

ORIGINAL ARTICLE

Characterization of the sensitization profile to lupin in peanut-allergic children and assessment of cross-reactivity risk

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Keywords

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Abstract

Background: Case reports of allergy to lupin, due to primary sensitization or cross-reactions with other legumes, are increasing as a consequence of the augmented use of lupin flour in bakery, pasta formulations and other food items. The main allergens that have been associated with the sensitization to lupin are α - and β -conglutins and, to a lesser extent, γ - and δ -conglutin, but no conclusive data are available so far. The aim of this study was to characterize the sensitization pattern to lupin in a group of 12 Italian children allergic to peanut and identify the specific lupin proteins involved in the cross-reactivity with peanut.

Methods: The immunochemical cross-reactivity among peanut and lupin was evaluated by both *in vitro* immunoblotting and *in vivo* fresh food skin prick test (FFSPT). **Results:** The results showed that β -conglutin was recognized by cutaneous IgEs from 7/12 peanut-allergic children in FFSPT and serum IgEs from 5/12 in immunoblotting, while 4/12 and 8/12 patients tested positive to γ -conglutin in FFSPT and immunoblotting, respectively. No significant immunoreactive responses were observed to α - and δ -conglutins under non-reducing conditions, but they were bound in FFSPT by the sera of 5/12 and 3/12 patients, respectively.

Conclusion: In this group of allergic children, β -conglutin has been identified as the major lupin allergen involved both *in vitro* and *in vivo* cross-reactivity with peanut proteins. The role of γ -conglutin in the cross-reactivity between lupin and peanut proteins was also relevant and clear, despite the observed unspecificity of the immunoblotting responses.

Lupin seed is an attractive source of proteins with a relevant content of essential amino acids, dietary fibre and a low concentration of starch. The growing use of lupin flour in bakery, pasta formulations, gluten-free products and other food items, is mostly due to its favourable nutritional and technological properties (1). Moreover, a number of health-promoting benefits have been ascribed to lupin seed components (2–6).

In recent years, reports on allergy to lupin, due to primary sensitization (7, 8) or to cross-reactions with other legumes, especially peanut (9–14), are increasing. A relatively recent large European study (15) showed that allergic cross-reactivity of lupin with peanut was around 15% for both children and adults.

Considering the increasing number of clinical cases of lupin allergy reported in the literature, in 2006, the European Union

included lupin among the allergens that must mandatorily be declared on the foodstuff labels (16).

As all edible legume seeds, the major protein fraction of lupin seeds is associated with storage proteins, including α - and β -conglutins as the two major globulin fractions and γ - and δ -conglutins in lower amounts (1).

The α -conglutin (legumin-like globulin or 11S globulin) consists of an heterogeneous assortment of acidic subunits of molecular weight (Mr) ranging from 47 to 54 kDa and one major basic subunit (20 kDa), linked by disulphide bridges (1).

The β -conglutin (vicilin-like globulin or acidic 7S globulin) is also made up by a number of heterogeneous polypeptide chains of 20–80 kDa with no disulphide bridging them. Extensive post-translational proteolytic processing increases the number of β -conglutin polypeptides (1).

γ -conglutin (basic 7S globulin) contains two disulphide-linked 30 and 17 kDa polypeptides, whereas δ -conglutin (2S albumin) consists of two disulphide-linked polypeptide chains of 9 and 4 kDa (1).

In the last years, we have studied the sensitization pattern to lupin involved in the primary or secondary sensitization to lupin mostly through molecular approaches (9, 11, 17). From these and other works, performed with both lupin-allergic patients (18–21) and peanut-allergic patients (22), it appears that a number of polypeptides belonging to the three main lupin protein families (α -, β - and γ -conglutins) are involved in the allergenic *in vitro* and *in vivo* responses. In particular, two polypeptide bands of 34.5 and 20 kDa corresponding to β -conglutin fraction and to α -conglutin basic subunit, respectively, were identified as major allergens of *Lupinus albus* and designated as Lup-1 and Lup-2 (19). Moreover, β -conglutin, corresponding to Lup an one, has been referred to as the major allergen of *Lupinus angustifolius*, as being identified by IgE of most of the sera collected from 12 lupin-allergic individuals (18).

The increased availability of lupin protein sequences in the databases has improved the knowledge in the field, allowing the conclusion that most lupin proteins are potential allergens. However, due to the intrinsic heterogeneity of the polypeptide chains, which is extremely acute for lupin proteins with respect to other legume seeds, and to the large extent of post-translational modifications, including glycosylation (1), lupin protein allergenicity is not easily amenable to specific sequences. Moreover, a certain extent of geographical distribution in the responses prevents a clear-cut definition of lupin allergens and epitopes. For these reasons, the need of augmenting the number of reports aimed at identifying and characterizing the sensitization pattern to lupin in various groups of allergic subjects is acute. To this purpose, in this work, we present the clinical and molecular data obtained with the sera of 12 peanut-allergic individuals.

Materials and methods

Lupin samples

A *Lupinus albus* protein concentrate supplied as freeze-dried powder was used in fresh food skin prick test (FFSPT) and immunoblotting tests.

Purified lupin proteins

α -, β - and γ -conglutins were purified from the total lupin protein extract using gel filtration chromatography to remove undesired components combined with ion-exchange chromatography to fractionate the three protein families, as detailed by Magni et al. (22). δ -conglutin was purified using selective precipitation steps, as described by Sironi et al. (23).

Patients

The design and aims of the study were authorized by the institutional review board of the Macedonio Melloni Hospital

(Milan, Italy). Informed consent was obtained from the parents of all children participating in this study.

Twelve patients (11/1, male/female) admitted for immediate symptomatic onset after ingestion of peanut or peanut derivatives between 1 December 2006 and 30 June 2007 were considered. According to hospital internal protocols, children were diagnosed as allergic to peanut in case of:

- 1 Positivity both to oral challenge and to skin prick test (SPT) and/or immuno-CAP to peanut proteins;
- 2 or, when oral challenge was contraindicated, positive SPT and/or immuno-CAP and a history of anaphylactic reaction after ingestion of peanut (24).

Fresh food skin prick test (FFSPT)

Skin prick tests with lupin was performed using freshly prepared allergens, as commercial lupin reagents were not available. Lupin protein concentrate and purified lupin proteins were suspended in the SPT vehicle (2% mannitol, 5.9% NaCl, 58% glycerol and 0.4% phenol; sterilized by microfiltration) to obtain a 5% final protein concentration.

Fresh food skin prick tests were performed by percutaneous lancing through a drop of allergen extract immediately wiped off with adsorbent paper using 1-mm-tipped lancets (Dome-Hollister-Stier, Warwickshire, UK). A solution of histamine phosphate in 50% glycosaline (10 mg/ml) and the vehicle were used as positive and negative controls, respectively. Wheal diameters were measured by a caliper disc marked in millimetres 15 min after testing, and a test was considered positive when the wheal diameter was wider than that of a negative control by at least 3 mm (25).

SDS-PAGE and immunoblotting

The pattern of specific IgE binding to single lupin proteins was determined by immunoblotting using patients' sera.

Lupin protein concentrate and purified lupin proteins were suspended in sample buffer with or without β -mercaptoethanol at different final concentrations. The proteins were then separated on a gradient 9–19% polyacrylamide gel according to Restani et al. (26) and electro-transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon P, Millipore, Bedford, USA). Polyvinylidene difluoride membranes were blocked with 1% gelatin and washed three times with 0.25% gelatin solution in 150 mM NaCl, 5 mM EDTA, 50 mM Tris and 0.05% Triton-X to prevent non-specific adsorption of the immunological reagents. Membranes were then immersed in 10 ml of 0.25% gelatin solution, containing 0.3 ml of allergic patients' serum. Antigen-IgE complexes were detected using goat anti-human IgE labelled with alkaline phosphatase (Sigma Aldrich, Italy). After incubation in the bromochloro-indolyl-phosphate/nitroblue tetrazolium solution, an intense precipitate developed at the site of the complex formation.

Quantitative evaluation of the immunoreactive bands was performed by a gel scanner (Sharp JX-330, Pharmacia Biotech) and the Image Master 1D Software. Protein bands were quantified by calculating the average density of pixels across the band length and integrating over the band width. Classes of

positive reactions were defined on the basis of an arbitrary scale of densitometric values, and six classes of reactivity were identified (Class ≥ 3 was considered clinically significant).

Moreover, immunoblottings were performed with (i) negative control sera; (ii) secondary antibody alone and (iii) positive-control serum/alkaline phosphatase without membrane being incubated with the secondary antibody, to identify any non-specific protein binding that could be misinterpreted as a positive result.

Results

The electrophoretic profiles of lupin protein concentrate and purified lupin conglutins under non-reducing and reducing conditions are reported in Figs 1 and 2, respectively.

Under non-reducing conditions, the lupin protein concentrate yielded a complex protein pattern, showing protein bands with a molecular weight from ~15 to 80 kDa (Fig. 1a, lane Lpc). α -conglutinin appeared as a group of various polypeptides of approximately 70 kDa, consisting in the acidic (47–54 kDa) and basic (20 kDa) subunits linked by disulphide bridge/s (Fig. 1a, lane α CN, black arrow). They can be separated upon disulphide bridge reduction using β -mercaptoethanol into two bands, as shown in Fig. 2a (lane α CN, black arrows). For β -conglutinin, which does not contain disulphide bridges, no differences in the electrophoretic pattern were observed under both analytical conditions (Figs 1a and 2a, lane β CN). On the other hand, γ -conglutinin appeared as a 47 kDa polypeptide under non-reducing conditions (Fig. 1a, lane γ CN, white arrow). A large-sized polypeptide, well visible under these experimental conditions (Fig. 1a, lane γ CN, black arrow), was previously shown to consist in the γ -conglutinin dimer (11). However, when the basic 7S globulin was treated with β -mercaptoethanol, two disulphide-linked polypeptide chains of approximately 30 and 17 kDa were separated (Fig. 2a, line γ CN, black arrows), with the concomitant and almost complete disappearance of large aggregates. A similar reducible behaviour was observed for δ -conglutinin, which predominantly exists as a dimer of a 14 kDa polypeptide (Fig. 1a, lane δ CN, black arrow). Each 14 kDa monomer consists of two disulphide-linked chains of about 9 and 4 kDa (1). Under

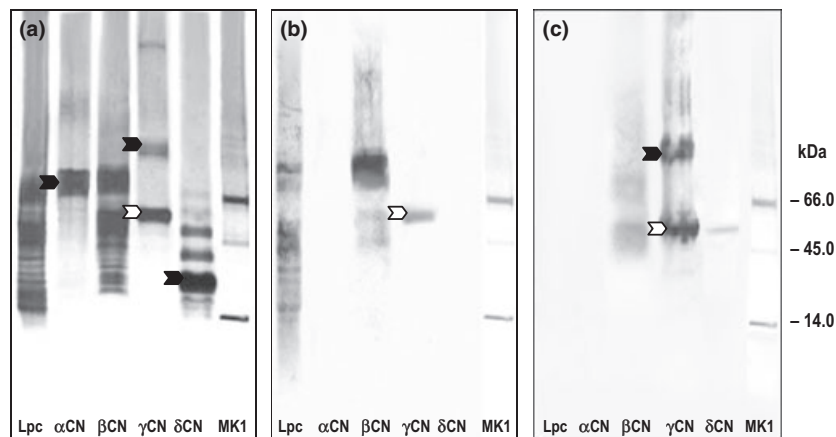
reducing conditions, only the 9 kDa is visible (Fig. 2a, lane δ CN, black arrow) because the 4 kDa chain was hardly blocked in the gel structure, due to its low Mr.

Panels B and C of Figs 1 and 2 show the different immunoreactivity of specific IgEs from the sera of two of the most representative peanut-allergic subjects (Children 2 and 9) included in this study. Child 2 was one of the two subjects tested positive at Double-Blind Placebo-Controlled Food Challenge (DBPCFC) when challenged with lupin-enriched pasta in a previous clinical study (11), whereas child 9 was among the subjects who did not show any clinical reaction at oral challenge with lupin pasta. As it is evident from Fig. 1b, the serum from child 2 significantly reacted to the lupin protein concentrate (Fig. 1b, lane Lpc). Interestingly, no immunoreaction was detected towards unreduced α -conglutinin (Fig. 1b, lane α CN), whereas specific IgE immunoreactivity, clearly associated with the 20 kDa basic subunit, was observed vs. this protein under reducing conditions (Fig. 2b, lane α CN, black arrow). The same behaviour vs. α -conglutinin was observed using the serum from child 9. In this case, the reactivity was exclusively towards the basic subunit (Fig. 2c, lane α CN, black arrow) as observed in most of sera from the children included in this study (data not shown). It can be noted that other bands in the region of 60 kDa (Fig. 2b, lane α CN, white arrow) were revealed with child 2 serum, likely belonging to β -conglutins, which often contaminate isolated α -conglutins. As a matter of facts, these bands are not visible with child 9 serum, which did not react with those proteins.

With regard to β -conglutinin, no differences in the immunoreaction responses under the two different analytical conditions were observed in both children (Figs 1 and 2, panels B-C, lane β CN), results consistent with the identical behaviour of this protein under reducing and non-reducing conditions. The sera of both subjects showed a high immunoreactivity vs. reduced and unreduced γ -conglutinin (Figs 1 and 2, panels B-C, lane γ CN, white arrows). In the case of child 9, reactivity was strong to the dimeric form of the protein, too (Fig. 1c, line γ CN, black arrow).

Eventually, the sera from both children were not immunoreactive against δ -conglutinin at both different working conditions.

Figure 1 SDS-PAGE under non-reducing conditions stained with Coomassie blue (a) and immunoblotting (b and c) of purified lupin proteins incubated with serum of two of the twelve children allergic to peanut (children 2 and 9, respectively). Lpc, lupin protein concentrate; α CN, α -conglutinin; β CN, β -conglutinin; γ CN, γ -conglutinin; δ CN, δ -conglutinin; MK1, molecular weight markers.



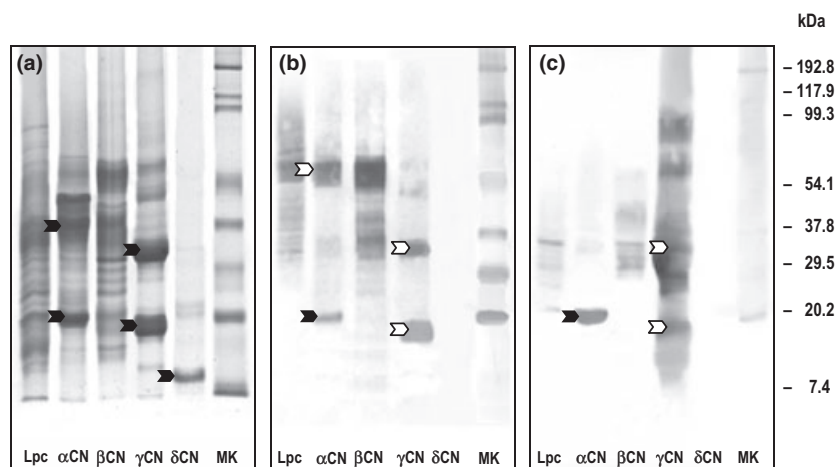


Figure 2 SDS-PAGE (a) and immunoblotting (b-c), under reducing conditions, of purified lupine proteins incubated with serum of two of the twelve children allergic to peanut. Lpc, lupine protein concentrate; αCN, α-conglutinin; βCN, β-conglutinin; γCN, γ-conglutinin; δCN, δ-conglutinin; MK, molecular weight markers.

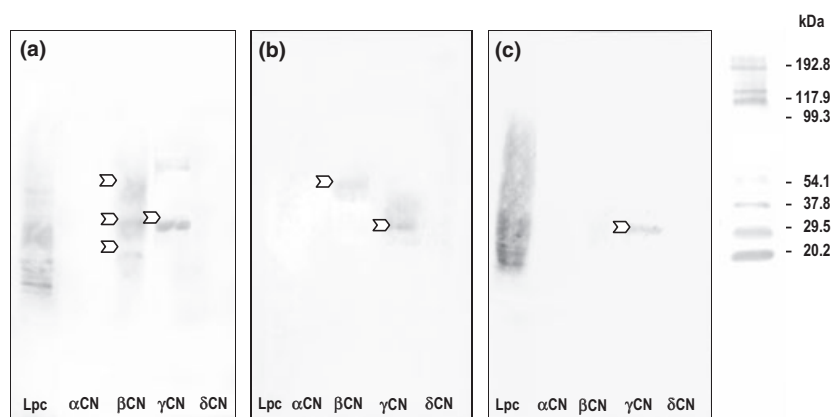


Figure 3 Immunoblotting of electrophoretic gels run under reducing conditions of purified lupine proteins incubated with negative control serum (a), secondary antibody alone (b), and positive-control serum reacted with the secondary antibody non-conjugated with alkaline phosphatase (c). Lpc, lupin protein concentrate; αCN, α-conglutinin; βCN, β-conglutinin; γCN, γ-conglutinin; δCN, δ-conglutinin.

To assess the extent of unspecific IgE reactivities, control membranes including a negative control serum (Fig. 3a), a secondary antibody-depleted membrane (Fig. 3b) and the serum of a patient allergic to peanut (child 9) treated with un-conjugated alkaline phosphatase secondary antibody (Fig. 3c) were set up. The results showed a certain unspecific response, especially with the lupin protein concentrate and γ-conglutinin (Figs 3a-c, lanes Lpc and γCN, white arrows). A slight immunochemical reactivity was also observed against β-conglutinin after incubation with the negative control serum and with the secondary antibody alone (Figs 3a and b, lane βCN, white arrows). These responses were, however, by far less intense than in the positive membranes of Figs 1 and 2 and in all other membranes not shown.

The results of immunoreactivity vs. purified lupin proteins evaluated by FFSPT and immunoblotting under non-reducing conditions are compared in Fig. 4. The intensity of the reactions obtained in immunoblotting was classified according to the densitometric analysis, as detailed in Method section.

Overall, the results obtained with the two different approaches using purified lupin proteins were consistent, with a substantial homogeneity of responses. In detail:

1 The sensitization to purified lupin conglutins was confirmed in 11 of 12 (11/12) children using FFSPT and in all children in immunoblotting.

2 The reactivity vs. purified lupin proteins at FFSPT was positive (wheal diameter ≥ 3 mm):

- i ain 5/12 children for α-conglutinin;
- ii bin 7/12 children for β-conglutinin;
- iii cin 4/12 children for γ-conglutinin;
- iv din 3/12 children for δ-conglutinin.

3 The sensitivity vs. purified lupin conglutins evaluated by immunoblotting performed under non-reducing conditions was positive (Class ≥ 3 was considered clinically significant):

- i ain 5/12 children for β-conglutinin;
- ii bin 8/12 children for γ-conglutinin.

No positive or clinically significant reactions in immunoblotting were found vs. unreduced α-conglutinin and δ-conglutinin in any child enrolled in this study.

Discussion

Sensitization to food allergens without allergic symptoms upon ingestion of the food items is common (27). Several studies have shown a significant risk of cross-reaction between peanut and lupin (9–14). This fact has also been confirmed in our previous study where 2 of 12 of the peanut-allergic children, included in a DBPCFC clinical trial, showed clinical symptoms when tested with lupin-enriched pasta (11), thus suggesting that

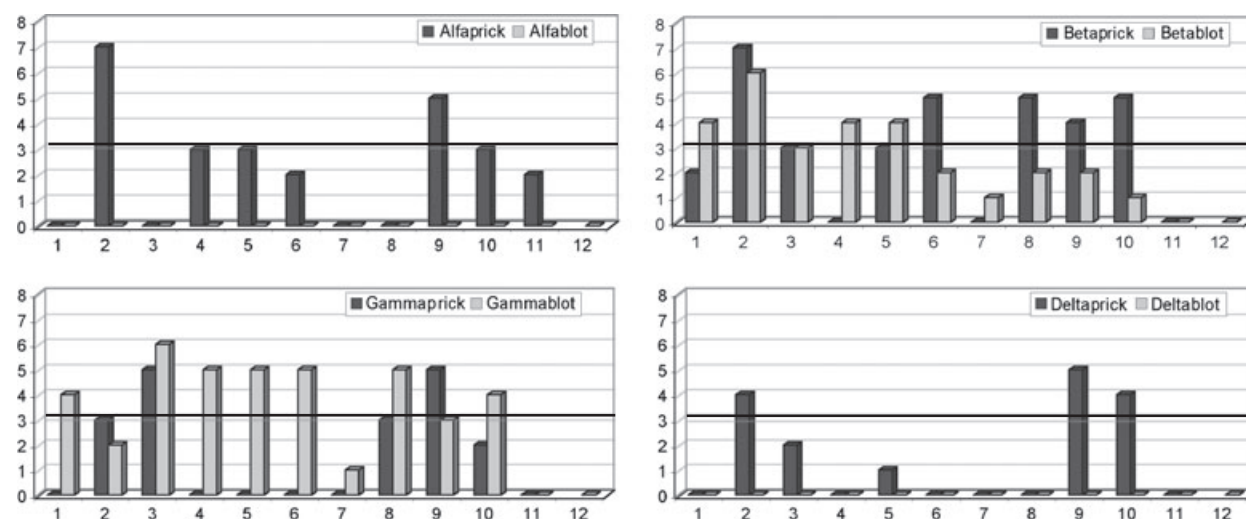


Figure 4 Sensitization patterns of the 12 patients evaluated by FFSPT and immunoblotting under non-reducing conditions. Each histogram represents the immunoreactivity vs. every single lupin protein. Black bar, FFSPT (wheal diameter in millimetres); Grey bar, immunoblotting, expressed as class of reactivity, see methods for details. Class ≥ 3 was considered clinically significant. alfa, α -conglutin; beta, β -conglutin; gamma, γ -conglutin; delta, δ -conglutin.

a specific alert in the labelling should be reported to avoid clinical reactions in consumers suffering from peanut allergy.

The present work aimed at the assessment of the specific pattern of sensitization to lupin proteins in the same group of 12 peanut-allergic children, to identify the role of each lupin protein in the development of IgE-sensitization.

In this work, special attention was paid to the conformational status of the proteins under investigation. Indeed, the immunoelectrophoretic analysis was performed also under non-reducing conditions to better correlate the outcomes of FFSPT, which does not use reducing conditions. This issue is especially relevant when the specific case of α -conglutin is considered, as already noted in previous papers (17, 20). Indeed, the immunoreactivity to α -conglutin at FFSPT was observed with 5 of the 12 sera from peanut-allergic children (including children 2 and 9), while the immunoblotting was negative for all children under non-reducing conditions, as it was showed for children 2 and 9 in Fig. 1. Conversely, under reducing conditions, the sera of the same five children showed well-defined IgE binding towards the basic subunit of α -conglutin, suggesting the presence of hidden linear epitopes in α -conglutin basic subunit. The complete unfolding of the protein upon reduction in disulphide bridges by β -mercaptoethanol and denaturation by SDS likely exposes these sequences to sera IgEs, thus causing a strong specific reactivity. This phenomenon was already described for other 11S globulins from peanut and soybean in patients allergic to peanuts (17) and from sesame in patients suffering for sesame allergy (28).

Our results suggest that β -conglutin is the major potential lupin allergen, because it was recognized by serum IgEs from 5 of 12 patients and cutaneous IgE from 7 of 12 peanut-allergic children. This finding is in agreement with those previously obtained by Goggin et al. (18), who found that IgE from most of the lupin-allergic subjects (8/12) studied recognized β -conglutin as the major allergen in *L. angustifolius* seeds. As a

matter of fact, lupin β -conglutin is now classified as a recognized allergen with the code Lup an 1 (18). β -conglutin has also been identified as one of the most important allergens in *L. albus* L. by a pool of sera from four lupin-allergic subjects and designated as Lup-1 (19). Nevertheless, Goggin et al. (18) showed that β -conglutin was not responsible for the cross-reaction between lupin and peanut, as none of the lupin-allergic subjects reacting to the acidic 7S globulin showed clinical responses upon ingestion of peanut. This fact was indirectly confirmed by Guillaumon et al. (19), who concluded that individuals allergic to peanut and lupin either react to different allergens or their IgEs bind to different epitopes of β -conglutin.

γ -Conglutin seems also to be involved in the allergenic responses to lupin proteins, because it was recognized by serum IgEs of 8/12 peanut-allergic children in immunoblotting under non-reducing conditions, including children 2 and 9, whereas only four patients tested positive towards the protein at FFSPT. A polypeptide of ~ 43 kDa, which might correspond with the unreduced γ -conglutin, has been identified as one of the major allergens in different works (12, 29, 30). However, our results showed a certain positive immunoblotting response towards γ -conglutin using the negative control serum, the secondary antibody alone and, the positive-control serum/unconjugated alkaline phosphatase secondary antibody. Thus, it can be assumed that the reactivity vs. this protein observed may in part be attributed to a non-specific binding, as it was previously hypothesized by Goggin et al. (18). However, the responses of positive children sera were dramatically more intense than the slight stains shown in Fig. 4.

Our immunoblotting results showed no reaction to δ -conglutin, which is considered a potent allergen in other seeds, in none of the patients' sera. These findings are in agreement with that previously reported by Magni et al. (17) in a study

involving the sera of five children suffering primary sensitization to lupin. However, a few positive responses (4/12) were observed towards δ -conglutin at FFSPT, suggesting that this protein may also be involved in the cross-reaction between lupin and peanut.

Conclusions

The extensive heterogeneity and complex electrophoretic pattern of white lupin proteins prevent the identification of sequential epitopes cross reacting with peanut-allergic sera.

Despite of this fact, this study has confirmed β -conglutin as the major lupin allergen in a population of peanut-allergic Italian children through *in vitro* and *in vivo* approaches. The role of α -conglutin in the cross-reactivity between lupin and peanut proteins seems to be mainly related to its basic subunit, which is highly homologous to the arachin cognate polypeptide; that of γ -conglutin was also relevant and clear, despite the observed unspecificity of the immunoblotting responses, while δ -conglutin did not seem to play a major role in the cross-reactivity between lupin and peanut.

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