

Short communication

IgE binding to almond proteins in two CAP-FEIA-negative patients with allergic symptoms to almond as compared to three CAP-FEIA-false-positive subjects

Background: Allergy to almonds has been frequently reported, but data on the identification of the almond allergens, as well as on the reliability of the methods for *in vitro* detection of specific IgE for these allergens, are scant. This study aimed to identify the almond allergens and to evaluate the reliability of the CAP-FEIA as the standard system for detection of almond-specific IgE with clinical significance.

Methods: Immunoblotting performed with an almond-protein extract was carried out on the sera of five patients who had previously been tested by the CAP-FEIA system; two of these patients had tested negative with the CAP-FEIA system but suffered life-threatening laryngeal edema after eating almonds, whereas the other three subjects, who had tested positive with CAP-FEIA, did not present any symptoms subsequent to almond ingestion.

Results: The sera of the two symptomatic CAP-FEIA-negative patients had IgE that bound only to a 37-kDa protein in immunoblotting. On the contrary, the sera of the three asymptomatic subjects all showed IgE binding to two almond proteins of 62 and 50 kDa, corresponding to the glycosylated components of the extract.

Conclusions: The results here presented suggest that, at least for the examined subjects, the positivity to almond, as measured with a standard laboratory method, is due to the presence of the 62/50-kDa glycoproteins with little or no immunologic significance, and not to the binding to the 37-kDa polypeptide, which appears to be a true almond allergen.

G. Pasini, B. Simonato

Dipartimento di Biotechnologie Agrarie, Università di Padova, Padova

M. Giannattasio

Dipartimento di Arboricoltura, Botanica e Patologia vegetale, Università di Napoli, Naples

C. Gemignani

Istituto di Medicina del Lavoro, Università di Padova, Padova

A. Curioni

Dipartimento Scientifico e Tecnologico, Università di Verona, Verona, Italy

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Prof. Andrea Curioni
Dipartimento Scientifico e Tecnologico
Università di Verona
Strada Le Grazie
37134 Verona
Italy

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Almonds are widely consumed in many forms, varying from raw seeds to roasted snacks, and as an ingredient in several foods of traditional and nontraditional preparation.

Allergy to almonds has been frequently reported (1, 2), making this tree nut one of the most common allergenic foods (3). However, data on the identification of the allergens responsible for IgE binding in allergic patients are very scant. Bargman et al. (4) were able to identify several protein bands bound by the IgE of almond-allergic patients' sera. Most of the binding occurred with proteins of molecular weights of 30–70 kDa, although a precise calculation of the M_r (and, thus, identification) of the single bands was not reported.

In vitro specific IgE detection and skin prick tests (SPT) are the diagnostic procedures commonly used to identify or exclude foods responsible for IgE-mediated

allergies. These tests are considered most valuable when negative because their negative predictive value is high (5). On the contrary, positivity to *in vitro* specific IgE detection and/or SPT has a low predictive value and correlates poorly with clinical reactivity.

Here we describe the cases of two patients with allergic symptoms to almonds who were negative in IgE-FEIA testing and whose IgE bound to a 37-kDa almond band in immunoblotting. Moreover, we examine the serum IgE-binding pattern of three subjects who were positive in IgE-FEIA testing, and who never experienced symptoms of allergy to almonds.

Material and methods

Patients

Patient C.O. A 31-year-old woman reported several

episodes of oral allergic syndrome (OAS) immediately after eating almonds. She suffered twice from angioedema and laryngeal edema and needed corticosteroid and antihistamine therapy. The patient, who suffered also from seasonal rhinitis, had positive skin prick tests to mite, Gramineae, and almond. Specific IgE to almond was undetectable.

Patient C.A. A 41-year-old man reported several episodes of angioedema and/or laryngeal edema with dyspnea and general malaise shortly (10–30 min) after ingestion of raw almonds. In these circumstances, he was treated at the emergency room with systemic corticosteroids and antihistamines, and improved within hours. He has also suffered several times from symptoms of lesser gravity (itching of the mouth and mild facial edema), which had disappeared spontaneously, after eating roasted almonds or almond pastries. Prick test with an almond extract was positive, but specific IgE was undetectable. The patient had a previous history of seasonal rhinoconjunctivitis. Skin prick tests and specific IgE for pollens of *Parietaria*, *Platanus*, and Gramineae mix were positive.

Patients T.R. and D.R. Two 30-year-old patients, suffering from latex allergy, had positive skin test also to Gramineae and tree pollens and almond. Detection of specific IgE to almond was positive (CAP score 3), but the patients did not report any symptoms after almond ingestion.

Patient P.A. A 42-year-old man with seasonal rhinitis had a positive skin test and specific IgE to Gramineae, *Parietaria*, and several foods of plant origin, including almonds (CAP score 2). He had never complained of symptoms related to almond ingestion.

Skin prick tests and specific IgE measurement

Skin prick tests were performed with a standard battery of glycerinated extracts (Lofarma, Milan, Italy). Histamine phosphate at 10 mg/ml and PBS were used, respectively, as positive and negative controls. The results of skin tests were evaluated at 15 min. Wheal diameters equal to or larger than 3 mm were considered positive in the absence of a reaction to PBS.

Specific IgE detection on patients' sera was performed by means of Pharmacia CAP-System IgE fluoroenzyme immunosorbent assay (CAP-System-FEIA, Pharmacia Diagnostic, Uppsala, Sweden). The results were expressed as CAP scores from class 0 to 6, according to the manufacturer's instructions.

Protein extraction

Raw whole almonds were ground in a coffee mill, defatted with petroleum ether, and extracted with 0.5 M NaCl in a solid-to-solvent ratio of 1:100 by stirring for 2 h at room temperature. After centrifugation (12 000 g for 15 min), 1 ml of the supernatant was added to 9 ml of cold acetone and left at 4°C for 30 min. The precipitated protein was then collected by centrifugation and resolubilized in 1.5 ml of a 0.2 M Tris-HCl buffer, pH 7.4, containing 2% (w/v) sodium dodecyl sulfate (SDS), 2.5% (v/v) 2-mercaptoethanol (2-ME), and 10% (w/v) glycerol. The protein solution was then heated in a boiling water bath for 5 min and used for SDS-PAGE.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and protein transfer

Electrophoresis in the presence of SDS was performed, according to Laemmli (6), in a 14% total polyacrylamide gel. A Mini-Protean II electrophoretic cell (Bio-Rad) was used. Electrophoresis was conducted at 50 mA constant current until the tracking dye bromophenol blue reached the bottom of the resolving gel. Gels were used for electroblotting or stained with Coomassie brilliant blue. Molecular weight (M_r) standard proteins (Bio-Rad) were phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), soy trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

Electrophoresed protein bands were transferred onto nitrocellulose membranes by semidry electroblotting, as previously described (7). M_r standard proteins on the membranes were marked by peroxidase spotting according to Eberini & Puglisi (8). Membranes were used either for immunodetection with patient sera or for glycoprotein staining.

Immunoblotting

Almond protein bands bound by serum IgE were detected by chemiluminescence, as previously described (9), with sera diluted 1:1 with phosphate saline buffer, containing 5% defatted powdered milk.

Glycoprotein detection

Glycosylated proteins were detected by treating membranes on which electrophoresed almond proteins had been transferred with concanavalin A (con A) and horseradish peroxidase, by the procedure of Weiss et al. (10).

Results

The clinical histories of patients C.O. and C.A. clearly indicated an allergy to almonds, although specific IgE to almond could not be detected by a standard

laboratory method. Immunoblotting experiments were carried out in order to detect serum IgE binding to almond salt-soluble proteins. The results obtained showed that the sera of both patients C.O. and C.A. contained IgE that bound only to a band with a SDS-PAGE mobility corresponding to 37 kDa (Fig. 1). No bands were recognized when a pool of sera of healthy subjects was tested (not shown).

The IgE-binding pattern of C.O. and C.A. was then compared to those of the three other atopic patients (T.R., D.R., and P.A.) that did not report any symptom after eating almonds, but who were positive to almonds in IgE-FEIA testing. An identical IgE-binding pattern to almond proteins was shown by immunoblotting for all these three subjects. In fact, all recognized two proteins with M_r of about 62 and 50 kDa, whereas no binding to the 37-kDa band was noted (Fig. 1).

For detection of glycosylated protein components, blots of almond proteins were treated with con A. This lectin binds mannose and glucose residues; therefore, it can be used to detect plant glycans carrying these sugars (10). Among all the proteins present in the blot, only two components were bound by Con A (Fig. 1), indicating their glycoprotein nature. Interestingly, these two con A-reactive components corresponded exactly to the two bands recognized by the three almond CAP-System-FEIA-positive patients.

Discussion

Here we describe the cases of two patients who suffered life-threatening laryngeal edema after eating almonds. This clinical manifestation seems to be the main danger deriving from allergy to nuts, including almonds (2). These patients were found to be negative in

CAP-System FEIA testing, but proved to have IgE that bound only a 37-kDa band in immunoblotting performed with an almond protein extract. A polypeptide with the same M_r was found to be a major component of the almond protein fraction extracted in water or salt solutions and is believed to correspond to one of the component subunits of amandin, the major globulin of almond (11). The 37-kDa almond polypeptide was shown to disappear completely from the extract after 15 days of storage, probably as a result of attack by endogenous proteinase(s). Moreover, in the same paper, it was also shown that the extractability of the various protein components from almonds is strongly dependent on the pH of the medium. In particular, the 37-kDa protein can be extracted only in the pH range from 5 to 8, with a maximum from pH 6.0 to 6.6 (11). Taking into account the particular behavior of this protein, the false negativity of C.O. and C.A. in the *in vitro* standard assay might be explained by the lability of the allergen and/or by its absence from the almond extract used for the preparation of the diagnostic kit.

On the contrary, the three people with no symptoms deriving from almond ingestion, but positive to this nut in IgE-FEIA, had IgE binding to almond proteins of 62 and 50 kDa, the only two glycosylated components of the extract. This may indicate the involvement of sugar residues carried by the reactive bands in the binding of serum IgE of these patients. The importance of sugar residues in the allergenic potential of plant proteins is controversial. Although these residues are often bound by IgE, it seems that they have only weak biologic activity, thus causing positive RAST results without apparent clinical effects (12–14). This may be also the case with the three IgE-FEIA-positive asymptomatic patients.

To the best of our knowledge, only one paper has been published to date on the IgE-binding pattern to almond proteins (4). In that paper, several bands were shown to be bound by individual sera in seven out of the eight RAST-positive patients tested. The major almond allergens, recognized by four patients, were identified as proteins of about 45–50 and 70 kDa, although the M_r was only roughly calculated. Taking into account the differences in M_r calculation between different experimental procedures, it is difficult to relate these bands to those identified by us. However, it is possible that some of them corresponded to the con A-reactive bands described here.

It has been recently shown that the IgE binding to one of the coconut allergens and to one of the walnut allergens is completely inhibited by an almond extract, suggesting a close similarity between the epitopes of the allergens of these tree nuts (15). It is interesting to note that the coconut and the walnut allergens have a M_r which is almost identical to that of the 37-kDa almond allergen here described,

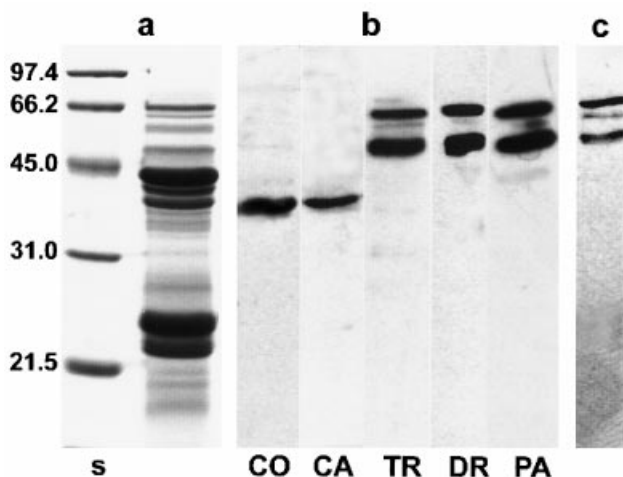


Figure 1. SDS-PAGE analysis of almond salt-soluble proteins. a) Coomassie-stained gel; b) IgE immunoblot with sera of patients C.O., C.A., D.R., T.R., and P.A.; c) glycoprotein detection by con A/peroxidase method. M_r standard proteins are in lane s.

suggesting also a close similarity of these proteins. Moreover, the two coconut-allergic patients were shown to recognize also a coconut band of 55 kDa, which is similar in M_r to the 50/62-kDa con A-reactive bands bound by the IgE of the three almond CAP-System-FEIA-positive asymptomatic patients. These findings should confirm the existence of homologous proteins in these seeds and give further indications regarding the involvement of legumin-type proteins in determining allergic responses (15).

The results presented here suggest that, at least for the subjects examined in this study, the positivity to

almond as measured with a standard laboratory method for specific IgE detection is due to the presence of the 50/62-kDa glycoproteins with little or no clinical significance, and not to the binding to the 37-kDa polypeptide. This implies that the allergen related to the severe symptoms of patients C.O. and C.A. is not detected in a routine laboratory test. Therefore, a negative *in vitro* response, which is commonly considered very reliable in ruling out an IgE-mediated reaction to a particular food, cannot have such a predictive value, at least for the diagnosis of almond allergy.

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