Allergic properties and differential response of walnut subjected to processing treatments

Beatriz Cabanillas a,b,* , Soheila J. Maleki c, Julia Rodríguez b, Hsiaopo Cheng c, Suzanne S. Teuber d, Mikhael L. Wallowitz d, Mercedes Muzquiz e, Mercedes M. Pedrosa e, Rosario Linacero f, Carmen Burbano e, Natalija Novak b, Carmen Cuadrado e, Jesús F. Crespo b

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Abstract

The aim of this study was to investigate changes in walnut allergenicity after processing treatments by in vitro techniques and physiologically relevant assays. The allergenicity of walnuts subjected to high hydrostatic pressure and thermal/pressure treatments was evaluated by IgE-immunoblot and antibodies against walnut major allergen Jug r 4. The ability of processed walnut to cross-link IgE on effector cells was evaluated using a rat basophil leukemia cell line and by skin prick testing. Susceptibility to gastric and duodenal digestion was also evaluated. The results showed that walnuts subjected to pressure treatment at 256 kPa, 138 °C, were able to diminish the IgE cross-linking capacity on effector cells more efficiently than high pressure treated walnuts. IgE immunoblot confirmed these results. Moreover, higher susceptibility to digestion of pressure treated walnut proteins was observed. The use of processed walnuts with decreased IgE binding capacity could be a potential strategy for walnut tolerance induction.

Introduction

Walnut consumption has been demonstrated to be a healthy dietary habit, due to its benefits in reducing serum cholesterol concentrations and oxidative stress (Banel & Hu, 2009). However, walnut is the most frequently involved food in anaphylactic reactions after peanut. Three of 32 fatal cases of anaphylactic reactions to foods, reported between 1994 and 1999 to the US register of allergies, were caused by walnuts (Bock, 2003). Walnut was the first cause of food anaphylaxis-related fatalities, were caused by walnuts (Bock, 2003). However, there are regional differences in walnut allergenicity patterns. Oral allergy syndrome after walnut consumption without other symptoms can be found in certain areas of Europe (Asero, 1999; Mielgo, Cabanillas, Crespo, & Rodríguez, 2007).

Most of nuts allergens described so far are seed storage proteins, such as vicilins (7S globulins), legumins (11S globulins) and 2S albumins. Other nuts allergens are profilins and pathogenesis-related protein homologues (Roux, Teuber, & Sathe, 2003). Allergens from different nuts belonging to the same protein family can present homologies in their amino acids sequences, which can contribute to the IgE cross-reactivity observed among tree nuts and between peanut and other tree nuts (Asero, Mistrello, Roncarolo, & Amato, 2004; de Leon et al., 2005, 2003; Goetz, Whisman, & Goetz, 2005). Recently, common linear and conformational IgE-binding epitopes have been described for some nut allergens (Barre, Jacquet, Sordet, Culerrier, & Rougé, 2007; Barre et al., 2005).
2008; Robotham et al., 2009). In the specific case of walnut, five allergens have been identified so far: Jug r 1 (2S albumin), Jug r 2 (7S globulin), Jug r 3 (LTP), Jug r 4 (11S globulin), and Jug r 5 (prolin). Immunologically and clinically, Jug r 4 is one of the major allergens in walnut with significant sequence homology with other allergenic 11S globulins, such as Cor a 9 (hazelnut), Ana o 2 (cashew nut) and Ara h 3 (peanut), contributing to their IgE cross-reactivity (Costa, Carrapotoso, Oliveira, & Mafra, 2014).

Currently, the only effective treatment for walnut allergy is avoidance of this tree nut in any form, however, due to its widespread use and the potential cross-contamination in bakeries, industrially processed food lines, etc., total avoidance is difficult.

Certain foods are subjected to processing methods in order to improve their quality, organoleptic properties, preservation and safety. These processing methods have the potential to modify food proteins, which could alter food allergenicity by disruption of IgE epitopes. In contrast, some treatments, such as roasting, can increase the allergenicity of certain foods through chemical reactions between proteins and reducing sugars which can generate neoallergens (Cabanillas Martin, Crespo, Burbano, & Rodríguez, 2010; Cuadrado et al., 2009; Maleki, Chung, Champagne, & Raufman, 2000). Among treatments, such as boiling, microwave heating and pressure cooking, processing based on pressure and heat seems to have an important impact on reducing IgE-binding capacity in vitro as measured via immunoblot and ELISA (Alvarez-Alvarez et al., 2005; Cabanillas et al., 2012; Cuadrado et al., 2009, 2011; Guillamon et al., 2008). However, physiologically relevant experiments analyzing the IgE cross-linking capacity of these processed foods on effector cells and the susceptibility to digestion have not been reported so far. Such studies are of utmost importance as a preliminary step prior to clinical oral challenge trials to confirm a possible decrease in allergenicity of these processed foods.

The use of processed food with diminished IgE binding capacity is also an attractive strategy for oral immunotherapy. Clinical trials have shown that around 70% of tested children, with milk and egg allergies, tolerated heated products with milk or egg with an association of increased levels of specific IgG4 antibodies and decreased in SPT wheals size (Lemon-Mulé et al., 2008; Nowak-Wegrzyn et al., 2008).

Since processing techniques able to reduce allergenicity of foods would be of considerable importance for consumers, we aimed to investigate, for the first time, in walnut, if a change in allergenicity can be produced by thermal/pressure treatments and whether similar effects could be obtained with other pressure processing methods, such as emerging technologies like high hydrostatic pressure (HHP) processing. We aimed to evaluate differences in IgE binding by means of in vitro techniques, such as immunoblot, but also to present new aspects on the evaluation of pressure treated foods using more physiologically relevant assays.

2. Materials and methods

2.1. Patients and sera

Sera from 5 patients (P1–P5) with walnut allergies, confirmed on the basis of either a history of recent documented severe anaphylaxis after walnut ingestion or a positive double-blind placebo-controlled food challenge (DBPCFC) with walnut, were used in this study. All subjects had a positive skin prick test (SPT) response to walnut (6.5–12.5 mm, median = 10.5 mm) performed according to standard methods (Malling, 1993) and a specific serum IgE level to walnut ranging from 0.6 to 51.7 kU/L (median = 5.3 kU/L) as quantified by the fluorescent enzyme immunoassay (CAP–FEIA system, Phadia, Uppsala, Sweden). After informed consent, 5 additional patients (P6–P10) sensitized to walnut underwent SPT with non-processed and processed walnut according to standard methods (Malling, 1993). Two healthy subjects were tested with the same samples as a control group. The study was approved by the Ethics Committee of the Hospital Universitario 12 de Octubre, Madrid, Spain (Permission No. 0312150129).

2.2. Plant material and processing

Walnuts (Juglans regia, variety Chandler) obtained from the Germoplasm Bank of Institut de Recerca i Tecnologia Agroalimentàries (IRTA-Mas de Bover, Tarragona, Spain) were used in the study. Whole walnut seeds were immersed in distilled water (1:5 w/v) and autoclaved using an autoclave Compact 40 Benchtop (Priorclave, London, UK) at 121 °C, 120 kPa, for 15 and 30 min and at 138 °C, 256 kPa, for 15 and 30 min. Untreated and autoclaved walnut seeds were ground and defatted with n-hexane (34 ml/g of flour) for 4 h, shaken, and air-dried after filtration of the n-hexane. Defatted flour from untreated walnut was the control for autoclaved samples.

The conditions for high-hydrostatic pressure treatment were established according to Omi, Kato, Ishida, Kato, and Matsuda (1996) and Kato, Katayama, Matsubara, Omi, and Matsuda (2000) who observed a release of allergenic proteins when rice or soybean were immersed in distilled water overnight and then subjected to high pressure. Therefore, in our study, walnut defatted flours were dissolved in distilled water (1:4 w/v) 20 h before HHP treatment. HHP using pressures of 300, 400, 500 and 600 MPa for 15 min in a multivessel high-pressure equipment (HHP, ACH, France) at 15 °C was applied. Defatted flour from walnut soaked for 20 h in distilled water and non-HHP-treated was the control for high-pressure samples. The nitrogen contents of the samples were determined by LECO analysis according to standard procedures based on Dumas method (AOAC, 1995). The total protein content was calculated as N × 5.3 (AOAC, 1995). The analyses were carried out in duplicate.

2.3. Protein electrophoresis and IgE immunoblot experiments

Protein electrophoresis of the defatted flours was carried out as previously described by Cabanillas et al. (2012). Briefly, defatted flours from untreated and treated walnut were dissolved by heating at 65 °C for 5 min in standard electrophoresis sample buffer, containing 2% sodium dodecyl sulfate (SDS) and dithiothreitol (DTT) (Invitrogen, Carlsbad, CA, USA). The samples in SDS-sample buffer were centrifuged at 5500g for 15 min. The supernatants were removed, aliquoted and stored at −20 °C. The same amount of protein (20 µg) calculated from LECO analysis from each sample was electrophoresed in 4–20% Tris–Glycine gel. Proteins were visualized with GelCode Blue Stain Reagent (Thermo Scientific, Waltham, MA, USA). For Western blot, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Bedford, MA, USA). After blocking with 5% dry milk in PBS, membranes were incubated overnight with individual sera from 5 patients with walnut allergy (P1–P5) (1:20 dilution) washed and then treated with Horseradish peroxidase (HRP) conjugated mouse anti-human IgE (1:5000 dilution for 30 min) (Southern Biotech, Birmingham, AL, USA). Detection of IgE-binding proteins was achieved by means of enhanced chemiluminescence, according to the manufacturer’s instructions (Amersham Biosciences, Piscataway, NJ, USA). The signal was measured using CCD camera system (Fuji Photo Film Co., Ltd., Duluth, GA, USA).
2.4. Anti Jug r 4 Western blot

Samples (20 μg of protein) were electrophoresed and transferred to a PVDF membrane as described in the previous section. After blocking with 5% dry milk in PBS, the membrane was incubated with a polyclonal IgY Chicken anti-Jug r 4 antibody (1:10,000 dilution) during 1 h at RT. The membrane was washed and then incubated with HRP-labelled anti-chicken IgY (1:100,000) (Sigma, Saint Louis, MO, USA) for 30 min at RT. Detection was achieved as described above.

2.5. Mediator release assay

To analyze the allergenic activity of untreated and treated walnut, the rat basophil leukemia cell line RBL-48 transfected with human FceRI/βx was used (Gilfillan et al., 1992). Cells were cultured in very low endotoxin RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum, 1% antibiotic/antimycotics and 500 μg/ml of active genetin (Merck Chemicals, Nottingham, UK). Cells at 1 x 10^5/ml were passively sensitized by incubation with 5 individual sera from patients with walnut allergy (P1–P5) [final dilution 1:20, 1:40 or 1:60 depending on sera availability] at 37 °C, 5% CO2 overnight in 96-well tissue culture plates (Corning Inc., NY, USA). Sensitized cells were exposed in triplicate for 60 min to 1 mg/ml of untreated and treated (autoclave 256 kPa, 15 or 30 min; HHP 500 or 600 MPa) walnut protein extracts obtained with buffered saline borate as a solvent (BSB; 0.1 M H3BO3, 0.025 M Na2B4O7, 0.075 M NaCl). β-Hexosaminidase release was measured as previously described (Kuehn, Radinger, & Gilfillan, 2010). RBL cells were lysed with 1% Triton X-100 for total β-Hexosaminidase release. The percentage of β-Hexosaminidase release induced by treated or untreated walnut was calculated as previously described (Kuehn et al., 2010). The decrease of β-Hexosaminidase release was calculated using the formula: (1 – RT/RC) x 100 where RT is the percentage of β-Hexosaminidase release obtained with untreated walnut challenge and RC is the percentage of β-Hexosaminidase release obtained with untreated walnut challenge.

2.6. In vitro digestion of treated and untreated walnut

Protein extracts from untreated, high-pressured (600 MPa) and autoclaved (120 kPa, 30 min) walnuts obtained with buffered saline borate as a solvent, were incubated in simulated gastric fluid, SGF: 0.084 M HCl, 0.035 M NaCl and 0.009 μM pepsin (Sigma, Saint Louis, MO, USA), pH = 1.2, final protein concentration 0.7 mg/ml, during 60 min at 37 °C. Digestion was stopped by addition of 100 mM NaHCO3 at a final pH of 6. Aliquots were taken at time 0 and after 60 min of digestion. Then, the gastric digests adjusted at pH 6 were incubated with 0.0122 μM trypsin and 0.0117 α-chymotrypsin (Sigma, Saint Louis, MO, USA) at 37 °C. Aliquots were taken for SDS–PAGE analysis at 1, 15, 30, 60, 120 min, and overnight (15 h) and heated at 80 °C for 5 min. A silver staining of proteins by traditional protein extraction methods (Schmitt, Nesbit, Hurlburt, Cheng, & Maleki, 2010) therefore defatted flours that were directly solubilized in sample buffer were used for the SDS–PAGE and immunoblot analysis carried out in this study. The results showed that electrophoretic migration patterns of high-pressured walnut proteins were similar to untreated walnut. Sensitized cells were exposed in triplicate to the same untreated walnut, high-pressured walnut at 600 MPa, previously unrecognized in the control walnut. Autoclaved walnut (120 and 256 kPa) showed less distinctly stained bands in SDS–PAGE (Fig. 1A) but an increase of a smear of proteins. IgE immunoblot using individual sera (Fig. 1B) showed a similar pattern to SDS–PAGE with an overall reduction in the distinct IgE-reactive bands as pressure and time of treatment increased.

3. Results

3.1. Sera from patients with walnut allergy recognize less IgE-binding proteins from autoclaved walnut than high-pressured walnut in immunoblot

The protein migration patterns of walnut before (control) and after pressure treatments are shown in Fig. 1A. Samples were also analyzed for differences in IgE binding by immunoblot using individual sera from 5 patients (P1–P5) with a clinical allergy to walnut (Fig. 1B). Previous studies have shown that certain physical processing can alter protein solubility affecting the extraction of soluble proteins by traditional protein extraction methods (Schmitt, Nesbit, Hurlburt, Cheng, & Maleki, 2010) therefore defatted flours that were directly solubilized in sample buffer were used for the SDS–PAGE and immunoblot analysis carried out in this study. The results showed that electrophoretic migration patterns of high-pressured walnut proteins were similar to untreated walnut. Sensitized cells were exposed in triplicate to the same untreated walnut, high-pressured walnut at 600 MPa, previously unrecognized in the control walnut. Autoclaved walnut (120 and 256 kPa) showed less distinctly stained bands in SDS–PAGE (Fig. 1A) but an increase of a smear of proteins. IgE immunoblot using individual sera (Fig. 1B) showed a similar pattern to SDS–PAGE with an overall reduction in the distinct IgE-reactive bands as pressure and time of treatment increased.

3.2. Jug r 4 recognition in high-pressured walnut differs from autoclaved walnut

Jug r 4 is a major walnut allergen which belongs to the legumin group of proteins. We used specific anti-Jug r 4 antibodies in order to identify Jug r 4 molecules in walnut before and after high-pressure and autoclave treatments. Immunoblot results (Fig. 2) confirmed the results obtained in SDS–PAGE (Fig. 1A) and IgE immunoblot (Fig. 1B). A decrease in the relative Jug r 4 levels could be observed in autoclaved walnut samples with increased pressure and time. In contrast, high-pressure treatments did not reduce the recognition of the acidic or basic subunits of Jug r 4, but caused a slight increase in the recognition of this allergen with 600 MPa treatment.

3.3. Processing at 256 kPa, 138 °C reduces the capacity of walnut allergens to trigger degranulation in a RBL mediator release assay

We sought to analyze the capacity of high-pressured or autoclaved walnut compared to control walnut to trigger degranulation of effector cells. To model this, we used the rat basophilic leukemia cell line RBL-48 passively sensitized with 5 sera from patients with walnut allergies (P1–P5). Sensitized cells were exposed to the same concentration of treated and untreated walnut protein extracts. Fig. 3 shows the percentages of β-Hexosaminidase release from RBL cells challenged with walnut processed at 256 kPa, 138 °C (A) and high-pressured walnut (B). The percentage of decrease of β-Hexosaminidase release is represented in a box plot (Fig. 3C). The results showed that a reduction of β-Hexosaminidase release could be achieved with autoclave treatments compared to high-pressure treatments.

3.4. Processing treatments make walnut more susceptible to digestion

An in vitro digestion system using gastric and duodenal enzymes was carried out in order to evaluate the effects of pressure in the digestibility of walnut proteins. Fig. 4 shows the
silver staining in polyacrylamide gels of untreated control, high-
pressured (600 MPa) and autoclaved (120 kPa, 30 min) walnut di-
gested with pepsin (60 min) and trypsin/α-chymotrypsin (1, 15,
30, 60, 120 min, and 15 h). The results showed that numerous pro-
teins from untreated and high-pressured walnut proteins were
quickly digested after 1 h of pepsin incubation; however, resistant
proteins with molecular weight under 15 kDa and between 55 and
100 kDa could be visualized even after overnight duodenal diges-
tion of untreated walnut. In contrast, fewer resistant proteins could
be detected in high-pressured walnut after 2 h of duodenal diges-
tion. Autoclaved walnut proteins showed higher susceptibility to
digestion since no bands after 120 min of sequential digestion
could be detected.

3.5. Autoclave treatments decrease the wheal size in SPT

SPT were performed in 5 patients sensitized to walnut (P6–
P10). In this test, the capacity of processed walnuts to crosslink

Fig. 1. SDS–PAGE (A) and IgE immunoblots (B) of untreated walnut (controls; lanes 1 and 6) and treated-walnut: autoclave at 120 kPa for 15 min (lane 2) or 30 min (lane 3);
autoclave at 256 kPa for 15 min (lane 4) or 30 min (lane 5); Hydrostatic high pressure at 300 MPa (lane 7), 400 MPa (lane 8), 500 MPa (lane 9) and 600 MPa (lane 10). IgE
immunoblots were carried out using individual sera from 5 patients allergic to walnut (P1–P5). Proteins in high-pressured walnut at 600 MPa unrecognized in control walnut
by patients sera are indicated by arrows.

Fig. 2. Anti-Jug r 4 Western blot of untreated walnut (control; lanes 1 and 6) and
treated-walnut: autoclave at 120 kPa for 15 min (lane 2) or 30 min (lane 3);
autoclave at 256 kPa for 15 min (lane 4) or 30 min (lane 5). Hydrostatic high
pressure at 300 MPa (lane 7), 400 MPa (lane 8), 500 MPa (lane 9) and 600 MPa (lane
10).

Fig. 3. Percentages of β-Hexosaminidase release from RBL cells sensitized with the
sera from 5 walnut allergic patients (P1–P5) and challenge with untreated control
walnut and autoclaved walnut (A) or high-pressured walnut (B). The percentages of
decrease of β-Hexosaminidase with autoclave and high pressure treatments were
calculated as described in Section 2 for the 5 patients. Results are represented in a
box plot (C). AU1: autoclave 256 kPa, 15 min; AU 2: autoclave 256 kPa, 30 min;
HP1: high pressure 500 MPa; HP2: high pressure 600 MPa.
specific IgE antibodies on the surface of mast cells causing the release of histamine and other mediators was evaluated. Fig. 5 depicts the results of SPT with untreated and processed walnut flours (autoclaved at 120 and 256 kPa, 30 min and high-pressured at 300 and 600 MPa). All patients had a positive SPT with high-pressured walnut and its untreated control. In patient P10, the wheal size was similar when control and high pressure-walnut were tested. Patient P7 showed a slight decrease in the size of the wheal with high-pressured walnut at 600 MPa. However, for patients P6, P8 and P9, an increase in the wheal size was observed with high-pressure walnut at 300 MPa (patients P8 and P9) and 600 MPa (patient P6). None of the patients reacted to autoclaved walnut at 120 kPa, 30 min, and four out of five patients showed no skin reactivity with autoclaved walnut at 256 kPa, 30 min. Patient P10 showed a 3 mm wheal with autoclaved walnut at 256 kPa, which was smaller than control walnut wheal (5.25 mm).

4. Discussion

In the present study, we have shown for the first time in walnut that IgE binding capacity of its proteins decreased after pressure treatment at 256 kPa, 138 °C. More specifically, we showed that the recognition of the major walnut allergen Jug r 4 decreased after such treatment. We also found that IgE-binding proteins from walnut were stable under high pressure processing and in some cases an increased IgE binding capacity could be observed. In accordance with these results, Han, Matsuno, Ikeuchi, and Suzuki (2002) found that high-pressure treatments (200–600 MPa) of beef extract did not show any significant changes in the IgE binding with sera from patients allergic to beef. However, in contrast, in another study the allergenicity of bovine gamma globulin, a beef allergen, was decreased by high-pressure treatment (Yamamoto et al., 2010). Hushand et al. (2011) found that neither thermal treatment alone (115 °C) nor high pressure alone (700 MPa) at an initial temperature of 20 °C caused changes in the immunoreactivity of the apple allergen, Mal d 3. However a combination of both, high pressure at 700 MPa and high temperature (115 °C), was the most effective method to reduce the allergenicity of this allergen. These findings are in agreement with the results obtained in the present study, where high pressure at low temperature did not produce changes in IgE binding proteins in walnut, whereas a combination of pressure and heat (autoclaving) was more efficient in decreasing them.

Besides in vitro experiments of immunoblot, in this study we aimed also to analyze the ability of pressure treated walnut to cross-link IgE and activate effector cells. Passively sensitized RBL cell lines have been widely used as a model of the allergic type I reaction to determine the biologic activity of allergenic extracts and to establish possible correlations with classical methods in allergen standardization (Hoffmann et al., 1999; Porterfield et al., 2009). The results indicated that a reduced mediator release in sensitized RBL cells could be achieved with autoclaved walnut compared to high-pressured walnut. Moreover, the wheal size in SPT with autoclaved walnut was largely diminished compared to untreated walnut; however one patient showed a 3 mm wheal with autoclaved walnut at 256 kPa. This result indicates that autoclaved walnut, although in a lower degree; still retains a certain capacity to crosslink specific IgE antibodies on the surface of effector cells.

Special attention was paid in the present study to the potential loss of protein solubility in walnut due to processing. It can be hypothesized that the decrease of IgE binding capacity after some treatments could be caused by a decrease of protein solubility. In order to address this point, in the in vitro experiments performed in this study, defatted untreated and treated walnut flours were directly solubilized in sample buffer and heated at 65 °C for SDS-PAGE/immunoblot in order to obtain the most efficient protein solubilization. When the use of protein extracts was necessary (RBL assays and in vitro digestion) buffered saline borate (BSB) was used as a solvent. This buffer was judged as the optimum solvent for nut protein extractions among 10 buffers and solutions in an extensive study carried out with nuts. In that study, protein solubility of walnut and pecan specifically improved by the mentioned buffer (Sathe et al., 2009). Moreover, here we additionally checked the insoluble fraction after BSB protein extraction and no IgE binding proteins in autoclaved walnut (256 kPa, 30 min)
were found (data not shown). Therefore, the large amount of modifications in walnut allergenic proteins observed after autoclave treatments at harsh conditions cannot be explained by a decrease of solubility but perhaps to a potential fragmentation of the proteins. This fragmentation would generate short peptides that although retaining a certain capacity to cross-link IgE as observed in the RBL assays and SPT, this capacity is largely diminished. Moreover, the higher susceptibility to physiological digestion of autoclaved walnut proteins obtained here could have a potential beneficial effect on preventing adverse responses. Further studies are necessary in order to evaluate sensorial properties of this processed walnut. In a previous report, Guillamon et al. (2010) found that autoclaved lupine flour at 256 kPa had similar breadmaking and sensorial properties to untreated flour and that autoclaving lupine flour at 256 kPa, 138 °C had a similar texture to untreated walnut, but an extensive study of the physical and sensory properties of this processed nut is required.

Application of pressure treatment at 256 kPa, 138 °C could be also potentially carried out in other nuts, such as hazelnut and cashew. Further studies would be necessary to evaluate the behaviour of allergens from these nuts after pressure treatment.

In conclusion, in this study we have shown that pressure treatment at 256 kPa, 138 °C was able to decrease IgE-binding of walnut proteins in vitro more efficiently than high pressure treatments at low temperature and was also able to largely diminish IgE cross-linking capacity on effector cells. The use of processed walnut with decreased IgE binding capacity could be a useful strategy for walnut tolerance induction. Evaluation of sensorial properties and clinical oral challenge trials are necessary to analyze organoleptic properties and to confirm the decrease of allergenicity of this processed nut.

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Conflict of interest

The authors declare no conflict of interest.

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