House dust extracts have both T_H2 adjuvant and tolerogenic activities

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Background: Although mechanisms remain a subject of controversy, there is general agreement that living environments influence allergic risk during the first years of life. We reasoned that sterile house dust extracts (HDEs) would have immunologic activities reflective of their environments of origin and therefore would be useful surrogates for investigations of how ambient exposures influence immune homeostasis.

Objective: These experiments determined how airway HDE exposures influence adaptive responses to a coadministered antigen and subsequent airway hypersensitivity responses to antigen challenge.

Methods: Mice received intranasal ovalbumin (OVA) vaccinations on a weekly basis. Select groups of mice also received intranasal HDE weekly with OVA; daily at one seventh the weekly dose, beginning 7 days before the first OVA sensitization; or both.

Results: Weekly intranasal vaccinations with OVA and HDE primed mice for the development of T_H2-biased immune and airway hypersensitivity responses. In contrast, daily low-dose intranasal HDE exposures protected against the immunologic and pathologic outcomes associated with weekly intranasal OVA/HDE vaccinations. The T_H2 adjuvant activities of HDEs were found to be dependant on MyD88, a molecule critical for signaling through a majority of Toll-like receptors. Moreover, the tolerogenic activity associated with daily intranasal HDE exposures could be replicated with LPS.

Conclusion: These investigations demonstrate that in addition to allergens, living environments contain immunomodulatory materials with both T_H2 adjuvant and tolerogenic activities.

Clinical implications: As the contents of HDEs are ubiquitous, these experiments might recapitulate and help explain clinically relevant immunologic events involved in the maintenance of aeroallergen tolerance and the dysregulated responses that lead to allergic respiratory diseases. (J Allergy Clin Immunol 2006;117:1074-81.)

Key words: *Hygiene hypothesis, toll-like receptor, house dust, allergy, asthma, tolerance, innate immunity*

Abbreviations used

BALF: Bronchoalveolar lavage fluid
BLN: Bronchial lymph node
HDE: House dust extract
ISS: Immunostimulatory sequence
McPC200: Provocative concentration of methacholine inducing a 200% increase in Penh from baseline
OVA: Ovalbumin
P-3-C: Lipopeptide Pam-3-Cys
Penh: Enhanced respiratory pause
TLR: Toll-like receptor

During the last century, asthma and other allergic diseases have become far more common in the industrialized world, whereas in underdeveloped countries, atopy rates remain low.[1,2] Although a topic of intense speculation and investigation, the basis for these trends has yet to be determined. Nonetheless, the rapidity with which allergic disease prevalence has increased in affected countries strongly suggests that environmental factors have played an important role.[1-5]

Aeroallergen exposure is a clear prerequisite for the development and persistence of respiratory allergic diseases.[6,7] Nonetheless, although for some allergens (ie, cockroach and mites) higher exposure levels have been associated with an increased risk of sensitization, this correlation has not been found for other allergens (dogs, cats, and molds).[6,8] Furthermore, for allergens associated with animals, increased levels of exposure have been associated with decreased atopic risk in several studies.[7,8] These and other observations suggest that aside from allergens themselves, other immunomodulatory elements within living environments influence the balance between immune homeostasis and dysregulation. In support of this view, endotoxin, an immunostimulatory product of gram-negative bacteria that signals through Toll-like receptor 4 (TLR4), has been reported to be present at higher concentrations in homes with regular exposures to animals than in homes without animal exposures.[9,10] Moreover, in several published reports infants raised in homes with high ambient levels of endotoxin were found to have a low relative risk for development of allergic hypersensitivities.[9,11]

In consideration of these findings, it is important to note that endotoxin-rich environments also generally contain increased levels of other immunostimulatory microbial products, including ligands for additional TLRs.[12,13] Furthermore, several man-made pollutants have been found to

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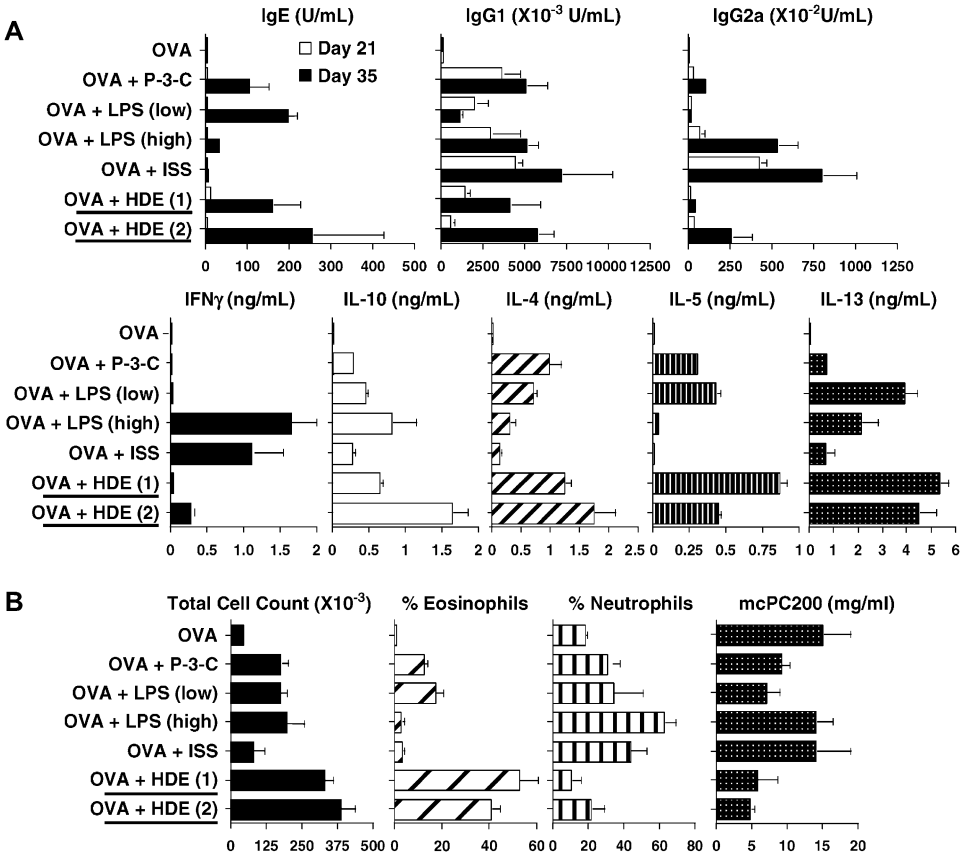

FIG 1. Adaptive and airway allergen challenge responses to weekly intranasal OVA/HDE vaccinations. Mice received 3 weekly intranasal immunizations with OVA (100 μg) alone or with P-3-C (50 μg), LPS (100 ng [low] or 20 μg [high]), ISS (10 μg), or HDEs (20 μL of 100 mg/mL solutions). Results are representative of those obtained with HDEs derived from 10 consecutive homes (see Table E2 in the Online Repository at www.jacionline.org). **A**, OVA-specific immune responses. **B**, Airway allergen challenge responses.

promote the development of allergic hypersensitivities.[14] Although much has been learned in recent years, such complexity in the content of daily exposures has hampered efforts to develop a holistic understanding of their impact on allergic risk. In this regard we reasoned that direct study of the immunologic activities of unpurified but clinically relevant environmental samples might prove enlightening. Sterile house dust extracts (HDEs) were chosen for investigation because we believed gravity would concentrate most, if not all, ambient immunomodulatory particulates present within living environments into settled dust. Furthermore, house dust allergen and endotoxin levels have already proven to be useful surrogate markers in epidemiologic studies of allergic risk.[6,9,11]

In previous studies we observed that bone marrow–derived dendritic cells produced cytokines and upregulated costimulatory molecule expression when cultured with HDEs.[15] Moreover, the capacity of HDEs to induce innate immune activation was found to be partially dependent on TLR2, TLR4, and TLR9 and almost completely dependent on signaling through MyD88, an intracellular docking protein required for signaling through most TLRs. Building on these observations, the present investigations characterized how airway HDE exposures influence adaptive responses to an experimental aeroallergen (ovalbumin [OVA]).

METHODS

Mice, OVA, and purified TLR ligands

Investigations received prior approval from our institution's animal welfare committee. Female mice aged 4 to 6 weeks were used for all studies. BALB/c and C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, Me), and MyD88 knockout mice (C57BL/6 background) were bred in our animal facility. Except for experiments with MyD88 knockout mice (Fig 2), BALB/c mice were used in all investigations. OVA (Grade VI; Sigma, St Louis, Mo), lipopeptide Pam-3-Cys (P-3-C; EMC microcollections, Tubingen, Germany), *Escherichia coli* 026-B6 LPS (Sigma), and immunostimulatory sequence (ISS) oligodeoxynucleotide (5′-TGACTGTGAACGTTCGAGATGA-3′; Trilink Biotechnologies, San Diego, Calif) were purchased from commercial vendors.

Preparation of HDEs

With approval from our institution's human subjects committee, dust samples were obtained by vacuuming a single carpeted bedroom in each of 10 suburban homes in San Diego County, California. Half

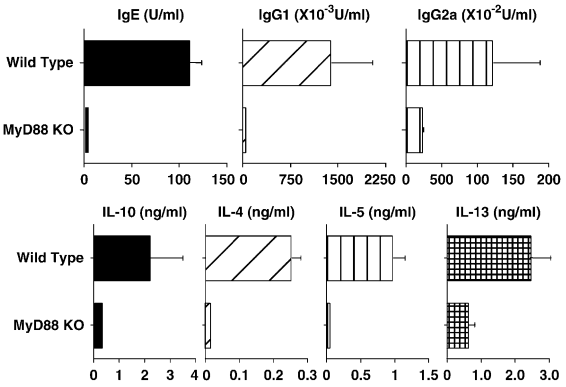

FIG 2. Adaptive responses of MyD88-deficient and control mice intranasally vaccinated with OVA and HDE on a weekly basis. Wild-type and MyD88-deficient mice received 3 weekly intranasal immunizations with OVA and HDE(5), as in Fig 1. IFN-γ was not detected in any culture supernatants. Similar results were obtained with 2 additional HDEs.

of the homes had regular exposures to indoor pets (dogs, cats, or both), and the rest had no identified animal exposures. Methods used for the collection and processing of house dust have been described in detail previously.[15] Briefly, study bedrooms were left unvacuumed for 1 week before exposed carpeting was vacuumed for 5 minutes with a Quick Broom (Hoover, Canton, Ohio). Collected house dust was then run through a coarse sieve to remove large particulate matter and suspended in sterile PBS at 1000 mg/mL or 100 mg/mL. House dust suspensions were then placed on a rotor at room temperature for 18 hours, filtered through glass wool, and finally filtered through 0.22-μm Steriflip filters (Millipore, Bedford, Mass) to obtain sterile HDEs. In studies presented herein, HDE concentrations refer to the amount of house dust added per milliliter of PBS suspension before filtration. Parentheses were used to identify individual HDEs in figures, tables, and legends.

Determining the endotoxin content, sterility, and toxicity of HDEs

HDE endotoxin levels were determined with the QCL-1000 kit (Bio-Whittaker, Walkersville, Md), according to the manufacturer's instructions. HDE stock solutions were assayed in duplicate at 25,000-fold, 125,000-fold, and 625,000-fold dilutions. By using previously described techniques, all HDEs were determined to be sterile and nontoxic before use.[15]

Vaccinations

For intranasal delivery, mice (n = 4 per group) were lightly anesthetized (isoflurane; Abbott Laboratories, North Chicago, Ill). Reagents were administered in a final volume of 30 μL of saline divided equally between the nares. Mice received 3 weekly intranasal immunizations with OVA (100 μg) alone or with P-3-C (50 μg), LPS (100 ng or 20 μg), ISS (10 μg), or HDEs. In experiments described in Table E1 (available in the Online Repository at www.jacionline.org), 1000 mg/mL HDE solutions were used (0.2-20 μL). For experiments described in Table E2 (available in the Online Repository at www.jacionline.org) and Figs 2 and 3, mice received 20 μL of 100 mg/mL HDE solutions. For Table E2 (available in the Online Repository at www.jacionline.org), an additional group of mice received intraperitoneal immunizations with OVA and alum (1 mg). In experiments described in Figs 3 and 5, select groups of mice were intranasally exposed to HDEs or LPS on a daily basis, beginning 7 days before the first and ending with the last OVA immunization. In these experiments mice were given one seventh the weekly dose of HDE (2.86 mL of 100 mg/mL HDE solutions) or LPS (14.3 ng).

Asthma challenge protocol

In select experiments mice were intranasally challenged with OVA (10 μg) on 2 occasions, spaced 5 days apart, beginning 3 weeks after the last OVA immunization. Twenty-four hours after the second dose of intranasal OVA, mice were exposed to increasing concentrations of nebulized methacholine, followed by measurements of pulmonary resistance (enhanced respiratory pause [Penh]) by means of body-box plethysmography (Buxco, Troy, NY), as previously described.[16,17] Methacholine responses are presented as the dose of methacholine leading to a doubling (200% increase) of the baseline Penh (McPC200). After methacholine challenge, mice were killed, bronchoalveolar lavage fluid (BALF) was obtained, and BALF cellularity was assessed by using standard techniques.[16,17]

OVA-specific Ig and cytokine responses

Sera were obtained 21 and 35 days after the first intranasal immunization with OVA to measure antigen-specific IgG1, IgG2a, IgE levels by means of ELISA. Samples were compared with high-titer anti-OVA IgG1, IgG2a, and IgE standards (end-point titration of 1.2×10^7 for IgG1, 1.5×10^5 for IgG2a, and 512 for IgE). To remove IgG and improve the sensitivity of the OVA-specific IgE ELISA, serum samples were preincubated with protein G sepharose beads (Pharmacia, Piscataway, NJ). Subsequent Ig ELISA techniques were routine and have been previously described.[16,17]

OVA-specific bronchial lymph node (BLN) cytokine responses were determined 4 weeks after mice received their vaccinations, as previously described.[16,17] Briefly, BLN cells were cultured in triplicate at 1×10^6 cells/mL in media with or without OVA (50 μg/mL) for 72 hours before harvesting supernatants. IL-4, IL-5, IL-10, IL-13, and IFN-γ levels in culture supernatants were compared with cytokine standards by means of ELISA with PharMingen reagents, according to the manufacturer's recommendations.

Assessment of HDE-induced desensitization

In Fig 4 experiments mice (n = 4 per group) received intranasal HDE daily (2.86 μL) for 1 week or as a single dose (20 μL). Twenty-four hours after the final intranasal HDE exposure, BALF samples were collected, and splenocytes were harvested. For cellular analyses, BALF samples were processed as previously described. BALF IL-6 and IL-12p40 levels were determined by means of ELISA with PharMingen reagents, according to the manufacturer's instructions. Splenocytes were cultured in triplicate at 2.5×10^6 cells/mL in media alone or with P-3-C (10 μg/mL), LPS (100 ng/mL), ISS (10 μg/mL), or HDE (1 mg/mL). Twenty-four hours later, culture supernatants were harvested, and IL-6, IL-10, and IL-12p40 levels were measured by means of ELISA.

RESULTS

HDEs are T_H2-biasing mucosal adjuvants in mice

Adaptive responses to airway antigen encounter are generally weak, and repeated exposures in the absence of adjuvant can induce tolerance.[18] Because immunostimulatory materials with adjuvant potential are present in both house dust and ambient air,[9,13,19] we determined whether airway HDE exposures could provoke an adaptive response to a codelivered antigen that by itself was poorly immunogenic. In initial dose-response experiments (see Table E1 in the Online Repository at www.jacionline.org),

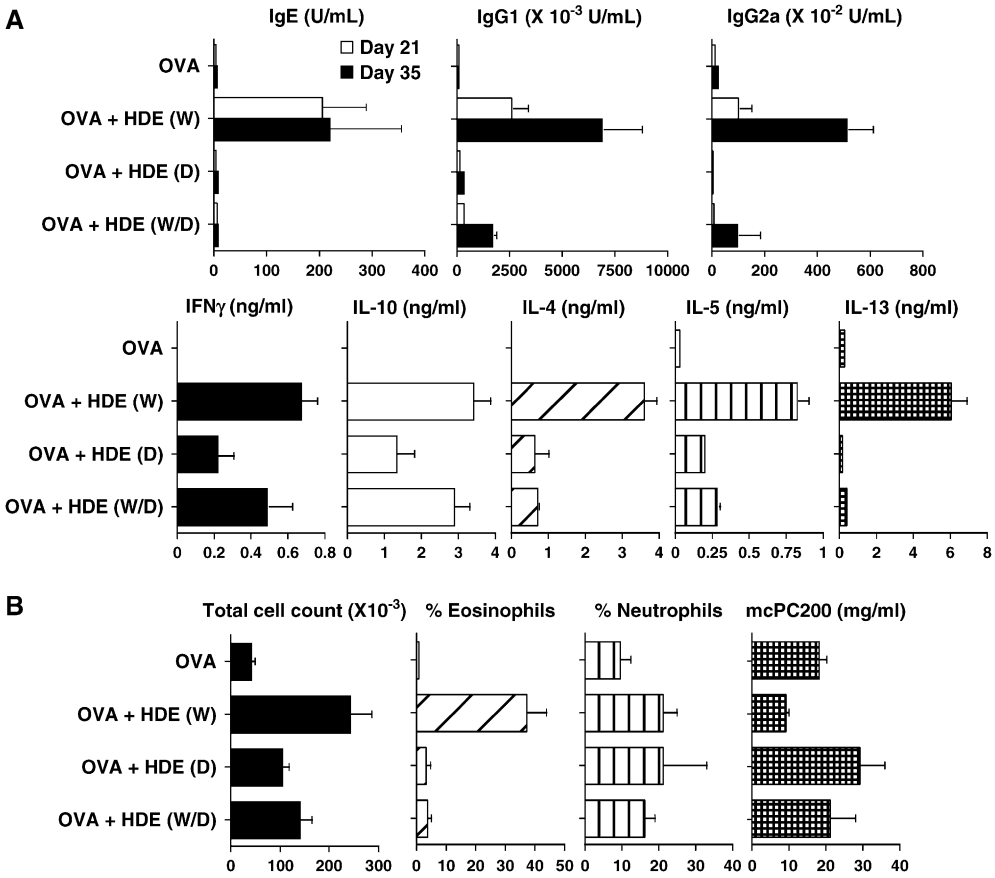

FIG 3. Influence of daily intranasal HDE exposures on adaptive and airway allergen challenge responses to weekly intranasal OVA/HDE vaccinations. Mice received 3 weekly intranasal immunizations with OVA, as in Fig 1. Select groups of mice were also treated with intranasal daily *(D)* low-dose HDE(2) (2.86 μL/d of a 100 mg/mL solution) for 21 days, beginning 7 days before the first and continuing through the day of the last OVA vaccination (60 μL total); weekly (*W*; 20-μL dose; 60 μL total) with OVA, as in Fig 1; or both (*W/D*; 120 μL total). Similar results were obtained in separate experiments with 2 additional HDEs. **A**, OVA-specific immune responses. **B**, Airway hypersensitivity responses.

we found that although mice intranasally immunized with OVA on a weekly basis had weak adaptive responses, mice coimmunized with bioactive doses of HDE had robust humeral responses characterized by increased serum levels of both OVA-specific IgE (T_H2 associated) and IgG2a (T_H1 associated). However, although OVA-stimulated BLN cells from mice receiving intranasal OVA/HDE produced detectable levels of IFN-γ, the hallmark cytokine of T_H1 cells, T_H2 cytokines, including IL-4, IL-5, and IL-13, were consistently secreted at higher levels.

Results described in Table E1 allowed us to identify an optimal HDE dose, which was used to compare the adjuvant activities of HDEs derived from 10 consecutive homes. At this dose (20 μL of 100 mg/mL solutions), HDEs contained from 11 to 132 ng of LPS, as determined by means of Limulus amebocyte lysate assay. Therefore control mice were intranasally immunized with OVA and LPS (TLR4) at 100 ng (low dose). Additional groups of mice were intranasally immunized with OVA and Pam-3-C (P-3-C; TLR2), high-dose LPS, or ISS (TLR9; Fig 1, *A*) or received intraperitoneal vaccinations with OVA and alum (see Table E2 in the Online Repository at www.jacionline.org), according to the same vaccination schedule. Consistent with previous reports,[16,20-22] OVA vaccination with alum, P-3-C, or low-dose LPS drove the development of T_H2-biased responses, whereas OVA vaccination with high-dose LPS or ISS favored development of T_H1-biased adaptive responses. Compared with these adjuvants, all HDEs studied were found to drive T_H2-polarized adaptive responses (Table E2 and Fig 1, *A*). It should also be noted that independent of the proinflammatory cytokines produced, OVA-stimulated BLN cells from mice coimmunized with OVA and HDE, P-3-C, LPS, or ISS also secreted IL-10 (Fig 1), an anti-inflammatory cytokine thought to be important in mucosal tolerance induction through the airways.[18]

Weekly intranasal vaccinations with OVA and HDE prime mice for experimental asthma

Mice described in Fig 1, *A*, were intranasally OVA challenged, beginning 3 weeks after their last immunization. Compared with mice intranasally immunized with

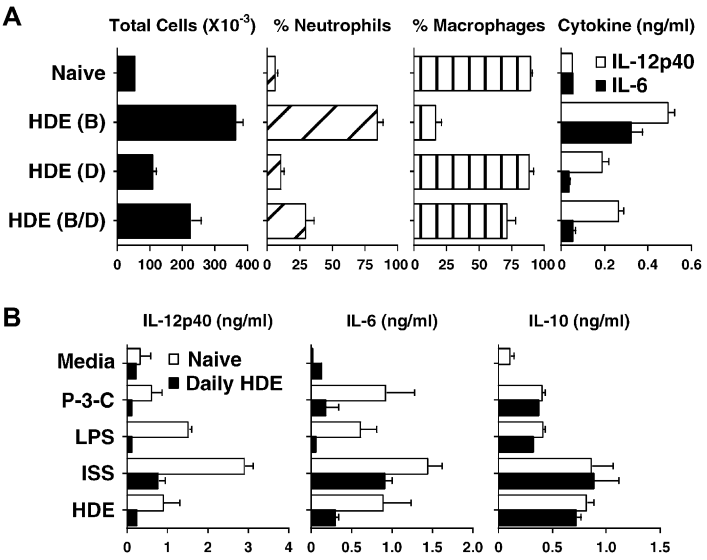

FIG 4. Influence of daily intranasal HDE exposures on airway and splenocyte responses to subsequent HDE challenge. Results are representative of independent experiments with 3 HDEs. **A**, Mice received nothing, intranasal low-dose HDE(1) daily *(D)* for 7 days, as a single high-dose bolus *(B)* on day 7, or both *(B/D)* at the same doses as used in Fig 2. On day 8, mice were killed, and BALF samples were collected for analyses. **B**, Mice received nothing or daily intranasal HDE, as in Fig 3, *A*. Splenocytes were harvested on day 8 and cultured with various stimuli. Supernatants were harvested for cytokine analyses 24 hours later.

OVA alone, mice receiving intranasal OVA vaccinations with P-3-C or low-dose LPS had stronger airway inflammatory responses characterized by higher BALF total cell counts with increased frequencies of eosinophils and neutrophils (Fig 1, *B*). In contrast, mice intranasally coimmunized with OVA and high-dose LPS or ISS had neutrophil-rich but eosinophil-poor airway inflammatory responses. In comparison with these control groups, intranasal OVA/HDE-vaccinated and airway allergen–challenged mice had the highest BALF cell counts and BALF eosinophil percentages (Fig 1, *B*). Although less sensitive and specific than invasive measures of bronchial hyperresponsiveness, which were unavailable at the time of these investigations, Penh measurements further suggested that mice immunized with OVA and P-3-C, low-dose LPS, or HDEs, but not high-dose LPS or ISS, had increased sensitivity to inhaled methacholine.

The T_H2 adjuvant activities of HDEs are MyD88 dependant

We did not initially anticipate that airway exposures to HDEs would prime mice for the development of T_H2-biased adaptive responses and airway hypersensitivities because HDEs contain TLR ligands, which, until recent reports,[16,20-22] were generally considered to protect against the development of allergic diseases.[1,9,11,17,23] Therefore the adjuvant activities of HDEs were next assessed in MyD88-deficient mice. Compared with wild-type control mice, MyD88-deficient mice were found to have markedly attenuated adaptive responses to intranasal OVA/HDE vaccination (Fig 2), confirming that the adjuvant activities of HDEs were TLR dependant.

Daily intranasal HDE exposures tolerize mice to their T_H2 adjuvant activities

Experiments presented thus far in this article might be construed to suggest that many, if not all, living environments intrinsically promote the development of T_H2-biased airway hypersensitivities. However, these studies fall short of modeling clinical airway exposures in several ways. For example, endotoxin and other immunostimulatory elements are likely to be ubiquitous in inspired air,[9,11,19,24] suggesting that physiologic airway exposures are fairly continuous, whereas in our experiments (Tables E1 and E2 and Fig 1) mice were exposed to HDEs only once a week. Moreover, healthy human airways have relatively few neutrophils,[25] whereas the dose of HDE used for weekly vaccinations induced marked neutrophilic inflammation (Fig 4, *A*). Therefore in experiments designed to better approximate clinical circumstances, mice received 3 weekly intranasal vaccinations with OVA, as in Fig 1, whereas HDEs were intranasally delivered daily at one seventh the weekly immunization dose (2.86 μL; 100 mg/mL HDE solution), beginning 7 days before the first and finishing with the last OVA immunization (21 doses or 60 μL total), weekly (20 μL per dose or 60 μL total) with OVA, or daily and weekly (120 μL total). As seen in Fig 3, *A*, daily intranasal HDE delivery had little adjuvant effect on OVA-specific responses. Furthermore, daily airway HDE exposures attenuated T_H2-biased immune responses to concurrent weekly intranasal OVA/HDE immunizations, whereas BLN IFN-γ and IL-10 responses were relatively preserved (Fig 3, *A*). Finally, daily airway HDE exposures protected weekly intranasal OVA/HDE-immunized mice from

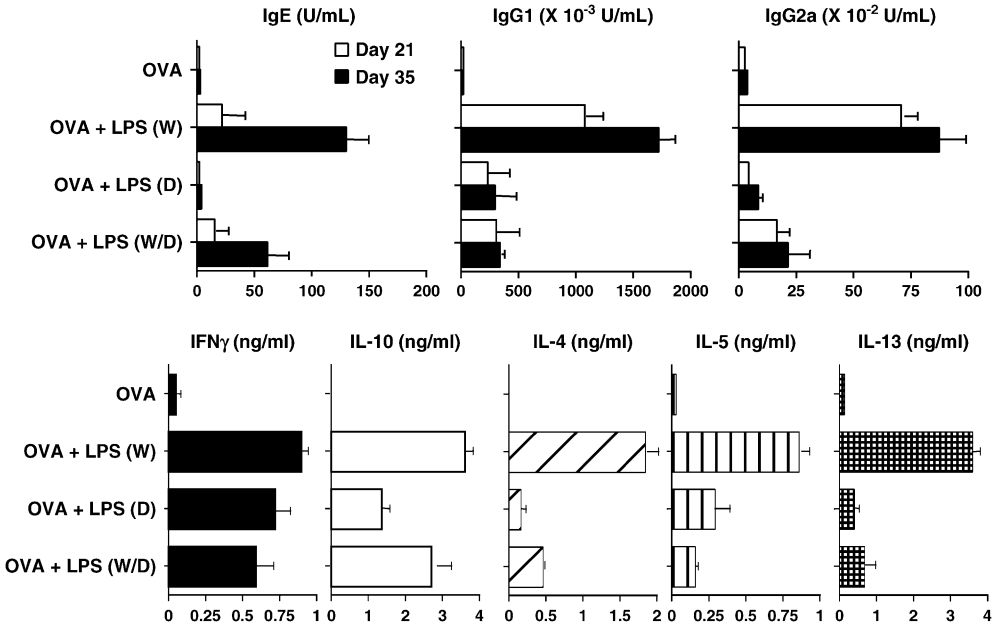

FIG 5. Influence of daily intranasal LPS exposures on adaptive responses to weekly intranasal OVA/LPS vaccinations. Mice received 3 weekly intranasal immunizations with OVA, as in Fig 1. Select groups of mice were also treated with daily *(D)* intranasal LPS (14.3 ng/d) for 21 days, beginning 7 days before the first and continuing through the day of the last OVA vaccination (300 ng total); weekly (*W*; 100-ng dose; 300 ng total) with OVA; or both (*W/D*; 600-ng total dose). Similar results were obtained in a replicate experiment.

having T_H2-biased airway hypersensitivity responses on subsequent intranasal OVA challenge (Fig 3, *B*).

Airway HDE exposures attenuate subsequent HDE responsiveness

Considered in a larger context, the results presented in Fig 3 could be explained if daily airway HDE exposures led to the desensitization of HDE responsive immunocytes. To determine whether innate airway responses to weekly high-dose HDE exposures were attenuated by daily low-dose exposures, mice were treated with HDEs, according to the delivery schedules outlined in Fig 3, until day 8, at which point BALF samples were collected to characterize airway inflammatory responses. Increased BALF cellularity was seen in all intranasally HDE-treated mice (Fig 4, *A*). However, mice receiving a single high-dose bolus of HDE delivered 24 hours before BALF recovery had the highest cell counts and a far greater percentage of neutrophils than those obtained from mice receiving both a high-dose bolus and daily HDE or daily HDE alone. None of the HDE delivery schedules selectively increased BALF eosinophil or basophil counts. In addition to their increased cellularity, BALF samples from mice treated with a single high dose of HDE consistently had higher IL-6 and IL-12p40 levels than samples from the other treatment groups (Fig 4, *A*).

Further studies determined whether daily intranasal HDE exposures induced HDE hyporesponsiveness distally in the spleen. Spleens were harvested from untreated mice and mice receiving daily intranasal HDE for 7 days. Splenocytes were then restimulated with purified TLR ligands or HDEs *in vitro* for 24 hours before culture supernatant cytokine levels were determined. Although daily intranasal HDE treatment was found to attenuate splenocyte IL-6 and IL-12p40 responses, IL-10 production was relatively unaffected (Fig 4, *B*). This systemic immunomodulatory influence of daily intranasal HDE exposures was not explained by changes in the cellular composition of spleens because flow cytometric analyses demonstrated that relevant cell types (plasmacytoid and myeloid dendritic cells, B cells, CD4 cells, CD8 cells, and natural killer cells) were present in the spleens of untreated and daily intranasal HDE-treated mice at similar frequencies (data not shown).

LPS mimics the T_H2 adjuvant and tolerogenic activities of HDEs

Because the bioactivities of HDEs appeared to be TLR dependant, a final series of experiments assessed whether they could be mimicked with purified LPS. Mice received 3 weekly intranasal OVA immunizations, whereas LPS was delivered either weekly at 100 ng (a dose shown to drive T_H2-polarized responses in Fig 1), daily at one seventh the weekly immunization dose (14.3 ng), or both in studies analogous to those described in Fig 3. As seen in Fig 5, compared with weekly delivery, daily intranasal LPS delivery had little adjuvant effect on OVA-specific responses. Moreover, as with HDEs, daily intranasal LPS exposures inhibited the development of T_H2-biased adaptive responses with concurrent weekly intranasal OVA/LPS vaccinations.

DISCUSSION

The effect of environmental exposures on the genesis of allergic respiratory diseases has been the focus of some investigation and far more controversy. Allergens present in ambient air play a role in driving the development of specific hypersensitivities.[6-8] However, evidence suggests that living environments are also rich in additional factors (ie, TLR ligands and industrial pollutants) that directly activate innate immunity and thereby indirectly influence responses to airway allergen encounter.[9,11,14,15] Results presented herein might help elucidate how innate immune activation by living environments contributes to the maintenance of aeroallergen tolerance and the development of respiratory allergic diseases.

The finding that weekly vaccinations with OVA and HDE elicited T_H2-polarized adaptive responses (see Tables E1 and E2 in the Online Repository at www.jacionline.org and Fig 1, *A*) and airway hypersensitivities (Fig 1, *B*) would at first appear to conflict with the popular belief that environmental TLR ligand exposures inherently protect against allergic hypersensitivities. One possible explanation is that HDEs have fundamentally different adjuvant activities in mice and human subjects. However, arguing against this, purified TLR ligands with T_H1- and T_H2-polarizing activities in mice[16,21,23] have generally been shown to have similar bioactivities in human studies.[20,26,27] Furthermore, if the immunostimulatory contents of HDEs promoted T_H1-biased, rather than T_H2-biased, responses in human subjects, one might predict that T_H1-biased airway hypersensitivity states would be common and T_H2-biased aeroallergen hypersensitivities would be rare, whereas epidemiologic studies have established the reverse to be true.[1-3]

TLR2, TLR4, and TLR9 ligands have been shown to be ubiquitous in homes and ambient air.[12,13,15,19,24] Of these, TLR2 and TLR4 ligands act as T_H2 adjuvants under at least some vaccination conditions (Fig 1, *A*),[16,22,28] whereas TLR9 ligands have consistently been described as T_H1 adjuvants.[16,17,23] These considerations implicate but certainly do not prove that TLR2 ligands, TLR4 ligands, or both were responsible for the T_H2 adjuvant activity associated with HDEs. The current results further suggest that living environments do not protect against the development of respiratory allergic diseases by driving the development of protective T_H1 responses. As previously reported with purified LPS,[28] Fig 2 demonstrates that MyD88 has a central role in mediating the T_H2 adjuvant activities of HDEs. Finally, consistent with previous[22,29] and current murine investigations with LPS (Fig 5), Fig 3 demonstrates that the dose and frequency of delivery significantly affects whether airway HDE exposures promote or antagonize the development of aeroallergen hypersensitivities.

In considering the clinical relevance of these studies, it should be noted that HDE delivery to the airways at doses providing T_H2 adjuvant activity also induced neutrophilic airway inflammation (Fig 4, *A*), a feature not characteristic of healthy human airways.[25] In contrast, intranasal low-dose HDE delivery on a daily basis failed to induce a neutrophilic airway inflammatory response, indicating that this delivery schedule might be more reflective of physiologic exposures to immunostimulants in inspired air (Fig 4, *A*). Furthermore, daily airway HDE exposures not only provided little adjuvant activity but also attenuated the development of T_H2 airway hypersensitivities with concurrent weekly OVA/HDE vaccinations (Fig 3). These results suggested that daily low-dose HDE exposures led to a global dampening of HDE responsiveness. In line with this view, daily intranasal HDE treatment also blunted both innate inflammatory responses to high-dose intranasal HDE challenge (Fig 4, *A*) and splenocyte responses to secondary *in vitro* HDE stimulation (Fig 4, *B*). Interestingly, Braun-Fahrlander et al[9] have similarly reported that PBMCs from children raised in homes with high ambient endotoxin levels had attenuated responses to LPS stimulation *in vitro* relative to PBMCs from children raised in homes with low ambient endotoxin levels.

Although these investigations established that daily low-dose airway HDE exposures render mice resistant to their adjuvant activities, they did not identify the molecular basis for this phenomenon. Nonetheless, because LPS mimicked the tolerogenic activities of HDEs (Fig 5), it seems likely that TLR4 and possibly other TLRs played a role. It has long been recognized that LPS has the capacity to desensitize the innate immune system to its immunostimulatory activities and the immunostimulatory activities of other TLR ligands.[30,31] Furthermore, as previously described with LPS,[18,32,33] daily airway exposures to HDEs inhibited splenocyte IL-12p40 and IL-6 (proinflammatory cytokines) responses to *in vitro* HDE stimulation (Fig 4, *B*), whereas production of IL-10, a cytokine important in the development of allergen-specific tolerance, was unaffected. In addition, daily intranasal HDE delivery inhibited the development of T_H2 hypersensitivities with concurrent weekly intranasal OVA/HDE immunization, whereas OVA-specific IL-10 responses were relatively preserved (Fig 3). These observations implicate IL-10 as having a role in mediating the tolerogenic influence of daily airway HDE exposures. However, although IL-10 can render cells hyporesponsive to LPS stimulation *in vitro*,[32,34] mononuclear cells from IL-10–deficient mice have been observed to exhibit LPS tolerance.[34] Therefore it seems probable that IL-10 contributes but is not the central regulatory molecule responsible for the inhibitory influence of daily HDE exposures on innate and adaptive immunity.

Recognizing that immunostimulatory elements are ubiquitous in inspired air[19,24] but that levels vary widely, the present results suggest a new paradigm by which ambient exposures affect airway immunity and allergic risk during the first years of life. According to this model, basal exposures to endotoxin and other immunostimulatory materials present in environments of daily living are generally not sufficient to provide adjuvant activity in the airways but rather serve to attenuate innate responsiveness. However, periodic exposures to ambient air with high

levels of immunostimulatory elements might provide sufficient adjuvant activity to induce a breakdown in allergen tolerance if *a priori* immunologic dampening by basal exposures is inadequate. Although far from proven, this model provides an alternative view of how ambient environmental exposures to materials with adjuvant activities can also provide protection against the development of hypersensitivities. Furthermore, because HDE bioactivities appeared to be mediated by TLRs, these observations offer a novel rationale for proposing that daily inhaled pharmacotherapy with low doses of TLR ligands could prove effective for maintaining aeroallergen tolerance in children with high allergic risk.

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