

Rapid publication

Persistent protective effect of heat-killed *Escherichia coli* producing "engineered," recombinant peanut proteins in a murine model of peanut allergy

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Background: Peanut allergy (PNA) is a life-threatening food allergy for which there is no definitive treatment.

Objective: We investigated the long-term immunomodulatory effect of heat-killed *Escherichia coli* producing engineered (mutated) Ara h1, 2, and 3 (HKE-MP123) administered rectally (pr) in a murine model of PNA.

Methods: Peanut-allergic C3H/HeJ mice received 0.9 (low dose), 9 (medium dose), or 90 (high dose) μ g HKE-MP123 pr, HKE-containing vector (HKE-V) alone, or vehicle alone (sham) weekly for 3 weeks. Mice were challenged 2 weeks later. A second and third challenge were performed at 4-week intervals.

Results: After the first challenge, all 3 HKE-MP123 and HKE-V-treated groups exhibited reduced symptom scores ($P < .01$, .01, .05, .05, respectively) compared with the sham-treated group. Interestingly, only the medium- and high-dose HKE-MP123-treated mice remained protected for up to 10 weeks after treatment accompanied by a significant reduction of plasma histamine levels compared with sham-treated mice ($P < .05$ and .01, respectively). IgE levels were significantly lower in all HKE-MP123-treated groups ($P < .001$), being most reduced in the high-dose HKE-MP123-treated group at the time of each challenge. IL-4, IL-13, IL-5, and IL-10 production by splenocytes of high-dose HKE-MP123-treated mice were significantly decreased ($P < .01$; .001, .001, and .001, respectively), and IFN- γ and TGF- β production were signifi-

cantly increased ($P < .001$ and .01, respectively) compared with sham-treated mice at the time of the last challenge.

Conclusions: Treatment with pr HKE-MP123 can induce long-term "downregulation" of peanut hypersensitivity, which might be secondary to decreased antigen-specific T_H2 and increased T_H1 and T regulatory cytokine production. (J Allergy Clin Immunol 2003;112:159-67.)

Key words: Peanut allergy, murine model, novel immunotherapy, persistent effect

Food allergy affects up to 6% of children younger than 4 years of age and about 2% of the US population beyond the first decade of life.¹ It is the single leading cause of anaphylaxis treated in hospital emergency departments in the United States and many "westernized" countries. Extrapolating from a survey in Olmstead County, Minnesota,² it is estimated that food allergy accounts for approximately 30,000 anaphylactic reactions and 150 deaths each year in the United States, with peanut (PN) and tree nut allergies accounting for the most severe reactions.^{3,4} A national survey indicated that approximately 3 million Americans are allergic to PNs, tree nuts, or both.⁵ Because of the apparent increasing prevalence of food allergy and the frequency and severity of food-induced anaphylactic reactions, food allergy research is receiving increased attention. However, there is still no treatment for this disorder.⁶

Antigen-specific immunotherapy is used to generate tolerance to specific allergens, but unlike traditional immunotherapy for inhalant and bee sting allergy, the benefit/risk ratio for injections of standard PN extracts was found to be unacceptable.⁷ In a trial of standard rush immunotherapy in 6 patients with peanut allergy (PNA), Nelson and colleagues⁷ observed an adverse reactions rate of 23% during the "buildup" phase and 39% during the "maintenance" phase, rates higher than those seen with aeroallergens and insect venom. Consequently, other forms of immunotherapy have been sought to treat food allergy.

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Abbreviations used

CT:	Cholera toxin
DNP:	Dinitrophenol
HKE:	Heat-killed <i>Escherichia coli</i>
HKE-MP123:	A mixture of equal proportions of HKE-mAra h1, HKE-mAra h2, and HKE-mAra h3
HKLM:	Heat-killed <i>Listeria monocytogenes</i>
ig:	Intragastric
MP123:	A mixture of equal proportions of mAra h1, mAra h2, and mAra h3
PNA:	Peanut allergy
PN:	Peanut
pr:	Per rectal
sc:	Subcutaneously
SPC:	Splenocyte

One approach for PNA is to generate engineered (mutated) PN proteins, modified (m)Ara h1, mAra h2, and mAra h3 (mAra-h123) in which IgE-binding epitopes have been altered by a critical amino acid to eliminate, or drastically reduce, IgE binding to the protein and therefore reduce the risk of eliciting allergic reactions. Our preliminary studies in a murine model of PN-induced anaphylaxis showed that the effect of subcutaneously (sc) administered engineered proteins was partially protective (Li et al, unpublished data). Other investigators demonstrated that bacterial adjuvants, such as heat-killed *Listeria monocytogenes* (HKLM), shifted antigen-specific T_H2 responses to T_H1 -like responses and enhanced the response to allergen immunotherapy in a murine model of allergic asthma.¹⁰ Recently, we demonstrated that the sc coadministration of HKLM and engineered PN proteins (HKLM-mAra h123) in PN-sensitized mice significantly reduced plasma histamine levels and anaphylactic symptom scores after intragastric (ig) PN challenge compared with sham-treated mice. IgE levels were significantly reduced, and IgG2a levels were significantly increased.¹¹ Because our engineered PN proteins are generated in *Escherichia coli*, which itself might serve as an adjuvant to promote a T_H1 response, we investigated the efficacy of a mixture of heat-killed *E coli* (HKE) producing mAra h1, 2, and 3 (HKE-MP123) and found that administering this mixture sc or rectally (pr), but not ig, suppressed PNA (Li et al, manuscript in preparation). The sc route of HKE-MP123 was abandoned, because it induced skin inflammation (Li et al, unpublished data) and was unlikely to be acceptable for human use. Because the rectal mucosa is heavily colonized with *E coli* and thousands of other organisms, it was believed that the pr administration of HKE-MP123 could provide an acceptable alternative. We recently compared the effects of pr administration of HKE-MP123 with a mixture of the 3 purified modified PN allergenic proteins (MP-123) and found that HKE-MP123, but not MP-123, suppressed PN-allergic reactions and did not induce local inflammatory reactions (Li et al, manuscript submitted for publication).

In this study, we examined the long-term protective effect of pr HKE-MP123 in the treatment of PN-allergic mice. PN-allergic C3H/HeJ mice received various doses of HKE-MP123 or vehicle alone (sham) and then were challenged on several occasions over a prolonged period. The medium and high doses of HKE-MP123 were found to provide the best protection. This novel immunotherapeutic approach seems to provide long-lasting protection and a broad immunoregulatory effect, which might benefit PN-allergic patients.

METHODS

Mice and reagents

Five-week-old female C3H/HeJ mice were purchased from the Jackson Laboratory (Bar Harbor, Me) and maintained on PN-free chow under specific pathogen-free conditions. Standard guidelines for the care and use of animals were followed.¹²

Freshly ground, whole-roasted PN and crude PN extract were prepared as previously described¹³ and used as antigens. Cholera toxin (CT) was purchased from List Biological Laboratories, Inc (Campbell, Calif). Modified recombinant Ara h1, 2, and 3 were prepared as previously reported.¹⁴ Concanavalin A and albumin-dinitrophenol (DNP-albumin) were purchased from Sigma (St Louis, Mo). Antibodies for ELISAs were purchased from the Binding Site Inc (San Diego, Calif) (sheep anti-mouse IgE, and biotinylated donkey anti-sheep IgG). Anti-DNP IgE and IgG2a were purchased from Accurate Scientific Inc (Westbury, NY).

Preparation of HKE-MP123

To prepare HKE-MP123, *E coli* clones BL21 (DE3) expressing mAra h1, mAra h2, and mAra h3, or carrying the pET24 (a)+ vector alone, were generated individually as previously described.¹⁵ One hundred milliliters of medium containing 30 μ g/mL of kanamycin was inoculated with a volume of the overnight bacterial culture containing approximately 2 optical units at 600 nm. The bacterial culture was grown at 37°C, ~240 rpm, for $\geq$ 2.5 hours until the optical density reached 0.6 optical units at 600 nm. Protein expression was induced by adding isopropyl β -D-1-thiogalactopyranoside at 1 mmol/L, and the bacteria were incubated for an additional 14 to 16 hours. The bacteria were inactivated by incubation in a water bath at 65°C for 30 minutes in aliquots of 50 mL or less, cooled on ice for ~20 minutes, and then collected by centrifugation. The bacteria were washed and resuspended in an appropriate volume of ice-cold PBS. The final HKE mixture containing approximately equal amounts of the 3 modified PN proteins was prepared. An equal amount of mAra-containing bacteria was used to produce HKE-MP123 (eg, high dose – 30 μ g each of mAra h1, 2, and 3 = MP-123, total 90 μ g protein), as measured by optical units at 600 nm and was used to prepare the modified PN protein mixtures and the HKE-carrying vector alone. The specific protein expression level was determined by comparing immunoblot analyses of the HKE-MP123 with serial dilutions of known quantities of bacterial cell lysates. The effectiveness of the heat-inactivation procedure was determined by plating bacterial aliquots taken from the induced culture and the final HKE suspensions on an LB-agar plate. Relative to the original induced bacterial cultures, less than 0.02% viable cells were detected in the final heat-treated bacterial suspensions. The integrity of the heat-treated bacteria was verified by analyzing the specific protein content in both the bacterial cell pellet and in the culture supernatant obtained by centrifugation (4000g). An immunoblot of the aliquot separated on an SDS polyacrylamide gel was prepared and was probed with either human polyclonal serum IgG antibodies (for modified Ara h1 and

Ara h3), or with an anti-modified Ara h2 mouse polyclonal IgG antibody. More than 99% of the specific recombinant protein was shown to be present in the bacterial cell pellet fraction.

Ig antigen sensitization, challenge, and HKE-mAra h1 to h3 treatment

Mice were sensitized with PN and CT as previously described.¹¹ As depicted in Fig 1, treatment began at week 10 after the initial PN sensitization. Six groups (n = 12/group) were involved: Group 1 (sham) received the methylcellulose vehicle; group 2 received HKE-MP123, 0.9 μ g (low dose); group 3 received HKE-MP123, 9 μ g (medium dose); and group 4 received HKE-MP123, 90 μ g (high dose); group 5 received HKE-V (vector alone); and group 6 was neither sensitized nor treated (naive). Treatments with HKE were administered in 90 μ L of methylcellulose as vehicle pr 3 times at weekly intervals. Mice were challenged ig 2, 6, and 10 weeks after therapy (weeks 14, 18, and 22 after initial sensitization). After each challenge, 4 mice were killed to collect samples for immunologic studies.

Assessment of hypersensitivity reactions

As shown in Fig 2, PN-sensitized mice given ig whole PN experienced anaphylactic reactions. Anaphylactic symptoms were evaluated 30 to 40 minutes after the second challenge dose using a scoring system as previously described¹³: 0 = no symptoms; 1 = scratching and rubbing around the snout and head; 2 = puffiness around the eyes and snout, diarrhea, pilar erecti, reduced activity, and/or decreased activity with increased respiratory rate; 3 = wheezing, labored respiration, cyanosis around the mouth and the tail; 4 = no activity after prodding, or tremor and convulsion; 5 = death. Scoring of symptoms was performed in a blinded manner.

Measurement of plasma histamine levels

Plasma histamine levels were obtained 30 minutes after the second ig challenge dose and analyzed using an enzyme immunoassay kit (ImmunoTECH Inc., Marseille, France) as described by the manufacturer.¹⁶

Measurement of serum PN-specific IgE and IgG2a levels

Venous blood samples were obtained from the tail vein throughout sensitization/boosting, 1 day before treatment, during treatments, and 1 day before challenge. Sera were collected and stored at -80°C until analyzed. Levels of PN-specific IgE and IgG2a levels were determined as previously described.^{13,17} Plates were coated with crude PN extract, incubated overnight at 47°C , and then blocked and washed. Serum samples (1:10 dilutions) were added to the plates, incubated overnight at 4°C , and then the plates were washed thoroughly. To determine IgE levels, sheep anti-mouse IgE (0.5 μ g/mL) was added to the wells, incubated for 1 hour, and the plates were again washed thoroughly. Finally, biotinylated donkey anti-sheep IgG (0.5 μ g/mL) was added to the wells and incubated at room temperature for 1 hour. After thorough washing, avidin-peroxidase was added for an additional 15 minutes at room temperature. For IgG2a measurements, biotinylated rat anti-mouse IgG2a monoclonal antibodies (0.25 μ g/mL) were used as the detection antibodies. Subsequent steps were the same as those in IgE measurement. Equivalent concentrations of PN-specific IgE and IgG2a were calculated by comparison with a reference curve generated with anti-DNP IgE and IgG2a mouse monoclonal antibodies, as described previously.^{13,17} Serum PN-specific IgE values were calculated by comparison with a reference curve generated with anti-DNP IgE mouse monoclonal antibodies, as described previously.^{13,17}

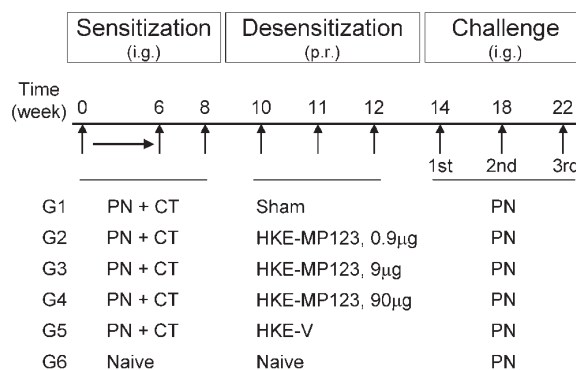


FIG 1. Experimental protocol. C3H/HeJ mice were sensitized intragastrically with freshly ground, whole roasted peanut together with cholera toxin over an 8-week period. Treatment was initiated at week 10. Six groups (n = 12/group) were involved: Group 1 (sham) received methylcellulose vehicle only; group 2 received HKE-MP123 0.9 μ g (low dose); group 3 received HKE-MP123, 9 μ g (medium dose); group 4 received HKE-MP123, 90 μ g (high dose); group 5 received HKE-vector; and group 6 was naive. The treatments were administered rectally in methylcellulose as vehicle 3 times at weekly intervals. Mice were challenged 2, 6, and 10 weeks after the termination of therapy (week 14, 18, and 22, respectively, after desensitization). After each challenge, 4 mice were killed for collection of blood and tissue samples.

Cell culture and cytokine measurements

Immediately after evaluating anaphylactic reactions, 4 mice from each group were killed and splenocytes (SPCs) were isolated and cultured in RPMI 1640 containing 10% FBS, 1% penicillin/streptomycin, and 1% glutamine. SPCs were cultured in 24 well plates (4×10^6 /well/mL) in the presence or absence of crude PN extract (50 μ g/mL) or concanavalin A (2 μ g/mL). Supernatants were collected after 72 hours of culture, and aliquots were stored at -80°C until analyzed. IFN- γ , IL-4, IL-5, IL-13, IL-10, and TGF- β levels were determined by ELISA according to the manufacturer's instructions (R&D systems, Minneapolis, Minn, for IL-13, PharMingen, San Diego, Calif, for all others).

Statistical analysis

Data were analyzed using SigmaStat statistical software package (SPSS Inc, Chicago, Ill). For histamine, IgE, and cytokine levels, the differences between the groups were analyzed by 1-way ANOVA followed by Bonferroni's *t* test for all pairwise comparisons, because the data passed the normality test. For symptom scores, the differences between the groups were analyzed by Kruskal-Wallis 1-way ANOVA on ranks followed by all pairwise comparison procedure (Dunn's), because the data failed to pass the normality test. *P* values < .05 were considered significant.

RESULTS

HKE-MP123 confers long-lasting protection against PN-induced anaphylaxis after oral PN rechallenge

To determine whether HKE-MP123 can provide a long-lasting effect on PNA, PN-sensitized mice were treated with 3 different weekly doses of pr administered HKE-MP123 in a methylcellulose carrier. Mice were then challenged ig with PN 2 weeks later (week 14 after

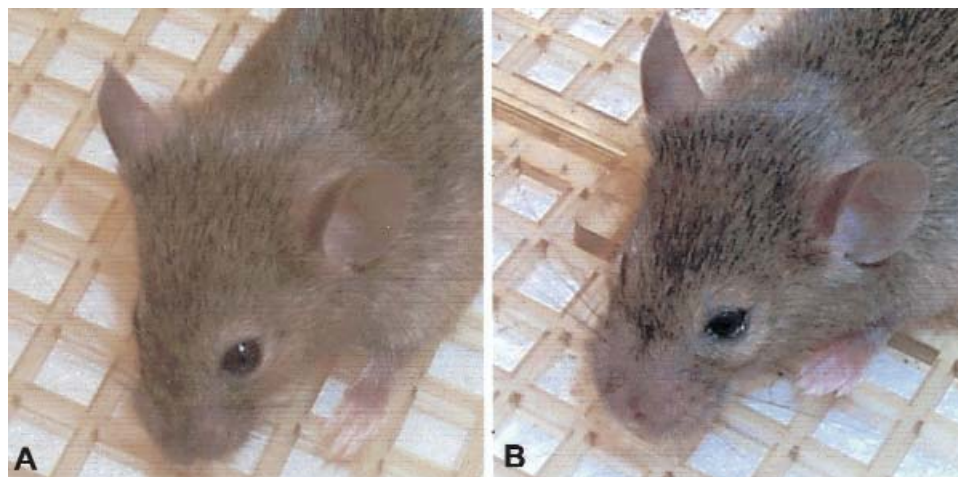


FIG 2. Mouse experiencing anaphylaxis. **A**, Normal C3H/HeJ mouse. **B**, Peanut-sensitized C3H/HeJ mouse experiencing an anaphylactic reaction after intragastric feeding of peanut; note swelling about the eyes and snout, pilar erecti, and cyanosis of the ears and feet.

the initial sensitization) and again 4 and 8 weeks later (weeks 18 and 22, respectively, after the initial sensitization). Anaphylactic symptom scores were evaluated 30 minutes after challenge. After the first challenge, all 3 HKE-MP123-treated groups exhibited significantly lower anaphylactic symptom scores compared with the sham-treated group (low-, medium-, and high-dose HKE-MP123-treated groups versus sham: $P < .01$, $.01$, and $.01$, respectively, Fig 3, A). No dose-response difference was observed among the HKE-MP-123-treated groups at the time of the first challenge. Surprisingly, HKE-V also significantly reduced symptom scores compared with the sham-treated group ($P < .05$), although the symptom scores tended to be greater in this group compared with the HKE-MP-123 treated groups. After the second challenge (week 18), anaphylactic symptom scores were reduced significantly only in the medium-dose and high-dose HKE-MP-123-treated groups ($P < .05$ and $P < .01$, respectively, Fig 3, B). Similarly, at the third challenge (week 22), only mice receiving the medium and high doses of HKE-MP-123 were protected from anaphylactic reactions ($P < .01$, Fig 3, C). These results demonstrate that higher doses of HKE-MP123 in a methylcellulose carrier resulted in persistent protection lasting at least 10 weeks.

HKE-MP123 has a long-lasting inhibitory effect on PN-induced histamine release

Because histamine is associated with the anaphylactic reactions, we also measured plasma histamine levels 30 minutes after PN challenge. We found that after the first challenge at week 14, plasma histamine levels were significantly reduced in all 3 HKE-MP-123-treated groups compared with the sham-treated group ($P < .01$, Fig 4, A). Interestingly, reduction of plasma histamine levels in the HKE-V-treated group failed to reach statistical significance. After the second challenge, only the high-dose HKE-MP123-treated group had significantly lower plas-

ma histamine compared with the sham-treated group ($P < .01$, Fig 4, B). After the third challenge, histamine levels were significantly lower in both the medium-dose and high-dose-HKE-MP123-treated groups compared with sham-treated group ($P < .01$, Fig 4, C). Mice treated with a low dose of HKE-MP-123 and HKE-V did not show a significant reduction in plasma histamine levels compared with the untreated group after the second and the third challenge (Fig 4). These results parallel the clinical findings in that HKE-MP123 (at medium and high doses) has a long-lasting suppressive effect on histamine release, which lasted for at least 10 weeks

HKE-MP123 has a long-lasting inhibitory effect on PN-specific IgE production

PN-specific IgE levels were monitored during sensitization/boosting, desensitization, and after treatment. IgE levels increased markedly over the 8-week sensitization/boosting in each group of mice after PN sensitization and were similar among the groups before treatment at week 10. After treatment, IgE levels were significantly reduced in all HKE-MP123-treated groups at the first, second, and the third challenge ($P < .001$, Fig 5, A), being lowest in the high-dose treatment group. IgE levels were also reduced in the HKE-V-treated group at the time of the third challenge ($P < .05$) but were significantly greater than in the high-dose HKE-MP123-treated group ($P < .05$). IgG2a levels were significantly increased in the HKE-MP123 medium and high dose-treated groups at the first ($P < .01$ and $P < .001$ respectively), the second ($P < .05$ and $P < .01$ respectively), and the third challenges ($P < .05$ and $.001$ respectively) compared with the sham-treated group (Fig 5, B). IgG2a levels in the low-dose HKE-MP123-treated group were also significantly greater than in the sham-treated group at the time of the first and second challenge, but not at the third challenge. IgG2a levels in the HKE-V-treated group were not significantly different than those of the

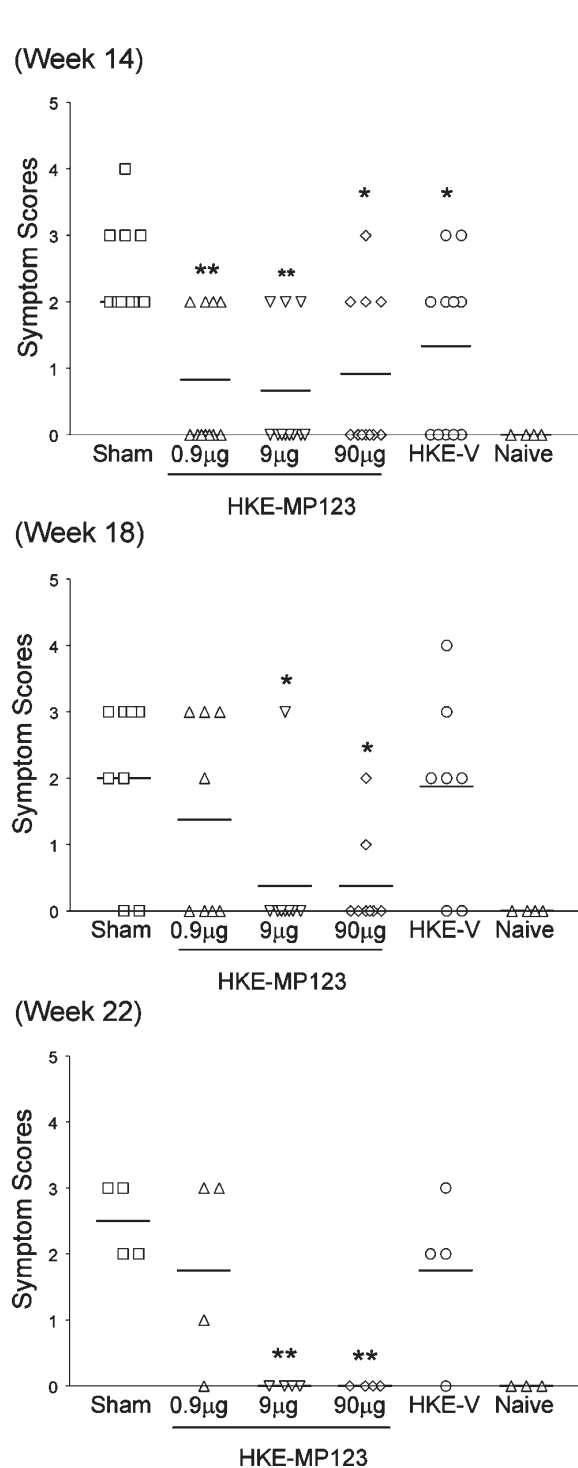


FIG 3. Persistent protection against peanut-induced anaphylactic reactions by HKE-MP123. Mice were challenged at weeks 2 (A), 6 (B), and 10 (C) after the last HKE-MP123 treatment. Anaphylactic symptom scores were determined 30 minutes after challenge. Each point indicates an individual mouse. Bars indicate the median of 12 mice (A), 8 mice (B), and 4 mice (C) in each group. * $P < .05$, and ** $P < .01$ vs sham.

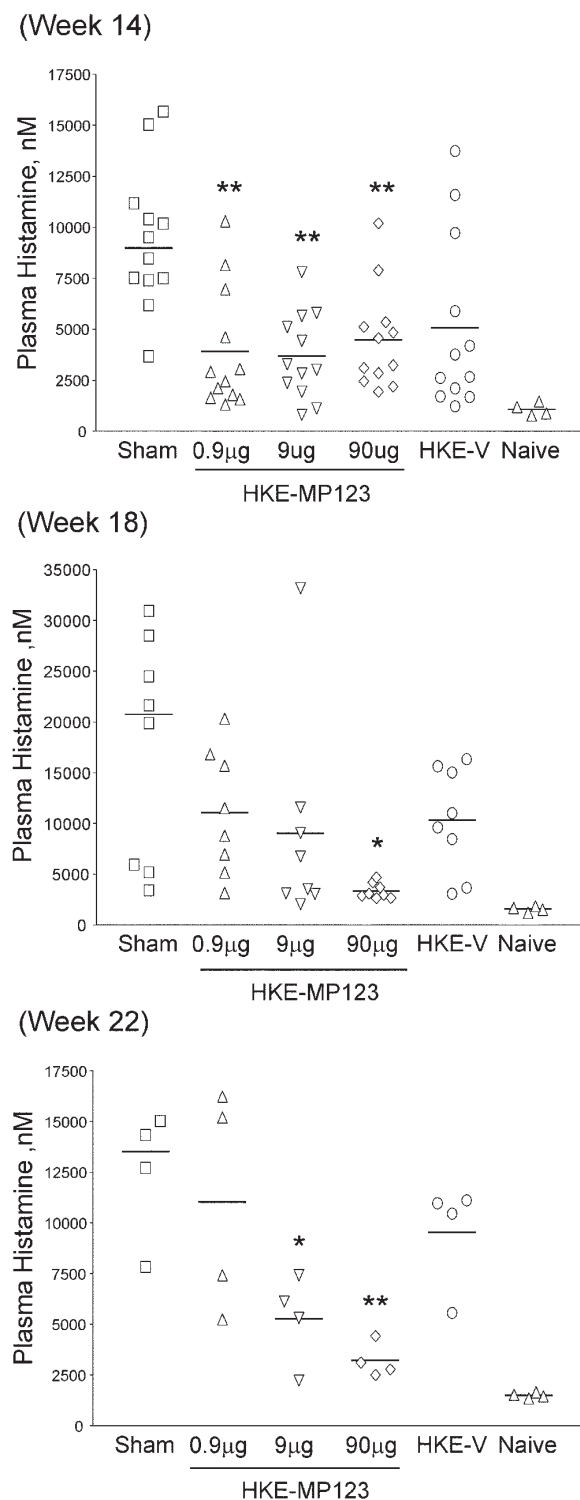


FIG 4. Effect of therapy on plasma histamine levels. Thirty to 40 minutes after challenge, blood was collected and plasma was obtained. Histamine levels were measured by use of an enzyme immunoassay kit. Data are means $\pm$ SEM for each group of 12 mice (A), 8 mice (B), and 4 mice (C) in each group. * $P < .05$, and ** $P < .01$ vs sham.

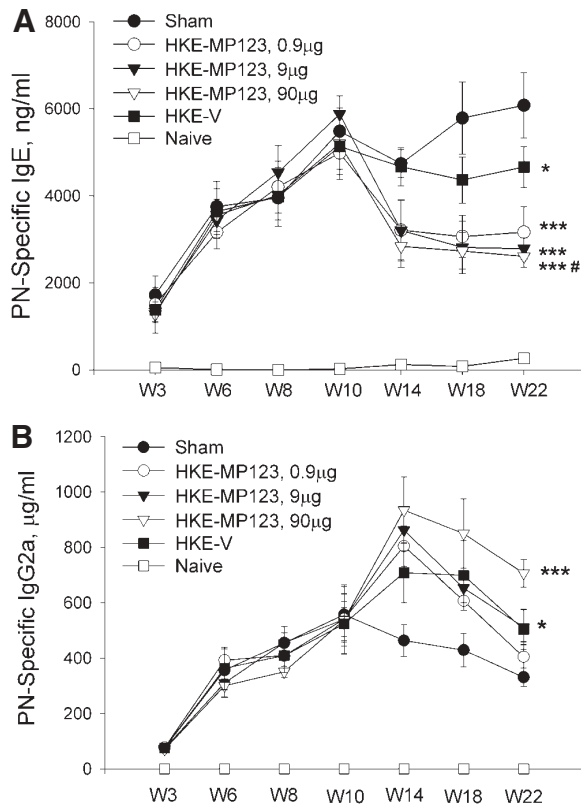


FIG 5. Effect of therapy on peanut-specific IgE levels. Sera from all groups of mice were obtained during sensitization (3, 6, and 8 weeks), before treatment, and 1 day before each challenge (weeks 14, 18, and 22). Peanut-specific IgE (**A**) and IgG2a (**B**) levels were determined by ELISA. Data are mean $\pm$ SEM for each group as in Fig 2. * $P < .05$ vs sham; *** $P < .001$ vs sham.

sham-treated group at the time of the first and the second challenge. These results indicate that HKE-MP123 suppresses IgE and increases IgG2a production. This effect lasted at least 10 weeks after discontinuing therapy, and the high doses of HKE-MP123 seemed to be the most effective.

HKE-MP123 modulation of T_H1 , T_H2 , and T-regulatory cytokines

To determine whether the HKE-MP123-mediated long-lasting protection against PNA was associated with altered SPC cytokine profiles, we analyzed cytokine levels in SPC culture supernatants from each group of mice after the last challenge. IL-4 levels were significantly lower in the low-, medium-, and high-dose HKE-MP123-treated and HKE-V-treated groups compared with the sham-treated group ($P < .01$, $.05$, $.01$, and $.05$, respectively; Fig 6, A). IL-13 levels also were significantly decreased in the medium-dose and high-dose HKE-MP123 groups and the HKE-V group ($P < .01$, Fig 6, B). However, significant reduction of IL-5 levels was only seen in the medium and high HKE-MP123-treated groups ($P < .001$, Fig 6, C). IFN- γ levels were increased in all HKE-MP123-treated and HKE-V-treated groups

($P < .01$, $.001$, $.001$, and $.001$, respectively; Figs 5 and 6). IL-10 is a classic T_H2 cytokine believed to be involved in the induction of oral tolerance¹⁸ and the downregulation of the allergic response.¹⁹ TGF- β is also believed to be important in the development of oral tolerance to food allergens.²⁰ We found that IL-10 was reduced in all HKE-MP123-treated and HKE-V-treated groups compared with the sham-treated group, being lowest in the HKE-MP123 high-dose-treated group ($P < .01$, Fig 6, E). In contrast, TGF- β levels were significantly increased only in the HKE-MP123-treated groups in a dose-dependent manner ($P < .05$, $P < .01$, and $P < .01$; Fig 6, F). These results demonstrate that HKE-MP123 has a broad immunoregulatory effect on T_H1 , T_H2 , and T-regulatory cytokines, which might contribute to its beneficial effect on PNA.

DISCUSSION

PNA is a life-threatening food allergy for which there is no satisfactory treatment. Monthly injections of humanized recombinant anti-IgE antibodies, which reduces mast cell and basophil-bound IgE, seem to be effective in reducing allergic responses in PN-sensitive subjects, at least to small amounts of PN protein.²¹ However, this treatment cannot "cure" food allergy, and continuous monthly injections are necessary to maintain protection. Consequently, additional therapeutic approaches for the treatment of food allergy are needed.

In the past several years, we have focused on developing novel immunotherapeutic approaches for PNA. In 1 approach, we have evaluated the efficacy of "engineered" recombinant protein-based immunotherapy in our murine model of PN-induced anaphylaxis.^{11,22} The IgE-binding sites on 3 major PN allergens, Ara h1 to h3, were identified and altered by a single amino acid, MP-123. This change led to marked abrogation or elimination of IgE binding without significantly affecting the T-cell activating capacity of these "engineered" proteins. Although intranasal or sc administration of the engineered proteins partially desensitized PN-allergic mice, leading to a marked decrease in anaphylactic symptoms after PN challenge, the addition of bacterial products (eg, *L monocytogenes* or *E coli*) induced more substantial protection. Recently, we found that administering HKE expressed the engineered proteins, HKE-MP123 significantly reduced anaphylactic reactions, and pr administration seemed to be safe and highly efficacious in the murine model of PNA (Li et al manuscript, submitted for publication). In this study, we have demonstrated that 3 pr treatments with HKE-MP123 at medium (9 μ g) or high doses (90 μ g) provided PN-allergic mice with significant protection from anaphylaxis for at least 10 weeks after the discontinuation of therapy. Low-dose (0.9 μ g) HKE-MP123 and HKE-V alone induced temporary protection (ie, protection against the first challenge) but not subsequent challenges. These results demonstrate that the pr administration of HKE-producing engineered PN proteins might be efficacious for treating PNA.

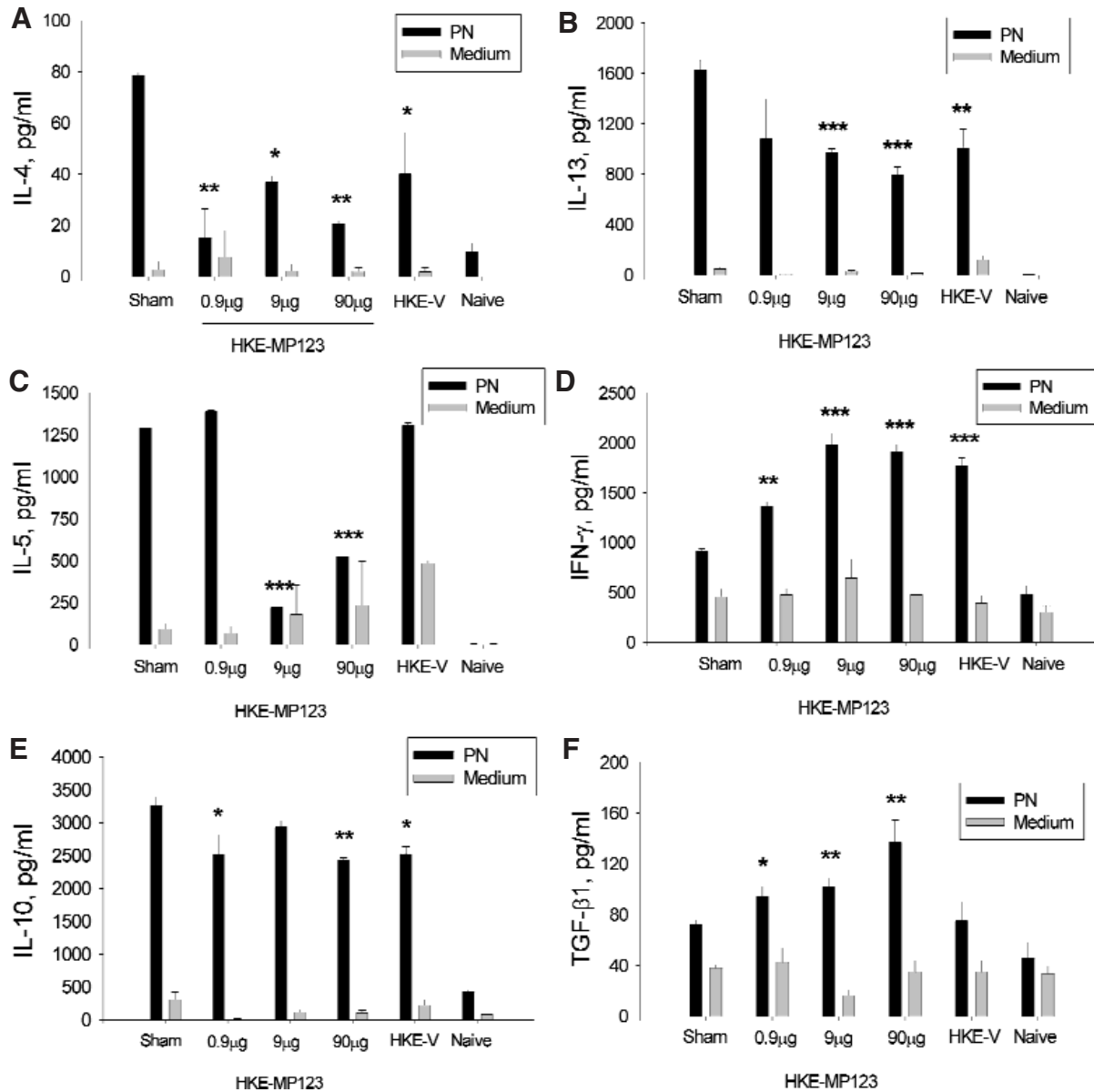


FIG 6. Effect of therapy on cytokine production. Cell suspensions were cultured in complete culture medium in the presence of crude peanut extract, medium alone, or concanavalin A (data not shown). Supernatants were collected 72 hours later, and cytokine levels were determined by ELISA. Results are expressed as mean $\pm$ SEM of 2 duplicate cultures of 4 mice. * $P < .05$; ** $P < .01$; *** $P < .001$ vs sham.

In addition to suppressing clinical symptoms, we found that HKE-MP123 produced the long-lasting suppression of histamine release after PN challenge and a decrease in PN-specific IgE levels. Interestingly, PN-specific IgE levels were also reduced in the HKE-V-treated group but were significantly greater than that seen in the high-dose HKE-MP123-treated group. This might have been due to the effect of CpG motifs in the plasmid vector. IgE levels were not significantly different in mice treated with the different doses of HKE-MP123, suggesting that the reduction in IgE is not solely responsible for the long-lasting protection mediated by HKE-MP123. IgG2a levels

were significantly increased for at least 10 weeks in the medium-dose and high-dose HKE-MP123-treated groups and were associated with the long-lasting protection in these 2 groups. IgG2a, a T_H1 -driven antibody²³⁻²⁶ generally considered to be a "blocking antibody,"²⁷ was enhanced by HKE-MP123 treatment and might have been at least in part responsible for the long-lasting beneficial effect of immunotherapy in this model.

Numerous studies have demonstrated that T_H2 cytokines play a central role in the pathogenesis of allergic disorders, including food allergy. IL-4 and IL-13 promote B cell switching to IgE production and mast cell

activation, whereas IL-5 has been shown to have a potentially autocrine effect on mast cells, in addition to its recognized paracrine effects on eosinophils.^{28,29} IFN- γ , on the other hand, inhibits T_H2 cell activation and mast cell/basophil mediator release on re-exposure to antigen.^{30,31} Schade et al³² recently demonstrated that T-cell clones generated from infants with cow milk allergy produced high levels of IL-4, IL-5, and IL-13 and low levels of IFN- γ , whereas T-cell clones produced from infants without cow milk allergy had high levels of IFN- γ and low levels of IL-4, IL-5, and IL-13. In addition, decreased IFN- γ was correlated with increased IgE levels in PN-allergic patients, and T_H2 clones have been generated from patients with PNA.^{33,34} Allergen-based immunotherapies are believed to re-establish immunologic tolerance to allergen by redirecting T-cell immune responses from a T_H2-type to a T_H1-type response.³⁵ In this study, we found that 10 weeks after therapy, SPCs from mice treated with the higher doses of HKE-MP123 induced significant reductions of T_H2 cytokines and increases in IFN- γ , suggesting a shift from T_H2 responses to T_H1 responses. The low-dose HKE-MP123-treated group and the HKE-V-treated group both showed induction of IFN- γ and selective suppression of IL-4 and/or IL-13 but no effect on IL-5 production. These results suggest that higher doses of HKE-MP123 are more effective in regulating T_H1 and T_H2 responses, which might be associated with the long-lasting therapeutic effect on PNA.

Although the counterregulatory effect between T_H1 and T_H2 responses remains an important paradigm, an appreciation of the regulatory role of TGF- β , a T_H3 cytokine, and IL-10, defined as a T-suppressor cytokine, has developed for both T_H1-mediated autoimmune and T_H2-mediated allergic responses.^{18,19,36-38} Colostrum TGF- β concentrations were found to be lower in samples from mothers of infants with IgE-mediated cow milk allergy than in samples from mothers of infants with non-IgE mediated cow milk allergy.³⁹ A recent study found that IL-10 was essential in parasite infection-mediated protection against PNA in a murine model.⁴⁰ However, any relationship between TGF- β , IL-10, and allergen immuno-therapy-mediated regeneration of oral tolerance to food antigen has not been demonstrated. In this study, we found that TGF- β levels were significantly increased in all HKE-MP123-treated groups but not the HKE-V-treated group and seemed to be dose dependent. These results suggest that the induction of TGF- β might also be important for the long-lasting therapeutic benefit of HKE-MP123 on PNA in this model. In addition, we found that IL-10 levels were reduced in all 3 HKE-MP123-treated and HKE-V-treated groups compared with the sham-treated group, being lowest in the high-dose HKE-MP123-treated group. These results suggest that IL-10 might play a less significant role in the HKE-MP123-mediated protective effect on PNA. We and others recently found that increased IL-10 production seemed to be associated with induction of PNA in this model.^{40,41} In a study that used the coadministration of HKLM and ovalbumin in ovalbumin-sensitized mice, the suppression of IL-4 and increase in IFN- γ production was associated with a

reduction of IL-10.¹⁰ The key cytokine(s) and cellular mechanisms responsible for the long-lasting protection against PN anaphylaxis induced by HKE-MP123 in this study are unknown. Further work is needed to determine whether IFN- γ and/or TGF- β are the key cytokines in re-establishing the long-lasting oral tolerance mediated by HKE-MP123, to define the effector T cells, and to determine which antigen presenting cells are involved in initiating the immunoregulatory responses.

Of interest, the HKE-V treatment also induced statistically significant protection (although less than HKE-MP123) at the first challenge, which might be due to vector CpGs within the *E coli*, resulting in switching the T_H2 response to a T_H1 response. However, the HKE-V effect on PN allergy is unlikely attributable to the vector alone, because mock DNA (plasmid DNA alone) had essentially no effect on allergy in our previous study.⁴²

In conclusion, we have demonstrated that the administration of high-dose HKE-MP123 has a potent and persistent therapeutic effect on PNA in this model of PN hypersensitivity. Protection lasted for at least 10 weeks and was accompanied by persistent reduction of PN-specific IgE and plasma histamine levels after challenges. The precise mechanisms associated with this long-lasting protection are not fully understood, but our study suggests that the protective effect is likely related to the downregulation of T_H2 cytokines, perhaps resulting from upregulation of IFN- γ and TGF- β . These studies suggest that in the future, patients who are allergic to PN might be successfully "desensitized" by use of a suppository-like form of HKE-MP123.

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