Initial High-Dose Nasal Allergen Exposure Prevents Allergic Sensitization to a Neoantigen[1]

Marc A. Riedl,[2]* Elliot M. Landaw,† Andrew Saxon,* and David Diaz-Sanchez*

Primary allergic sensitization—IgE formation after Ag exposure—is fundamental in the development of allergic respiratory disease. With the rising prevalence of asthma and allergic rhinitis, improved understanding of the determining factors for allergic sensitization is needed. Human epidemiologic studies suggest high-dose allergen exposure may paradoxically protect against sensitization. Prospective human studies of allergen dose effect on primary allergic sensitization are lacking. We prospectively examined the effect of respiratory Ag dose exposure on the rate of primary allergic sensitization to a neoantigen, keyhole limpet hemocyanin, using a unique model of human nasal allergic sensitization. Atopic human subjects were exposed to 0.1-, 10-, 1,000-, or 100,000-μg doses of intranasal keyhole limpet hemocyanin in conjunction with adjuvant intranasal diesel exhaust particles. Ag-specific IgE, IgG, and IgG4 were measured in nasal lavage samples at the conclusion of the sensitization protocol. Allergic sensitization rates for the 0.1-, 10-, 1,000-, and 100,000-μg dose groups were 0, 100, 57, and 11%, respectively. All subjects produced Ag-specific IgG with the highest levels observed in the high-dose group. These results provide direct evidence that primary allergic sensitization may be prevented by initial high levels of respiratory Ag exposure through induction of a modified, nonallergic immune response. This Ag dose effect was capable of overcoming the well-established allergic adjuvant effects of diesel exhaust particle exposure. Whether this immune response represents durable allergic tolerance is not yet known. Studies investigating the molecular mechanisms of this non-IgE response may be useful in developing therapy to prevent allergic sensitization. ***The Journal of Immunology*****, 2005, 174: 7440–7445.**

The ability of an individual to form an allergic response and its subsequent severity is dependent on a combination of intrinsic and extrinsic factors. Intrinsic traits such as age and genetic background have been shown to have a strong influence on the development and severity of allergic disease. In addition, a multitude of experimental and epidemiological studies have demonstrated that environmental factors such as infections, air pollution, and indoor allergens may affect the symptoms of established allergic disease. Less is known about specific environmental factors leading to primary allergic sensitization in humans, i.e., the initial formation of Ag-specific IgE following Ag exposure. Although conventionally and intuitively increased allergen exposure is considered a risk factor for allergic sensitization in genetically predisposed individuals (1), recent epidemiological data suggest the development of allergen tolerance in young children with environmental exposure to high levels of cat allergen (2, 3). Such tolerance is defined by the presence of Ag-specific IgG and IgG4 in the absence of Ag-specific IgE. This induction of allergic tolerance rather than allergic sensitization to respiratory Ags has been well demonstrated in animal models (4–8), but there is little direct experimental evidence to support induction of allergen tolerance by initial high-dose respiratory exposure as a means to prevent primary allergic sensitization in Ag-naive humans. We have established previously a novel model of human primary allergic nasal sensitization to a neoantigen, keyhole limpet hemocyanin (KLH)[3] (9). This human model uses the proallergic adjuvant effects of diesel exhaust particles (DEPs) and reliably induces Ag-specific nasal IgE production in human subjects. Our previous work has demonstrated that subjects enrolled in the protocol will make IgE Abs to KLH following intranasal immunization with an optimal "low dose" of KLH plus adjuvant DEPs (10). In contrast, subjects exposed to intranasal KLH alone, in the absence of DEPs, produce KLH-specific IgG but not IgE in nasal lavage samples. Using this human model, we sought to investigate prospectively the dose-response effect of initial Ag exposure level on the rate of allergic sensitization as a way to test the hypothesis that initial high-dose Ag exposure would lead to a diminished or absent subsequent allergic response.

Materials and Methods

Human subjects

Fifty-one healthy nonsmoking atopic subjects (29 females, 22 males, ages 18–55 years) were recruited in Los Angeles, California. Subjects were volunteers who responded to flyers displayed on the campus of the University of California–Los Angeles and to advertisements in local newspapers from January 1, 1999, to July 31, 2003. Atopic status was determined by a positive skin prick test response to at least one aeroallergen on a standard screening panel. Thus, individuals were selected who might under the appropriate circumstances mount an allergic (IgE) response to respiratory Ag exposure. Subjects with active symptoms of allergic rhinitis or asthma at the time of screening or with a history of allergy to fish, arthropods, or mollusks were excluded. The use of nasal corticosteroids was prohibited during the duration of the study. All study activities were approved by the Human Subject Protection Committee of the University of California–Los Angeles. Because this was a dose-ranging investigation, subjects were allocated to one of four KLH dose-exposure groups in a

*Hart and Louis Lyon Laboratory, Division of Clinical Immunology and Allergy, Department of Medicine, and †Department of Biomathematics, David Geffen School of Medicine, University of California, Los Angeles, CA 90095

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[2] Address correspondence and reprint requests to Dr. Marc Riedl, 10833 Le Conte Avenue, 52-175 CHS, Los Angeles, CA 90095. E-mail address: mriedl@mednet.ucla.edu

[3] Abbreviations used in this paper: KLH, keyhole limpet hemocyanin; DEP, diesel exhaust particle.

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Table I. *Protocol schedule for nasal allergic sensitization to KLH*[a]

Day −1	Nasal lavage, DEPs nasal spray (0.3 mg)
Day 0	KLH nasal challenge (0.1, 10, 1,000, or 100,000 μg)
Day 13	DEPs nasal spray (0.3 mg)
Day 14	KLH nasal challenge (100 μg)
Day 27	Nasal lavage, DEPs nasal spray (0.3 mg)
Day 28	KLH nasal challenge (100 μg)
Day 29	Nasal lavage
Day 33	Nasal lavage

[a] Subjects were immunized with intranasal DEP and KLH. Initial KLH dose varied by 100-fold for each group.

nonrandomized fashion with each dose group completed consecutively. One subject in the highest dose group was found to have Abs that bound KLH in the baseline nasal lavage sample and was excluded from analysis.

Keyhole limpet hemocyanin

Purified, lyophilized, and endotoxin-free (≤12 EU/mg) KLH was purchased from Calbiochem. KLH solutions for 0.1-, 10-, and 1000-μg doses were prepared by dilution in sterile saline to the appropriate concentration for delivering the desired dose in two metered sprays (200 μl) from commercially available metered nasal spray bottles. For the 100,000-μg dose, lyophilized KLH was delivered directly by nasal insufflation because it would not have been feasible to use as a solution due to the volumes of fluid that would have been needed. DEPs were a kind gift from Dr. M. Sagai (Aomori University, Aomori, Japan). The particles were collected, processed, and preserved as described previously (11). Immediately before nasal challenge, the DEPs were sonicated for 10 min in a 50-W sonic disrupter (TeledyneTekmar).

Nasal washes and challenge procedures

Subjects performed a baseline nasal lavage with 40 ml of sterile saline on day −1 using previously described methods (12). Nasal lavage fluid was centrifuged at 350 × *g* for 15 min at 4 °C. The aqueous supernatants were removed from the cell pellets and stored at −30°C until needed. Subjects received 0.3 mg of DEPs in 200 μl of saline intranasally on day −1. In previous studies, this dose of DEPs has been shown to produce optimal IgE production in vivo. KLH was administered intranasally on day 0. The initial intranasal KLH dose differed 100-fold among 4 groups: 0.1-, 10-, 1,000-, and 100,000-μg KLH doses were used. The first 21 subjects were assigned to the 1000-μg group, the next 10 to the 10-μg group, the next 10 to the 0.1-μg group, and the final 10 to the 100,000-μg group. Subjects received 0.3 mg of DEPs intranasally on days 13 and 27 with 100 μg of KLH intranasally on days 14 and 28. Nasal lavage fluid was collected on days 27, 29, and 33 of the protocol. The challenge and testing schedule is shown in Table I.

Ab measurements

KLH-specific IgE and IgG levels were measured in the nasal lavage supernatant samples at the completion of each dose group. IgG4 levels were measured at a later date using stored specimens. Measurement of IgG4 levels was not possible for the 0.1-μg dose due to an insufficient amount of sample fluid. All Ig measurements were performed by modifications of the Ag and isotype-specific ELISAs, as described previously (10, 13). Assay results are expressed as units/ml, and units are specific for each Ig class. The lower detection limit of the KLH-specific Ig assays was 0.05 U/ml for IgE, IgG, and IgG4.

Statistical analysis

ANOVA and Fisher's exact test were used to compare the distribution of ages and sex, respectively, among the dosing groups. Rank order correlation coefficients (Kendall's τ) were used to assess correlations between continuous variables. A preliminary analysis demonstrated no significant imbalance in ages or female:male ratio among the dosing groups and no important association of either sex or age to KLH-specific Ig levels within any group. Therefore, sex and age were not used in any additional analyses. Fisher's exact test was used to compare group rates of allergic sensitization. Comparisons among the three highest dose groups of the day 33 nasal lavage KLH-specific Ig (E, G, and G4) levels and ratios of levels were assessed by Kruskal-Wallis tests. Comparisons of continuous variables between consecutive dosing levels were performed by the rank-sum test with Bonferroni correction for multiple comparisons.

Results

Mucosal IgE production to KLH

The formation of IgE Abs to an allergen is considered the hallmark of allergic sensitization. KLH-specific IgE levels in nasal lavage fluids at the end of the study period (day 33) for each group is shown in Table II. Data from previous investigation with intranasal KLH alone is provided for comparison. All subjects in the lowest dose-exposure group responded with KLH-specific IgG at day 33 (mean ± SD: 13.9 ± 7.62 U/ml), but no KLH-specific IgE was detectable in any subject of this group. IgG4 levels were not measured in this group.

KLH-specific IgE mucosal sensitization differed markedly across the remaining groups (Fig. 1): 10 of 10 (100%) subjects at the 10-μg KLH dose; 12 of 21 subjects (57%) at 1,000 μg; and 1 of 9 (11%) subjects at 100,000 μg ($p = 0.0003$). Mean KLH-specific IgE levels decreased with increasing doses of initial Ag exposure (Fig. 2). Mean IgE levels for the 10-, 1,000-, and 100,000-μg doses were 29.2, 11.5, and 1.2 U/ml, respectively (Kruskal-Wallis test, overall $p = 0.001$; 10 vs 1,000 μg, $p = 0.05$; 1,000 vs 100,000 μg, $p = 0.034$).

Mucosal IgG and IgG4 production to KLH

KLH-specific IgG and IgG4 levels in nasal fluids at the end of the study period for each group are shown in Table II. All subjects participating in the study developed detectable KLH-specific IgG levels, and 39 of 40 (98%) developed detectable KLH-specific IgG4 levels. KLH-specific IgG levels demonstrated a trend for increased levels with increasing dose. Mean IgG levels were 17.9, 32.1, and 44.0 U/ml for the 10-, 1,000-, and 100,000-μg dose groups, respectively (overall $p = 0.003$; 10 vs 1,000 μg, $p = 0.02$; 1,000 vs 100,000 μg, $p = 0.17$).

Mean KLH-specific IgG4 levels were comparable for the two lower dose groups, but significantly higher in the high dose group.

Table II. *Summary statistics*[a] *for nasal lavage Ig levels at day 33*

	Very Low Dose 0.1 μg, $n = 10$	Low Dose 10 μg, $n = 10$	Medium Dose 1,000 μg, $n = 21$	High Dose 100,000 μg, $n = 9$	KLH Alone 1,000 μg, $n = 10$
Female/Male	5/5	6/4	13/8	4/5	6/4
Age (years)	29.2 ± 8.7 (28.5, 20–49)	27.2 ± 9.9 (23, 18–53)	29.1 ± 8.0 (27, 20–55)	29.1 ± 10.3 (26, 22–54)	24.7 ± 4.4 (23, 21–34)
IgE (units/ml)[b]	Not detectable	29.2 ± 24.8 (29.1, 0.3–66.2)	11.45 ± 13.83[c] (8.7, 0–42.9)	1.21 ± 3.63[d] (0, 0–10.9)	Not detectable
IgG (units/ml)[b]	13.9 ± 7.62 (12.4, 5.6–29.7)	17.9 ± 9.1 (15.9, 7.4–39.7)	32.1 ± 17.6[c] (28.8, 4.8–79.3)	44.0 ± 18.2 (42.1, 18.7–69.3)	16.2 ± 8.4 (17.5, 4.3–29.6)
IgG4 (units/ml)[b]	Not performed	10.7 ± 8.1 (8.6, 0.9–23.2)	9.41 ± 5.96 (8.6, 0–19.3)	18.1 ± 9.57[d] (15.6, 8.9–38.0)	1.49 ± 1.98 (0, 0–4.3)
IgG4:IgG ratio	Not performed	0.65 ± 0.55 (0.50, 0.12–1.6)	0.36 ± 0.28 (0.25, 0–1.14)	0.46 ± 0.24 (0.46, 0.15–0.80)	0.18 ± 0.32 (0, 0–1.0)
IgE:IgG ratio[b]	Not performed	1.83 ± 1.70 (1.42, 0.04–4.76)	0.48 ± 0.66[c] (0.31, 0–2.54)	0.02 ± 0.07[d] (0, 0–0.22)	Not performed
IgE:IgG4 ratio[b]	Not performed	2.34 ± 1.30 (2.31, 0.33–4.90)	1.01 ± 0.98[c] (1.14, 0–2.65)	0.03 ± 0.10[d] (0, 0–0.29)	Not performed

[a] Expressed as mean ± SD (median, range).
[b] Kruskal-Wallis test $p < 0.05$ for three highest doses.
[c] A value of $p \leq 0.05$ compared with 10-μg group.
[d] A value of $p < 0.05$ compared with 1,000-μg group.

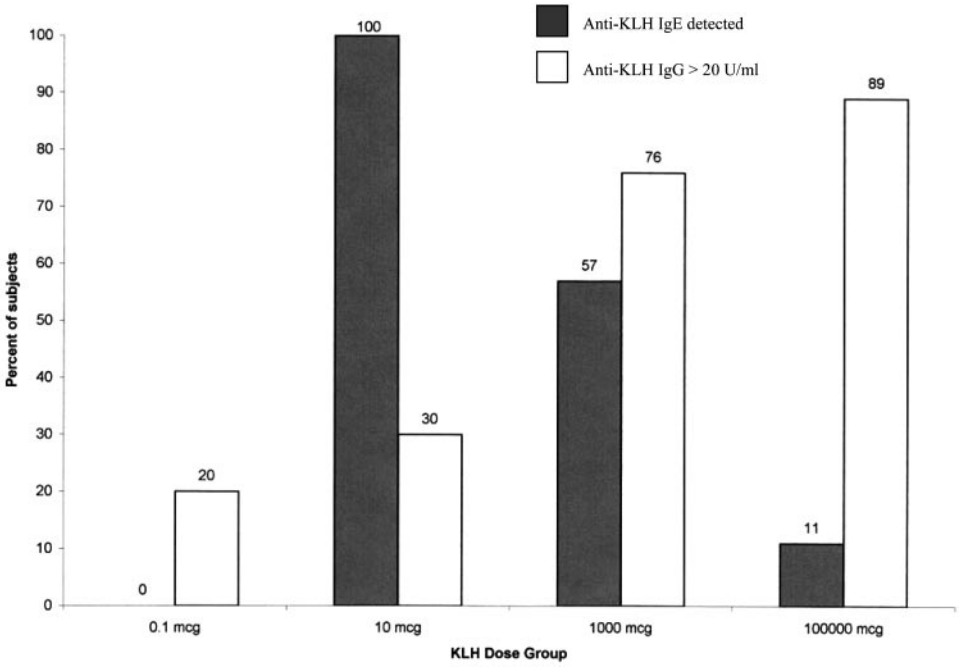

FIGURE 1. Prevalence of KLH-specific IgE and IgG by dose group. The prevalence of detectable KLH-specific IgE (▨) and of KLH-specific IgG > 20 U/ml (□) is shown for four KLH dose-exposure groups. The rates of allergic sensitization are highest among groups with moderate-dose neoantigen exposure.

Mean IgG4 levels in increasing dose order for the three groups were 10.7, 9.4, and 18.1 U/ml (overall $p = 0.04$; 10 vs 1,000 μg, $p > 0.5$; 1,000 vs 100,000 μg, $p = 0.02$).

Relationship among IgE, IgG, and IgG4 levels

The mean IgG4:IgG ratio did not vary significantly among the three groups, with ratios of 0.65, 0.36, and 0.46 for the 10-, 1000-, and 100,000-μg groups, respectively (overall $p = 0.35$). However, IgE:IgG and IgE:IgG4 ratios decreased significantly with increasing dose (Table II). Mean IgE:IgG ratios in increasing dose order were 1.83, 0.48, and 0.02, respectively (overall $p = 0.0004$; 10 vs 1,000 μg, $p = 0.02$; 1,000 vs 100,000 μg, $p = 0.03$). Mean IgE:IgG4 ratios in increasing dose order were 2.34, 1.01, and 0.03, respectively (overall $p = 0.0002$; 10 vs 1,000 μg, $p = 0.015$; 1,000 vs 100,000 μg, $p = 0.02$). Within each dosing group there was no significant correlation between KLH-specific IgE and IgG levels, but positive correlations were observed between IgE and IgG4 levels (Fig. 3). The rank order correlation coefficients were significant for the low- and medium-dose groups (0.750 and 0.513, respectively; both $p = 0.002$ under the hypothesis of no association between IgE and IgG4 levels).

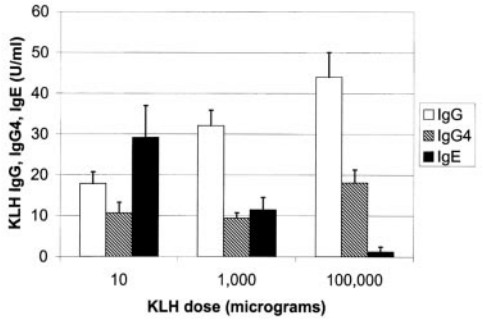

FIGURE 2. Group means of KLH-specific Abs. Group mean KLH-specific Ab levels in nasal lavage specimens of subjects at day 33 demonstrate a trend of increasing IgG levels and decreasing IgE levels associated with increasing dose exposure to nasal KLH. Error bars indicate SEM.

IgE responders vs nonresponders

To compare differences between IgE responders (IgE + IgG) vs nonresponders (IgG only), rank-sum testing was used to compare IgG, IgG4, and IgG4:IgG ratios for these two populations in the medium-dose (1000 μg) exposure group. KLH IgG levels did not differ; however, mean IgG4 levels were significantly higher in the IgE-sensitized subgroup (12.1 vs 5.8 U/ml, $p = 0.01$). Likewise, the IgG4:IgG ratio was significantly higher in the IgE-sensitized subgroup than the subgroup responding with IgG alone (0.49 vs 0.20, $p = 0.023$). The IgE:IgG4 ratio for the subgroup of 12 responders in this medium dose group was 1.69 ± 0.65 (mean ± SD) and was not distinguishable statistically from the 2.34 ± 1.30 ratio for the low-dose group ($p = 0.2$).

Discussion

The results of this prospective human study provide direct experimental evidence to support the hypothesis that initial high-dose

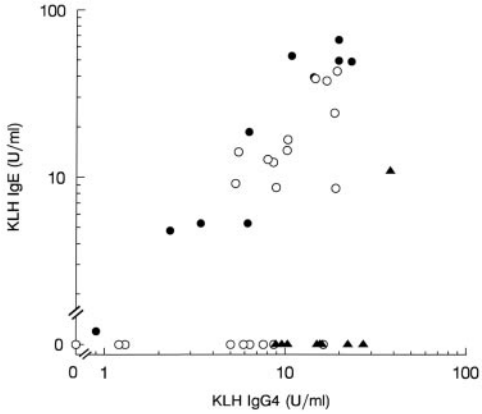

FIGURE 3. KLH-specific IgG4 and IgE correlation by dose group. Correlation between anti-KLH IgG4 and IgE Abs from day 33 nasal lavage for low-dose exposure (●), medium-dose exposure (○), and high-dose exposure (▲) subjects. Both axes on a log scale from 1 to 100 U/ml, with broken axes to include undetectable (0) levels.

exposure to a neoantigen (100–10,000 times higher than the moderate and low-dose exposures) is more likely to result in a lack of primary allergic sensitization; for example, higher levels of Ag may induce a humoral immune response that lacks an IgE component. Notably, this dose effect on the mucosal immune response was capable of overcoming the proallergic adjuvant effects of DEPs that otherwise result in primary allergic sensitization to a neoantigen.

We observed a bell-shaped, dose-response curve when plotting Ag dose vs allergic sensitization (Fig. 1). Exposure to respiratory allergens in individuals with the appropriate atopic genetic background is generally thought to result in allergic sensitization. This hypothesis is supported by observational studies reporting a linear dose-response curve for allergic sensitization during childhood with exposure to dust mite and cockroach allergen (14–18). However, other observational data has suggested an alternative phenomenon occurring with childhood exposure to cat allergen: a bell-shaped dose response with maximal rates of specific IgE sensitization at moderate exposure and a modified nonallergic immune response at the highest dose exposures (2, 3). Differences between the nature and dose of cat vs dust mite and cockroach Ag are likely important in these observations as discussed below in the penultimate paragraph. Ag dose has also been shown to be an important variable in murine models of allergic sensitization, i.e., low-dose Ag produces optimal IgE production in most strains with higher doses stimulating IgG alone in the same strains (19).

The induction of a non-IgE humoral immune response, or allergic tolerance, through respiratory Ag exposure has been demonstrated previously in murine models of allergic sensitization (5). Whether such allergic tolerance occurs in a similar fashion in humans is not clear. Woodfolk and Platts-Mills (20) have described the protective "modified Th2 response" as a serologic profile with high titer Ag-specific IgG Ab, including IgG4, with absent or low levels of Ag-specific IgE. A strong correlation between this modified Th2 response and levels of Ag exposure has been suggested for cat allergen based on observational studies (21). Our results with nasal KLH exposure support the hypothesis that respiratory Ag dose influences the initial humoral immune response (allergic vs nonallergic).

The mechanism of respiratory immune tolerance induction has not been elucidated clearly. Based on numerous animal models and supported by our own work in humans, Ag dose is an important factor in eliciting a tolerant immune response. Ligand density related to Ag concentration may play a role in determining T cell activation threshold and signal transduction for cytokine production (22). Currently, it appears that respiratory Ag dose has important qualitative effects on the function of effector cells such as dendritic cells and $CD4^+$ T cells (23–25). Such effects may include increased apoptosis of IL-4-producing Th cells, as well as altered cytokine production favoring a tolerant rather than allergic immune response. There is also evidence that IL-10 plays an important role in the induction of respiratory immune tolerance (20, 21). According to this model, early high-dose allergen exposure leads to a moderate level of IFN-γ production coupled with increased IL-10 production from regulatory $CD4^+$ T cells. The resulting humoral immune response is elevated Ag-specific IgG and IgG4 and a nonallergic phenotype with subsequent Ag exposure. Other cytokines such as TGF-β also appear to promote immune tolerance of respiratory Ags (26). A link between the innate and adaptive immune system via Notch signaling or TLR effects on Th cells may be involved in immune tolerance induction (23, 27, 28). Such signaling may be epitope nonspecific based on the presence or absence of other inflammatory stimuli.

Our data confirms a correlation between increased levels of Ag-specific IgG and the absence of Ag-specific allergic sensitization (IgE formation). In our study, the group with the highest level of Ag exposure responded with significantly higher IgG levels and an allergic sensitization rate of only 11%. We observed a positive correlation between IgG4 and IgE levels within the low- and medium-dose groups, with the size of the IgE:IgG4 ratio similar for IgE responders in both groups. This is consistent with previous observations that allergic patients often produce high levels of allergen-specific IgG4 (29, 30) and considerable evidence that IgE and IgG4 share common molecular regulatory mechanisms (31–34). However, we also noted a "disparity" in IgG4 and IgE levels, i.e., elevated IgG4 and undetectable IgE, for approximately half of the medium-dose subjects and the vast majority of high-dose subjects (Fig. 3). This suggests an additional event or control mechanism that inhibits IgE production while driving IgG4 production against the inhaled Ag. Some evidence suggests that IL-10 may be capable of suppressing IgE while maintaining IgG and IgG4 production (35).

The mechanism(s) underlying the modified nonallergic response or tolerance in the high-dose KLH subjects is of considerable clinical interest. Future studies are planned to examine additional biomarkers (IL-10, IL-6, IFN-γ, and T-regulatory cell expression) in nasal lavage samples for high-dose vs low- to moderate-dose Ag exposure cohorts at critical time points 24–72 h after Ag exposure. Such data may delineate mechanisms underlying an IgG vs IgE response to a neoantigen and identify pathways by which high-dose Ag exposure modifies the mucosal immune response. Furthermore, it will be important to identify possible genotypic factors associated with the observed modified Th2 response and to assess the durability and reproducibility of the tolerant response. Demonstration of consistent, durable allergic tolerance induction with high-dose primary exposure to a neoallergen would present a plausible technique for the prevention of allergic sensitization. If effective, such controlled exposures could be developed into immunization programs aimed at preventing aeroallergen sensitization in individuals with a high genetic risk for allergic sensitization.

A potential concern regarding our study might be that our human system uses DEPs as an adjuvant to induce allergic sensitization. However, these adjuvant effects may be quite relevant to the development of allergic respiratory disease in the general population. Human exposure to respiratory allergens commonly occurs in conjunction with other respired particles and chemicals (36–38). Considerable evidence supporting the complex interactions between allergens and particulate air pollution is outlined elsewhere (39, 40). Thus, the model used in our study provides a representation of "real-world" allergic sensitization, particularly for individuals living in urbanized areas that are exposed to inhaled allergens in conjunction with particulate air pollution.

We used KLH as the neoantigen while remaining fully cognizant that it may not be representative of the "natural" inhaled Ags. KLH was selected based on the following characteristics: 1) well-defined properties as a potent immunogen; 2) historical safety in human studies; 3) value as a true neoantigen; and 4) minimal risk of future health effects from allergic sensitization. Different respiratory allergens are recognized to have individual biologic properties that may influence the immune response to them. Such allergen difference is exemplified by comparisons of cat and dust mite allergen. Because of physical properties, ambient exposure to cat allergen is generally thought to be up to 100-fold greater than exposure to dust mite allergen (1, 41, 42). Environmental dust mite and cockroach exposure studies showing a linear dose-sensitization relationship simply may not have achieved the allergen levels where allergic tolerance occurs as with cat allergen exposure.

However, dust mite allergen(s) also possesses potent enzymatic properties not present in cat allergen (43). It has been suggested that allergens with enzymatic activity nonspecifically activate basophils and mast cells to release histamine, IL-4, and other immunomodulating molecules that deviate the resulting immune response toward Th-2 polarization (44, 45). As a result of differing enzymatic activities, the observed dose-response effects for the allergens may differ considerably with respect to allergic sensitization. Whether the observed allergen dose-immune response for dust mite vs cat is due to intrinsic biologic properties of the individual allergens, dose, or other factors is presently not known.

We believe our study data demonstrates a direct proof of principle: initial high-dose respiratory Ag exposure may induce an Ag-specific immune response in humans that is deviated away from the production of allergic Abs. Clearly, at the present time, extrapolation of the bell-shaped allergen dose-IgE immune response concept demonstrated in our study to all respiratory allergens would be inappropriate. However, our results provide a format for future randomized studies to test this principle and its application to relevant allergens and represent an important step in moving primary prevention therapy for allergic disease from animal models to human intervention.

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Disclosures

The authors have no financial conflict of interest.

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