Engineered Recombinant Peanut Protein and Heat-Killed *Listeria monocytogenes* Coadministration Protects Against Peanut-Induced Anaphylaxis in a Murine Model

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Engineered Recombinant Peanut Protein and Heat-Killed 
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Peanut-Induced Anaphylaxis in a Murine Model

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Peanut allergy (PNA) is the major cause of fatal and near-fatal anaphylactic reactions to foods. Traditional immunotherapy using peanut (PN) protein is not an option for PNA therapy because of the high incidence of adverse reactions. We investigated the effects of s.c. injections of engineered (modified) recombinant PN proteins and heat-killed Listeria monocytogenes (HKLM) as an adjuvant on anaphylactic reactions in a mouse model of PN allergy. PN-allergic C3H/HeJ mice were treated s.c. with a mixture of the three major PN allergens and HKLM (modified (m)Ara h 1–3 plus HKLM). The effects on anaphylactic reactions following PN challenge and the association with Ab levels and cytokine profiles were determined. Although all mice in the sham-treated groups exhibited anaphylactic symptoms with a median symptom score of 3, only 31% of mice in the mAra h 1–3 plus HKLM group developed mild anaphylaxis, with a low median symptom score of 0.5. Alterations in core body temperature, bronchial constriction, plasma histamine, and PN-specific IgE levels were all significantly reduced. This protective effect was markedly more potent than in the mAra h 1–3 protein alone-treated group. HKLM alone did not have any protective effect. Reduced IL-5 and IL-13, and increased IFN-γ levels were observed only in splenocytes cultures from mAra h 1–3 plus HKLM-treated mice. These results show that immunotherapy with modified PN proteins and HKLM is effective for treating PN allergy in this model, and may be a potential approach for treating PNA. The Journal of Immunology, 2003, 170: 3289–3295.

Food allergy, which affects ~6–8% of children younger than 4 years of age and ~2% of the U.S. population, is now the leading single cause of anaphylactic reactions treated in hospital emergency departments in the United States (1). Allergy to peanuts (PN) accounts for the majority of fatal and near-fatal anaphylactic reactions to food (2), and the incidence appears to be increasing. At the present time, there is no satisfactory treatment for PN or other food allergies. Because traditional immunotherapy is not an option for patients with PN allergy (PNA) due to the high incidence of adverse reactions and low rate of maintenance of tolerance (3, 4), the only way to manage PNA is by strict dietary avoidance. However, because PN is a hidden ingredient in a number of processed foods, accidental ingestions are common (5, 6). One study reported that up to 55% of PN-allergic children experienced reactions following accidental ingestion within a 5.4-year period (7). In view of these characteristics and because most PN-allergic reactions occur following inadvertent ingestion, PNA has a severe negative effect on the quality of life of children and their families (8).

Ara h 1 and Ara h 2, the two major PN allergens, are recognized by 95% of PN-allergic patients (9, 10), and Ara h 3, the minor PN allergen, is recognized by 40% (11). Recently, engineered (modified) recombinant proteins with altered primary amino acid sequences of IgE-binding epitopes on Ara h 1, 2, and 3 (modified (m)Ara h 1–3) have been developed (12–14). These modified proteins bind minimal IgE Abs from PN-allergic patients although they do promote T cell proliferation comparable to that of native PN proteins (13, 14).

Re-establishment of immunologic tolerance is believed to be induced by redirecting T cell immune responses from a Th2 to a Th1 type response (15). Thus, these modified proteins may be favorable for PN allergen immunotherapy, which may reduce or eliminate the risk of anaphylaxis while retaining T cell immunoregulatory properties. We recently found that intranasal immunization with mAra h 2, but not wild-type Ara h 2, reduced PN hypersensitivity reactions (4). However, desensitization required frequent administration (three times weekly for 4 wk). s.c. administration, the standard route for immunotherapy, produced no significant protection (X.-M. Li, K. Srivastava, and H. A. Sampson, unpublished data).

Heat-killed Listeria monocytogenes (HKLM) has been used as an adjuvant in immunotherapeutic desensitization of mice. For example, Yeung et al. (16) showed that coadministration of HKLM and keyhole limpet hemocyanin (KLH) to KLH-sensitized mice reduced Th2 cytokines and IgE production, and increased IFN-γ production. Hansen et al. (17) showed that coadministration of

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Abbreviations used in this paper: PN, peanut; PNA, PN allergy; m, modified; HKLM, heat-killed Listeria monocytogenes; KLH, keyhole limpet hemocyanin; PEP, peak expiratory flow; CPE, crude PN extract; CT, cholera toxin; i.g., intragastric.
HKLM and OVA prevented and reversed established airway hyperreactivity in a murine model of allergic asthma. The finding that HKLM is a strong Th1 adjuvant suggested that HKLM might enhance the efficacy of modified PN protein-based immunotherapy.

In this study, we investigated the effect of a novel immunotherapy using modified PN proteins (mAra h 1, 2, and 3) together with HKLM as an adjuvant (mAra h 1–3 plus HKLM) on a murine model of PN hypersensitivity. This model immunologically and physiologically mimics human PN allergy (18). We found that, following PN challenge, all mice in sham and HKLM alone-treated groups developed anaphylactic reactions (median symptom scores of 3 and 2.7, respectively), whereas only 31% of mice in the mAra h 1–3 plus HKLM-treated group developed anaphylactic symptoms (median score of 0.5). Changes in core body temperatures and peak expiratory flows (PEF) were markedly reduced in the mAra h 1–3 plus HKLM-treated group as compared with the sham-treated group. IgE levels were significantly reduced, and IgG2a levels were significantly increased in the mAra h 1–3 plus HKLM-treated group as compared with the sham-treated group. These results suggest that novel immunotherapy with modified PN protein and HKLM as an adjuvant may be a potential approach for treating PN hypersensitivity.

Materials and Methods

Mice and reagents

Five-week-old female C3H/HeJ mice purchased from The Jackson Laboratory (Bar Harbor, ME) were maintained on PN-free chow, under specific pathogen-free conditions. Standard guidelines for the care and use of animals were followed (19).

Freshly ground whole PN, and crude PN extract (CPE) were prepared as previously described (18) and used as Ags. Cholera toxin (CT) was purchased from List Biological Laboratories (Cambell, CA). HKLM was prepared as described previously (16, 17). Engineered Ara h 1, 2, and 3 were prepared as previously reported (14). Con A and DNP-albumin were purchased from Sigma-Aldrich (St. Louis, MO). Abs for ELISAs were purchased from the Binding Site (San Diego, CA) (sheep anti-mouse IgE and biotinylated donkey anti-sheep IgG) and BD PharMingen (San Diego, CA) (sheep anti-mouse IgE–conjugated biotin). Abs for IFN-γ, IL-4, IL-5, and IL-13 production were obtained from Ratigen (San Diego, CA) (biotinylated anti-mouse IgG2a). Anti-DNP IgE and IgG2a were purchased from Accurate Scientific (Westbury, NY).

Intragastric (i.g.) Ag sensitization and challenge, and HKLM–mAra h 1–3 treatment

PN sensitization and challenge followed the previously described protocol (18) with minor modification. Mice were deprived of food for 2 h and given 300 µl of 1.5% NaHCO3 30 min before feeding to neutralize stomach acid. Sensitization was then performed by i.g. administration of 10 mg of freshly ground whole PN together with 20 µg of CT on day 0, then boosted weekly for 6 wk and again at week 8 (Fig. 1). Treatment was initiated at week 10. Groups 1–4 received the following: 1) PBS (sham); 2), HKLM only (1 × 108); 3), a mixture of HKLM (1 × 108) and the modified recombinant three major PN allergens proteins three times at weekly intervals; and 4), mAra h 1–3 alone (30 µg each, mAra h 1–3) three times per week for 4 wk. Group 5 (naïve) served as normal control. All groups were challenged at week 14.

Assessment of hypersensitivity reactions

Anaphylactic symptoms were evaluated 30–40 min after the second challenge dose using a scoring system from a previous report (18): 0, no symptoms; 1, scratching and rubbing around the nose and head; 2, puffiness around the eyes and mouth, diarrhea, pilo 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; and 5, death. Scoring of symptoms was performed in a blinded manner.

Measurement of immediate airway reactions and core body temperatures

Immediate airway responsiveness was monitored in conscious unrestrained mice using an unrestrained plethysmograph chamber (Kent Scientific, Litchfield, CT). Immediately after evaluation of symptom scores following i.g. challenge, mice were placed in the unrestrained plethysmograph chamber without any direct restraints such as neck seals that cause stress. Bias airflow was passed through the chamber and allowed to exit through a direct airflow sensor. Signals were viewed and parameters were derived with a Kent RSP001 respiratory data acquisition system (Kent Scientific). PEF (milliliters per minute) was used as a parameter to determine the severity of bronchial constriction.

Fifteen to 20 min following challenge, temperatures were measured using a rectally inserted thermal probe (Harvard Apparatus, Holliston, MA).

Measurement of plasma histamine levels

Plasma histamine levels in blood samples collected 30 min after the second i.g. challenge dose were determined using an enzyme immunoassay kit (Immunotech, Marseilles, France) as described by the manufacturer (22).

Measurement of serum PN-specific IgE and IgG2a levels

Tail vein blood was obtained during sensitization/boosting, before treatment, during treatments, and 1 day before challenge. Sera were collected and stored at −80°C. Levels of PN-specific IgE (18) and IgG2a were measured by ELISA as described previously (23). Brieﬂy, plates were coated with CPE incubated overnight at 4°C, and then blocked and washed. Samples (1/10 dilutions for IgE; 1/500 dilution for IgG2a) were added to the plates and incubated overnight at 4°C. For IgE measurement, plates were washed and sheep anti-mouse IgE was added and incubated for 1 h. After washing, biotinylated donkey anti-sheep IgG was added for 1 h. After washings, avidin peroxidase was added for an additional 15 min at room temperature. The reactions were developed with ABTS (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and read at 405 nm. For IgG2a measurement, biotinylated rat anti-mouse IgG2a mAbs (0.25 g/ml) were used as the detection Abs. Subsequent steps were the same as those used 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 mAbs, as described previously (18, 23).

Cytokine measurements

Splenocytes were isolated from pooled spleens removed from each group of mice, which had been sacrificed immediately after evaluation of the
anaphylactic reactions, and cultured in RPMI 1640 containing 10% FBS, 1% penicillin/streptomycin, and 1% glutamine. Splenocytes were cultured in 24-well plates (4 × 10^6/well/ml) in the presence or absence of CPE (50 μg/ml) or Con A (2 μg/ml). Supernatants were collected after 72 h of culture and aliquots were stored at −80°C until analyzed. IFN-γ, IL-4 and IL-5, IL-13, and IL-10 levels were determined by ELISA according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN, for IL-13; BD PharMingen for all others).

Statistical analysis

Data were analyzed using SigmaStat 2.03 statistical software package (SPSS, Chicago, IL). For histamine, IgE, IgG2a, and cytokine levels, the differences between the groups were analyzed by one-way ANOVA followed by Bonferroni’s t test for all pairwise comparisons, because the data passed normality test. For symptom scores, rectal temperatures, and PEF, the difference between the groups were analyzed by Kruskal-Wallis one-way ANOVA on ranks followed by all pairwise comparison procedure (Dunn’s), as the data failed to pass the normality test. Values of p < 0.05 were considered.

Results

mAra h 1–3 plus HKLM immunization had a protective effect on PN-induced anaphylactic reactions

Anaphylactic reactions were evaluated and the severity of anaphylactic symptoms were scored 30–40 min following i.g. PN challenge. As depicted in Fig. 2A, 100% of mice in the sham-treated group exhibited symptoms; 32% of mice developed fatal or near-fatal anaphylactic reactions. In contrast, only 31% of mice in the mAra h 1–3 plus HKLM-treated group showed mild symptoms, and no fatal or near-fatal anaphylaxis was observed. Symptom scores in the mAra h 1–3 plus HKLM-treated group were significantly lower than in the sham-treated group (median score 0.5 vs 3, p < 0.01). In the mAra h 1–3 alone-treated group, 57% of mice developed anaphylactic symptoms, but there were no fatal or near-fatal reactions. Symptom scores were also significantly lower than in the sham-treated group (p < 0.05), but higher than in the mAra h 1–3 plus HKLM-treated group (median score: 1.8 vs 0.5 in mAra h 1–3 and mAra h 1–3 plus HKLM, respectively, although this difference did not reach statistical significance, p < 0.09). One hundred percent of mice in the HKLM alone-treated group developed anaphylaxis with a median score of 2.7 and were not different from the sham-treated group.

Because decreased core body temperature is inversely correlated with the severity of systemic anaphylaxis in mice (24), we also used this parameter to objectively measure the consequence of anaphylactic reactions. Rectal temperatures of the sham-treated group were significantly lower in the naive mice (Fig. 2B). Mean rectal temperatures in the mAra h 1–3 plus HKLM-treated mice were significantly higher than in the sham-treated group (p < 0.05) and not different from the naive group. Temperatures in mAra h 1–3 alone-treated groups were slightly higher than in the sham-treated group, but the difference did not reach statistical significance. Temperatures in the HKLM alone-treated group were not different from those in the sham-treated group.

Bronchial constriction is a severe consequence of systemic anaphylaxis. Therefore, we conducted PEF measurements to monitor the degree of bronchial constriction following challenge. The mAra h 1–3 plus HKLM-treated group had significantly higher PEF values than did the sham-treated group (p < 0.05, Fig. 2C). PEF values were slightly but not significantly higher in the mAra h 1–3 alone-treated group. No difference was observed between HKLM and sham-treated groups. These results demonstrate that mAra h 1–3 plus HKLM treatment protected PN-allergic mice from PN-induced anaphylactic reactions. mAra h 1–3 alone treatment, despite nine more administrations, was less effective. No effect was detected in the HKLM alone-treated group. These results show that coadministration of modified PN proteins and HKLM was required to produce significant effective protection.

mAra h 1–3 plus HKLM treatment reduced plasma histamine levels

Mast cell degranulation and histamine release are major mechanisms underlying anaphylactic reactions. We (18) and others (25) previously found that plasma histamine levels are correlated with the severity of anaphylactic reactions in this model. Therefore, we
determined the effect of mAra h 1–3 plus HKLM on plasma histamine levels 30 min after challenge. Consistent with previous findings (25, 26), plasma histamine levels were markedly elevated in the sham-treated group (Fig. 3). Plasma histamine levels in the mAra h 1–3 plus HKLM treatment group were significantly lower compared with sham-treated group (p < 0.001). Plasma histamine levels in mice treated with mAra h 1–3 alone were also significantly lower than in the sham-treated group (p < 0.01), but were significantly higher than in the mAra h 1–3 plus HKLM-treated group (p < 0.05). HKLM alone did not reduce histamine levels.

mAra h 1–3 plus HKLM treatment reduced PN-specific IgE and increased IgG2a levels

PN-induced hypersensitivity is IgE mediated in this model (18, 25). Therefore, we determined whether mAra h 1–3 plus HKLM treatment reduced PN-specific IgE by monitoring serum IgE levels following sensitization, during treatment, and at the time of challenge. As shown in Fig. 4A, 3–8 wk following the initial sensitization, PN-specific IgE levels were elevated in all sensitized groups and were not different between the groups. Two weeks after treatment (wk 12), PN-specific IgE levels were significantly lower in the mAra h 1–3 plus HKLM-treated group as compared with the sham-treated group, and remained significantly lower at the time of challenge (wk 14). However, no reduction in PN IgE was observed in either the HKLM only- or the mAra h 1–3-treated groups. IgG2a levels were not different following sensitization at the initiation of treatment, but were significantly higher in the mAra h 1–3 plus HKLM-treated group following treatment and at time of challenge. No significant increases in IgG2a levels were observed in HKLM alone- or mAra h 1–3-treated groups.

Reduction of Th2 cytokines and increase in Th1 cytokines

To determine any association between the protective effect of mAra h 1–3 plus HKLM on PN hypersensitivity and cytokine profiles, we measured IL-4, IL-5, IL-13, and IFN-γ production by splenocytes from each group of mice. Significant decreases in IL-4, IL-5, and IL-13, and increased IFN-γ were found in the mAra h 1–3 plus HKLM-treated group (Fig. 5). Although moderate reductions in IL-4 were observed in groups treated with HKLM alone and mAra h 1–3 alone, these decreases failed to reach statistical significance. No decreases in IL-5 and IL-13, and increased IFN-γ, were observed in groups treated with HKLM alone or mAra h 1–3 alone. In addition, IL-10 levels were significantly elevated in all PN-sensitized groups and were essentially the same in all treatment groups. These results suggest that the therapeutic effect of mAra h 1–3 plus HKLM on PN hypersensitivity in this model is due to alterations in IL-4, IL-5, IL-13, and IFN-γ.

Discussion

In this model of PN hypersensitivity, s.c. coadministration of modified PN allergen protein and HKLM markedly reduced PN-induced anaphylaxis. Treatment with mAra h 1–3 plus HKLM not only reduced the incidence of anaphylactic symptoms from 100 to 31%, but also reduced the severity of anaphylaxis (fatal and near-fatal consequences) from 32 to 0%. Core body temperatures and lung function parameters were also significantly improved. The incidence and the severity of anaphylaxis in mAra h 1–3 proteins alone-treated group were also reduced, but to a less degree than in the mAra h 1–3 plus HKLM-treated group. In addition, this group showed no significant improvement in body temperature or lung function measurements despite nine additional treatments. In contrast, HKLM alone treatment was without effect. These results demonstrate that a combination of modified PN proteins and HKLM is much more effective in protecting against PN anaphylaxis than modified proteins alone, and may be a potential therapeutic approach for PN hypersensitivity. Although no adverse reactions to treatment were observed in this study, further investigation of long-term efficacy and safety is required.
PNA is an IgE-mediated type I hypersensitivity in which allergen-specific IgE Abs that bind to high affinity receptors (FcεRI) on the surface of mast cells and basophils is central to the immunopathology of food allergy. In patients with food allergy, re-exposure to the relevant foods triggers degranulation of mast cells/basophils resulting in the release of histamine and other mediators, which provoke symptoms of anaphylaxis. In this model of PNA hypersensitivity, as demonstrated by ourselves (18) and others (25), PNA-specific IgE levels were markedly elevated following PNA plus CT sensitization. Anaphylactic reactions and markedly increased plasma histamine levels were provoked in PNA-allergic mice following oral PNA challenge. In the present study, reductions in IgE and histamine levels by mAra h 1–3 plus HKLM treatment coincided with marked reduction in both incidence and severity of anaphylactic reaction, suggesting that reduced IgE-mast cell activation and histamine release were responsible for the protective effect of mAra h 1–3 plus KLH. IgG2a is a Th1-driven Ab (27–30), and has been suggested to be a blocking Ab that is responsible in part for the beneficial effect of immunotherapy (31). In the present study, IgG2a levels were increased by mAra h 1–3 plus KLH treatment. Induction of PNA-specific IgG2a may also have been beneficial in this model. No significant reduction in PNA IgE levels or increase in IgG2a levels was detected in the mAra h 1–3 only-treated group; however, plasma histamine levels were somewhat reduced.

FIGURE 5. Cytokine levels in splenocyte cultures. Cell suspensions were cultured in complete culture medium in the presence of CPE, or medium alone. Supernatants were collected 72 h later, and IL-4, IL-5, IL-13, IL-10, and IFN-γ were determined by ELISA. Results are expressed as mean ± SEM of three duplicate cultures from three separate experiments as Fig. 2. *, p < 0.05; **, p < 0.01; ***, p < 0.001 vs sham.
mRNA h 1–3 direct blocking of mast cell degranulation in this model. It was observed that mRNA h 1, 2, and 3 reduced histamine release by basophils from some patients with PNA compared with wild-type Ara h 1, 2, and 3 (K. Jarvinen, K. Beyer, W. Shreffler, and H. Sampson, unpublished data).

Th2 cytokines play a central role in the pathogenesis of allergic diseases. A Th2-skewed response has also been demonstrated in food allergy patients and animal models (22, 25, 32). For example, T-cell clones generated from cow milk–allergic infants released high levels of IL-4, IL-5, and IL-13, and low levels of IFN-γ, whereas cells from infants without food allergy released high levels of IFN-γ and low levels of IL-4, IL-5, and IL-13 (32). Furthermore, decreased IFN-γ production correlated with increased IgE levels in PN-allergic patients. Th2 clones have also been generated from patients with PNA (33). In the present study, IL-4 levels in splenocyte cultures from both m Ara h 1–3- and m Ara h 1–3 plus HKLM-treated groups were reduced. This finding suggests that reduction in IL-4 was not sufficient to block PN hypersensitivity in this model. IL-10, another classical Th2 cytokine, has been recently suggested to be important in suppression of allergic inflammation (34). However, we found no difference in IL-10 production by splenocytes from m Ara h 1–3 plus HKLM-treated mice. This finding is in agreement with a previous finding that, although anti-IL-10 Ab abrogated helminth infection protection against PN hypersensitivity, anti-IL-10 did not alter PN-induced IgE synthesis and hypersensitivity reaction following oral challenge (25). Thus, IL-10 may not play a role in m Ara h 1–3 plus HKLM protection against PN allergy in this study. The finding that IL-5 and IL-13 levels were markedly reduced, and IFN-γ levels were markedly increased only by m Ara h 1–3 plus HKLM treatment, suggests that specific reduction of IL-13 and IL-5 and induction of IFN-γ may be directly linked to m Ara h 1–3 plus HKLM suppression of PN-specific IgE and histamine release and the subsequent protection against PN-induced anaphylaxis in this model. IL-13 and IL-4 share a common receptor subunit, IL-4Ra chain, and induce similar biological activities (35). IL-13 is a more potent inducer of IgE than IL-4 (36). Thus, IL-13 is an important regulator of Th2 commitment. Reduction of IL-13 by helminth infection is associated with inhibition of PN-specific IgE and anaphylaxis (25). IL-5, in addition to its well-known paracrine effects on eosinophils, also has an autocrine effect on mast cells (37, 38).

In contrast, IFN-γ cross-regulates Th2 cytokines. The intestinal immune system preferentially responds to food Ags with a dominant Th1 profile (39), in the seemingly normal state (40). According to the hygiene hypotheses, the increase in allergy in westernized societies over the last decades (41, 42) may, to some extent, be explained by a reduced microbial load early in infancy (42–44), which results in too little Th1 cell activity and therefore an insufficient level of IFN-γ to optimally cross-regulate Th2 responses (45). Therefore, it is possible that the reduction of Th2 cytokines by m Ara h 1–3 plus HKLM is primarily due to increased IFN-γ production.

In conclusion, we demonstrated that m Ara h 1–3 plus HKLM markedly reduced PN-specific IgE and plasma histamine levels and protected against systemic anaphylaxis in this model of PN hypersensitivity. The precise mechanisms associated with protection are not fully understood, but the protective effect was associated with up-regulation of Th1 and down-regulation of Th2 responses. Although animal models are not identical with human diseases, the present study suggests that modified PN protein-based immunotherapy together with HKLM as adjuvant may have potential for treatment of PNA.