofen, Germany]); chlorpropham stock solution (1 mg chlorpropham [Dr. Ehrenstorfer, Augsburg, Germany] + 300 µl methanol + 9.7 ml isotonic NaCl solution); thiram stock solution (1 mg thiram [Dr. Ehrenstorfer, Augsburg, Germany] + 300 µl methanol + 9.7 ml isotonic NaCl solution). If not otherwise indicated all chemicals were obtained from Merck, Darmstadt, Germany.

EAST examination (coupling of the extracts). Round paper bromine cyanide activated disks (Ceska & Lundkvist, 1972), 6 mm (Schleicher & Schuell, Dassel, Germany); apple protein solution (20 mg ml⁻¹, 0.2 mg ml⁻¹, 0.02 mg ml⁻¹, and 0.002 mg ml⁻¹) in methanol (Dr. Ehrenstorfer, Augsburg, Germany); EAST-testkit (Allergopharma, Reinbek, Germany).

SDS-PAGE/immunoblotting. Chlorpropham solutions (2 mg ml⁻¹ and 20 mg ml⁻¹) in water/methanol (60:40, v:v) (Dr. Ehrenstorfer, Augsburg, Germany); methanol.

Equipment

Histamine release. Centrifuge Megafuge 1.0 R (Heraeus, Hanau, Germany); osmometer model 3 MO plus (Advanced Instruments, Norwood, USA); pH-meter CG 801; pycnometer 1 ml (Schott, Mainz, Germany); shake water bath 1083 (GFL, Hannover-Vinnhorst, Germany); ultrasound bath RK 510 S (Badelin Sonorex, Berlin, Germany); microscope Bio-Med (Leitz, Wetzlar, Germany). Histamine and leukotriene C₄ (LTC₄) analysis was performed by HPLC (Beil *et al.*, 1998).

Examination of chlorpropham-apple protein binding. HPLC pump 64; degasser 3312; dynamic mixing chamber No. 21564; UV/VIS-detector Soma 3702 (ERC, Alteglofsheim, Germany); autosampler promis II (Spark, Emmen, The Netherlands); column RP-8 spherisorb, 250×4 mm i.d., 5 µm (Ziemer, Mannheim, Germany); integrator D-2000 (Merck-Hitachi, Darmstadt, Germany); compressor model minor (Jun-Air, Nørresundby, Denmark); disposable syringe filters Minisart RC 15, 0.45 µm (Sartorius, Göttingen, Germany); drying cupboard KTG 900 (Heraeus, Hanau, Germany).

EAST examination. ELISA-reader MRX (Dynex, Sullyfield Circle, Chantilly, USA); microtitre plates 96 holes; EAST-immuno tubes Minisorb 75 × 12 mm (Nunc, Roskilde, Denmark).

SDS-PAGE/immunoblotting. Main power supply unit 1200/200; electrophoresis chamber desaphor HF (Desaga, Heidelberg, Germany); cryostat RMT 6 (mgw Lauda, Lauda-Königshofen, Germany); water bath 2 (Haake, Saddle Brook, USA); gel carrier films GelBond-PAGfilm, 124×258 mm (Pharmacia, Uppsala, Sweden); electrode bridges (Vileda, Weinheim, Germany); blotting electrodes nova blot 2117–250 (LKB, Bromma, Sweden); blot membrane nitrocellulose, 5×10 mm (Schleicher & Schüll, Dassel, Germany).

Methods

Histamine release

Enrichment. The enrichment of basophil granulocytes from heparinized human blood was performed by density gradient centrifugation as described in the literature (Warner *et al.*, 1987; Schroeder & Hanrahan, 1990).

Incubation. 350 µl Tris buffer were mixed with 100 µl enriched cell suspension and an equilibration was carried out at 37°C for 5 min. 50 µl of the stimulus solutions (anti-IgE, apple allergen extract, pesticides, as well as combinations of these substances) of different concentrations were added. The incubation performed at 37°C for 45 min was stopped by centrifugation (249 × g, 20°C, 10 min). Total releasable histamine was measured in the cell pellet resuspended in dist. water and lysed by ultrasound bath/freezer shock treatment. Spontaneous mediator release was determined in nonstimulated suspensions (Dvorak & Harold, 1979). Anti-IgE was used as positive control to determine releasability (Kleine-Tebbe *et al.*, 1992).

Cell vitality. To exclude cytotoxic effects from enrichment or incubation, vitality tests using trypan blue staining were carried out (Gilbert & Ornstein, 1975).

Cell counting. The basophil counting was performed by astra blue staining and for nonspecific cell counting cytopreparations with May-Grünwald's eosin-methylene blue solution were made (Inagaki, 1969; Zeile, 1980).

TABLE 1. HPLC conditions of the determination of chlorpropham

Column	Reversed-phase-8, spherisorb, 25.4 mm i.d., 5 μ m
Column temperature	25°C
Eluent	Acetonitrile/water (70:30, v:v)
Elution	Isocratic
Flow	1 ml min ⁻¹
Injection volume	20 μ l (sample loop)
Detection	Ultraviolet, 220 nm
Retention time of chlorpropham	3.7 min

Mediator analysis. The mediator analysis was performed by HPLC. Histamine and LTC₄ were captured by pre-column derivatization with *o*-phthaldialdehyde. The separation of the indole derivatives occurred on a reversed-phase-8-column by gradient elution with a phosphate/methanol mixture. The detection was fluorimetrically performed (excitation wavelength: 340 nm, emission wavelength: 455 nm) (Beil *et al.*, 1998).

Preparation of apple allergen extracts. Apple protein extracts of the variety 'Golden Delicious' from organic cultivation were self-prepared by low-temperature extraction with acetone/dry ice and their protein content as well as their suitability for allergological examinations were determined (Laemmli, 1970; Bensadoun & Weinstein, 1976; Westermeier, 1990; Vieths *et al.*, 1992).

Binding of chlorpropham to apple protein The apple protein extract of the concentration 2.6 μ g ml⁻¹ was spiked with chlorpropham concentrations which were partly determinable at the detection level by HPLC and ultraviolet detection.

The chlorpropham concentrations were 1.67, 0.84 and 0.33 μ g ml⁻¹. Pure chlorpropham solutions of the same concentrations served as reference standards. The spiking occurred just by mixing apple protein solutions with chlorpropham solutions. After incubation at 50°C for 45 min in a drying cupboard or immediately after spiking chlorpropham was measured in the supernatants (HPLC conditions Table 1).

EAST examination For each bromine cyanide activated paper disk 0.3 ml apple protein solution were added. 1 ml of the chlorpropham solutions for each spiking experiment and 1 ml of methanol as solvent control were used. The mixed solutions were stirred overnight at 4°C. The coupling and the EAST were performed according to the instructions (Ceska & Lundkvist, 1972).

SDS-PAGE/immunoblotting The lyophilized apple protein extract was solved in twice-distilled water, in chlorpropham solutions (2 mg ml⁻¹ and 20 mg ml⁻¹) and in a solvent mixture of water and methanol (60:40, v:v). The same volume of sample buffer was added and the mixtures were reduced for 3 min at 90°C in a water bath. The resulting protein concentrations were 260 μ g ml⁻¹. The relations of the apple protein and the chlorpropham concentrations were about 1:4 and 1:40. Because it was impossible to prepare pure aqueous solutions of chlorpropham for these concentrations (1 mg ml⁻¹ and 10 mg ml⁻¹) a solvent mixture was used. To exclude denaturing effects of the methanol a solvent control apple protein in water/methanol (60:40, v:v) was utilized. A total of four different experiments for each patient serum were performed.

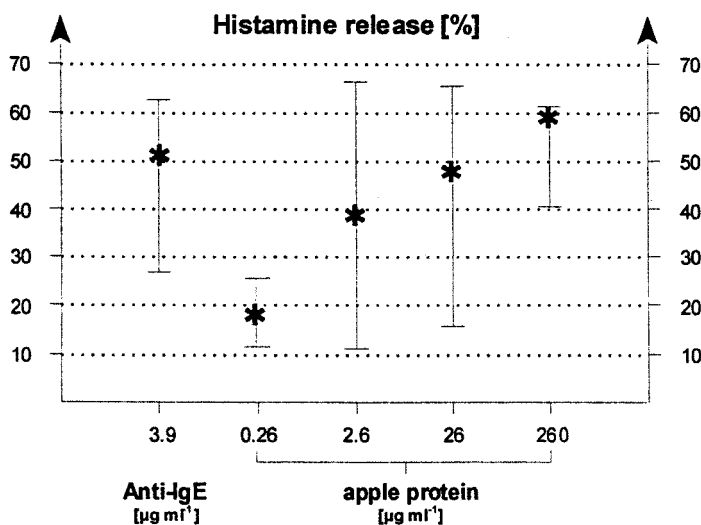


FIG. 1. *In vitro* histamine release of five apple-allergic patients after stimulation with apple-allergen extracts and anti-IgE as control (*median value and range).

RESULTS

In vitro Mediator Release

Mediator release induced by apple-allergen extracts. First dose-response studies were performed in order to determine optimal allergen concentrations for histamine release. Suitable protein concentration steps were found at 0.26, 2.6, 26 and 260 $\mu\text{g ml}^{-1}$ (Figure 1). Anti-IgE in the concentration of 3.9 $\mu\text{g ml}^{-1}$ served as positive control to determine releasability of basophil granulocytes.

The apple-allergic patients showed the lowest histamine release (12.9–25.2%) after stimulation with the lowest protein concentration of 0.26 $\mu\text{g ml}^{-1}$. Dependent on the protein concentration the median values increased up to 59.7%.

A concentration dependent LTC₄ secretion could not be observed in any apple-allergic patient. The values measured were in the range of 30 and 40 ng 10^{-6} basophil granulocytes. The six control persons did not show any mediator release, neither histamine nor LTC₄, after stimulation with apple extracts, as expected. In all incubation experiments the vitality rate determined by trypan blue staining was above 94.6%.

In the following spiking experiments a self-prepared apple-allergen extract in the concentration 0.26 $\mu\text{g ml}^{-1}$ was used to examine possible modulating effects of pesticides on histamine release.

Mediator release induced by pesticides

Mediator release induced by chlorpropham. In the chlorpropham-induced histamine release using chlorpropham concentrations of 0.1, 1, 10 and 100 $\mu\text{g ml}^{-1}$ and basophil granulocytes of four apple-allergic patients and two controls no concentration dependence could be observed. Chlorpropham proved to be a weak direct histamine liberator both in apple-allergic patients and controls with the highest histamine release values at the highest chlorpropham concentration (100 $\mu\text{g ml}^{-1}$). The release was between 9.4 and 27.6% with a median value of 11.3%. LTC₄ secretion could not be detected in any case. The vitality rates for each incubation experiment were higher than 95.1%.

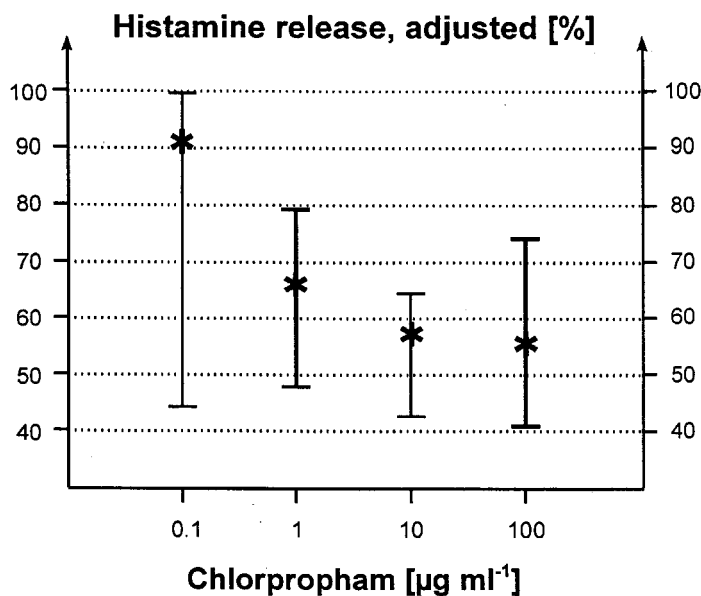


FIG. 2. *In vitro* histamine release of four apple-allergic patients after stimulation with apple-allergen extract (protein content $0.26 \mu\text{g ml}^{-1}$) and apple-allergen extract spiked with chlorpropham (*median value and range of the percentual histamine release in comparison to the non-spiked extract set at 100%).

Mediator release induced by thiram. The incubation with thiram in the concentration range as described above was performed with basophil granulocytes of two persons, one apple-allergic patient and one control. No mediator release was observed.

Mediator release induced by apple-allergen extracts combined with pesticides

Mediator release induced by apple-allergen extracts plus chlorpropham. For stimulation the apple protein concentration of $0.26 \mu\text{g ml}^{-1}$ was used. The spiking of the apple extract was done with chlorpropham in concentrations of 0.1, 1, 10 and $100 \mu\text{g ml}^{-1}$ by mixing extract and pesticide solutions immediately before incubation. Figure 2 shows the percentual histamine release from basophils of four apple-allergic patients in comparison to the non-spiked extract set at 100%.

In all experiments there was a decrease in apple-induced histamine release after spiking with highest chlorpropham concentrations. The patients also showed a slightly chlorpropham concentration dependent decrease in histamine release from median values of 91.2% down to 56.0%. The LTC_4 secretion with values of 30 and $50 \text{ ng } 10^{-6}$ basophils was not influenced by chlorpropham in any patient. The cell vitality rate was higher than 97.1%.

Mediator release induced by apple-allergen extracts plus thiram. The incubation of basophil granulocytes with apple protein combined with thiram as described above was performed with two apple-allergic patients. No difference in the mediator release compared to the non-spiked apple protein of the concentration $0.26 \mu\text{g ml}^{-1}$ was observed. The vitality rates in each experiment were higher than 97.3%.

Binding of Chlorpropham to Apple-Protein (HPLC-Analysis). The HPLC determination of chlorpropham was performed with apple protein solutions of $2.6 \mu\text{g ml}^{-1}$ which had been

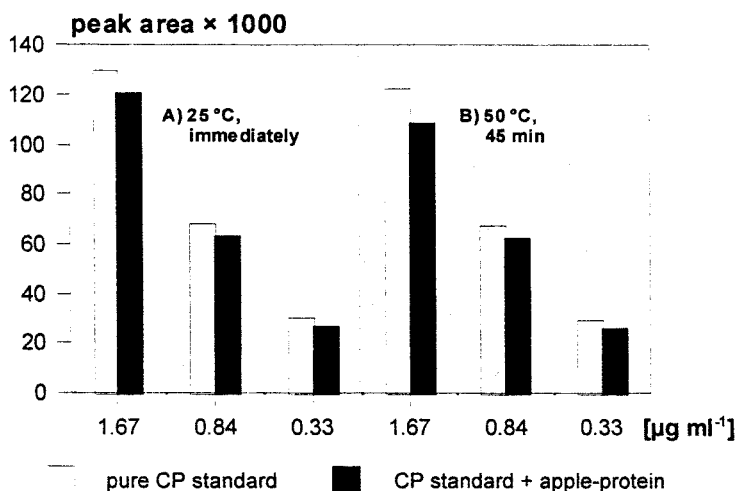


FIG. 3. Binding of chlorpropham (CP) to apple-protein. HPLC-determination of CP in supernatants compared to the standard. (A) determination immediately after spiking, 25°C; (B) determination after 45 min, 50°C. Protein concentrations: each $2.6 \mu\text{g l ml}^{-1}$, CP concentrations: 1.67, 0.84 and $0.33 \mu\text{g ml}^{-1}$. Presentation of peak areas after integrator evaluation.

spiked with chlorpropham concentrations of 1.67, 0.84 and $0.33 \mu\text{g ml}^{-1}$. In comparison to the pure chlorpropham standards, identically treated, a possible apple protein/chlorpropham interaction was expected (Figure 3). The figure shows that chlorpropham bound to apple protein. This is leading to a decrease in HPLC peak areas of up to 13%. No direct time and temperature dependence could be indicated.

EAST Examination

In the EAST the contents of specific IgE in U ml^{-1} were computed by calibration curves in double determinations. All 12 apple-allergic patients had specific IgE antibodies to apple protein. All controls had a negative EAST. The spiking of the apple extract with different chlorpropham concentrations caused a concentration dependent decrease of the IgE antibody binding (Figure 4).

SDS-PAGE/Immunoblotting

In this examination the possible modulating effect of chlorpropham on antibody binding of the major apple-allergen *Mal d 1* was determined by immunoblot detection (Figure 5). The typical experiment showed a dose-dependent band weakening by chlorpropham. All examined apple-allergic patients showed this tendency. In controls no bands were detected.

DISCUSSION

The results of this study show that some pesticides may modulate allergy-relevant reactions. Chlorpropham has been found to be a weak direct histamine liberator. It has an inhibiting effect on the apple induced histamine release, in contrast to thiram. The histamine release induced by pure chlorpropham is probably not an IgE mediated allergic reaction because the newly generated mediator LTC_4 was not released in any patient (Lichtenstein, 1993). One possible explanation for the mechanism of histamine release could be the enclosure of chlorpropham in the cytoplasm membrane of the basophil

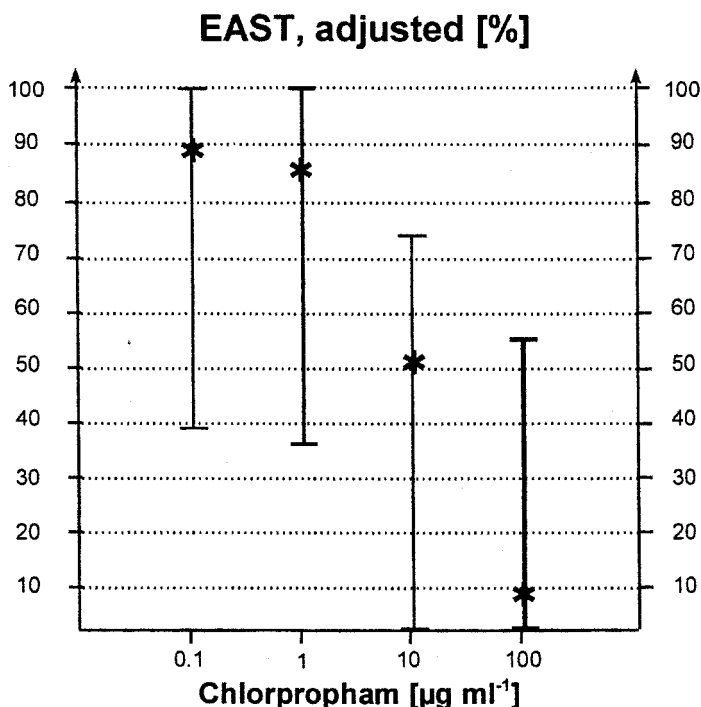


FIG. 4. EAST: Binding of specific IgE antibodies of 12 apple-allergic patients to apple-allergen extracts (protein $5.2\mu\text{g}$) nonspiked and spiked with chlorpropham (*mean values and ranges of the percentual IgE-binding in comparison to the non-spiked extract set at 100%).

granulocytes. This could activate calcium channels leading to an increased permeability for calcium ions (Antunes-Madeira & Madeira, 1979, 1982; Rohr *et al.*, 1985, 1989; Diel *et al.*, 1998; Sato *et al.*, 1998).

In contrast to the histamine release of the pure pesticide, the chlorpropham spiked apple protein showed an opposite effect. If high chlorpropham concentrations were used, the histamine release was always the lowest and clearly different from the pure apple allergen induced histamine release. With decreasing chlorpropham concentration the histamine release increased up to a value which was also caused by the non-spiked apple protein. To explain this phenomenon two hypotheses could be made:

Firstly, an interaction of chlorpropham with the apple protein is possible weakening the allergenic potency. The toxicity of chlorpropham as a carbamate is primarily based on the inhibition of the acetylcholinesterase at the synapses of the nerves. The carbamate in particular reacts to the hydroxyl group of the amino acid serine in the active center of the enzyme (WHO, 1986; Dikshith, 1991). Therefore a reaction of chlorpropham to the apple allergen (*Mal d 1*) is conceivable. Direct reactions to the epitopes or induced conformation changes of all apple proteins are also possible.

Secondly, structural changes of IgE antibodies on the basophil surface caused by chlorpropham could lead to the phenomenologically same result.

For conformation of the first hypothesis the chlorpropham binding to the apple protein was studied. A reaction between chlorpropham and the apple protein was determined using HPLC, but the kind of chemical binding could not be clarified.

In the EAST and immunoblotting a reduced specific IgE antibody binding by high chlorpropham concentrations was determined, which confirmed both the result of the

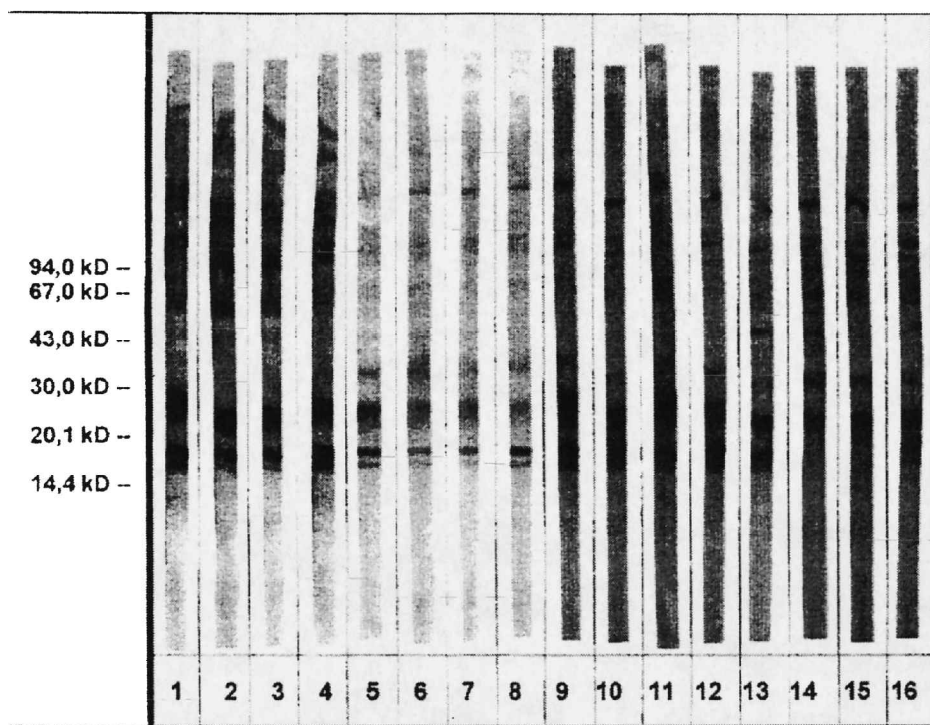


FIG. 5. Immunoblot – apple and apple plus chlorpropham (CP). lanes 1–4, 5–8, 9–12 and 13–16 are related to distinct patients. lanes 1, 5, 9 and 13 – pure apple; 2, 6, 10 and 14 – apple + CP (1:40); 3, 7, 11 and 15 – apple + CP (1:4); 4, 8, 12 and 16 pure apple (solvent control).

preceding examination of histamine release and the first hypothesis. The results of these *in vitro* investigations support the first hypothesis, because it appears most improbable that free chlorpropham residues remain able to perform a structural modification of IgE antibodies after the electrophoretic separation. An additional direct influence of chlorpropham on the IgE antibody structure *in vivo*, however, can not be finally excluded (second hypothesis).

In order to investigate possible interactions between pesticides and allergens high chlorpropham concentration had to be used. These free chlorpropham concentrations are not to be found in commercially available apples. But one has to be taken into consideration that the pesticide concentration during the agricultural application is much higher than at the point of time of sale. Nevertheless it can not be excluded that other pesticides in a lower dimension of concentration are able to react likewise or contrarily.

For the evaluation of the clinical relevance of these results clinical studies including provocation tests are necessary. Further investigations in this field with the object of other pesticides in a sensible combination with foods could discover similar or contrary effects. In addition, combinations of pesticide active substances should be examined for allergy modulating effects. Investigations on apples treated with pesticides under strongly strict conditions would be of particular interest. Because of that more realistic and more controllable conditions could be performed and the later spiking of the protein extracts could be dropped.

If epitope structures of food allergens are known, specific experiments are possible after isolation and enrichment or synthesis of these molecules to examine the kind and position of the chemical binding with pesticides.

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