First case report of anaphylaxis caused by Rajgira seed flour (*Amaranthus paniculatus)* from India: A clinico-immunologic evaluation

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Summary

The prevalence of food allergy is reported to be 3-4% in adults and about 6% in children. However food allergy across different countries accounts for 35-50 % all cases of anaphylaxis to foods. In the present study, we have reported a case of anaphylaxis to Amaranth grain *(Amaranthus paniculatus*) commonly known as Rajgira (Ramdana) in India. A 60 year old female suffered anaphylaxis after consuming Rajgira seed flour generally consumed during fasting. Food allergy to Amaranth seeds is not reported so far. The patient reported to hospital with complaints of itching in mouth, choking throat, redness and swelling of face and burning abdomen within 5 min of consuming Rajgira flour.

Clinical and immunological investigations revealed SPT and oral challenge positivity beside high allergen specific IgE in the serum of the patient. Three IgE binding protein fractions were detected in roasted Rajgira seed flour extract which could be considered to be allergenically important for triggering anaphylaxis. *(Asian Pac J Allergy Immunol 2013;31:79-83)*

Key words: Amaranthus, Rajgira, anaphylaxis, allergy, India

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Introduction

Food allergy, a type I hypersensitivity reaction, is an adverse immune response to food protein. They are distinct from other adverse responses such as food intolerance, pharmacological reactions, and toxin-mediated reactions. Food allergic reactions are responsible for a variety of symptoms involving the skin, gastro-intestinal tract and respiratory tract and may be due to IgE mediated /Non-IgE mediated mechanisms. Symptoms may vary from rhinitis, atopic dermatitis, urticaria, asthma, to severe anaphylactic shock.[1] The prevalence of IgE mediated food allergies is reported to be about 3-4% in adults and 6% in children.[2] However, perceived adult food hypersensitivity varies (1.3–19.1%) largely across different countries .[3] Food allergies account for 35% to 50% of all cases of anaphylaxis.[4]

Here we report the first case of food allergy to Amaranth grain (*Amaranthus paniculatus*) commonly known as "Rajgira" in India in a 60 year old female who had anaphylaxis after consuming the Rajgira flour. Amaranth grain, commonly known as royal grain, (Rajgira and Ramdana) is consumed as a substitute to cereals by people during fasting in India. *Amaranthus paniculatus* , which belongs to the plant family Amaranthaceae (Figure 1A), produces the most nutritious grain (Figure 1B) due to the balanced amino acids composition of its proteins and the fact that it is rich in vitamins and minerals.[5-6] Amaranth seeds contain respectable amounts of lysine and methionine[7] and they also contain tocotrienols (a form of vitamin E) which has cholesterol-lowering activity in humans. Amaranth grain can be cooked as a cereal, ground into flour, popped like popcorn, sprouted, or toasted in confectionary in India.[8]

Allergy to pollen of *Amaranthus spinosus*, a wild growing weed, has been reported by different workers.[9-11] However food allergy from cultivated amaranth seeds (Rajgira) has not been reported to date, according to our literature search. We therefore aimed to investigate the causative role of Rajgira

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Figure 1. *Amaranthus paniculatus* (Rajgira) twig with inflorescence (A) and seeds kept in petriplates (B).

seeds in precipitating an anaphylaxis reaction in the above patient after its consumption.

History of the patient

Ingestion of roasted Rajgira flour meal by a 60-year-old female who had no atopic history caused an anaphylactic shock, which needed hospitalization. The patient came to the hospital in October, 2009 with the complaints of itching in the mouth, a choking sensation in the throat, redness and swelling of her body, red eyes, palpitations, a burning sensation in the abdomen within 5 minutes of consuming "Rajgira flour". She had consumed the Rajgira flour meal, rather than whole roasted grain, for the first time. Before she came to us she had reported to a nearby doctor by whom she was administered an injection of Dexamethazone 2mg. and an injection of chlropheniramine 1mg., after which she had recovered. After relieving her of symptoms we investigated the patient in detail.

The patient gave the history of anaphylaxis twice earlier, the first time in October, 2007 and second episode in April, 2008 after consumption of Rajgira whole meal roasted grains during fasting and was treated by a local general physician for her symptoms.

Preparation of Rajgira extract

Healthy raw seeds of Rajgira were crushed to powder and defatted in diethyl ether at 4°C. The extraction was carried out by continuous stirring for 8 h at 4°C in 1:20 w/v ammonium bicarbonate buffer, 50 mM, pH 8.0 as described earlier.[12] The extract was centrifuged at 13000 rpm and the supernatant was filtered through a 0.22-µm membrane, lyophilized and stored at -70°C. In addition to raw seeds, extraction of Rajgira antigen was also done from roasted seeds. The protein content of the extracts was determined by Lowry's method with slight modification by precipitation of proteins using phosphotungstic acid.[13]

Skin prick tests

Skin prick tests (SPT) were performed with a lyophilized extract reconstituted in 50% glycerinated phosphate buffered saline (PBS). A drop of the extract (1:20 w/v) was placed on the volar aspect of the forearm and the skin was pricked with a 26 G sterile needle. SPTs were also performed with a panel of food and inhalant allergens procured commercially along with raw and roasted Rajgira extract. Glycerinated (50%) PBS was used as a negative control and histamine diphosphate (5 mg/ml) as a positive control. The skin test results were graded after 20 min in comparison with the wheal diameter of the positive control. The reactions with wheal diameter equal to that of the positive control or more (≥3 mm) were considered to be positive skin reactions.

Oral Food Challenge

Following skin prick tests, the patient underwent an open oral food challenge with a roasted preparation of Rajgira meal. The challenge protocol was developed and adapted from published methods involving food challenge.[14] The patient was allowed to ingest 200 mg, 500mg and 1000mg. of roasted seeds. The patient was monitored carefully after ingestion of Rajgira and the challenge test was terminated as soon as initial symptoms of allergy appeared after ingestion of 1000 mg. of roasted seeds. Medication was given immediately to the patient to relieve her symptoms.

Patient Sera

Venous blood was drawn from patient and also from 5 healthy non-allergic volunteers to act as controls. Consent was obtained from the patients and volunteers for participation in the study, as per Institutional guidelines. Estimation of total serum IgE antibody was carried out.

Total serum IgE, which is considered to be a marker of atopy, was estimated in the sera of the patient by using a kit procured from Bethyl Laboratories (USA). The protocol followed was similar to that reported earlier.[13] The Total IgE values were expressed in IU/ml (1 IU/ml = 2.4 ng/ml).

Estimation of Rajgira specific IgE antibodies

Specific IgE was determined by ELISA against Rajgira extract in the serum of the patient as described earlier.[15] In short, a polysterene 96-well microtiter plate (Nunc AS Rockslide, Denmark) was

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coated with Rajgira extracts (20µg/well), incubated overnight at 4^0C and then blocked with 1% BSA and incubated overnight at 4°C with 1:10 diluted sera. The plate was washed and incubated with antihuman IgE-horse radish peroxidase 1:1000 v/v for 4 h at 37°C. Colour was developed using orthophenylene diamine.

IgE inhibition assay

The allergenic potency of Rajgira extract was determined by ELISA inhibition. Raw Rajgira extract was coated (20 µg/ well) onto a microtitre plate. The Rajgira-hypersensitive patient's sera (1:10 v/v) was pre-incubated with 10, 50, 100, 500 and 1000 ng of Rajgira protein at 4°C and this pre-inhibited sera was then added to the Rajgira extract coated ELISA plate. A pool of normal human sera was used as a control. The self protein required for 50% inhibition or more for IgE binding was calculated using the equation given below

$$1- \frac{\text{OD of sample with inhibitor}}{\text{OD of sample without inhibitor}} \times 100$$

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Immunoblot

To study the protein profile, raw and roasted Rajgira extract (20 µg protein per lane) was loaded onto a 12% reducing gel.[16] The proteins resolved on SDS-PAGE were stained with Coomassie brilliant blue R-250.

For immunoblot, the resolved proteins were transferred on to a nitrocellulose membrane (NCM), as described by Towbin et al.[17] The unbound sites were blocked by 3% BSA for 3 h at 37°C. The NCM strips were washed and incubated with 1:10 v/v Rajgira-hypersensitive patient's sera at 4°C. Normal human sera pools were used as controls. The strips were washed with PBS–Tween 20 and incubated with 1:1000 diluted antihuman IgE-peroxidase (Sigma, USA). The IgE binding was detected by adding diaminobenzidine with hydrogen peroxide in sodium acetate buffer (pH 5.0).

Simulated Gastric Fluid (SGF) Digestion

The digestibility of the processed and unprocessed Rajgira proteins was examined in the SGF, as described by Astwood et al.[18] Briefly, a protein sample (680 µg of extract proteins) was treated with 200 µL of pre-warmed SGF (US Pharmacopoeia) containing 0.32 w/v percentage of pepsin A (Sigma Chemical Co). Digestion was carried out at 37°C with continuous shaking and an aliquot (20 µL) of this digest was periodically withdrawn at 0.5, 1, 5, 15, 30, 45, and 60 minutes for analysis. These aliquots were quickly mixed with 26 µL of a sample buffer (containing 2% β-mercaptoethanol and 4% SDS) for SDS-PAGE together with 6.0 µL of Na_2CO_3 solution (200 mmol/L). The mixture was then boiled for 5 minutes and stored at –20°C for further analyses. As a control, each protein sample was treated with SGF that did not contain pepsin and then processed as described above. The digestibility of two known allergens namely, milk lactoglobulin and bovine serum albumin (BSA), was examined to assess the activity of the SGF. These two purified proteins were purchased from Sigma Chemical Co (USA).

Results

Skin Prick Tests

The result of the SPT with Rajgira extract showed a wheal reaction of more than 8 mm with erythema and was considered markedly positive. Interestingly, none of the other allergens tested showed a positive reaction.

Oral Food Challenge

As soon as the patient consumed the roasted Rajgira flour preparation meal she developed mild symptoms of itching and swelling of the lips, indicating a positive provocation test. The test was terminated after mild symptoms had appeared.

Protein profile Amaranth grain (Rajgira) extract

The Amaranth grain (Rajgira) contained 84 mg protein/g of dry powder in extract. SDS-PAGE resolved raw Amaranth grain extract into 28 Coomassie stained protein bands ranging from 14 to 98 kDa. (Figure 2A Lane 1) However, the profile of roasted Rajgira revealed only 2 faint protein bands of 28 & 66 kDa with smear around 14 kDa. (Figure 2A Lane 2)

Total and Specific Serum IgE

The patients' serum showed highly raised total serum IgE (416 IU/ml) and specific IgE against raw Amaranth grain (Rajgira) was found to be OD 2.9 and for roasted Amaranth grain (Rajgira) it was OD 0.53.

Immunogenic proteins of Amaranth (Rajgira) extract

The IgE binding components of extracts prepared from raw and roasted Rajgira seeds were analyzed by western blot with sera from Amaranth grain (Rajgira)-sensitive patient. Sixteen IgE binding protein fractions in the following range were identified -13, 16, 18, 20, 22, 24, 27, 36, 38, 41, 43,

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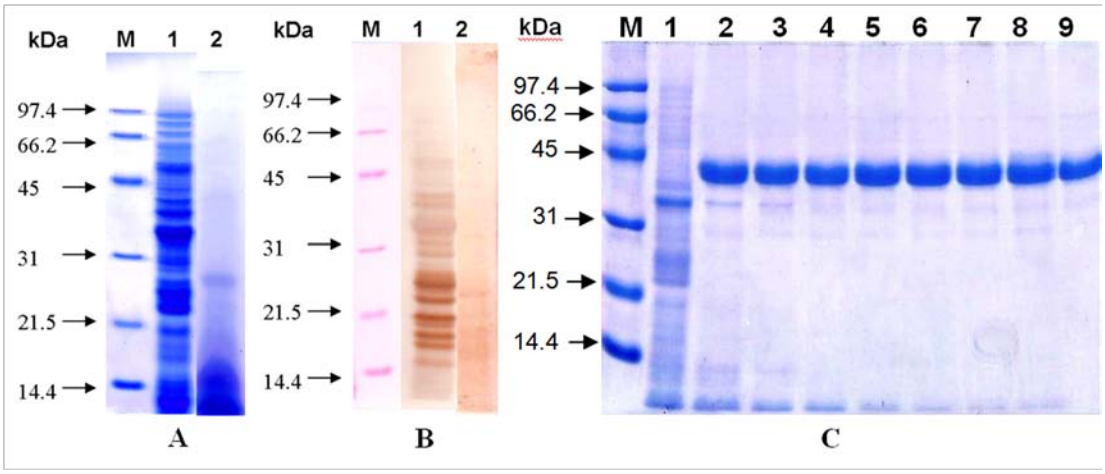

Figure 2. SDS-PAGE profile (A) of raw amaranth seed (rajgira) extract (Lane 1) and roasted amaranth seed (rajgira) extract (Lane 2). Immunoblot (B) using patient's sera with raw amaranth seed (rajgira) (lane 1), roasted amaranth seed (rajgira) (lane 2). SDS-PAGE profile of SGF digest of Amaranth (rajgira) seed (C). Lane 1: undigested, lane 2-8 treated for 0.5, 1, 5,15, 30, 45, 60 min in SGF, lane 9: pepsin, M: Molecular weight markers. (D) IgE ELISA inhibition of Amaranth seed extract using self raw protein, roasted protein and heterologous extracts as inhibitor. Amaranth seed (rajgira) positive patient's sera (1:10 v/v) was pre incubated with 10, 50, 100, 1000, 10000ng of soybean, peanut and *A. spinosus* pollen extracts as inhibitors.

46, 54, 57, 67 and 72 kDa (Figure 2B, Lane 1). Immunoblotting with roasted Rajgira revealed protein bands of 14, 28, 66 kDa. (Figure 2B, Lane 2). The strips incubated with pooled normal control human sera did not show any binding.

Allergenic potency and cross reactivity of Amaranth grain (Rajgira) extract

ELISA inhibition was carried out to determine the potency of raw and roasted Rajgira extracts. The raw extract was highly potent since it required only 80 ng of self protein to achieve 50% inhibition of IgE binding in ELISA, on the other hand 1100 ng of roasted Rajgira extract was required for 50% inhibition in IgE binding.

Cross reactivity of Rajgira with other extracts (soybean, peanut and *Amaranthus spinosus*), was performed but none of the three inhibitors could exhibit 50% inhibition of solid phase Rajgira extract, indicating absence of cross reactivity of Rajgira with soya bean, peanut and Amaranth pollen.

Simulated Gastric Fluid (SGF) Digestion

Rajgira extract was mixed with SGF fluid and incubated at 37°C. The raw Rajgira proteins degraded readily, as followed by SDS-PAGE. All the proteins were digested within 30 minutes. (Figure 2C, Lane 6 to 8)

Discussion

Food allergy is apparently increasing in different parts of the world, including India. Primary care and emergency medical healthcare providers are called upon to diagnose and treat such patients.[19] Food allergies account for around 35% to 50% of all cases of anaphylaxis.[4] In the present study we have reported the first case of severe allergy to Rajgira seed flour *(Amaranthus paniculatus).* Allergies to different species of *Amaranthus* pollen, growing wild, have been reported from different parts of the world.[9, 11, 12] However, allergy to Amaranth (Rajgira) seed has not been reported to date according to our literature search. SPT revealed sensitization to Rajgira seed extract but the patient was found to be SPT negative to other foods and aeroallergens, including *Amaranthus spinosus* pollen, suggesting no cross reactivity of Amaranth (Rajgira) seed extract with pollen of other species of Amaranth and indicating specific antigenic moieties present in cultivated Amaranth seeds. It is interesting that we did not find pollen food cross-reactants as reported in literature.[20-21] An oral food challenge test with Amaranth (Rajgira) seed powder further confirmed allergy to the suspected Rajgira seeds.

SDS-PAGE resolved raw Rajgira grain extract into 28 Coomassie stained proteins among which 16 were found to have IgE binding capability. Roasting decreases the number of protein bands (in

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SDS-PAGE) and IgE binding fractions (in immunoblot), as only 3 faint IgE binding fractions were seen. The results of specific IgE and inhibition assay suggested 6 and l4 folds reduction in allergenicity of Rajgira extract after roasting. However, clinically this reduction in IgE binding is not sufficient to prevent the patient from anaphylaxis, as the patient had consumed the pure Rajgira flour preparation. This might be because the protein may get precipitated by roasting and doesn't come in the aqueous extract but unfortunately, people eat whole grains of Amaranth and many other allergens (even the precipitated form) are available to the immunological system of gut.

SGF digestion of the Amaranth (Rajgira) proved that almost all the proteins get digested within 30 minutes. The result of SGF clearly established that even the allergenic proteins are easily digestible. This could be due to the rapid gastric emptying time and active bowel movement that occurs during fasting and this in turn facilitated absorption of proteins before they were totally digested . Thus, the myth that only stable proteins are allergenic may not be completely true.

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