

Nasal challenge with diesel exhaust particles can induce sensitization to a neoallergen in the human mucosa

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Background: Diesel exhaust particles (DEPs) increase in vivo IgE and cytokine production at the human upper respiratory mucosa, exacerbating allergic inflammation.

Objective: We examined the ability of DEP exposure to lead to primary sensitization of humans by driving a de novo mucosal IgE response to a neoantigen, keyhole limpet hemocyanin (KLH).

Methods: Ten atopic subjects were given an initial nasal immunization with 1 mg of KLH followed by 2 biweekly nasal challenges with 100 µg of KLH. Identical nasal KLH immunization was then performed on 15 different atopic subjects, but DEPs were administered 24 hours before each KLH exposure.

Results: Exposure to KLH alone led to the generation of an anti-KLH IgG and IgA humoral response, which was detected in nasal fluid samples. No anti-KLH IgE appeared in any subjects. In contrast, when challenged with KLH preceded by DEPs, 9 of the 15 subjects produced anti-KLH-specific IgE. KLH-specific IgG and IgA at levels similar to that seen with KLH alone could also be detected. Subjects who received DEPs and KLH had significantly increased IL-4, but not IFN-γ, levels in nasal lavage fluid, whereas these levels were unchanged in subjects receiving KLH alone.

Conclusion: These studies demonstrate that DEPs can act as mucosal adjuvants to a de novo IgE response and may increase allergic sensitization. (*J Allergy Clin Immunol* 1999;1183-8.)

Key words: Diesel exhaust particles, allergic sensitization, IgE regulation, pollution, atopy

The ability of certain environmental contaminants to modulate the immune system has been demonstrated in animal and human models both in vitro and in vivo. Recently, there has been an increased focus on the role of fossil fuel combustion products, in particular diesel exhaust particles (DEPs), in exacerbating allergic inflammation.¹ It is clear that DEPs can provide a mucosal

Abbreviations used

DEP: Diesel exhaust particle

KLH: Keyhole limpet hemocyanin

PAH: Polyaromatic hydrocarbon

adjuvant effect and thereby enhance ongoing allergic inflammation,² including IgE production, which is considered the hallmark of atopy in humans. Furthermore, coexposure to DEPs plus an allergen in the human upper respiratory tract markedly increased specific IgE levels to the allergen while skewing the cytokine pattern toward a T_{H2}-like pattern.³ DEPs were also shown to interact with ragweed allergen to drive in vivo isotype switching to IgE in the nasal mucosa.⁴

Although these prior studies demonstrated that DEPs have a variety of effects on their own and can strongly enhance mucosal allergic inflammation and specific Ig responses in already sensitized subjects, a key question remained unanswered. Would mucosal exposure to DEPs lead to the production of IgE to a neoantigen (sensitization) under conditions whereby exposure to the neoantigen alone would not? We directly addressed this question in human subjects by using the neoantigen keyhole limpet hemocyanin (KLH), a glycoprotein isolated from the blood of marine mollusk (*Megathura crenulata*), which has no known cross-reacting antibodies in human subjects. The immunogenicity of KLH and its role as a neoantigen in humans has been well established.^{5,6} Using in vivo nasal human challenges, we demonstrate that although exposure to KLH alone resulted in an IgG and IgA anti-KLH response, mucosal challenge with DEPs plus KLH exposure elicited an IgE anti-KLH response as well, thereby demonstrating that DEPs were able to drive sensitization to a neoantigen.

METHODS

Subjects

Twenty-five healthy nonsmoking volunteers (11 men and 14 women), ranging in age from 21 to 55 years, were recruited in Los Angeles, California. Atopic subjects were chosen as individuals who might, under appropriate circumstances, mount an IgE response to a foreign protein when exposed through the airway. All subjects had a positive skin prick test response to at least one aeroallergen but not to dust mite allergens. Additionally, all subjects had a history of prior seasonal airway allergies but were asymptomatic. All studies were performed outside their regular allergy season. No sub-

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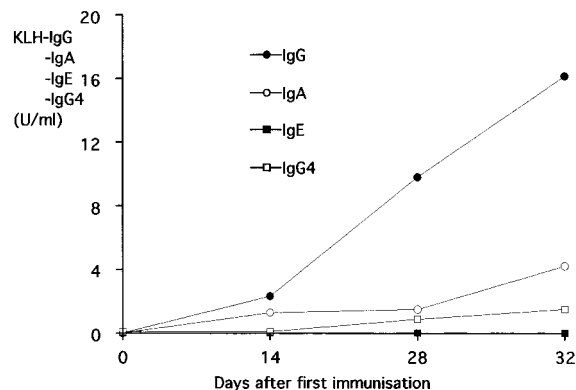


FIG 1. Appearance of antibodies to KLH in nasal fluid samples after nasal immunization with KLH. Subjects were immunized on day 0, 14, and 28, and the levels of anti-KLH IgE, IgG, IgA, and IgG4 were measured (see "Methods" section). Units are different for each isotype measured and cannot be compared. Mean of $n = 10$ is shown.

ject complained of chronic or perennial symptoms. The subjects did not take any medication for the 3 days before or at any time during the duration of the study. All atopic subjects were outside their allergy season. None had any known allergy to any type of fish or shellfish. None of the volunteers had any out of the ordinary exposure to air pollutants. Throughout the period of the study there were no pollution alerts in the Los Angeles area. All studies were approved by the Human Subject Protection Committee of the University of California at Los Angeles.

Materials

DEPs were obtained by collecting the exhaust from light-duty diesel passenger cars onto a cyclone impactor equipped with a dilution-tunnel, constant-volume sampler system and were the kind gift of Dr Masaru Sagai (National Institute for Environmental Studies, Tsukuba, Japan). These particles were collected, processed, and preserved as previously described.⁷ Immediately before challenge, the DEPs underwent sonication for 10 minutes in a 50-W sonic disrupter (Tekmar, Cincinnati, Ohio). Purified KLH was purchased from Calbiochem (La Jolla, Calif), dialyzed, and tested to ensure it was free of endotoxin.

Nasal washes and challenge procedures

Nasal washes were performed as previously described.^{2,8} The combined lavage fluid was centrifuged at 350g for 10 minutes at 4°C, and the aqueous supernatants were separated from the cell pellets and stored at -70°C until needed. Subjects were challenged with either 200 μ L of saline or 0.3 mg of DEPs in 200 μ L of saline. This dose of DEPs had previously been shown to produce optimal IgE production in vivo.^{2,8} KLH was administered as an aerosol at either 1 mg or 100 μ g in 200 μ L of saline. The challenge and testing schedule is shown in Table I. KLH was administered a total of 3 times. When DEPs were administered, they were given 24 hours before each KLH dose.

Ig and cytokine measurements

The levels of the KLH antibodies of different isotypes (IgE, IgG, IgG4, and IgA) in the supernatants obtained from nasal fluid samples were measured by modifications of the antigen and isotype-specific ELISAs, as previously described.^{3,9} Briefly, the plates were coated overnight with the appropriate anti-Ig antibody: IgE mAb (4.15 and 7.12) from our laboratory⁹ and other mAbs from Kierge-

TABLE I. Immunization protocols used in this study

Intranasal immunization with KLH		
Day 0	Lavage	1 mg KLH
Day 14	Lavage	100 μ g KLH
Day 28	Lavage	100 μ g KLH
Day 29	Lavage	
Day 32	Lavage	
Intranasal immunization with KLH and DEPs		
Day 1	Lavage	0.3 mg DEP
Day 0		1 mg KLH
Day 13		0.3 mg DEP
Day 14	Lavage	100 μ g KLH
Day 27		0.3 mg DEP
Day 28	Lavage	100 μ g KLH
Day 29	Lavage	
Day 32	Lavage	

Subjects were immunized by nasal provocation with either KLH or 0.3 mg of DEPs followed by KLH.

gard and Perry (Gaithersburg, Md). The plates were washed and blocked with 1% BSA-PBS, and samples that had been concentrated with a Centricon plus-20 filter concentrator (Amicon, Beverly, Mass) were added overnight. For each Ig isotype, a standard curve was constructed by using serial dilutions of high-titer sera pooled from subjects immunized intradermally with KLH provided as a kind gift from Dr Neil Sidell (Emory University, Atlanta, Ga).¹⁰ After washing, KLH, which had been conjugated with alkaline-phosphatase according to standard methodology,¹¹ was added (1:500) overnight to the plates. Color was developed by using an enzyme amplification system (Gibco BRL, Gaithersburg, Md), as per the manufacturer's instructions. For the anti-KLH IgE assay, the standard was absorbed against anti-IgE antibody and then used in the assay as a control to affirm that no signal could be measured, and therefore the assay was isotype specific. All samples were run in duplicate and repeated if there was more than a 5% variation between the duplicates. The amounts of KLH-specific antibodies are expressed in units per milliliter. It should be stressed that these units are different for each isotype measured and cannot be used for comparison between isotypes.

IL-4 and IFN- γ were measured in nasal lavage supernatants by using commercial ELISA kits (Endogen, Boston, Mass), following the manufacturer's instruction. The sensitivity of these assays was 0.2 pg/mL. Cytokine levels were measured in nasal lavage fluid collected at baseline and 24 hours after the last KLH exposure (day 29) because optimal production of cytokines has been shown to occur 18 to 24 hours after allergen challenge in this model.⁸

Statistical analysis

The Statview II computer package (Abacus concepts, Berkeley, Calif) for the Macintosh was used for all analysis. Comparisons within groups were done by using paired t tests. Comparisons between groups were performed by using unpaired t tests or the Wilcoxon rank-sum test. A P value of less than .05 was considered statistically significant.

RESULTS

Mucosal Ig response to KLH alone

Initially it was necessary to establish an intranasal KLH immunization regimen that would elicit a local humoral immune response. Fig 1 shows the mucosal antibody response to KLH in 10 atopic subjects after 3 intranasal administrations with the antigen alone. No

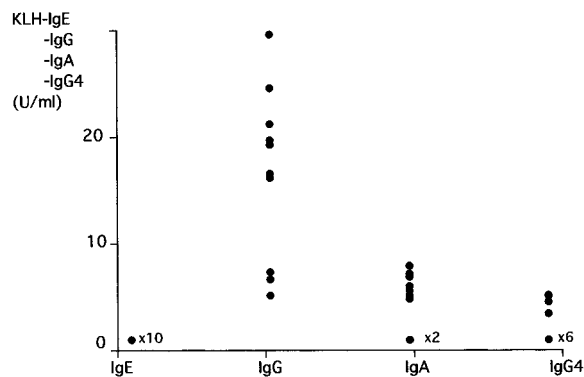


FIG 2. Individual antibody responses to KLH as measured at day 32 (4 days after the last immunization). Units are different for each isotype measured.

detectable antibodies against KLH of any isotype were observed in nasal washes performed before challenge. After primary immunization with KLH, allergen-specific IgG and IgA were already detectable after 14 days, and with each subsequent challenge, these antibody levels increased further. Formation of KLH-specific IgG4 was not detected 14 days after the initial immunization but was observed in nasal fluids performed at day 28 and at day 32. In contrast, no IgE anti-KLH antibodies could be detected at any time when the subjects were exposed to KLH alone. Not unexpectedly, there was considerable variation in the antibody response to KLH between individuals. Fig 2 shows the antibody response for each Ig isotype at day 32 (4 days after the last immunization). Although IgG antibody responses were detected in all subjects, IgA was detected in 8 of 10 subjects, IgG4 in only 4 of 10 subjects, and IgE in 0 of 10 individuals. All those subjects in whom IgG4 was detected also had detectable IgA levels. There was a significant correlation between high levels of IgG and the ability to make both an IgA and an IgG4 response ($P < .05$, Wilcoxon rank-sum test).

Sera collected from the subjects failed to reveal the presence of KLH-specific antibodies at any time (data not shown).

Production of mucosal IgE in subjects immunized with KLH in the presence of DEPs

The ability of DEPs to drive IgE production (sensitization) to KLH in subjects was tested by repeating the above challenge protocol but with coadministration of DEPs as per the immunization schedule shown in Table I. IgE anti-KLH antibodies were present in nasal lavage samples collected at both day 28 and day 32 (Fig 3) after the KLH plus DEP immunization regimen in contrast to what was observed in subjects challenged with allergen alone. Allergen-specific total IgG levels were detected in all lavage fluid samples after the combined challenges, with levels of anti-KLH IgG similar to those seen in subjects receiving KLH alone (eg, day 32 mean = 17.5 ± 9.4

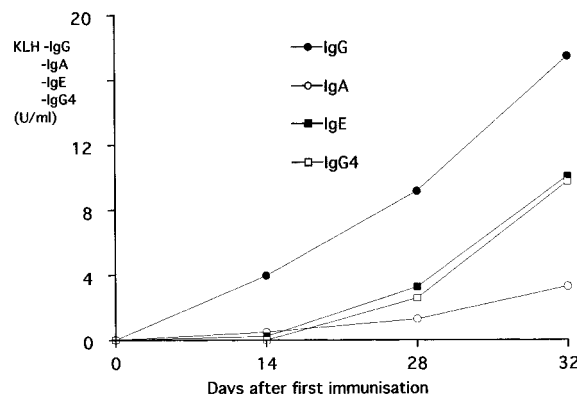


FIG 3. Appearance of antibodies to KLH in nasal fluid samples after nasal immunization with KLH plus DEPs. Subjects were immunized on days 0, 14, and 28, and the levels of anti-KLH IgE, IgG, IgA, and IgG4 were measured (see "Methods" section). Units are different for each isotype measured and cannot be compared. Mean of $n = 15$ is shown.

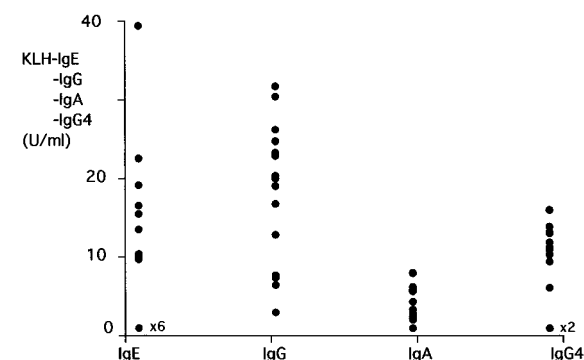


FIG 4. Individual antibody response to KLH on day 32 (4 days after the last immunization) within each isotype. Units are different for each isotype measured.

vs 16.2 ± 8.4 U/mL, $P > .05$). Nasal anti-KLH IgA levels were also not significantly different between the 2 groups. By comparison, subjects coadministered DEPs and KLH had significantly higher IgG4 levels (mean = 9.8 ± 5.0 U/mL) than when DEPs were absent (mean = 1.5 ± 1.3 U/mL, $P < .01$). Fig 4 shows antibody levels for each individual at day 32 and again illustrate that the antibody response was highly variable between subjects. At this time point, 9 of 15 subjects had measurable KLH-specific IgE, with the levels in those sensitized persons ranging from 9 to 39 U/mL (mean = 10.1 ± 11.2 U/mL). As with KLH alone, all subjects made detectable levels of KLH-IgG, whereas IgA was present in 14 of the 15 subjects. Compared with IgG4 antibodies, which were observed in only 40% (6/15) of subjects immunized with KLH alone, in those immunized with both DEPs and KLH, this number increased to 87% (13 of 15). Once again no KLH antibodies were detected in the subjects' sera.

Cytokine levels after immunization

The levels of 2 cytokines that are believed to play a central role in allergic sensitization, IL-4 and IFN- γ ,

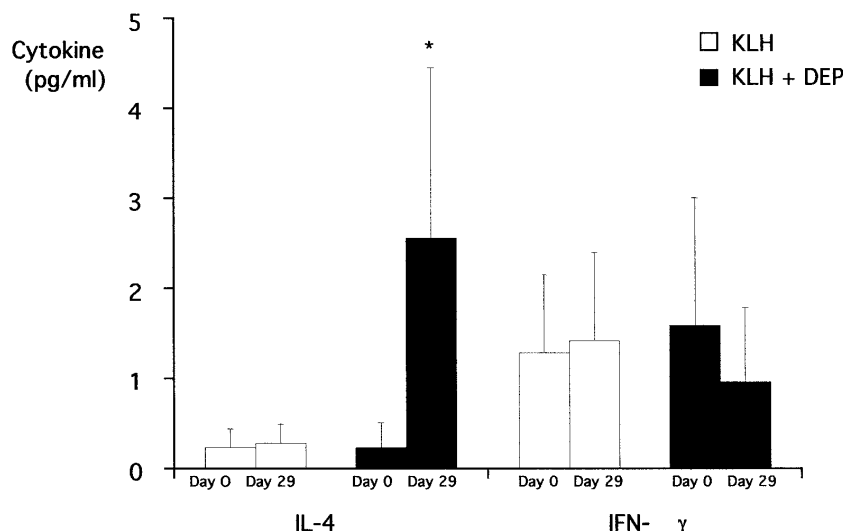


FIG 5. Cytokine response to nasal immunization with KLH ($n = 10$) or with DEPs plus KLH ($n = 10$). IL-4 and IFN- γ protein levels in the nasal lavage fluid samples were measured at baseline (day 0) and 24 hours after the last immunization (day 29). Means $\pm$ 1 SD are shown. * $P < .01$ compared with baseline (time 0).

were measured in nasal fluid samples obtained before immunization (day 0) and 24 hours after the final nasal challenge (day 29) with either KLH or KLH plus DEPs. Fig 5 shows that at day 29, subjects coadministered DEPs and KLH produced significantly more IL-4 than the group immunized with KLH alone (2.56 vs 0.28 pg/mL, $P > .01$) or than at day 0 (baseline). In contrast, although IFN- γ production was detectable after challenge with KLH plus DEPs, it was not significantly different from preimmunization levels. In subjects immunized with KLH alone, there was no significant difference in the amount of either IL-4 or IFN- γ between the 2 time points measured (day 0 and day 29).

DISCUSSION

DEPs, as environmental pollutants, have become the focus of intense investigation as a model to elucidate the interaction between fossil fuel combustion products, the immune response, and disease outcomes.^{1,12,13} Studies in animal and human models have indicated that DEPs have mucosal adjuvant properties at the molecular and cellular level. In particular, the ability of DEPs to exacerbate allergic inflammation and augment production of allergic antibody has been demonstrated both in animal and human models.^{2,3,7,14-16} In this study we demonstrate, for the first time in humans, that DEP exposure can lead to sensitization of atopic subjects to a neoantigen (KLH) under conditions during which the neoantigen alone does not have this effect.

The production of specific IgE antibodies against inhaled protein allergens is the hallmark of the sensitization phase of allergic airway disease (allergic rhinitis and asthma); subsequent seasonal exposure leads to a secondary immune response.¹⁷ We have previously shown that DEPs can strongly boost secondary antibody

responses to aeroallergens in a mucosal human model. Challenge of subjects allergic to ragweed with DEPs plus ragweed allergen resulted in a 16-fold increase in ragweed-specific IgE compared with that induced by ragweed alone.³ It could be argued from these studies that DEPs simply expanded a population of memory antigen-specific cells that arose as a result of previous exposure to the antigen. Similarly, although DEPs by themselves can cause a significant rise in total IgE, ϵ messenger (m)RNA production, and the number of IgE-producing cells present in the nasal mucosa in the absence of antigen, it is possible that DEPs simply expand the numbers of IgE-committed cells or cause increased migration of these cells. However, an alternative explanation is that DEPs act on B cells activated by allergen challenge or by low-level ambient allergen to increase the frequency of their isotype switching to antigen-specific IgE. There is evidence to support this latter interpretation. Numerous animal models have been established that demonstrate that initial injection of adjuvant plus allergen followed by repeated allergen exposure induces ϵ isotype switching in vivo. Although initial in vitro studies with DEPs alone failed to find evidence for induction of IgE isotype switching,¹⁸ subsequent in vivo studies in human subjects with combined nasal challenge with DEPs and allergen demonstrated isotype switching to IgE in vivo. In those studies circular Ig switch DNA produced when 2 switch (S) region sequences were looped out (so-called deleted switch circles, which are markers of active Ig isotype switching) was shown to appear after the combined challenge with DEPs plus ragweed allergen.⁴

In this study production of IgE (sensitization) to KLH did not occur in the absence of DEPs. Repeated exposure to KLH alone led to the formation of a mucosal response characterized by the production of KLH-specific IgA and IgG only. Other studies have also succeeded in eliciting

humoral immune responses by localized challenge with KLH alone. A local antigen-specific response was observed by Schuyler et al¹⁹ when 500 µg of KLH was instilled into the human lung. KLH-specific IgA, IgG, and IgM antibodies were detected in bronchoalveolar lavage fluid from the lobes of immunized subjects. Similar to our study, no anti-KLH IgE could be detected, although they did detect anti-KLH IgG, IgA, and IgM in the sera of their subjects 10 days after immunization. Systemically detectable responses did not occur in our study. This is probably because intrapulmonary instillation permits much greater systemic access than intranasal challenge. Weissman et al²⁰ estimate that the amount of serum antibody after intrapulmonary deposition of 500 µg of KLH is similar to that produced after 100 µg of KLH administered subcutaneously. It is also possible that the doses used by us were too low to elicit a systemic response. Thirty years previously, Salvaggio et al,²¹ using intranasal immunizations of KLH, detected IgM and IgG in the sera when administering 0.3 mg of KLH per week for 2 months followed by a 0.6-mg booster. Regardless, it is clear that at least through nasal immunization, the generation of IgE antibodies to a neoantigen requires an adjuvant or other factor, such as DEPs.

In ragweed-sensitive subjects challenged with ragweed plus DEPs, DEPs have a pronounced inhibitory effect on IFN-γ production while inducing an enhanced IL-4 response.³ Similarly, in the present study, after nasal provocation with DEPs plus KLH, IL-4 production was augmented, whereas that of IFN-γ was not. As we have previously observed, IFN-γ could be detected in baseline nasal fluid samples, probably because of the constant bombardment and stimulation of the nasal airways with organic, bacterial, and inorganic material found in the atmosphere. The ability of DEPs to augment IL-4 secretion provides an explanation for the ability of DEPs to sensitize subjects to a protein antigen present at the same time. IL-13 may also play an important role in local IgE responses in the nasal mucosa; unfortunately in this study we were unable to detect IL-13 protein either before or after immunization, probably because of the sensitivity of the assays used (data not shown). In this study we show that sensitization to a neoallergen when DEPs are preadministered is characterized by the formation of a T_{H2}-type cytokine pattern. This is in agreement with previous studies showing that DEPs can synergize with low doses of allergen, which in themselves are insufficient to initiate a T_{H2} cytokine response. Within hours of exposure to DEPs plus allergen, IL-4 secretion occurs from CD117⁺ cells residing in the nasal mucosa (presumably mast cells or basophils).²² Presumably, this early-appearing IL-4 deviates the subsequent response toward a T_{H2} pattern by expanding the repertoire of IL-4-producing cells in the local microenvironment.

Another potential mechanism for the effect of DEPs in promoting sensitization is its ability to enhance antigen presentation. Several studies have shown that DEPs can enhance the role of macrophages as antigen-presenting cells. Kerkvliet and Oughton²³ showed that polyaromat-

ic hydrocarbons (PAHs) present in DEPs can enhance MHC-II gene expression in murine macrophages. Nel et al¹ have shown that CD80 (B7-1) protein and message can be upregulated *in vivo* and *in vitro* by DEPs or by PAHs.¹ Additionally PAHs can also affect macrophage-derived cytokines, such as IL-1 and TNF-α,²⁴ that may play a role in allergic sensitization by inducing adhesion molecules, initiating cytokine cascades, and recruiting proinflammatory cells. Much work still remains to elucidate these and other possible mechanisms involved.

The results presented here show that DEPs, under experimental conditions, have the ability to drive sensitization to a neoantigen, sensitization that did not occur with exposure to the neoantigen alone. Thus DEPs not only may play a role in exacerbating pre-existing allergic antibody responses and allergic inflammation, but DEPs can also be a factor in driving primary sensitization to inhaled allergens in human subjects. Indeed, a recent epidemiologic study by Heinrich et al²⁵ shows that sensitization to common aeroallergens as assessed by skin prick test results and allergen-specific serum IgE levels was more common for children in a German county strongly affected by industrial pollution than in a nearby nonpolluted county. DEPs share many characteristics with other xenobiotics (eg, tobacco smoke and fly ash), including having a particulate phase and the presence of many similar PAHs. The increased presence of DEPs and similar airborne materials therefore may cause those with the appropriate genetic predisposition to become sensitized to allergens to which they may not otherwise have become sensitized.

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Correction

The following correction applies to the article by Molet et al entitled "Adoptively transferred late allergic response is inhibited by IL-4, but not IL-5, antisense oligonucleotide," which appeared in volume 104, number 1, p 205-14, 1999, of The Journal.

The phosphorothioate oligonucleotide sequences reported on p 206 were incorrect and should be, instead:

5'-TGAGACCCATCAATAGCTCTCTACAGTCAG-3'	IL-4
5'-AACTTAATCCCCAATAAAGTGAAGAAAA-3'	IL-5
5'ATAGAGCGAGGGTCAGTCCA-3'	NPY