

Pepsin-digested peanut contains T-cell epitopes but no IgE epitopes

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Background: Peanuts are a common cause of food-induced anaphylaxis and fatalities. Previous studies have demonstrated that rush immunotherapy to crude peanut extract reduces clinical symptoms triggered by oral peanut challenges, but the immunotherapy was associated with an unacceptably high incidence of systemic allergic reactions. One approach to reduce the frequency of allergic reactions would be to use a modified peanut antigen with low allergenic properties. **Objective:** We sought to determine the immunologic characteristics of crude intact peanut extract before and after pepsin digestion.

Methods: We used IgE immunoblotting and assessment of T-lymphocyte responses to intact and peptic digests of peanut extracts.

Results: Western blot analysis of sera from 5 subjects with peanut allergy showed multiple IgE-reactive proteins in crude intact peanut extract that were eliminated after pepsin treatment of the peanut extract. In contrast, pepsin-digested peanut induced significant T-cell proliferation responses (stimulation index = 30) in vitro in PBMCs from 7 subjects with peanut allergy, albeit at lower levels than that induced by intact peanut (stimulation index = 66). Furthermore, IFN- γ production was induced by intact peanut and pepsin-digested peanut in a concentration-dependent manner. Importantly, T-cell lines generated in response to intact peanut also reacted to pepsin-digested peanut, indicating cross-reactive T-cell epitopes in intact and pepsin-digested peanut.

Conclusion: These findings suggest that pepsin-digested peanut may be useful in peanut immunotherapy because pepsin digestion eliminates IgE reactivity but maintains T-cell reactivity. (*J Allergy Clin Immunol* 1999;104:473-7.)

Key words: Peanuts, food allergen, T cells, pepsin

Immediate hypersensitivity reactions to peanuts are a common cause of anaphylactic reactions and fatalities in patients with food allergy.¹⁻³ Sensitivity to peanuts occurs early in life and, once established, persists indefi-

nately.⁴ There are currently no curative therapies for peanut allergy. Current management of such patients consists of symptomatic relief of allergen-induced reactions and avoidance of peanuts. Unfortunately, because peanuts frequently contaminate foods in the diet, the majority of patients diagnosed with peanut allergy experience accidental ingestion of peanuts despite attempts to avoid this food allergen.⁴

We have recently reported 2 controlled trials demonstrating that rush immunotherapy to crude peanut extract significantly increases the tolerance of subjects with peanut allergy to oral ingestion of peanuts.^{5,6} However, injections resulted in repeated systemic reactions in most patients, even during maintenance injections. Thus clinical application of rush immunotherapy would require a modified peanut extract with a low allergenic potential. Enzymatically derived peptide fragments of ragweed pollen have been reported to contain T-cell reactive epitopes required for effective immunotherapy but no B-cell epitopes recognized by IgE in the sera of atopic subjects. More importantly, a controlled trial of patients with ragweed allergy that compared the effect of peptic-digested fragments of ragweed with a conventional ragweed preparation found that both preparations of ragweed were clinically efficacious, and peptic-digested ragweed allergen had minimal side effects.⁷

The feasibility of using pepsin-digested allergen for peanut immunotherapy is unknown, particularly because crude peanut extracts contain multiple complex allergenic epitopes.^{8,9} The purpose of this study was therefore to determine the effect of pepsin digestion on the immunologic characteristics of crude peanut extracts, particularly their effect on IgE binding and T-cell stimulatory antigenic epitopes.

METHODS

Subjects

Seven adult patients with peanut allergy were studied. All 7 of these subjects had a positive immediate skin prick test reaction to peanut and a history of peanut-induced anaphylaxis. None of these patients were receiving steroids orally. Written informed consent was obtained from each subject before entry into the study.

Peanut preparations

Lyophilized crude peanut extract was provided by Greer Laboratories (lot No. XPF171-H5; Lewistown, NC). Pepsin-digested peptide fragments were prepared from crude peanut extract as follows. Crude peanut extract at a concentration of 1 mg of protein/mL was adjusted to pH 1.8 by using 0.1 mol/L HCl. This solution was then sterile filtered with a 0.22- μ m Nalgene syringe filter. Pepsin immobilized to agarose (Sigma, 40 U/mg solid) was added to the diluted

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Supported by Public Health Services Research grant AR41256 and General Clinical Research Center grant 5 MO1 RR00051 from the Division of Research Resources.

Received for publication Feb 10, 1999; revised May 3, 1999; accepted for publication May 3, 1999.

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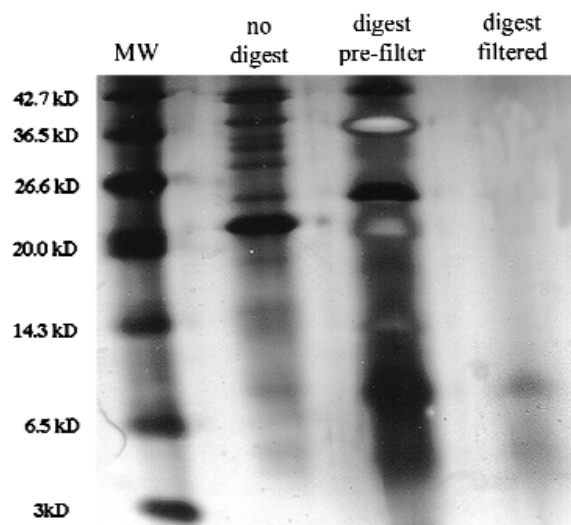


FIG 1. Molecular size of pepsin-digested peanut fragments. A 16.5% TRIS-tricine SDS gel was run and silver stained as described in the "Methods" section. The representative gel shows undigested peanut extract (lane 2), pepsin-digested peanut extract before filtering (lane 3), and pepsin-digested and centrifuge-filtered peanut extract (lane 4). Lane 1 contains a broad range of molecular weight markers.

peanut extract at a 3:1 ratio (3 mg of peanut protein to 1 mg of pepsin). The solution was incubated for 24 or 48 hours at room temperature on a rocking platform. The extract was centrifuged at 10,000g for 20 seconds to pellet the agarose. The extract was removed from the agarose, and the pH was then raised to 7.5 by the addition of 0.1 mol/L NaOH to stop any additional pepsin digestion. The digest was filtered through a Centricon filter to obtain pepsin-free peanut peptide fragments in the ultrafiltrate with a molecular size of approximately 3000 to 6000 d. The concentrations of peanut proteins and their peptide fragments were determined by using a standard colorimetric BCA assay kit obtained from Pierce (Rockford, Ill), which was used following the manufacturer's protocol.

A 16.5% Tris-tricine SDS gel was run by using undigested, digested and unfiltered, and digested and filtered samples to determine the approximate molecular weight of the peanut fragments (Fig 1). The gel was stained following standard protocols by using the Daiichi (Owl Separation Systems, Portsmouth, NH) silver stain kit.

IgE immunoblotting

Twenty-microliter samples of peanut extract (5 µg) or pepsin-digested peanut fragments (20 µg) were electrophoresed on 4% to 20% gradient polyacrylamide gels at 15 mA of constant current until the tracking dye reached the bottom of the gel. The gels were equilibrated in transfer buffer (0.024 mol/L TRIS) before overnight transfer to nitrocellulose at 8 V of constant voltage. The nitrocellulose was blocked in "blotto" for 30 minutes before incubation (3 hours) with the patient sera (diluted 1:10 in blotto). The nitrocellulose was washed (3×) with TRIS-buffered saline-blotto and then incubated for 1 hour with horseradish peroxidase-labeled goat anti-human IgE (Kirkegaard & Perry; diluted 1:2500 in 1% blotto). Blots were developed by using the TMB substrate system.

Lymphocyte stimulation studies

Donor PBMCs were isolated from heparinized venous blood by density gradient centrifugation on Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ), washed 3 times in HBSS (Gibco,

Grand Island, NY), and resuspended at 1.0×10^6 cells/mL in AIM media (Gibco) supplemented with 2 mmol/L L-glutamine, 1.5 mmol/L sodium pyruvate, 15 mmol/L Hepes, 50 µg/mL streptomycin, and 100 U/mL penicillin but no serum. A tritiated (^3H) thymidine assay was used to measure cellular proliferation. In these experiments PBMCs were cultured with antigen at 37°C in a 5% CO_2 humidified atmosphere for 6 days. These assays were performed in triplicate in 96-well, round-bottomed microtiter plates (Nunc, Denmark) by using 1×10^5 cells per well in a total volume of 200 µL. Proliferation was measured by ^3H -thymidine incorporation (1 µCi/well, 6.7 Ci/mmol; Amersham, Arlington Heights, Ill) during the last 6 hours of culture. Proliferation response was measured as cpm incorporated or stimulation index by using the following formula: cpm incorporated into antigen-stimulated cultures/cpm incorporated into medium control $\times 100$.

Peanut-specific T-cell lines

Peanut-specific T-cell lines were generated as follows. PBMCs (2×10^6) were cultured with 50 µg/mL crude peanut in RPMI media with 10% autologous plasma. Cells were restimulated after 1 week with crude peanut, 20 U of IL-2/mL, and irradiated autologous monocytes. After 2 weeks, cells were harvested, and their proliferation response to 50 µg/mL crude peanut versus pepsin-digested peanut was carried out in a 3-day assay by using 4×10^4 T cells and 4×10^4 feeder cells.

IFN-γ production

In these experiments 2×10^6 PBMCs/mL were cultured with the appropriate antigen in a 12 $\times$ 75-mm Falcon tube (Becton Dickinson). After 6 days of activation, supernatants were harvested, spun at 1000 rpm for 10 minutes to remove any remaining cells, placed in aliquots, and stored at -80°C before testing. IFN-γ levels in the supernatants of activated PBMCs were determined by using a commercial ELISA (Endogen) specific for IFN-γ with a sensitivity of 5 pg of IFN-γ/mL.

Statistical analysis

Nonparametric analysis (Student *t* test after log transformation) was used to determine significant differences in cell proliferation and cytokine production between subjects with peanut allergy versus nonallergic control subjects. The paired Student *t* test was used to determine significant differences between the stimulation of crude peanut extract, pepsin-free peptide peanut, and tetanus toxoid. Differences associated with *P* values of less than .05 were considered significant.

RESULTS

Western blot analysis of sera from 5 subjects with peanut allergy showed multiple IgE-reactive proteins in crude intact peanut extract that were eliminated after pepsin treatment of the peanut extract (Fig 2). In contrast, as shown in Fig 3, pepsin-digested peanut induced significant ($P < .05$ compared with medium control) T-cell proliferation responses *in vitro* in PBMCs from 7 subjects with peanut allergy at concentrations of 5 µg/mL, 10 µg/mL, and 50 µg/mL. Although the proliferation responses to pepsin-digested peanut were lower than those to intact peanut, there was no statistical significance between the 2 sets of stimuli.

Significant levels of IFN-γ production was induced by intact peanut and pepsin-digested peanut and increased in a concentration-dependent manner (Fig 4). However,

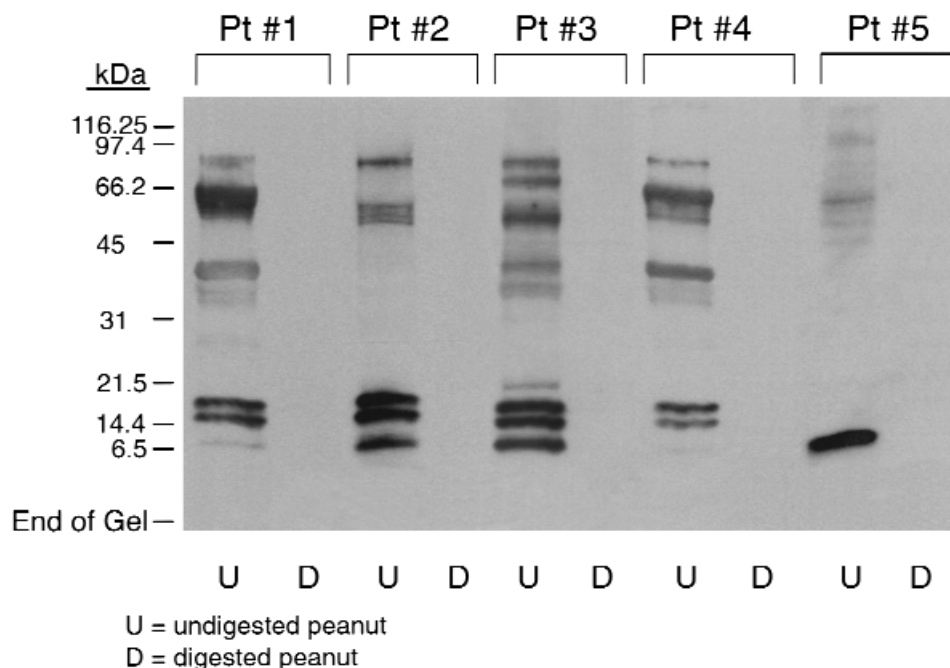


FIG 2. Western blot analyses of sera from 5 subjects with peanut allergy for IgE-reactive proteins in crude peanut extract versus pepsin-treated peanut.

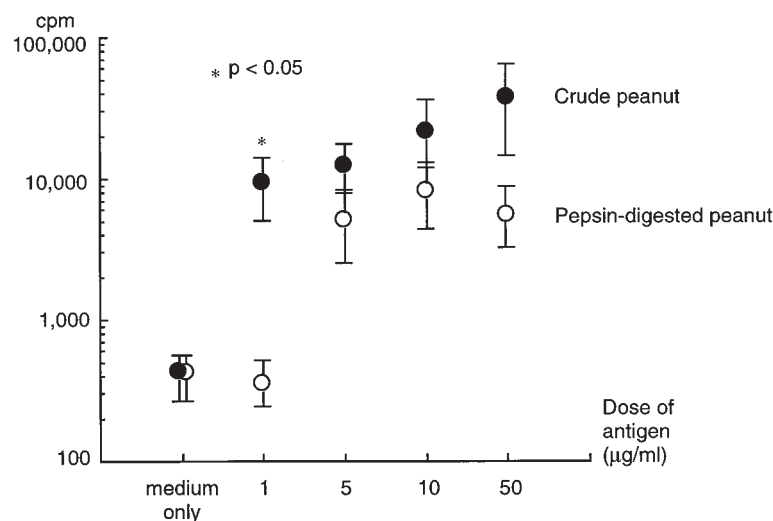


FIG 3. Mean cellular proliferation responses (cpm) by PBMCs of 7 patients with peanut allergy after stimulation by crude peanut extract (filled circles) versus pepsin-digested (open circles) peanut.

IFN- γ production of pepsin-digested peanut was less than that of crude intact peanut extract in subjects with peanut allergy ($P < .05$).

T-cell lines were generated to intact peanut and assessed for their capacity to react with pepsin-digested peanut versus intact peanut. As shown in Table I, these T-cell lines were found to react with pepsin-digested peanut (mean stimulation index of T-cell proliferation = 10.2), indicating that there were cross-reactive T-cell epitopes in intact and pepsin-digested peanut.

DISCUSSION

The majority of allergic reactions to peanuts are believed to be IgE mediated. After a patient with peanut allergy ingests peanut, the allergen cross-links peanut-specific IgE bound to mast cells or basophils to induce the release of multiple mediators involved in immediate hypersensitivity reactions.⁹ T_H2 cells play a key role in the induction of IgE responses, as well as modulating the nature and magnitude of allergic inflammatory responses.

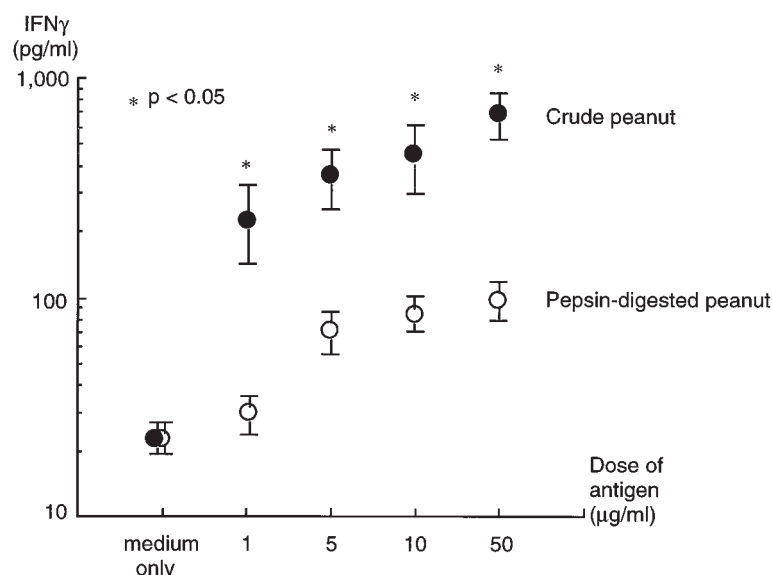


FIG 4. Mean IFN- γ production by PBMCs from 7 patients with peanut allergy after stimulation by crude peanut extract (filled circles) versus pepsin-digested (open circles) peanut.

TABLE I. Proliferation response (stimulation index) of peanut-specific T-cell lines after stimulation with crude peanut versus pepsin-digested peanut

Stimulus (50 μ g/mL)	T-cell lines			Mean
	1	2	3	
Undigested peanut	9.5	6.9	9.6	8.6
Pepsin-digested peanut	4.7	5.1	5.8	5.2

Values represent the mean stimulation index of peanut T cells after stimulation with either undigested peanut or pepsin-digested peanut.

In patients with allergic rhinitis, allergic asthma, and insect venom anaphylactic hypersensitivity, it has been found that conventional immunotherapy to intact allergens reduces clinical symptoms and modulates T_{H2}/T_{H1} cell reactivity to specific allergens.¹⁰⁻¹⁴ We have recently reported that rush immunotherapy to crude peanut extract significantly increases the tolerance of subjects with peanut allergy to oral ingestion of peanuts and decreases immediate skin test reactivity by endpoint titration to peanut extracts.^{5,6} However, injections resulted in repeated systemic allergic reactions in most patients, particularly as doses of the peanut allergen were increased or attempts were made to place patients on maintenance immunotherapy with increasing time intervals between allergen injections.⁶

Induction of T-cell unresponsiveness with peptides is therefore of particular interest for the treatment of peanut allergy because immunotherapy with intact peanut allergens is limited in its usefulness. This is because of the elicitation of potentially life-threatening IgE-mediated side effects.¹⁵ However, peanuts potentially contain multiple allergens to which IgE-directed responses can develop in patients. Although 3 major peanut allergens,

Ara h 1, Ara h 2, and Ara h 3, belonging to the vicilin, conglutin, and glycinin families of seed storage proteins, respectively, account for the majority of allergenic activity, there are potentially over 30 different molecules in peanut extracts that could act as allergens in any given individual.⁹ Furthermore, IgE-binding epitopes are located throughout the length of the Ara h 1, Ara h 2, and Ara h 3 allergens. Thus an approach relying on the identification of a single or limited number of dominant peptides, as has been attempted for Fel d 1, is unlikely to be successful in the treatment of peanut allergy by immunotherapy.

We therefore selected enzymatic degradation of peanut proteins to create multiple small immunoregulatory peptides that would presumably contain multiple T-cell epitopes with decreased IgE reactivity. Such enzymatically derived allergen fragments would have the potential to induce T-cell responses and allergen-blocking antibodies more effectively because large doses could be administered. Indeed, Standring et al¹⁶ reported that chymotrypsin treatment of rye grass pollen induced potent T-cell responses, but no B-cell responses, in a murine model. In a study with pepsin-derived fragments of BSA, the preserved T-cell epitopes in such BSA fragments favored immune suppression rather than helper cell function. This suppression was mediated by T cells.¹⁷

The clinical relevance of this approach is supported by a study by Litwin et al,⁷ who demonstrated that pepsin-digested ragweed extract was as effective as crude ragweed in the treatment of ragweed-sensitive patients. In this study the immediate skin test activity of the peptic fragments was 1000-fold less than that of the original crude ragweed material. The peptic fragment mixture, however, was more effective than the original intact undi-

gested extract in relieving clinical symptoms caused by ragweed presumably because injections of the peptic fragment mixture could be tolerated at doses substantially higher than those of the crude ragweed. Both preparations were effective in preventing the seasonal rise in ragweed-specific IgE antibodies. Despite the fact that the group receiving peptide fragments reached a concentration of allergen injection 14 times higher than the mean concentration final dose achieved by the group receiving crude ragweed, both groups had a relatively low prevalence of adverse events.

With these previous studies in mind, we undertook the current preliminary study to determine whether pepsin digestion could eliminate IgE-binding epitopes in crude peanut extracts. Our results demonstrate that after pepsin digestion, IgE-binding epitopes can be eliminated, but the remaining peptic fragments of peanut peptides still contained T cell-stimulating epitopes. At present, there is no direct proof that the current T-cell reactivity measured against the extract and digested material is directed exclusively against the relevant allergens. Nevertheless, on the basis of previous clinical and animal model studies of pepsin-digested allergens, our data suggests a promising approach for immunotherapy in the treatment of patients with peanut allergy.

We thank Maureen Sandoval for help in preparation of this manuscript.

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