

Peanut oral immunotherapy modifies IgE and IgG₄ responses to major peanut allergens

Brian P. Vickery, MD,^{a*} Jing Lin, PhD,^b Michael Kulis, PhD,^{a*} Zhiyan Fu, PhD,^b Pamela H. Steele, MSN, CPNP,^{a*} Stacie M. Jones, MD,^c Amy M. Scurlock, MD,^c Gustavo Gimenez, BSc,^b Ludmilla Bardina, MSc,^b Hugh A. Sampson, MD,^b and A. Wesley Burks, MD^{a*} Durham, NC, New York, NY, and Little Rock, Ark

Background: Patients with peanut allergy have highly stable pathologic antibody repertoires to the immunodominant B-cell epitopes of the major peanut allergens Ara h 1 to 3.

Objective: We used a peptide microarray technique to analyze the effect of treatment with peanut oral immunotherapy (OIT) on such repertoires.

Methods: Measurements of total peanut-specific IgE (psIgE) and peanut-specific IgG₄ (psIgG₄) were made with CAP-FEIA. We analyzed sera from 22 patients with OIT and 6 control subjects and measured serum specific IgE and IgG₄ binding to

epitopes of Ara h 1 to 3 using a high-throughput peptide microarray technique. Antibody affinity was measured by using a competitive peptide microarray, as previously described. **Results:** At baseline, psIgE and psIgG₄ diversity was similar between patients and control subjects, and there was broad variation in epitope recognition. After a median of 41 months of OIT, polyclonal psIgG₄ levels increased from a median of 0.3 µg/mL (interquartile range [25% to 75%], 0.1–0.43 µg/mL) at baseline to 10.5 µg/mL (interquartile range [25% to 75%], 3.95–45.48 µg/mL; $P < .0001$) and included *de novo* specificities. psIgE levels were reduced from a median baseline of 85.45 kU_A/L (23.05–101.0 kU_A/L) to 7.75 kU_A/L (2.58–30.55 kU_A/L, $P < .0001$). Affinity was unaffected. Although the psIgE repertoire contracted in most OIT-treated patients, several subjects generated new IgE specificities, even as the total psIgE level decreased. Global epitope-specific shifts from IgE to IgG₄ binding occurred, including at an informative epitope of Ara h 2. **Conclusion:** OIT differentially alters Ara h 1 to 3 binding patterns. These changes are variable between patients, are not observed in control subjects, and include a progressive polyclonal increase in IgG₄ levels, with concurrent reduction in IgE amount and diversity. (J Allergy Clin Immunol 2012;■■■:■■■–■■■.)

Key words: Peanut allergy, oral immunotherapy, IgE, IgG₄, peptide microarray, epitope, B cell, antibody affinity

From ^athe Division of Pediatric Allergy and Immunology, Duke University School of Medicine, Durham; ^bPediatric Allergy and Immunology, Mount Sinai School of Medicine, New York; and ^cPediatric Allergy and Immunology, University of Arkansas for Medical Sciences, Little Rock.

*These authors are currently affiliated with the Division of Allergy, Immunology, Rheumatology, and Infectious Disease, Department of Pediatrics, University of North Carolina, Chapel Hill, NC.

Supported by the National Institutes of Health/National Institute of Allergy and Infectious Diseases.

Disclosure of potential conflict of interest: B. P. Vickery has received research support from the National Institutes of Health (NIH)/National Institute of Allergy and Infectious Diseases (NIAID); the American Lung Association; Cephalon; the Foundation of the American College of Allergy, Asthma & Immunology (ACAAI); and the Thrasher Research Fund and has received consultancy fees from Mead Johnson. M. Kulis has received research support from the NIH/NIAID. S. M. Jones has received research support from the National Peanut Board, the NIH, and the Food Allergy & Anaphylaxis Network; is on the Food Allergy & Anaphylaxis Network medical advisory board; has received lecture fees from Abbott Nutrition International, the Kentucky Society for Allergy, Asthma & Immunology, the New England Allergy Society, the ACAAI, Indiana University Medical School and Riley Children's Hospital, the Spanish Society of Allergy & Clinical Immunology (Madrid, Spain), and the Oregon Allergy Asthma & Immunology Society; is on the NIAID Safety Monitoring Committee and the Arkansas Medicaid Drug Review Committee; and participated in ad hoc review for the NIAID Study Section. H. A. Sampson has received research support and travel support from the NIAID; is on the Danone Scientific advisory board; has received consultancy fees from Allertein Therapeutics and Food Allergy Initiative; is employed by Mount Sinai Medical School; receives royalties from Elsevier-Wiley; and is a 42.5% owner of Herbs Springs, LLC. A. W. Burks has received research support and travel support from the NIH/NIAID; is on the board for the American Academy of Allergy, Asthma & Immunology (AAAAI), the Food Allergy & Anaphylaxis Network, the US Food and Drug Administration, the *Journal of Allergy and Clinical Immunology*, and the NIH/HAI; has received consultancy fees from Dannon Co Probiotics, Exploramed Development, Intelliject, McNeil Nutritionals, Merck & Co, Novartis, Nutricia, Pfizer, Portola Pharmaceuticals, and Schering-Plough; is employed by Duke University Medical Center and UNC North Carolina Children's Hospital; has received research support from the Food Allergy & Anaphylaxis Network, the Food Allergy Initiative, the NIH, the National Peanut Board, Scientific Hospital Supplies (Nutricia North America), and the Wallace Research Foundation; has received royalties from UpToDate; has received payment for developing educational presentations from Current Views; has stock/stock options in Allertein and MastCell; and has received travel support from the AAAAI, the European Academy of Allergy & Clinical Immunology, and the ACAAI. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication February 13, 2012; revised October 29, 2012; accepted for publication October 30, 2012.

Corresponding author: Brian P. Vickery, MD, UNC Department of Pediatrics, CB#7231, Chapel Hill, NC 27599. E-mail: bvickery@email.unc.edu.

0091-6749/\$36.00

© 2012 American Academy of Allergy, Asthma & Immunology

<http://dx.doi.org/10.1016/j.jaci.2012.10.048>

Peanut allergy is a serious immunologic disorder characterized by the production of highly pathogenic IgE antibodies that can mediate life-threatening anaphylaxis. No treatment is currently available to modify the natural history of the disease, although several experimental interventions are currently in clinical trials. One such intervention, oral immunotherapy (OIT), appears to induce an altered state of clinical reactivity called desensitization.^{1–3} Mechanistically, desensitization is complex and might involve multiple mechanisms, including suppression of effector cells and allergen-specific T_H2 responses, possibly through induction of regulatory T cells, and alterations in the amount of circulating allergen-specific antibody.⁴ Specifically, peanut-specific IgE (psIgE) levels increase early in OIT when administered allergen doses are low but subsequently plateau and then begin to decrease, continuing to decrease to less than pretreatment values. This progressive decrease in psIgE levels is similar to the trend seen in patients who naturally outgrow peanut allergy. Consistent with other forms of immunotherapy, the repeated oral administration of allergen also leads to increased production of specific IgG, IgG₄, and an inhibitory IgG-dependent serum factor that inhibits the binding of allergen to IgE.¹ Thus previous studies have established that OIT affects the quantity of allergen-specific antibodies, but it is not known whether the diversity, specificity, and affinity of such antibody repertoires change during treatment.

Abbreviations used

FDR: False discovery rate
 HSA: Human serum albumin
 OIT: Oral immunotherapy
 PBS-T: PBS containing 0.05% Tween 20
 psIgE: Peanut-specific IgE
 psIgG₄: Peanut-specific IgG₄

The potential of any food allergy treatment to alter these repertoires can be critical, given that antibody-binding patterns have been shown to associate with peanut allergy phenotypes.⁵⁻⁷ Specifically, IgE recognition of certain immunodominant regions within the major peanut allergens Ara h 1 to 3, as well as broad IgE epitope specificity overall, correlates with persistent disease and more severe reactions. These antibody repertoires appear durable and remain stable over time, although the longest interval studied thus far was 20 months.^{7,8} It is important to note that these data were generated from studies of patients receiving standard-of-care treatment with allergen avoidance.

Here we use a peptide microarray system to examine, for the first time, the longitudinal antibody repertoires of subjects undergoing treatment with OIT. We show that in patients receiving OIT but not elimination diets, the amplitude, specificity, and diversity of the psIgE and peanut-specific IgG₄ (psIgG₄) responses are altered in a complex. These alterations in binding patterns are individualized, occur broadly across the major peanut allergens, and affect informative epitopes within immunodominant regions.

METHODS**Subject recruitment and selection**

Patients with peanut allergy aged 1 to 16 years were recruited from the Allergy and Immunology Clinics at Arkansas Children's Hospital and Duke University Medical Center or surrounding community physicians' offices. These subjects were enrolled in an institutional review board–approved open-label trial of peanut OIT. Protocol details and interim results of this study have been published previously.¹ Serum samples at baseline and throughout the study were collected and frozen at -20°C . Twenty-two subjects for whom multiple time points were available were included in this study. Six control samples, managed with diet restriction alone, were obtained from an institutional review board–approved biorepository of untreated subjects with peanut allergy recruited from the clinic of one of the investigators (A.W.B.).

Conventional measurement of IgE and IgG₄

psIgE and psIgG₄ levels were measured in serum samples in the laboratory of one of the investigators (A.W.B.) by using the ImmunoCAP 100 instrument (Phadia AB, Uppsala, Sweden), according to the manufacturer's instructions.

Peptide microarray analyses

Identified serum samples were sent to Mount Sinai for microarray analysis. A library of peptides, consisting of 15 amino acids overlapping by 12 (3-offset), corresponding to the primary sequences of Ara h 1, Ara h 2, and Ara h 3, was commercially synthesized by JPT Peptide Technologies (Berlin, Germany). Peptides were printed in 2 sets of triplicates onto Arrayit SuperEpoxy glass slides (Arrayit, Sunnyvale, Calif), as previously described.⁹

Immunolabeling was performed as previously described, with some modifications.^{10,11} In brief, the slides were blocked with 400 μL of 1% human serum albumin (HSA) in PBS containing 0.05% Tween 20 (PBS-T) for 60 minutes at room temperature, followed by incubation with 250 μL of the patient's serum diluted 1:5 in PBS-T/HSA for 24 hours at 4°C . Slides were then washed with

PBS-T and incubated for 24 hours at 4°C with a cocktail of several mAbs, including 3 biotinylated anti-human IgE mAbs, one from Invitrogen (Carlsbad, Calif) diluted 1:250, one from BD Biosciences PharMingen (San Jose, Calif) diluted 1:250, and one as a gift from Phadia that was biotinylated in our laboratory and diluted 1:1000, and 1 monoclonal anti-human IgG₄–fluorescein isothiocyanate antibody (Southern Biotech, Birmingham, Ala) diluted 1:1000 in PBS-T/HSA. Slides were then incubated for 3 hours at 31°C with a cocktail of anti-biotin Dendrimer Oyster-550 (350; Genisphere, Hatfield, Pa) and anti-fluorescein isothiocyanate Dendrimer Oyster-650 (350; Genisphere) in Dendrimer Buffer (Genisphere), both at 0.6 $\mu\text{g}/\text{mL}$, with the addition of 0.02 $\mu\text{g}/\text{mL}$ salmon sperm DNA (Invitrogen), followed by a wash with PBS-T, 15 mmol/L Tris, $0.1 \times$ PBS, and $0.05 \times$ PBS. Slides were centrifuged dried and scanned with a ScanArrayGx (PerkinElmer, Waltham, Mass). Images were saved in TIF format.

The fluorescence signal of each spot was digitized with the ScanArray Express Microarray Analysis System (PerkinElmer), exported as comma-delimited (CSV) files, and transformed into robust *z* scores, as previously described.¹² An individual peptide is considered positive if its *z* score is greater than 3, meaning that the signal was greater than the background with a *P* value of less than .003.

A competition assay was performed by immunolabeling the slide as described above, with an additional incubation using 1 mg/mL peanut extract at 16°C for 1 hour after serum incubation and before application of the second antibody. Peanut extract was prepared from commercially available roasted peanut, as described previously.¹³ Protein concentrations were determined by using BCA protein assays (Pierce, Rockport, Ill).

Statistical analyses

Descriptive statistics, including medians, means, and measures of variance, were tabulated for all study parameters. Nonparametric methods, including 2-sample Wilcoxon tests, Spearman correlation, and simple linear regression models, were used to analyze the results (Prism 5 for Windows; GraphPad Software, La Jolla, Calif). Wilcoxon signed-rank pairwise comparisons were used where appropriate for pre-post values obtained from the same subject. A *P* value of less than .05 was considered significant. Pooled IgE and IgG₄ binding peptides/regions were identified by using TileMap, a tool for tiling array analysis,¹⁴ based on a hierarchic empiric Bayes model with a moving average method. Because hundreds of peptides were analyzed simultaneously, the false discovery rate (FDR) was calculated to adjust for multiple comparisons.

RESULTS**Reduction in psIgE levels during OIT is polyclonal and might preferentially affect IgE that binds with high intensity**

The demographics of the study and control populations are shown in Table E1 in this article's Online Repository at www.jacionline.org. Whereas psIgE levels remain stable in untreated control subjects, they decrease from a median of 85.5 kU_A/L at baseline to 7.8 kU_A/L after a median of 41 months (range, 24-53 months) of OIT treatment ($P < .0001$; Fig 1, A). To account for differences in age between the study populations, we performed a nonparametric analysis of pre-post differences in psIgE levels using median regression while adjusting for baseline age. In the control group there was no significant difference in pre-post psIgE levels when controlling for age ($P = .77$), whereas in patients receiving OIT, the pre-post difference remained significant even after adjustment ($P = .002$). Although the total production of psIgE, as measured by using CAP-FEIA, is reduced while receiving OIT, the median number of total Ara h 1 to 3 epitopes bound by IgE did not change over time in either group (Fig 1, B), suggesting that the quantitative reduction in psIgE levels observed during OIT is broad in scope and not due to the elimination of a few specific B-cell clones. Although the control group appeared to have a more diverse IgE repertoire, this difference did not achieve statistical significance.

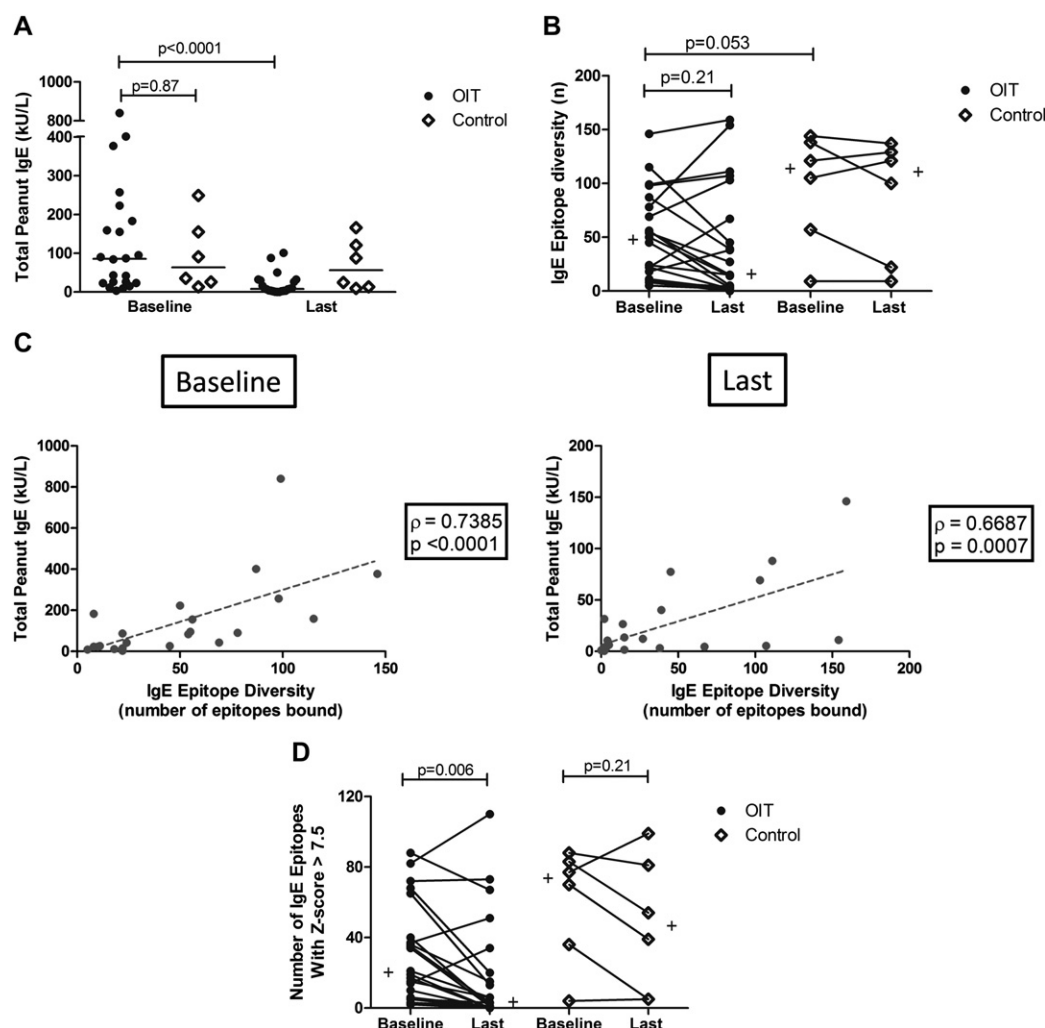


FIG 1. OIT alters the peanut-specific immunoglobulin response in quantity and intensity. In these figures each data point represents 1 subject. *Lines or plus signs* indicate medians. **A**, psIgE, as measured by using conventional CAP-FEIA, decreases from a median of 85.5 kU_A/L at baseline to 7.8 kU_A/L after OIT treatment ($P < .0001$). **B**, The overall IgE repertoire remains stable, even as psIgE levels decrease. All array spots with a z score of greater than 3 were considered positive. **C**, IgE level and epitope diversity are strongly correlated at baseline and at the time of the last measurement after a median of 41 months of treatment. **D**, Fewer peanut peptides are bound by IgE with high intensity over the course of OIT treatment.

psIgE concentrations and repertoire diversity were highly correlated at baseline (correlation coefficient $\rho = 0.7385$, $P < .0001$) and remained so at 41 months ($\rho = 0.6687$, $P = .0007$), further confirming the overall preservation of IgE epitope diversity while total psIgE changes during OIT (Fig 1, C). Because we did not see significant numbers of peptides in which IgE binding converted from positive to negative (ie, z score < 3), we next assessed whether OIT could reduce high-intensity binding on the array. When we focused on areas of Ara h 1 to 3 that bound IgE with a z score of greater than 7.5 at baseline, we observed a reduction in the number of such epitopes bound over time from a median of 20 at baseline that decreased to 3 ($P = .006$) after treatment (Fig 1, D).

OIT induces a polyclonal expansion of psIgG₄ production, which includes novel specificities

As shown in Fig 2, A, a small repertoire of psIgG₄ is detectable at baseline in both groups, but only in treated subjects does

the production of psIgG₄ diversify over time, increasing from a median of 1.5 array spots at baseline to 18 array spots after a median treatment length of 41 months ($P < .0001$). This is concurrent with a progressive increase in the total amount of psIgG₄ produced, from a median 0.3 $\mu\text{g/mL}$ (interquartile range [25% to 75%], 0.1–0.43 $\mu\text{g/mL}$) at baseline to 10.5 $\mu\text{g/mL}$ (interquartile range [25% to 75%], 3.95–45.48 $\mu\text{g/mL}$; $P < .0001$; see Fig E1 in this article's Online Repository at www.jacionline.org).

To understand whether the IgG₄ that emerges during OIT has a similar specificity as the original sensitization profile, we compared the binding patterns of IgG₄ at the last measurement with the IgE-binding patterns at baseline. Peptides were defined as new if IgE did not bind them at baseline and concordant if they were bound. As shown in Fig 2, B, the IgG₄ repertoire in untreated control subjects generally does not expand to include new specificities. In contrast, in the sera of 8 (36%) of 22 patients receiving OIT, the number of *de novo* peptides recognized by the IgG₄

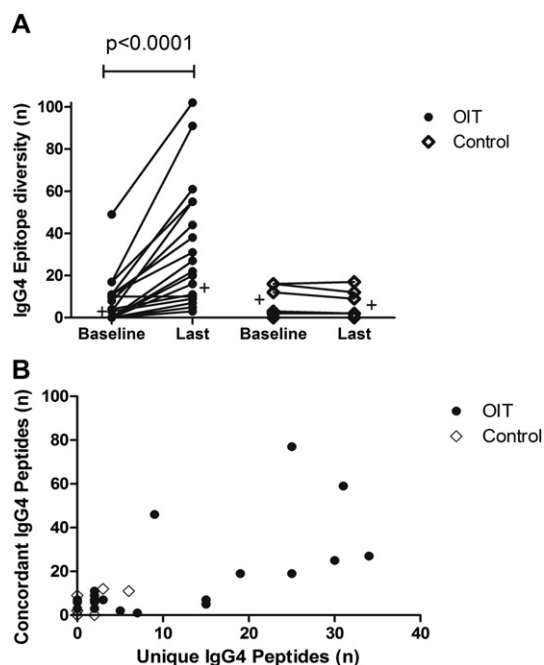


FIG 2. OIT increases the production of broadly diverse IgG₄ antibodies, some of which have *de novo* specificity. **A**, A polyclonal expansion of IgG₄ production occurs during OIT but not during an allergen elimination diet. Plus signs indicate median values. **B**, The specificity of the IgG₄ response after treatment includes both peptides recognized before OIT and new specificities. Each circle or diamond represents 1 subject.

repertoire expands by greater than 10. At the last time point measured, IgG₄ of the 22 patients receiving OIT recognized 584 total peptides (mean, 26.54 peptides per subject), of which 228 (39%) were new, compared with 45 total peptides among 6 control subjects (mean, 7.5 peptides per subject), of which 11 (24.4%) were new ($P = .056$, Fisher exact test).

Binding affinity of pslgE and pslgG₄ is stable over time and largely unaffected by OIT

Because high-intensity IgE binding appeared to be preferentially reduced over time, paralleled by an emerging polyclonal IgG₄ response, we considered the possibility that alterations in antibody affinity can occur through affinity maturation during OIT. To investigate this, we performed competition experiments in a subset of subjects who had detectable IgE binding at both baseline and at the last time point. As shown in Fig 3, compared with the standard protocol, antibody binding to peptides was slightly reduced when peanut extract was added as a competitor. The overall decrease in IgE binding achieved a statistically significant difference. However, the competition effect was weak for both IgE and IgG₄ binding, and no obvious difference between time points could be observed.

Diversity of the pslgE repertoire varies by subject during OIT and might change independently of the pslgE level

To further assess OIT's effect on the IgE repertoire, we investigated individual variation by calculating the difference in

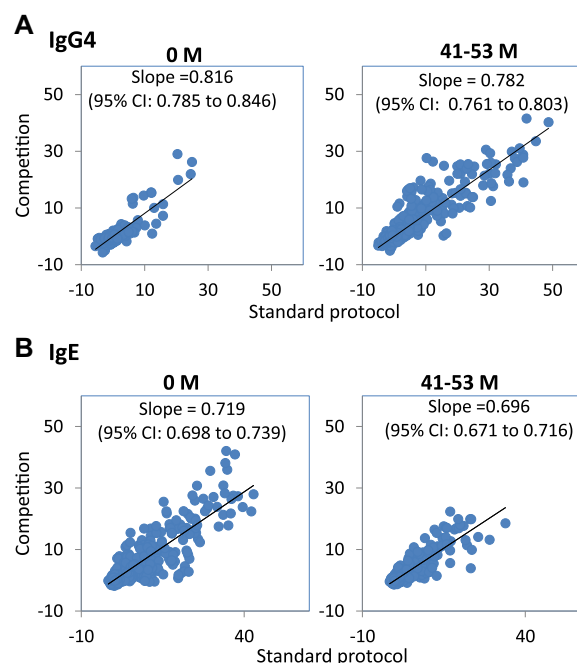


FIG 3. Scatter plot comparing IgG₄ (**A**) and IgE (**B**) binding to the array between the standard protocol (*x*-axis) and the competition assay (*y*-axis) from baseline and the last time points of 5 subjects. Each spot represents antibody binding (represented as a z score) to 1 peanut peptide on the array.

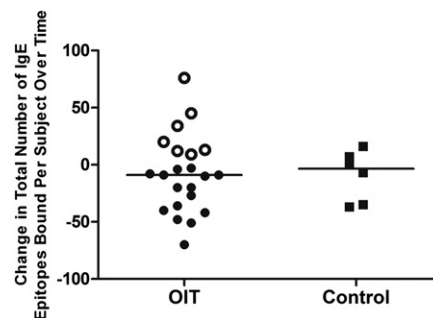


FIG 4. The peanut IgE repertoire contracts in most but not all subjects treated with OIT. The number of array spots bound by IgE was compared from baseline with that at the last available measurement for each subject.

the number of Ara h 1 to 3 epitopes bound by individual subjects' IgE at baseline and after a median of 41 months of OIT. As demonstrated in Fig 4, the number of epitopes bound by IgE was reduced in 15 (68%) of 22 subjects, indicating a tapering of the antibody repertoire over time. However, an increase in the diversity of IgE binding was observed in 7 (32%) of 22 subjects. Interestingly, in all 7 subjects, because the IgE repertoire was expanding, the total pslgE level decreased, from a median of 90.3 kU_A/L (interquartile range [25% to 75%], 15-377 kU_A/L) at baseline to 9.1 kU_A/L (interquartile range [25% to 75%], 4.5-88 kU_A/L) after a median of 41 months (data not shown). In contrast, repertoires and total IgE levels of control subjects remained stable. These data suggest that new B-cell clones could emerge and produce novel epitope-specific IgE during the course of OIT, even as the overall pslgE response is being suppressed.

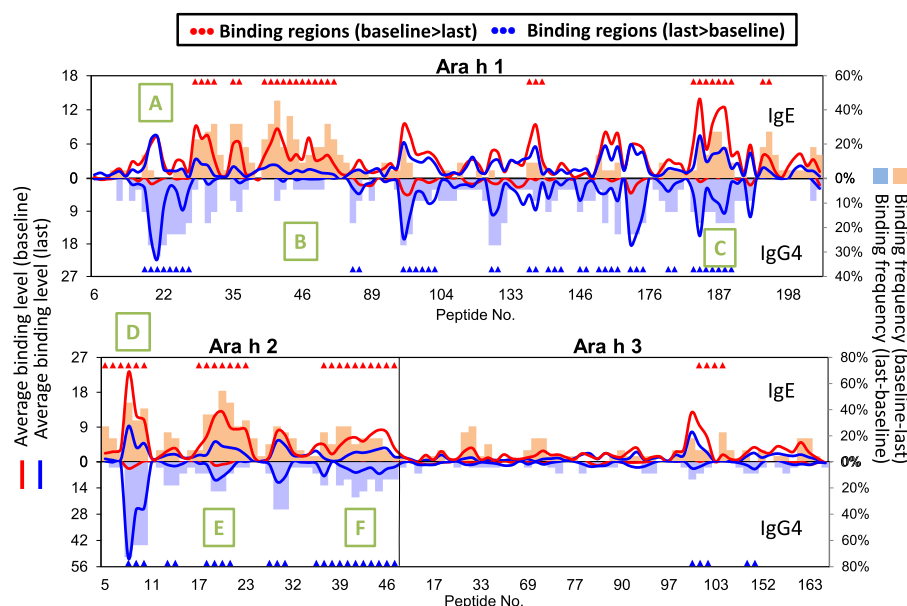


FIG 5. Comparison of IgE and IgG₄ binding of peanut OIT-treated patients to peptides of Ara h 1 to 3 between baseline and the last time point. The x-axis shows the overlapping peptides. The left y-axis shows the average IgE and IgG₄ binding level (represented as the z score) to each peptide. The right y-axis shows the difference in the percentage of patients with positive binding to each peptide between time points. Binding for IgE is indicated above the x-axis line, and binding for IgG₄ is indicated below the line. Regions/peptides with significantly ($P < .01$, FDR < 0.1 , as identified by using TileMap) higher binding at baseline than at the last time point are indicated with red triangles, whereas regions with significantly higher binding at the last time point than baseline are indicated with blue triangles. Green boxes labeled A-F indicate regions at which varied concordance of IgE and IgG₄ binding is of interest.

OIT induces shifts in global binding patterns involving informative epitopes, with varying degrees of concordance

To assess global epitope-binding patterns and observe any possible relationship between epitope-specific IgE and IgG₄ production, we constructed epitope maps of binding patterns for all subjects in the study. As previously reported, we observed recognition of immunodominant regions and significant variation in binding patterns between individual subjects (data not shown). The frequencies of antibody binding across the primary sequence of Ara h 1 to 3, as well as the average z score for each peptide, and the changes over time in each parameter are represented in Fig 5 for all treated subjects. For these subjects, 10 IgE-binding regions consisting of 57 peptides (Ara h 1, 30 peptides/6 regions; Ara h 2, 23 peptides/3 regions; and Ara h 3, 4 peptides/1 region) were identified by TileMap as having significantly greater IgE binding ($P < .01$, FDR < 0.1) at baseline than at the last time point measured. Similarly, 18 IgG₄ binding regions consisting of 69 peptides (Ara h 1, 41 peptides/11 regions; Ara h 2, 23 peptides/5 regions; and Ara h 3, 5 peptides/2 region) were identified as having significantly greater IgG₄ binding ($P < .01$, FDR < 0.1) at the last time point than at baseline. No region was found to have significantly greater IgE binding at the last time point than baseline or, conversely, significantly greater IgG₄ binding at baseline than at the last time point. Interestingly, reduction in IgE binding was discordant with specific IgG₄ production in 2 regions of Ara h 1, as indicated by the green boxes labeled A and B in Fig 5. However, in another immunodominant region of the protein (box C), the reduction in IgE binding was inversely associated with IgG₄ recognition. Similarly, the changes in Ara h 2-binding patterns (boxes D-F) were much more concordant, including importantly at epitopes previously

identified to be “informative (box D).” Binding patterns did not significantly change over time in control subjects (see Fig E2 in this article’s Online Repository at www.jacionline.org).

DISCUSSION

Although several studies have now examined in detail the epitope-specific antibody repertoires of untreated subjects with food allergy, few have had longitudinal designs, and no prior studies have investigated whether OIT can change the specificity and diversity of such repertoires. To address this knowledge gap, we used a high-throughput peptide microarray system¹² to investigate the longitudinal effects of peanut OIT on the specificity and diversity of the antibody repertoire directed against the 3 major peanut allergens Ara h 1 to 3.

As previously reported,⁶ we observed marked variation in IgE- and IgG₄-binding patterns between subjects. We confirmed the finding that patients with peanut allergy on allergen elimination diets have highly stable antibody repertoires by studying untreated control subjects over an average follow-up duration of 58 months (data not shown), far longer than the 20 months previously reported.⁷ However, in contrast to these control subjects, we show that the antibody repertoire undergoes dynamic and individualized changes during OIT. In our previous studies of peanut OIT, we have shown that the overall quantity of pIgE is reduced on treatment while the concentration of pIgG₄ is concurrently increased and that, over time, a serum factor (presumed to be IgG₄) emerges that can functionally inhibit IgE binding *in vitro*.¹ These data were consistent with the hypothesis that functionally inhibitory “blocking” antibodies of the IgG class develop during immunotherapy.^{15,16} Some of the findings of the current study support

this hypothesis, such as a progressive isotype shift during OIT in some subjects, in which a reduction in IgE binding occurs at the same epitopes at which IgG₄ binding gradually increases. Importantly, we observed this shift toward IgG₄ predominance occurring especially within Ara h 2 and involving critical “informative” epitopes previously shown to be predictive of natural tolerance acquisition.⁵ Studies with SPOTS membranes,⁵ immunoblotting,^{8,17} and component-resolved techniques¹⁸ have all previously demonstrated the importance of IgE binding to Ara h 2 in patients with peanut allergy compared with control subjects who outgrew peanut allergy or were sensitized to peanut but not allergic.

However, several additional findings from this study suggest that the antibody response to OIT is more complex than simply the gradual induction of epitope-identical blocking antibodies of a different isotype. First, the IgG₄ response was broadly expanded in a polyclonal fashion during OIT, including induction of novel specificities. At certain peptides, this IgG₄ production had no apparent effect on IgE binding. In addition, the clonality of the IgE repertoire was generally preserved, even while total pIgE levels decreased, suggesting that the diversity and amplitude of the allergen-specific antibody responses to OIT are differentially regulated. Indeed, we unexpectedly also observed a concomitant expansion of the IgE repertoire in roughly one third of our subjects, even while an overall reduction in pIgE levels occurred in each of them. The induction of *de novo* specificities in both the IgE and IgG₄ compartments had been previously shown with the aid of recombinant test antigens in a study of specific subcutaneous immunotherapy with grass pollen.¹⁹ It has been proposed that low-affinity IgE with novel specificities might emerge early in the course of specific immunotherapy when doses are low and that because these clones bind allergen with less relative avidity than antibody produced by hypermutated long-lived memory B or plasma cells, they are not likely to be clinically deleterious.²⁰ Remarkably, in our study the IgE repertoire expansion occurred over a period of almost 4 years and not just during the early phases of treatment. This paradoxical expansion of IgE diversity concurrent to the reduction in amplitude of allergen-specific IgE production has never been shown in a study of specific immunotherapy.

Finally, using peanut extract, we were unable to outcompete IgE or IgG₄ binding to target epitopes in the sera of subjects treated with OIT for several years, suggesting that the affinity of these antibodies is and remains high. This might be unique to peanut allergy and this form of therapy because of the use of crude whole-antigen preparations for treatment, the complex process of food preparation, digestion, and absorption, and/or differences in the immunogenic potential of food antigens in patients with more severe forms of food allergy. A previous study of subjects with milk allergy similarly found that patients remaining allergic to unheated milk had a substantial fraction of high affinity antibodies when compared with those tolerant of heated milk or nonallergic control subjects.²¹

Several previous studies have used similar methodologies to analyze the epitope-binding patterns of patients allergic to peanut,^{6,7} milk,²¹⁻²³ shrimp,¹¹ and lentil.²⁴ Collectively, these studies have established (1) the individuality of epitope-binding patterns among patients, (2) the association between the diversity of the IgE repertoire and the persistence and severity of food allergy, and (3) the long-term stability of IgE- and IgG₄-binding patterns in patients with peanut allergy treated with elimination diets. Wang et al²¹ extended these observations to include the antibody responses of those patients who can tolerate a heated milk product, which is considered to be a form of partial tolerance, and

included affinity studies. However, all of these studies were observational, and most were conducted in patients practicing allergen avoidance using cross-sectional designs. A recent prospective study²³ followed patients with milk allergy over time and analyzed the evolution of antibody responses occurring as milk allergy was outgrown. Compared with those with persistent milk allergy, patients who recover spontaneously experience a longitudinal reduction in the diversity of the IgE repertoire and a decrease in intensity of IgE binding that is concurrent with an increase in IgG₄ binding intensity at similar epitopes. We observed similar changes in some but not all of our patients receiving OIT, suggesting that although the serologic response to OIT might share some overlap with natural tolerance induction, it could have its own unique features. In future analyses, we will investigate the relationship of particular binding patterns to clinical responses using oral food challenge outcomes. Intriguingly, Savi-lahti et al²³ used mathematic modeling to show that a signature that considered the IgE and IgG₄ binding to 15 regions across 4 main milk allergens at the time of diagnosis could predict whether milk allergy would resolve or persist, suggesting that this type of approach might have merit in tolerance evaluation.

We have previously used CAP-FEIA to show that peanut OIT changes the amplitude of the antibody response to peanut, and others have used peptide microarrays to study IgE and IgG₄ responses in children with food allergy on elimination diets. This article extends these previous studies by demonstrating for the first time that in patients undergoing peanut OIT but not those on elimination diets, the amplitude, specificity, and diversity of the antibody response to the major peanut allergens Ara h 1 to 3 is modified over time. These changes occur without evident effect on the affinity of the repertoire. Although the changes in binding patterns are individualized, they tend to affect the previously shown immunodominant regions, including at epitopes within Ara h 2 previously shown to be informative for naturally occurring tolerance. Future study will be necessary to further elucidate how these findings contribute to clinical tolerance.

We thank the subjects and their families. We thank Xiaohong Yue, Huamei Zhang, Anne Hiegel, Suzanne Carlisle, Henry Beresford, Meredith Ross, and Nikolas and Jan Kamilaris for their contributions to these studies. Brian Smith, MD, MPH, MHS, provided invaluable statistical expertise. We also thank the Duke Clinical Research Unit for providing the clinic space and staff support necessary to conduct our study.

Key messages

- Allergen avoidance does not change the highly stable peanut-specific antibody repertoires of patients with peanut allergy.
- In contrast, OIT induces dynamic and individualized changes in IgE- and IgG₄-binding patterns, with a minimal effect on antibody affinity.
- Some of these changes are consistent with those seen in other forms of specific immunotherapy or natural tolerance.

REFERENCES

1. Jones SM, Pons L, Roberts JL, Scurlock AM, Perry TT, Kulis M, et al. Clinical efficacy and immune regulation with peanut oral immunotherapy. *J Allergy Clin Immunol* 2009;124:292-300, e1-97.

2. Clark AT, Islam S, King Y, Deighton J, Anagnostou K, Ewan PW. Successful oral tolerance induction in severe peanut allergy. *Allergy* 2009;64:1218-20.
3. Blumchen K, Ulbricht H, Staden U, Dobberstein K, Beschorner J, de Oliveira LC, et al. Oral peanut immunotherapy in children with peanut anaphylaxis. *J Allergy Clin Immunol* 2010;126:83-91.e1.
4. Nowak-Węgrzyn A, Sampson HA. Future therapies for food allergies. *J Allergy Clin Immunol* 2011;127:558-73.
5. Beyer K, Ellman-Grunther L, Järvinen KM, Wood RA, Hourihane J, Sampson HA. Measurement of peptide-specific IgE as an additional tool in identifying patients with clinical reactivity to peanuts. *J Allergy Clin Immunol* 2003;112:202-7.
6. Shreffler WG, Beyer K, Chu TH, Burks AW, Sampson HA. Microarray immunoassay: association of clinical history, in vitro IgE function, and heterogeneity of allergenic peanut epitopes. *J Allergy Clin Immunol* 2004;113:776-82.
7. Flinterman AE, Knol EF, Lencer D, Bardina L, den Hartog Jager CF, Lin J, et al. Peanut epitopes for IgE and IgG4 in peanut-sensitized children in relation to severity of peanut allergy. *J Allergy Clin Immunol* 2008;121:737-43.
8. Flinterman AE, van Hoven E, den Hartog Jager CF, Koppelman S, Pasmans SG, Hoekstra MO, et al. Children with peanut allergy recognize predominantly Ara h2 and Ara h6, which remains stable over time. *Clin Exp Allergy* 2007;37:1221-8.
9. Flinterman AE, Knol EF, Lencer DA, Bardina L, Jager C, Lin J, et al. Peanut epitopes for IgE and IgG4 in peanut-sensitized children in relation to severity of peanut allergy. *J Allergy Clin Immunol* 2008;121:737-43.
10. Lin J, Bardina L, Bruni FM, Shreffler WG, Wang J, Sampson HA. Development of a reliable and sensitive peptide microarray for large-scale epitope mapping of milk allergens. *J Allergy Clin Immunol* 2008;121:949.
11. Ayuso R, Sánchez-García S, Lin J, Fu Z, Ibáñez MD, Carrillo T, et al. Greater epitope recognition of shrimp allergens by children than by adults suggests that shrimp sensitization decreases with age. *J Allergy Clin Immunol* 2010;125:1286-93.e3.
12. Lin J, Bardina L, Shreffler WG, Andreae DA, Ge Y, Wang J, et al. Development of a novel peptide microarray for large-scale epitope mapping of food allergens. *J Allergy Clin Immunol* 2009;124:315-22, e1-3.
13. Beyer K, Bardina L, Grishina G, Sampson HA. Identification of sesame seed allergens by 2-dimensional proteomics and Edman sequencing: seed storage proteins as common food allergens. *J Allergy Clin Immunol* 2002;110:154-9.
14. Ji HK, Wong WH. TileMap: create chromosomal map of tiling array hybridizations. *Bioinformatics* 2005;21:3629-36.
15. Wachholz PA, Durham SR. Mechanisms of immunotherapy: IgG revisited. *Curr Opin Allergy Clin Immunol* 2004;4:313-8.
16. Uermösi C, Beerli RR, Bauer M, Manolova V, Dietmeier K, Buser RB, et al. Mechanisms of allergen-specific desensitization. *J Allergy Clin Immunol* 2010;126:375-83.
17. Koppelman SJ, Wensing M, Ertmann M, Knulst AC, Knol EF. Relevance of Ara h1, Ara h2 and Ara h3 in peanut-allergic patients, as determined by immunoglobulin E Western blotting, basophil-histamine release and intracutaneous testing: Ara h2 is the most important peanut allergen. *Clin Exp Allergy* 2004;34:583-90.
18. Nicolaou N, Poorafshar M, Murray C, Simpson A, Winell H, Kerry G, et al. Allergy or tolerance in children sensitized to peanut: prevalence and differentiation using component-resolved diagnostics. *J Allergy Clin Immunol* 2010;125:191-7, e1-13.
19. Ball T, Sperr WR, Valent P, Lidholm J, Spitzauer S, Ebner C, et al. Induction of antibody responses to new B cell epitopes indicates vaccination character of allergen immunotherapy. *Eur J Immunol* 1999;29:2026-36.
20. Shamji MH, James LK, Durham SR. Serum immunologic markers for monitoring allergen-specific immunotherapy. *Immunol Allergy Clin North Am* 2011;31:311-23.
21. Wang J, Lin J, Bardina L, Goldis M, Nowak-Węgrzyn A, Shreffler WG, et al. Correlation of IgE/IgG4 milk epitopes and affinity of milk-specific IgE antibodies with different phenotypes of clinical milk allergy. *J Allergy Clin Immunol* 2010;125:695-702, e1-702.
22. Cerecedo I, Zamora J, Shreffler WG, Lin J, Bardina L, Dieguez MC, et al. Mapping of the IgE and IgG4 sequential epitopes of milk allergens with a peptide microarray-based immunoassay. *J Allergy Clin Immunol* 2008;122:589-94.
23. Savilahti EM, Rantanen V, Lin JS, Karinen S, Saarinen KM, Goldis M, et al. Early recovery from cow's milk allergy is associated with decreasing IgE and increasing IgG4 binding to cow's milk epitopes. *J Allergy Clin Immunol* 2010;125:1315-21.e9.
24. Vereda A, Andreae DA, Lin J, Shreffler WG, Ibáñez MD, Cuesta-Herranz J, et al. Identification of IgE sequential epitopes of lentil (Len c 1) by means of peptide microarray immunoassay. *J Allergy Clin Immunol* 2010;126:596-601.e1.

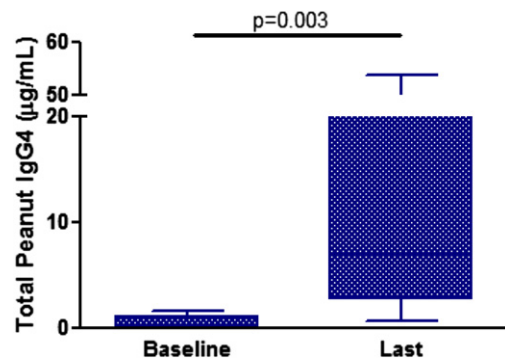


FIG E1. Peanut OIT induces production of pslgG₄. pslgG₄ levels were measured by using ImmunoCAP. *Box plots* represent 10th and 90th percentile data with medians. *Error bars* denote the entire range.

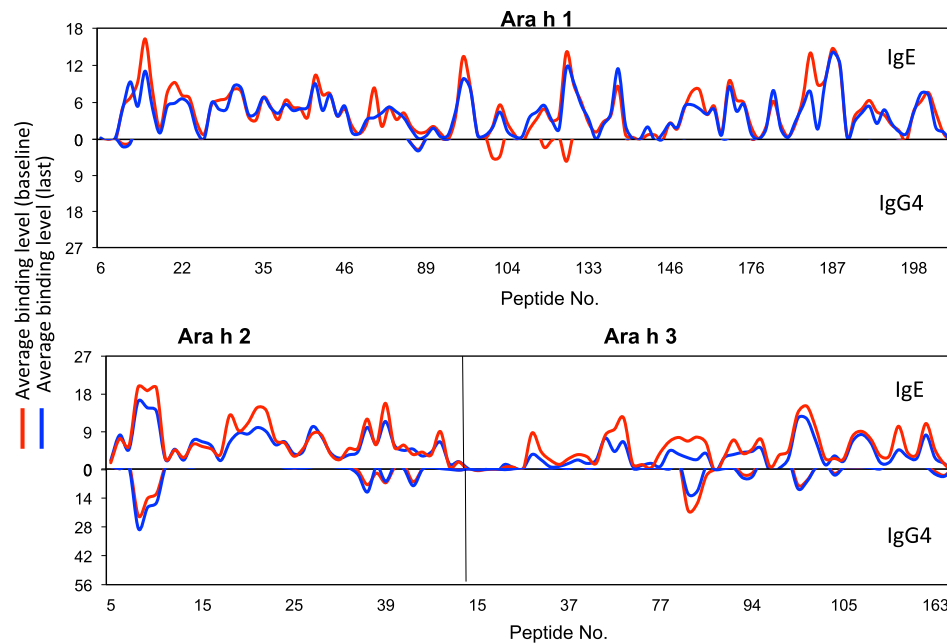


FIG E2. Comparison of IgE and IgG₄ binding of control subjects to peptides of Ara h 1 to 3 between baseline and the last time point. The *x-axis* shows the overlapping peptides, and the *y-axis* shows the average IgE and IgG₄ binding level (represented as the z score) to each peptide. Binding for IgE is indicated above the *x-axis* line, whereas binding for IgG₄ is indicated below the line.

TABLE E1. Demographics of the control and study populations

Subject ID	Race	Sex	Age at study entry (y)	Initial peanut reaction	Other current food allergies	Atopic comorbidities
Control subjects						
PEC01	White	M	4	Unknown	E, M, N, F	AD, A
PEC02	White	M	0.75	Unknown	E, M, S	AD
PEC03	White	F	24	Anaphylaxis	N, S	A
PEC04	White	M	45	Anaphylaxis	W	
PEC05	White	M	32	Anaphylaxis		
PEC06	White	F	35	Anaphylaxis	E, M, W	AD, A
Patients						
PN001	White	F	5	Anaphylaxis	N	A, AD
PN002	White	M	6	Anaphylaxis		AD, AR
PN003	White	M	2	Urticaria		A, AR, AD
PN004	White	F	4	Urticaria	E	AD, A
PN005	White	M	5	Angioedema	N	AD, A
PN007	White	M	5	Urticaria + angioedema		A, AR, AD
PN008	White	M	2	Urticaria	E	AD, AR
PN009	White	F	4	Anaphylaxis		A, AR
PN011	White	M	4.5	Anaphylaxis		AD, A, AR
PN012	White	F	4	Urticaria		A, AR
PN014	White	F	9	Urticaria		AD, AR
PN015	White	F	8	Anaphylaxis	N, S	A, AR
PN016	White	M	8	Anaphylaxis	A	AD, AR, A
PN018	White	F	2	Anaphylaxis		A, AR, AD
PN019	White/Asian	M	4	Urticaria, angioedema, rhinorrhea	E	A, AR, AD
PN021	White	M	5	Angioedema		AD, AR
PN023	White	M	5	Urticaria		A, AR
PN024	White	M	6	Urticaria	N	A, AD
PN026	White	M	9	Urticaria + abdominal pain	N	AD, A, AR
PN027	White	M	2	Anaphylaxis		AD
PN028	White	F	8	Anaphylaxis		A
PN029	White	M	4	Urticaria	E, M, N	AD, A, AR

Other current food allergies: A, Apple; E, egg; F, fish; M, milk; N, tree nut(s); S, shrimp; W, wheat. Atopic comorbidities: A, Asthma; AD, atopic dermatitis; AR, allergic rhinitis.