ORIGINAL ARTICLE

Basophil Activation Reveals Divergent Patient-Specific Responses to Thermally Processed Peanuts

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Abstract

Introduction: The impact of processing on the allergenicity of peanut (*Arachis hypogaea*) proteins has traditionally been studied using immunoglobulin (Ig) E binding assay. However, as this technique does not assess the potential of an allergen to trigger basophils and mast cells, studies based on it can hardly be considered complete. We evaluated the effect of processing on peanut allergenicity using flow-cytometric quantification of in vitro basophil activation (basophil activation test [BAT]).
Patients and Methods: Basophils from 10 patients with severe peanut allergy and 3 peanut-tolerant individuals were stimulated with extracts from 5 raw and thermally processed peanut varieties. Data were compared using protein staining (sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) and IgE immunoblotting.
Results: Stimulation with different extracts resulted in patient-dependent and variety-dependent effects on basophil activation. SDS-PAGE revealed a considerable loss of identifiable bands, especially for the South Africa Common Natal, Argentina Runner, and US Virginia varieties. The results of IgE immunoblotting in patients were similar, irrespective of the responses observed in the BAT.
Conclusions: The impact of thermal processing on the capacity of peanuts to trigger basophils seems highly divergent between patients and cannot be predicted using SDS-PAGE or IgE binding. BAT can be considered a complementary tool for the evaluation of food allergenicity.

Key words: Peanut allergy. Peanut processing. Basophil activation test. Allergy diagnosis.

Resumen

Introducción: El impacto del procesamiento en la alergenicidad de las proteínas del cacahuete (*Arachis hypogaea*) se ha estudiado tradicionalmente utilizando ensayos de unión de inmunoglobulina (Ig) E. No obstante, puesto que esta técnica no evalúa el potencial de un alérgeno de activar los basófilos y los mastocitos, los estudios basados en ella difícilmente pueden considerarse completos. En este estudio se evaluó el efecto del procesamiento sobre la alergenicidad de los cacahuetes por medio de la cuantificación por citometría de flujo de la activación in vitro de basófilos (test de activación de basófilos [TAB]).
Pacientes y métodos: Se estimularon los basófilos de 10 pacientes con alergia grave a los cacahuetes y de 3 voluntarios tolerantes a los cacahuetes con extractos de 5 variedades sin procesar y termoprocesadas de este fruto. Se compararon los datos con la tinción de proteínas (electroforesis en gel de poliacrilamida con dodecil sulfato de sodio [SDS-PAGE]) y la inmunotransferencia de IgE.
Resultados: La estimulación con diferentes extractos dio lugar a efectos dependientes del paciente y de la variedad sobre la activación de los basófilos. La técnica SDS-PAGE reveló una pérdida considerable de bandas identificables, especialmente en el caso de las variedades Natal Common sudafricana, Runner argentina y Virginia estadounidense. Los resultados de la inmunotransferencia de IgE en pacientes fueron similares, con independencia de las respuestas observadas en el TAB.
Conclusiones: El impacto del procesamiento térmico en la capacidad de los cacahuetes de activar los basófilos presenta grandes divergencias entre pacientes y no puede predecirse mediante SDS-PAGE o unión de IgE. El TAB puede considerarse una herramienta complementaria para la evaluación de la alergenicidad de los alimentos.

Palabras clave: Alergia a los cacahuetes. Procesamiento de cacahuetes. Test de activación de basófilos. Diagnóstico de alergia.

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Introduction

Thermal processing can significantly affect the allergenicity of peanut proteins [1]. Ideally, these effects are studied through double-blind placebo-controlled food challenge (DBPCFC). However, as recently addressed [2], DBPCFCs are hampered by various ethical and practical limitations that almost preclude their use for this purpose. Consequently, most of our current knowledge on the impact of thermal processing on the allergenicity of peanuts has been revealed by immunoglobulin (Ig) E binding studies [3-6]. However, IgE binding cannot be considered absolutely predictive of the residual allergenicity of processed food protein [2], since it does not assess the ability of an allergen to trigger basophils and mast cells.

The principles of flow-assisted analysis of basophils, known as the basophil activation test (BAT), have been detailed elsewhere [7-10].

As the BAT closely mimics the in vivo allergic reaction, we anticipated that it could help to determine the effect of thermal processing on the allergenicity of peanut.

Patients and Methods

Patients and Controls

This study was approved by the local ethics committee.

The study population comprised 10 patients with severe peanut allergy (5 males/5 females; median age, 12 years) and 3 peanut-tolerant healthy controls (2 males/1 female; aged 4, 12, and 29 years). Diagnosis of peanut allergy was based on a compelling history corroborated by peanut-specific IgE ≥14 kU_A/L (ImmunoCAP FEIA, Phadia, Uppsala, Sweden) and/or a peanut skin prick test result ≥8 mm (HAL Allergy, Haarlem, The Netherlands). These values have been shown to have a positive predictive value ≥95% [11-12]. Since these patients reported severe anaphylactic reactions, a DBPCFC was deemed unnecessary and unethical [13-14]. Furthermore, all patients except one (patient 8) showed concomitant triple sensitization to the peanut components Ara h 1 (vicillin), Ara h 2 (2S albumin), and Ara h 3 (legumin) (ImmunoCAP FEIA, Phadia), a combination that has been associated with more severe clinical disease [15-16].

Peanut Extracts (Table 1)

We tested 5 different raw and thermally processed peanut varieties, namely, Argentina Runner, US Jumbo Runner, South Africa Common Natal, US Virginia, and China Virginia (Institute for Reference Materials and Measurements, Geel, Belgium)

Table 1. Raw and Thermally Processed Peanut Varieties Tested in the Studyt

Origin	Variety	Heat Processing
Argentina	Runner	Dry hot air roasting, 140°C for 20 min
US	Jumbo Runner	Blanching, 100°C for 50 min
South Africa	Common Natal	Dry hot air roasting, 140°C for 13 min
China	Virginia[a]	Oil roasting, 140°C for 9 min
US	Virginia	Oil roasting, 145°C for 25 min

[a]Not available in unprocessed form, but blanched at 90°C for 20 minutes.

Protein extraction involved solubilization of 1 g of liquid nitrogen–ground peanut in 10 mL of 60°C phosphate-buffered saline (10 mM, pH 7.4) for 15 minutes before centrifuging (1900*g* for 10 minutes) at 4°C. The supernatant was filtered with a 5-µ filter and stored at –20°C until use [17].

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Protein fractions of different peanut extracts were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (Novex NuPage Bis-Tris gel 4-12%, Invitrogen, Carlsbad, California, USA). Thirty micrograms of protein were loaded into each well. Electrophoresis was performed in a Novex electrophoresis chamber (Invitrogen) at 200 V and 120 mA for 35 minutes. Gels were then stained with SYPRO Ruby (Invitrogen) and scanned with a ChemiDoc system (Bio-Rad, Hercules, California, USA).

BAT Experiments

The BAT was performed as detailed elsewhere [9].

Using data from preliminary dose-finding experiments, we selected an optimal stimulation concentration of 10 ng/mL of peanut protein (data not shown). All analyses were run in a single experiment to rule out variability of cell responses. Cells were stained with anti-CD63-FITC/anti-CD123-PE/anti-HLADR-PerCP (BD Biosciences, San Jose, California, USA) and analyzed on a FACSCanto II cytometer (BD Immunocytometry Systems, San Jose, California, USA). Percentages of CD63-positive cells of at least 500 basophils gated as low side-scatter, $CD123^+$, and HLA-DR– were measured.

Immunoblot Analysis

Individual sera from all 10 peanut-allergic patients and from 1 healthy control were used to study the effect of roasting on the IgE binding capacity of the Argentina Runner variety. Protein extract was separated using SDS-PAGE, as described above. Proteins were then blotted onto nitrocellulose (Protran B85, Schleicher & Schull, Dassel, Germany) using a Novex blot system (Invitrogen) and a Bio-Rad power supply (Bio-Rad) at 20 V and 160 mA. After blotting, the membrane was incubated in blocking buffer (Sigma-Aldrich, Bornem, Belgium) in Tris-buffered saline (10 mmol/L Tris, 150 mmol/L NaCl, pH =7.4) and cut into strips. These strips were incubated overnight at 4°C with patient's serum diluted one-quarter in blocking buffer. The blank strip was incubated with buffer instead of serum. Bound specific IgE was determined using mouse monoclonal antihuman IgE (Sigma-Aldrich), biotin-conjugated rabbit anti-mouse IgG (Sigma-Aldrich), and streptavidin-conjugated peroxidase. Super Signal West Dura (Thermo Scientific, Erembodegem, Belgium) was used as a substrate and gels were digitalized using a Chemi-Doc system (Bio-Rad).

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Results

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (Figure 1)

The most significant findings of these SDS-PAGE experiments were that dry hot air roasting of the South Africa Common Natal variety (lanes 5 and 6) and, to a lesser extent, of the Argentina Runner variety (lanes 1 and 2) considerably reduced the number and intensity of identifiable protein bands.

Similar effects were observed by oil roasting of the US Virginia variety (lanes 7 and 8), and clearly involved the most relevant peanut components Ara h 1, 2, and 3. In contrast, the effect of blanching the raw US Jumbo Runner variety (lanes 3 and 4) and roasting China Virginia variety (lanes 9 and 10) seemed less prominent.

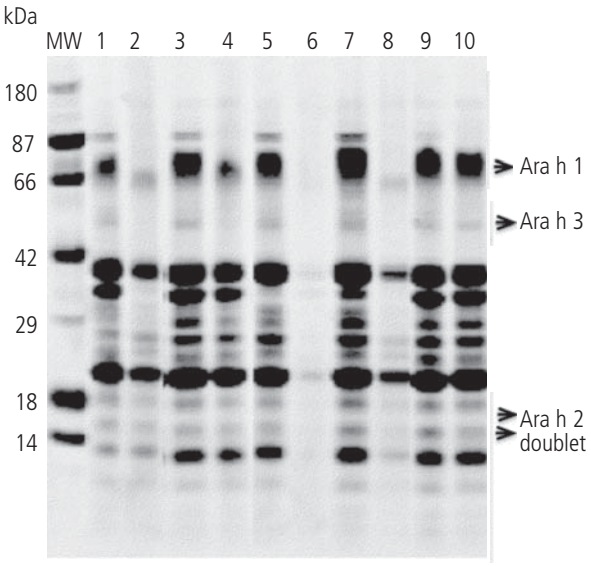

Figure 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of 5 raw and thermally processed peanut varieties. Argentina Runner raw (lane 1) and strong air roasted (lane 2), US Jumbo Runner raw (lane 3) and blanched (lane 4), South Africa Common Natal raw (lane 5) and mild air roasted (lane 6), US Virginia raw (lane 7) and strong oil roasted (lane 8), and China Virginia blanched (lane 9) and mild oil roasted (lane 10).

BAT Experiments

Patients and healthy controls presented comparable spontaneous and anti-IgE–induced CD63 upregulation (data not shown). No basophil activation by peanut was observed in healthy controls (data not shown). In 5 patients (patients 6 to 10), all BAT experiments were performed in duplicate and variability was below 2%.

Figure 2 shows the upregulation of $CD63^+$ on basophils from patients induced by the 5 peanut extracts tested. The effect

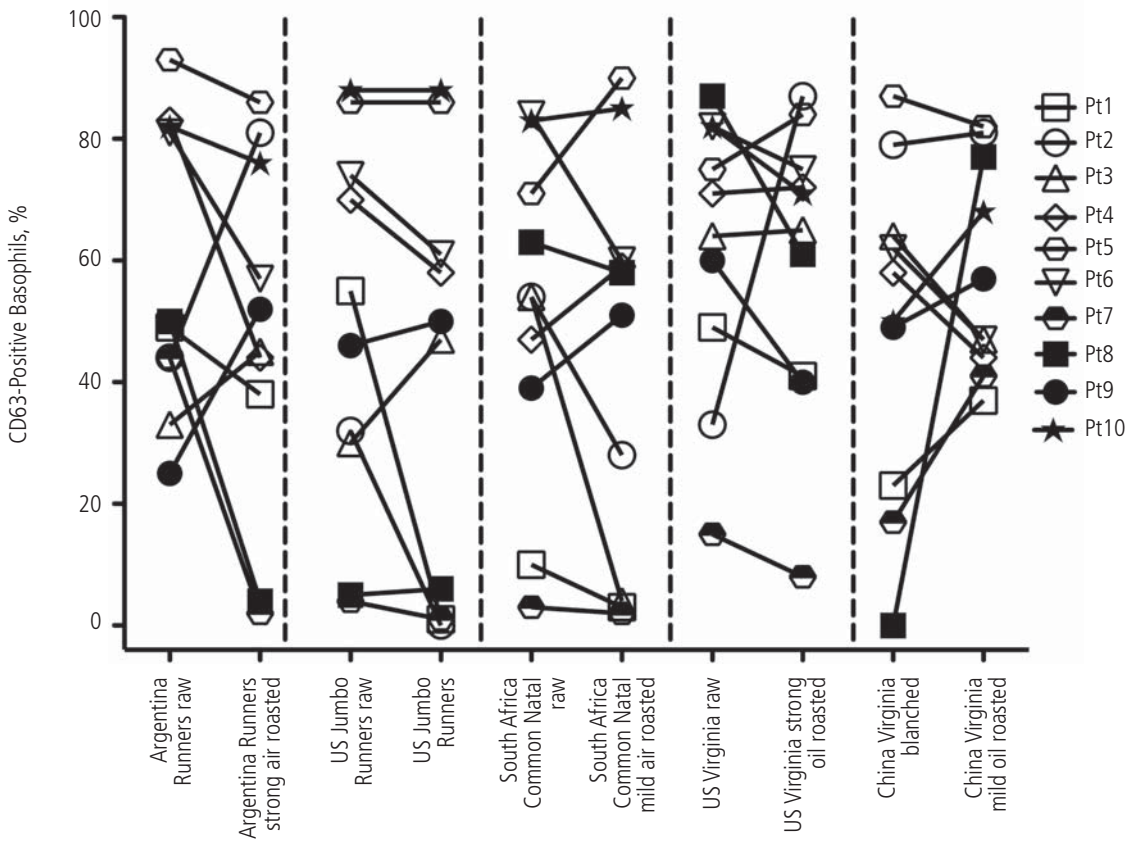

Figure 2. Results of the basophil activation test with 5 different raw and thermally processed peanut varieties. Individual results are plotted. The effect of thermal processing seems to be patient- and variety-dependent. No CD63 upregulation was appreciable in controls (data not shown).

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of thermal processing seems highly heterogeneous and depends on the individual patient, as well as on the studied peanut variety, and could resulted in an increased, unchanged, decreased, and even totally abolished capacity to stimulate the cells.

Notably, extracts from the 5 raw and thermally processed peanut varieties failed to elicit basophil activation in individual cases, as follows: raw South Africa Common Natal in patient 7; raw US Jumbo Runner in patients 7 and 8; roasted Argentina Runner in patients 7 and 8; blanched US Jumbo Runner in patients 1 and 2; roasted South Africa Common Natal in patients 1 and 3; and blanched China Virginia in patient 8. Again, these results appear to be patient- and variety-dependent.

Moreover, a comparison of SDS-PAGE and BAT data shows that the findings from the protein staining technique are not predictive of the residual capacity of an extract to trigger basophil activation. For example, although dry hot air roasting of the South Africa Common Natal and the Argentina Runner variety resulted in loss of identifiable components, this was not accompanied by a decrease in basophil activation.

Immunoblot Analysis

Figure 3 shows a representative example of the IgE immunoblot for the Argentina Runner cultivar (raw and roasted with dry hot air) with the individual sera of 4 patients (patients 2, 5, 6, and 7), which showed an increased, reduced, abolished, or unaltered basophil response for the thermally processed form of the legume. In parallel to the observations in SDS-PAGE, the IgE binding patterns do not seem to predict BAT results. Although 4 different responses were observed in the BAT, the IgE immunoblot patterns seem similar, and increased basophil responses would theoretically have been expected in all 4 patients as a result of enhanced IgE binding at the Ara h 2 doublet.

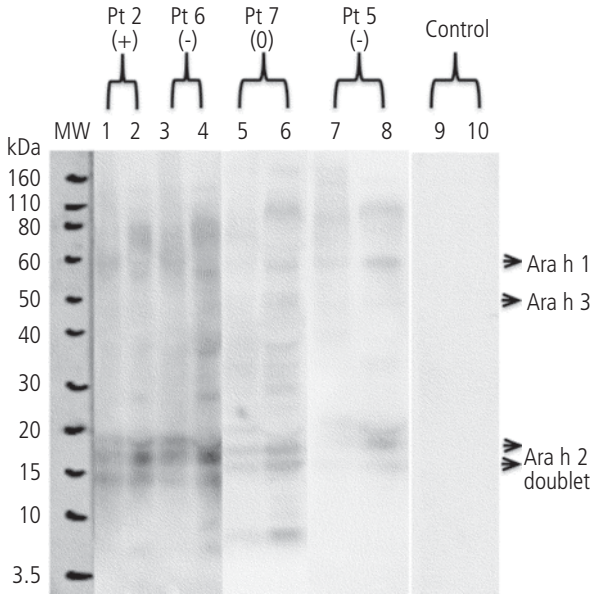

Figure 3. Immunoblot for anti–peanut-specific IgE against Argentina Runner (raw and air roasted). Representative examples from 1 patient with increased basophil activation (patient 2, lanes 1-2: raw vs air roasted), 1 patient with reduced basophil activation (patient 6, lanes 3-4: raw vs air roasted), 1 patient with abolished basophil activation (patient 7, lanes 5-6: raw vs air roasted), and 1 patient with unchanged basophil activation (patient 5, lanes 7-8: raw vs air roasted) after stimulation with air roasted peanut extracts. No significant differences in IgE reactivity were observed. No IgE reactivity was detectable in the peanut-tolerant controls (lanes 9-10: raw vs roasted). MW indicates standard molecular weight.

Discussion

The most striking observation of our study is that various methods of thermal processing can considerably enhance, reduce, leave unaltered, or totally abolish the capacity of peanuts to trigger basophils in patients with severe peanut allergy. However, the impact of thermal processing seems dependent on the cultivar and on the individual patient and cannot be predicted using SDS-PAGE. Protein staining and functional analysis using BAT can even yield opposite results. This was best demonstrated for the South Africa Common Natal, Argentina Runner, and US Virginia varieties. In these cultivars, air and oil roasting considerably reduced the number and intensity of clearly identifiable protein bands, although this finding was not accompanied per se by a lower potency to stimulate the cells of our patients. Actually, in some patients the residual allergenicity after roasting as assessed by the BAT increased. To some extent, these findings are consistent with the results obtained from a comparison to determine the ability of peanut extracts to induce positive skin test responses. Hefle et al [18] showed that peanut extracts with a lower protein content can still induce skin test responses comparable to those of extracts with a higher protein content.

A comparison between BAT and IgE immunoblotting for the raw and roasted Argentina Runner cultivar revealed that there is no correlation between these 2 techniques, thus stressing that IgE binding studies do not predict the potential of an allergen to trigger effector cell degranulation [19]. For peanut, the discrepancy between a positive immunoblotting result and negative BAT result could be due to sensitization to heat-resistant cross-reactive carbohydrate determinants, which constitute an important cause of positive IgE results with no clinical significance [20]. Conversely, as recently addressed in this Journal [17], we cannot rule out the possibility that the IgE binding assay failed to disclose traces of allergens that were still able to elicit basophil activation.

Our findings confirm that the cultivars chosen to manufacture in vitro or in vivo diagnostic tests may affect the predictive accuracy of these tests and hamper correct diagnosis of a potentially life-threatening allergy [21-23]. Moreover, we cannot rule out that this phenomenon has contributed to false-negative challenges in about 10% of patients with severe peanut allergy [24]. Actually, a negative response to a diagnostic test may be ascribed either to the different allergen profile in various extract preparations or to an inappropriate source material.

In conclusion, our findings stress that the evaluation of the effect of processing on food allergenicity results obtained with IgE binding techniques should be complemented by a more functional analysis. In this context, the BAT, which closely resembles the in vivo pathway leading to symptoms, could provide important additional information without endangering health.

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