

Letter to the Editor

Exploiting CD22 on antigen-specific B cells to prevent allergy to the major peanut allergen Ara h 2

To the Editor:

Oral, sublingual, and epicutaneous immunotherapies are under clinical study as potential food allergy therapies, yet the side effects, requirement for daily dosing, and lack of prolonged efficacy remain limitations in these human trials.^{1,2} Targeting the allergen-specific B cells may limit side effects and promote long-term tolerance.

Sialic acid-binding immunoglobulin-type lectins (Siglecs) are a family of immunomodulatory receptors with cell-specific expression.³ Inhibitory Siglecs, including CD22 expressed on

B cells, use immunoreceptor tyrosine-based inhibitory motifs to suppress activatory receptors, such as the B-cell receptor (BCR). Enforcing colocalization of CD22 with the BCR, with liposomes that codisplay an antigen and high-affinity CD22 ligand, not only prevents B-cell activation but also induces apoptosis of the antigen-reactive B cells, resulting in robust immunological tolerance due to depletion of the antigen-specific B cells from the B-cell repertoire.⁴ These Siglec-engaging tolerance-inducing antigenic liposomes (STALs) can be formulated with any antigen of choice. STALs displaying factor VIII (FVIII) inhibit antibody responses to exogenous FVIII, preventing bleeding in FVIII^{-/-} mice.⁴ Accordingly, STALs have the potential to prevent undesired B-cell responses and we were motivated to examine their potential for inducing immunological tolerance to a food allergen.

Peanut allergies are dominated by undesired IgE antibody responses to the 2S albumin Ara h 2 (Ah2),^{5,6} which induce

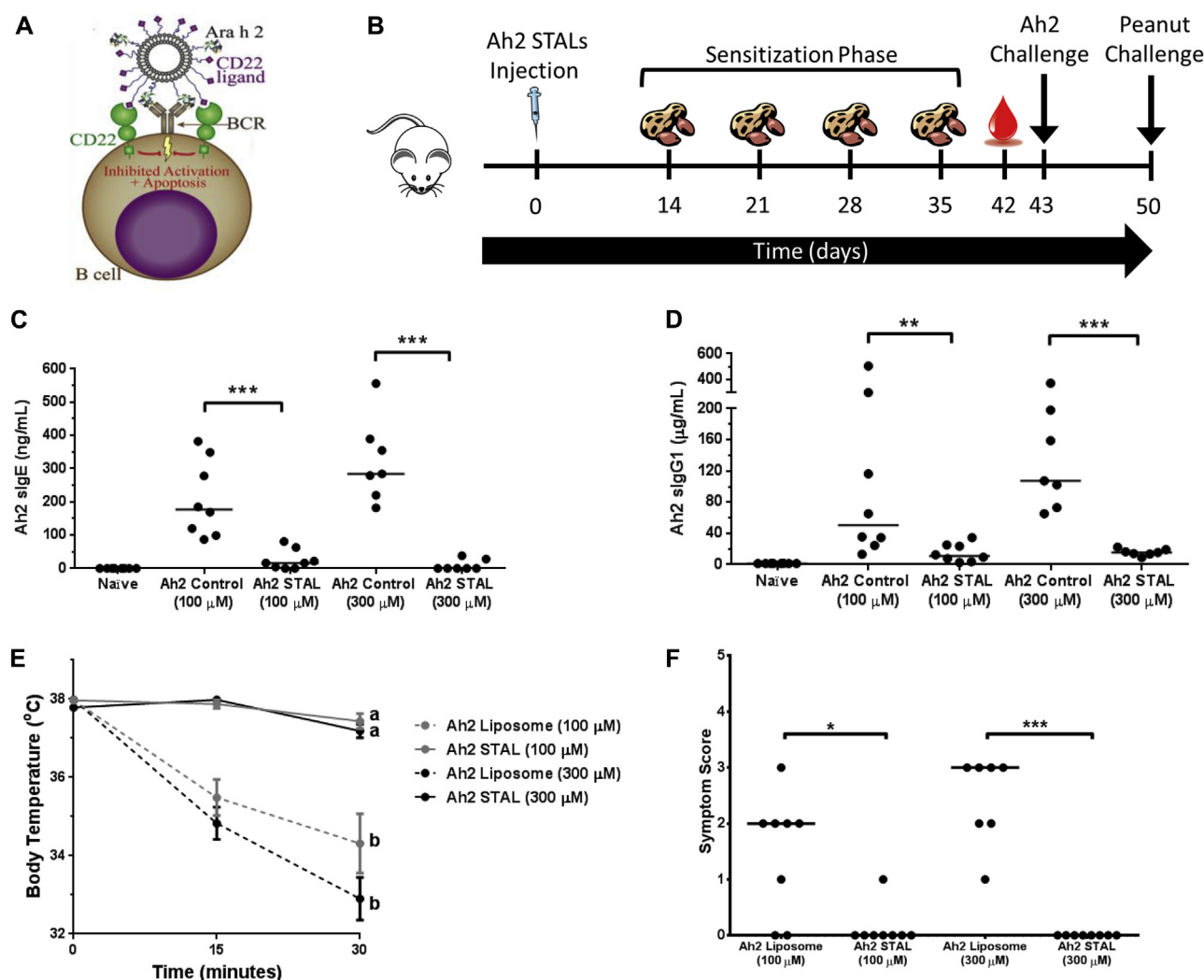


FIG 1. Ah2-specific immune responses are prevented in mice administered Ah2 STALs. Schematic of Ah2 STALs (A) and experimental protocol (B). Serum levels of Ah2 sIgE (C) and Ah2 sIgG₁ (D) following oral sensitization. Body temperatures (E) and symptom scores (F) after challenge with 200 μg Ah2 i.p. Lines "a" are statistically different from lines "b" ($P < .001$) at 15 and 30 minutes. i.p., Intraperitoneal. Mann-Whitney U test: * $P < .05$, ** $P < .01$, and *** $P < .001$.

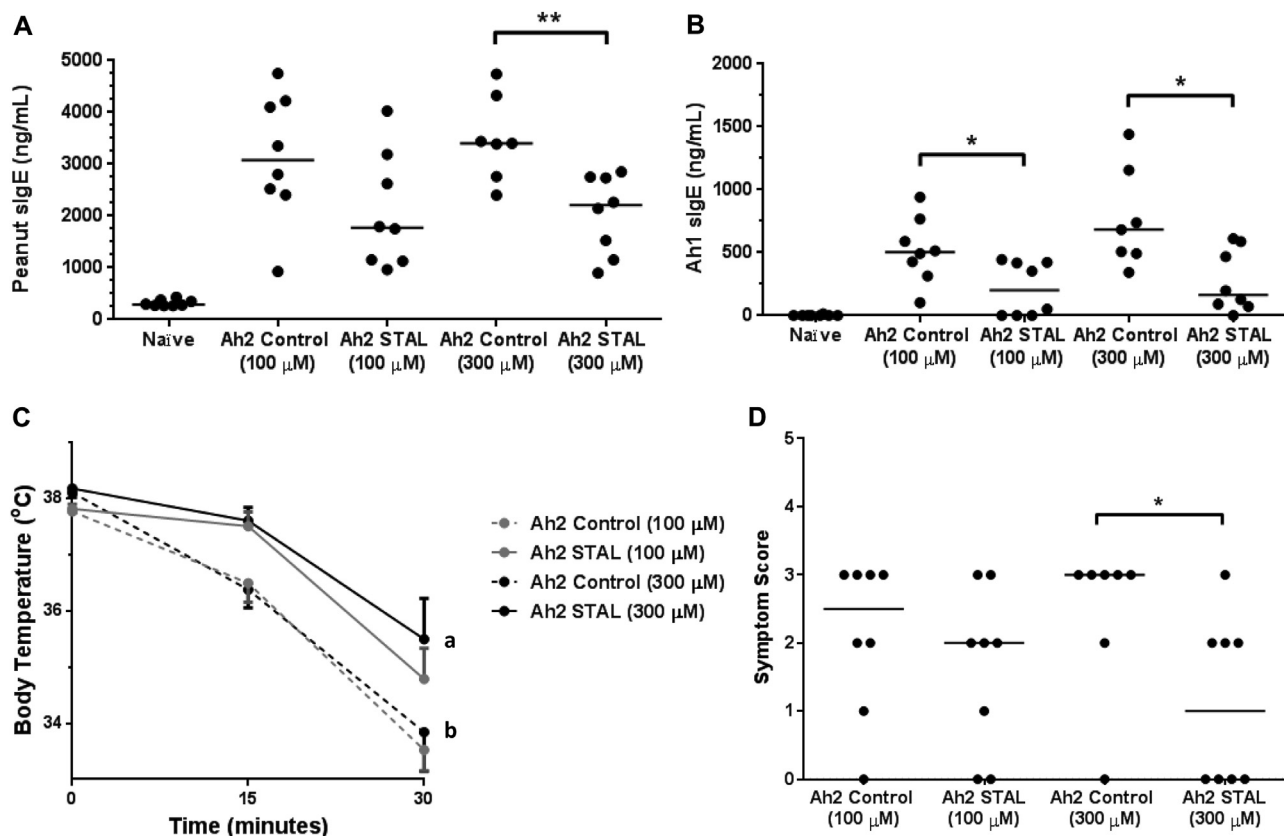


FIG 2. Immune responses to WPE and the peanut allergen Ah1 in mice treated with Ah2 STALs or immunogenic Ah2 liposomes. Peanut sIgE (**A**) and Ah1 sIgE (**B**) following oral sensitization with WPE and CT. Body temperatures (**C**) and symptom scores (**D**) after i.p. challenge with 750 μ g WPE. Line "a" is statistically different from line "b" ($P < .05$) at 15 and 30 minutes. i.p., Intraperitoneal. Mann-Whitney U test: * $P < .05$ and ** $P < .01$.

degranulation of effector cells on exposure to the antigen. We hypothesized that STALs displaying both a high-affinity and selective CD22 ligand and Ah2 (Fig 1, A) could be an attractive strategy to prevent sensitization and subsequent anaphylaxis to Ah2 and potentially whole peanut extract (WPE).

A schematic representing the experimental design is shown in Fig 1, B. All animal studies were approved by the University of North Carolina Institutional Animal Care and Use Committee and investigated under protocol number 13-216.0. Four-week-old female BALB/cJ mice (Jackson Laboratories, Bar Harbor, Me) were injected intravenously with 200 μ L of 100 μ M Ah2 STALs ($n = 8$), 300 μ M Ah2 STALs ($n = 8$), 100 μ M immunogenic Ah2 liposomes ($n = 8$), or 300 μ M immunogenic Ah2 liposomes ($n = 7$). All liposomes consisted of 0.03 mol% Ah2, which amounted to a dose of 0.12 μ g of Ah2 in the 100- μ M group. STALs also consisted of 1% BPA-Neu5Gc, the high-affinity and selective CD22 ligand.⁴ Two weeks following infusion of STALs, a time frame previously determined to maximize tolerance induction through STALs,⁴ the mice were orally sensitized, with 2 mg WPE and 10 μ g cholera toxin (CT) weekly for 3 weeks followed by a boost dose of 5 mg WPE and 10 μ g CT. A group of naive mice ($n = 8$) underwent the same protocol and were injected with PBS to determine baseline titers. Serum was collected 1 week later to quantify specific IgE (sIgE) and sIgG₁ levels to Ah2, WPE, Ah1, and CT by ELISA.⁶ Mice were initially challenged with 200 μ g Ah2 via an intraperitoneal injection. One

week later, mice were challenged intraperitoneally with 750 μ g WPE. To assess anaphylaxis during challenge, rectal temperatures were recorded for 30 minutes, and symptom scores were documented at 30 minutes using a 0- to 5-point scale, where 0 represents no symptoms and 5 represents death, as described previously.⁶ All methods are described in detail in this article's Online Repository at www.jacionline.org.

On day 42, before the challenge, Ah2 sIgE levels were significantly lower in animals injected with either 100 μ M or 300 μ M Ah2 STALs compared with those injected with the same dose of immunogenic controls (100 μ M, $P = .0002$; 300 μ M, $P = 0.0006$) (Fig 1, C). Pretreatment with Ah2 STALs also inhibited production of Ah2 sIgG₁ compared with controls (100 μ M, $P = .0047$; 300 μ M, $P = .0006$) (Fig 1, D). Upon challenge with 200 μ g Ah2, mice pretreated with either 100 μ M or 300 μ M Ah2 STALs were protected from hypothermia, an objective feature of anaphylaxis in mice, compared with mice pretreated with immunogenic controls that had severe reactions (Fig 1, E). The symptom scores, as defined previously,⁶ also reflected more severe reactions in animals pretreated with immunogenic Ah2 liposomes compared with those pretreated with Ah2 STALs (Fig 1, F; 100 μ M, $P = .0126$; 300 μ M, $P = .0002$). CT sIgE was not different among treatment groups (Fig E1), indicating that tolerance induction is antigen-specific and that no intrinsic differences in an ability to mount antibody responses were present between the groups.

These findings validated the results of a pilot study where 100 μ M but not 20 μ M Ah2 STALs led to significantly lower Ah2-specific IgE and IgG₁ levels. Taken together, these results suggest that Ah2 STALs induce antigen-specific tolerance toward the major peanut allergen, severely blunting Ah2 sIgE and sIgG₁ levels and reactions upon intraperitoneal challenge with Ah2.

Because Ah2 is only 1 of several related antigens in peanuts and sensitization was done with WPE, we also examined the impact of Ah2 STALs on sIgE levels to WPE (Fig 2, A) and to another peanut allergen, Ara h 1 (Ah1). Pretreatment with 100 μ M Ah2 STALs by intravenous injection resulted in lower peanut sIgE level compared with the immunogenic control mice, though differences were not statistically significant ($P = .1296$). However, mice that received 300 μ M Ah2 STALs (intravenous) had significantly less peanut sIgE than did their control counterparts ($P = .0037$). Interestingly, mice injected with Ah2 STALs had lower Ah1 sIgE levels than did their controls at each dose (100 μ M, $P = .0146$; 300 μ M, $P = .0140$; Fig 2, B). Extensive cross-reactivity between Ah1 and Ah2 has previously been demonstrated and is likely to account for this effect.^{7,8} Finally, these mice were challenged with 750 μ g WPE and, similar to the WPE sIgE results, the body temperatures of mice pretreated with 300 μ M Ah2 STALs were significantly greater than those of mice that received 300 μ M immunogenic controls, demonstrating that the STALs attenuated anaphylaxis to WPE (Fig 2, C). Symptom scores reflected similar results, with the 300 μ M immunogenic control mice reacting more severely than the Ah2 STALs mice ($P = .0350$). The groups treated with 100 μ M Ah2 STALs were not significantly different in body temperatures or symptom scores from the immunogenic control group, suggesting a dose effect. These results demonstrate for the first time that antigen-specific B cells for a single component can be selectively targeted to diminish an immune response to a complex mixture of several allergens.

In conclusion, our data demonstrate that liposomes simultaneously targeting CD22 and the BCR specific for the major peanut allergen, Ah2, can be used to prevent sensitization to Ah2. On the basis of previous studies using other antigens, we hypothesize that simultaneous engagement of CD22 and the Ah2-specific BCR leads to deletion of the Ah2 B cells. Future experiments include testing a combination of STALs that display several peanut antigens as well as using Ah2 STALs as a postsensitization therapy. These findings provide the foundation for the development of a novel therapy for peanut allergy using a highly targeted, antigen-specific approach.

Kelly A. Orgel, BS^{a,b,c}
Shiteng Duan, BS^{d,e,f}
Benjamin L. Wright, MD^{a,b,g}
Soheila J. Maleki, PhD^h
John C. Wolf, PhD^h
Brian P. Vickery, MD^{a,b}
A. Wesley Burks, MD^{a,b}

James C. Paulson, PhD^{d,e,f}
Mike D. Kulis, PhD^{a,b,*}
Matthew S. Macauley, PhD^{e,*}

From ^athe Department of Pediatrics, University of North Carolina School of Medicine, ^bUNC Food Allergy Initiative, and ^cthe Department of Cell Biology and Physiology, University of North Carolina, Chapel Hill, NC; Departments of ^dCell and Molecular Biology, ^eChemical Physiology, and ^fImmunology and Microbial Sciences, The Scripps Research Institute, La Jolla, Calif; ^gthe Department of Pediatrics, Duke University Medical Center, Durham, NC; and ^hthe US Department of Agriculture, Agricultural Research Service, Southern Regional Research Center, New Orleans, La. E-mail: mike.kulis@unc.edu.

*These authors contributed equally to this work as senior investigators.

This work was supported by the National Institutes of Health (grant no. T32 AI007062 to B.L.W.) and grants AI099141 and AI050143 (to J.C.P.).

Disclosure of potential conflict of interest: B. L. Wright has received a grant from the National Institutes of Health (NIH) (grant no. T32AI007062) and is employed by Mayo Clinic Arizona and Phoenix Children's Hospital. B. P. Vickery is employed by and has stock/stock options in Aimmune Therapeutics. A. W. Burks has received grants from the Food Allergy & Anaphylaxis Network, the NIH, and the Wallace Research Foundation; has served as the Research Chairman of Food Allergy Research & Education (2013-2015); has served as a board member for the NIH Administrative Training Committee Review Panel, NIH HAI Study Section, and the World Allergy Organization; has consultant arrangements with Adept Field Solutions, Aimmune Therapeutics, Inc, Dow AgroSciences, Epiva Biosciences, Inc, Genentech, GLG Research, Inc, Insys Therapeutics, Merck, Regeneron Pharmaceuticals, Inc, SRA International, Stallergenes, Valeant Pharmaceuticals North America, LLC, and Sanofi US Services; is an independent contractor for PPD Development, LP; has received royalties from the American Society for Microbiology; and is a minor stockholder in Allertein. J. C. Paulson has received grants from the NIH (grant nos. AI099141 and AI050143). M. D. Kulis is employed by the University of North Carolina at Chapel Hill and has received grants from the NIH. The rest of the authors declare that they have no relevant conflicts of interest.

REFERENCES

- Jones SM, Burks AW, Dupont C. State of the art on food allergen immunotherapy: oral, sublingual, and epicutaneous. *J Allergy Clin Immunol* 2014;133:318-23.
- Gorelik M, Narisety SD, Guerrero AL, Chichester KL, Keet CA, Bieneman AP, et al. Suppression of the immunologic response to peanut during immunotherapy is often transient. *J Allergy Clin Immunol* 2015;135:1283-92.
- Macauley MS, Crocker PR, Paulson JC. Siglec-mediated regulation of immune cell function in disease. *Nat Rev Immunol* 2014;14:653-66.
- Macauley MS, Pfrengle F, Rademacher C, Nycholat CM, Gale AJ, von Drygalski A, et al. Antigenic liposomes displaying CD22 ligands induce antigen-specific B cell apoptosis. *J Clin Invest* 2013;123:3074-83.
- Klemans RJ, van Os-Medendorp H, Blankstijn M, Bruijnzeel-Koomen CA, Knol EF, Knulst AC. Diagnostic accuracy of specific IgE to components in diagnosing peanut allergy: a systematic review. *Clin Exp Allergy* 2015;45:720-30.
- Kulis M, Chen X, Lew J, Wang Q, Patel OP, Zhuang Y, et al. The 2S albumin allergens of *Arachis hypogaea*, Ara h 2 and Ara h 6, are the major elicitors of anaphylaxis and can effectively desensitize peanut-allergic mice. *Clin Exp Allergy* 2012;42:326-36.
- Bublin M, Kostadinova M, Radauer C, Hafner C, Szepefalusi Z, Varga EM, et al. IgE cross-reactivity between the major peanut allergen Ara h 2 and the nonhomologous allergens Ara h 1 and Ara h 3. *J Allergy Clin Immunol* 2013;132:118-24.
- Smit JJ, Pennings MT, Willemsen K, van Roest M, van Hoffen E, Pieters RH. Heterogeneous responses and cross reactivity between the major peanut allergens Ara h 1, 2, 3 and 6 in a mouse model for peanut allergy. *Clin Transl Allergy* 2015;5:13.

<http://dx.doi.org/10.1016/j.jaci.2016.06.053>

METHODS

Peanut extract and Ah2 preparation

Peanut proteins were extracted by mixing peanut flour (12% fat light roast, 50% protein; Golden Peanut Co, Alpharetta, Ga) in a 1:5 (wt:vol) ratio of PBS with 1 mol/L NaCl. The solution was mixed for 2 hours while maintaining an alkaline pH (8.5). The solution was centrifuged at 14,000 rpm for 45 minutes at 4°C. The supernatant was collected and filter-sterilized through a 0.2- μ M filter. Protein concentration was determined by bicinchoninic acid assay (Pierce, Rockford, Ill). The final preparation is referred to in the article as WPE. Ah2 was purified according to work previously published by Sen et al.^{E1}

STAL and immunogenic liposome preparation

Lipid modification of Ah2. A frozen stock of Ah2 in dH₂O was initially buffer exchanged into PBS. The protein (2 mg/mL) was reacted with 2.5 molar equivalents of succinimidyl 3-(2-pyridyldithio) propionate (Thermo Fisher, Waltham, Mass) for 1 hour at room temperature, followed by desalting over Sephadex G-50 (GE Healthcare Life Sciences, Marlborough, Mass). Following deprotection with 25 mM dithiothreitol for 10 minutes at room temperature, the protein was again desalted. The thiol-modified protein was reacted with 10 equivalents of maleimide-PEG₂₀₀₀-distearoylphosphatidylethanolamine (DSPE; NOF America, Irvine, Calif) overnight under nitrogen. The following day, the reaction was passed over a Sephadex G-100 column (GE Healthcare) and the fractions containing the lipid-modified Ah2 in the void volume were pooled and stored at 4°C.

Liposome formulation and extrusion. Distearoylphosphatidylcholine (Avanti, Alabaster, Ala), cholesterol (Sigma-Aldrich, St Louis, Mo), and PEG₂₀₀₀-DSPE (NOF America) were dissolved in chloroform and combined at an approximate ratio of 57:38:5. The solvent was evaporated under nitrogen and 100 μ L of dimethyl sulfoxide (DMSO) was added. To formulate the STALs, 1 mol% of PEG₂₀₀₀-DSPE was replaced with the high-affinity CD22 ligand (6'-BPA-Neu5Gc-LacNAc-PEG₂₀₀₀-DSPE) and added from a DMSO stock. The lipid solutions in DMSO were lyophilized overnight to yield a fluffy powder. To hydrate the liposomes, the dried lipids were hydrated in the appropriate amount of PBS containing the Ah2-PEG-DSPE such that the mol% of the protein was 0.033 mol% of the total lipids. The lipids were hydrated in 1 mL to generate liposomes with a concentration of 5 mM total lipid. The hydrated lipids were sonicated a minimum of 5 times at 30-second intervals and then extruded using a hand-extruder (Avanti) 20 times through a 0.8- μ M filter, followed by 20 times through a 0.1- μ M filter. The final liposomes passed over a CL-4B column (Sigma) and pooled fractions were stored at 4°C before being diluted in PBS to the appropriate liposome concentration and administered to the mice.

Mouse model of peanut sensitization

Four-week-old female Balb/cJ mice purchased from Jackson Laboratory were maintained on peanut-free food under pathogen-free conditions. Mice

were housed with 4 animals per cage and separated into 5 groups: (1) naive, (2) 100 μ M Ah2 immunogenic liposomes, (3) 100 μ M Ah2 STALs, (4) 300 μ M immunogenic liposomes, and (5) 300 μ M Ah2 STALs. Naive mice were maintained throughout the experiment but received no treatments and were not sensitized. Mice in groups 2 to 5 received a single tail vein injection of the designated liposome or STAL. Mice were rested for 2 weeks before being sensitized. After the 2-week rest period, mice were sensitized with 2 mg peanut extract and 10 μ g CT (List Biological Laboratories, Campbell, Calif) by oral gavage once a week for 3 weeks. On the fourth week of sensitization, mice were boosted with 1 gavage of 5 mg peanut extract and 10 μ g CT. One week after the last sensitization dose, 200 μ L blood from each mouse was collected by submandibular bleed to measure sIgE and IgG₁ levels by ELISA. The day following bleeding, mice in groups 2 to 5 were challenged with 200 μ g intraperitoneal Ah2 and 1 week later were challenged intraperitoneally with 750 μ g peanut extract. Anaphylactic reactions were assessed by measuring core body temperatures with a rectal probe (Physitemp, Clifton, NJ) at 15-minute intervals. Symptoms were scored after 30 minutes using a pre-established 5-point scale: 0, no symptoms; 1, scratching around the nose and head; 2, puffiness around the eyes and mouth with reduced activity; 3, labored respiration and/or cyanosis around the mouth and tail; 4, no activity after prodding or tremor and convulsion; and 5, death.

IgE and IgG₁ measurements

sIgE and IgG₁ levels were measured by ELISA using a reference cure. Plates were coated with 20 μ g/mL WPE, 5 μ g/mL Ah2, 5 μ g/mL Ah1, or 5 μ g/mL CT in carbonate-bicarbonate buffer at pH 9.6 for 1 hour at 37°C. Plates were blocked with PBS containing 0.05% Tween 20 and 2% BSA for 2 hours at 37°C. Serum samples were added for 1 hour at 37°C. Detection of IgE was performed using sheep antimouse IgE (0.5 μ g/mL; Binding Site, Birmingham, United Kingdom), followed by biotinylated donkey antisheep IgG (0.5 μ g/mL; Accurate Chemical, Westbury, NY) and neutravidin-horseradish peroxidase (HRP; 0.2 μ g/mL; Pierce) for 1 hour at 37°C. IgG₁ was detected by HRP-conjugated goat anti-mouse IgG₁ (Southern Biotech, Birmingham, Ala) used at 1:40,000 for 1 hour at 37°C. HRP activity was measured by blue color development of Sure Blue TMB Microwell Peroxidase Substrate (KPL, Gaithersburg, Md). Plates were read on an Epoch Microplate Spectrophotometer (BioTek Instruments, Winooski, Vt).

REFERENCE

- E1. Sen M, Kopper R, Pons L, Abraham EC, Burks AW, Bannon GA. Protein structure plays a critical role in peanut allergen stability and may determine immunodominant IgE-binding epitopes. *J Immunol* 2002;169:882-7.

