

Defining a role for ambient TLR ligand exposures in the genesis and prevention of allergic diseases

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Abstract Environmental variables responsible for the increasing allergic disease burden observed in developed countries over the last century have yet to be adequately characterized. Meta-analyses of epidemiological studies presented in the first half of this paper demonstrate a correlation between farm-associated exposures (i.e., livestock, pets, unpasteurized milk, and endotoxin) and a reduction in allergic risk during childhood. Laboratory investigations discussed in the second half of the paper characterize the intrinsic immunostimulatory activities of living environments. Considered together, experimental findings presented herein suggest a novel paradigm by which early life home exposures to microbial products and other allergen-nonspecific immunostimulants modify allergic risk.

Keywords Hygiene Hypothesis · Toll-like receptor · Endotoxin · Environment · Allergy

Introduction

During the last century, asthma and other allergic diseases have become far more common in industrialized countries, while atopy rates remain low in most of the Third World [1–3]. Although reasons for these trends remain specula-

tive, the rapidity with which the prevalence of allergic diseases has increased in affected countries strongly suggests environmental factors have played a major role. Therefore, there is a great deal of interest in identifying ambient exposures responsible for the low and high allergic disease prevalence rates of poor and affluent countries, respectively.

Many investigators have considered the role of allergen exposure in the development of Th2-biased hypersensitivities. For some allergens (i.e., cockroach and house dust mite), the risk of developing hypersensitivities has been found to increase considerably when the home allergen burden increases above quantifiable threshold levels [4–6]. However, for other allergens (i.e., dogs, cats), increased levels of home exposure appear linked to a decreased risk of sensitization, both to the allergen of interest and to other unrelated allergens [6, 7]. These and other lines of evidence suggest that aside from allergens themselves, living environments contain additional molecules that influence the immunological balance between allergen-specific tolerance and hypersensitivity.

Epidemiological studies suggest that a number of allergen-independent environmental variables, including lifestyle choices (i.e., urban versus rural living) [8], diet [9], exposures to diesel exhaust and other man-made pollutants [10], and infectious and noninfectious exposures to microbes [1], influence allergic risk. One of the most consistent findings of these studies has been the reduced incidence of allergic hypersensitivities and diseases among children raised on farms [8, 11, 12]. However, the reason(s) why farm living reduces allergic risk remain speculative. As houses located on farms, particularly those with livestock, are rich in their microbial content [8, 13–15], it has been suggested that microbial stimulation educates host immunity in a manner that prevents dysregulated immune

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responses to ambient allergens. This theory, “The Hygiene Hypothesis”, is also supported by investigations in which other variables linked to microbial exposure, including pet ownership, family size, day-care attendance (community-acquired infections), vaccination status, antibiotic use, animal exposure, and infectious disease history were found to influence allergic disease risk [1, 8, 16].

Rural living, animal exposure, endotoxin, and allergic risk: meta-analyses of previously published investigations

As atopy prevalence rates are lower in farming than in nonfarming communities [8, 11, 12], a number of investigators have tried to identify specific farm-associated exposures that might protect against the genesis of allergic diseases both in rural and urban environments (i.e., animal and endotoxin exposures and unpasteurized milk ingestion). However, while associations between specific exposures and allergic risk have been found in some investigations, results of others have been inconclusive and/or inconsistent. To better understand the impact of farm, animal, and endotoxin exposures on allergic disease genesis, we conducted an exhaustive review of the literature and conducted meta-analyses of relevant studies. Three models were selected for the design of meta-analyses based on criteria that allowed maximization of the number of studies included in each analysis: (A) Farm (livestock and unpasteurized milk) and/or pet exposures versus atopy, (B) endotoxin exposure versus atopy, and (C) endotoxin exposure versus wheezing (non-atopic or atopic).

Initially, a MEDLINE search was conducted to identify articles published between 1966 to 2007 that assessed the influence of childhood environmental exposures on atopic risk, using the following search commands: “atopy, allergy, asthma, eczema, wheeze, or rhinitis” and “farm, endotoxin, dog, cat, livestock, or unpasteurized milk”. This search identified 6,549 papers of potential relevance to these meta-analyses. All abstracts were reviewed independently by two investigators. Abstracts obviously unrelated to the topic at hand were discarded. For the rest, copies of full articles were retrieved and reviewed. To be included in these meta-analyses, investigations were required to meet the following criteria: (1) they assessed for associations between environmental exposures during childhood and atopic risk, (2) they were considered to be of compatible design with other studies included in analyses, and (3) results were reported as odds ratios (ORs) to facilitate the execution of meta-analyses.

Of the initial 6,549 papers identified by MEDLINE search, 6,510 were excluded because they were considered

irrelevant, incompatible for comparative analyses with other selected studies, and/or did not report results as ORs, while results of 39 investigations were deemed appropriate for meta-analyses. Twenty-five studies that correlated farming lifestyle and/or pet ownership with the development of atopy were used for analysis A (Fig. 1). For analysis B, published results of 15 investigations were included (Fig. 2). Finally, for analysis C, 11 investigations were identified that used ORs to correlate endotoxin-exposure levels with atopic or non-atopic wheezing (Fig. 3).

Using ORs and confidence intervals (CIs) reported within individual investigations, we calculated pooled effects estimates (ORs and 95% CIs) using both fixed and random-effects models. Heterogeneity, a value which uses a chi-square test to determine goodness-of-fit, was calculated for each fixed-effects model. Significant heterogeneity was found between studies ($P < 0.1$) with fixed-effects modeling. Therefore, random-effects models were selected to calculate summary ORs and CIs presented within this paper, as these estimates tend to be more conservative, taking into account between-study and within-study sampling variability. Fixed-effects and random-effects models were run with the *R* statistical software package (Vienna, Austria) using the “rmeta” command.

Model A: farm and/or animal exposure versus atopy

Twenty-five studies were considered appropriate for inclusion in meta-analyses of associations between farm and/or animal exposures and the development of allergic stigmata (Fig. 1). In these investigations, atopic wheeze, eczema, allergic rhinitis, and/or conjunctivitis symptoms were used as criteria for defining atopy. Nine studies specifically considered whether living on a farm reduced the incidence of allergic manifestations. A meta-analysis of these studies confirmed that farm living had a substantial protective influence on the development of allergic stigmata (OR 0.74, 95% CI 0.61–0.91). When the meta-analysis was expanded to include ORs for all specific exposures (i.e., livestock on property, pet ownership, and unpasteurized milk ingestion) and their associated allergic risk, the summary OR did not change significantly (OR 0.72, 95% CI 0.65–0.81). However, when considered separately, livestock (cattle and pig) exposure and unpasteurized milk ingestion during childhood were associated with lower ORs of developing allergic stigmata (OR 0.54, 95% CI 0.36–0.81 and OR 0.67, 95% CI 0.59–0.75, respectively). Taken together, analyses considered in this section suggest that the protective influence of farm living on allergic risk is likely to be multifactorial.

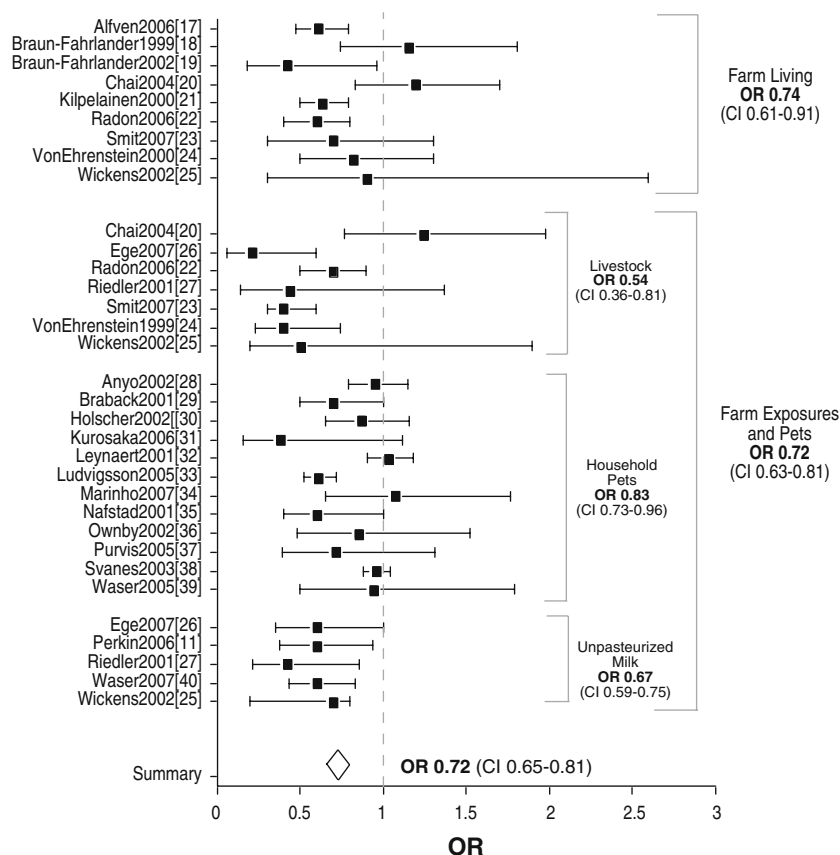


Fig. 1 Associations between farm-related exposures and allergic risk: Individual studies are identified by the first author and year of publication [11, 17–40]. ORs and 95% CIs are represented by *black squares with error bars*. If individual studies offered more than one relevant OR with CIs, summary ORs/CIs were calculated and

presented in the figure. Additional between-study summary ORs and CIs were determined for investigations identified within each meta-analysis. All ORs and CIs were calculated by variable-effects modeling

Model B: endotoxin and allergy

Ambient endotoxin levels can be easily quantified with the limulus amebocyte lysate assay and can be used as continuous rather than binary variables, making them an attractive molecular measure for use in epidemiological studies of allergic risk. Several research groups have shown that compared to control homes, endotoxin levels were higher on farms, particularly those with livestock [41], as well as in urban and suburban homes with pets [1, 42, 43]. These observations have prompted a number of investigators to determine if ambient endotoxin levels are predictive of allergic risk. Indeed, a number of studies have found that children raised in homes with high ambient endotoxin levels were less likely to develop allergic manifestations than children raised in homes with low endotoxin levels. However, other studies have not found a correlation between home endotoxin levels and allergic risk.

In our meta-analyses of ten relevant studies, subjects living in homes with high endotoxin levels were at a modestly reduced risk of having allergic symptoms (OR

0.86, 95% CI 0.73–1.0; Fig. 2). Even when stringent criteria (allergen skin prick test or radioallergosorbent test IgE results) were used to define atopy ($n=10$ studies), the association between home endotoxin levels and allergic risk remained essentially unchanged (OR 0.85, 95% CI 0.77–0.93). These meta-analyses suggest that living environments with high ambient endotoxin levels do not protect children from the development of allergic stigmata as effectively as farm-living environments and specific farm-associated exposures (i.e., livestock exposure and unpasteurized milk ingestion; Fig. 1). These findings further imply that either endotoxin is one of several molecules within living environments that protect against allergic stigmata or that endotoxin is a surrogate marker for other microbial products that are more effective at protecting against the allergic phenotype.

Model C: endotoxin and wheezing

Many investigations have considered endotoxin exposure as a risk factor for wheezing, with mixed results. This is not

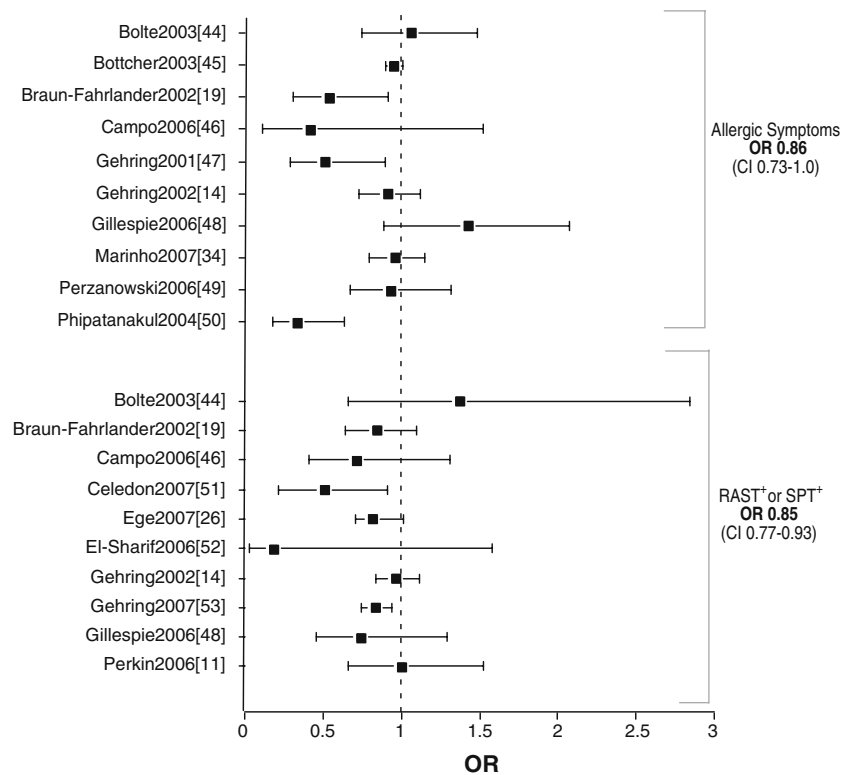


Fig. 2 Associations between endotoxin exposures and allergic risk: The Fig. 1 legend offers technical details relevant to the calculation of ORs presented in these meta-analyses [11, 14, 19, 26, 34, 44–53]

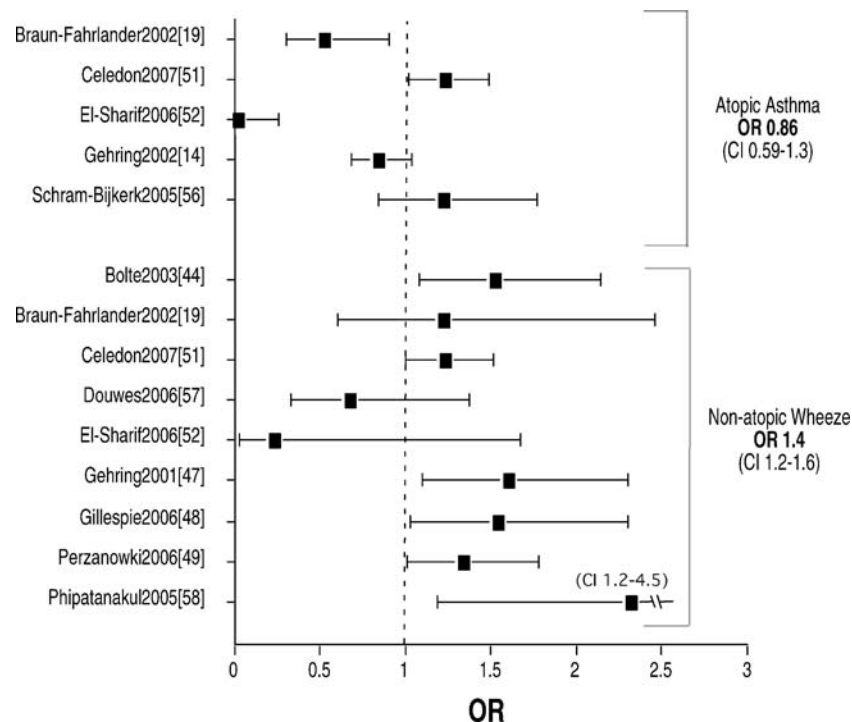


Fig. 3 Associations between endotoxin exposures and wheezing episodes: The Fig. 1 legend offers technical details relevant to the calculation of ORs presented in these meta-analyses [14, 19, 44, 47–49, 51, 52, 56–58]

completely surprising, given that while endotoxin exposures may influence development of allergic hypersensitivities and their associated clinical manifestations (Fig. 2), endotoxin is also a potent bronchospastic agent [54]. Consistent with these considerations, a number of studies have found that children exposed to high levels of endotoxin in their homes were less likely to have atopic wheezing episodes but more likely to have non-atopic wheezing episodes than children raised in low-endotoxin-exposure homes.

Our meta-analysis of five studies that used ORs to assess associations between home endotoxin levels and atopic wheezing suggests that high-endotoxin-exposure homes have a similar protective influence on atopic wheezing (OR 0.86, 95% CI 0.59–1.3; Fig. 3) as on other atopic manifestations (Fig. 2). Unfortunately, we could only identify a few epidemiological studies that specifically evaluated the influence of endotoxin-exposure levels on the wheezing risk of subjects known not to have allergic hypersensitivities. However, many studies have considered the effects of home endotoxin-exposure levels on wheezing in the first few years of life, a time when viral rather than allergen exposures are responsible for the vast majority of wheezing episodes [55]. Therefore, to consider the effects of endotoxin exposure on non-atopic wheezing, we combined studies with subjects specifically identified as nonallergic and studies in which subjects were aged 5 or less. In this meta-analysis of nine studies, children living in high-endotoxin-level homes were found to be at increased risk of having asthmatic episodes (OR 1.4, 95% CI 1.2–1.6).

Rationale for studying the immunological activities of house dust extracts

As previously discussed, evidence suggests that the immunomodulatory influence of living environments on allergic risk are multifactorial. Nonetheless, by design, a majority of investigations aimed at characterizing how living environments influence host immunity have made a priori assumptions about which exposures to pay attention to and which to ignore. As an alternative, we reasoned that the immunological “ether” associated with homes might be better understood by investigating the immunostimulatory activities of clinically relevant, sterile, but unpurified environmental samples. Logic suggests that gravity should concentrate immunostimulatory particulates into settled dust, and endotoxin levels have previously been found to be somewhat predictive surrogate markers of allergic risk (Fig. 2). Therefore, our laboratory has begun to characterize the immunostimulatory activities of sterile house dust

extracts (HDEs). Studies conducted to date have yielded provocative and reproducible results, which will be the focus of the following sections of this paper [59, 60, 62].

Dendritic cell activation by HDEs

In order to conduct experiments with HDEs, dust samples were first collected from bedrooms in 15 suburban homes in San Diego, California [60]. House dust samples were then processed by standardized techniques that included suspension in phosphate-buffered saline, physical agitation, and sterile filtration. The sterility of each HDE was confirmed by culturing an aliquot in bacterial growth medium and observing for the growth of microbes. In initial experiments, we determined whether HDEs could activate bone-marrow derived dendritic cells (BMDDCs) [60], as these cells have previously been shown to be highly responsive to purified toll-like receptor (TLR) ligands [61]. BMDDCs were cultured in serial dilutions of HDEs for 24 h before supernatant cytokine levels were assessed. BMDDCs cultured with Pam-3-Cys (P-3-C; TLR2), lipopolysaccharide (LPS; TLR4), or immunostimulatory sequence oligodeoxynucleotide (ISS; TLR9) were used as benchmarks for comparative analyses. While relative bioactivities varied widely, all HDEs studied induced concentration-dependent interleukin (IL)-6 and IL-12p40 responses by BMDDCs [60, 62]. Moreover, higher concentrations of most HDEs and optimized concentrations of TLR ligands elicited similar levels of IL-6 production. In contrast, LPS (TLR4) and ISS (TLR9) induced stronger IL-12p40 responses than any of the HDEs studied. In a subsequent study, we determined whether a sampling of HDEs induced the production of bioactive IL-12 (IL-12p70). HDE-induced BMDDC IL-12p70 responses were weak compared to those induced by LPS and ISS but similar to responses elicited by P-3-C [62]. Moreover, R848, a TLR7 ligand used as an additional control for this study, elicited BMDDC IL-12p70 responses that were tenfold greater than those induced by HDEs. Purified TLR ligands and HDEs also induced low levels of BMDDC IL-10 production, while IL-4, IL-13, and tumor necrosis factor (TNF)- α were not detected in any culture supernatants [60, 62]. In additional studies, HDE regulation of BMDDC costimulatory molecule expression was assessed. BMDDCs stimulated with HDEs displayed up regulation of CD40, CD80, CD86, and major histocompatibility complex (MHC) class II expression compared to unstimulated BMDDCs [60]. Moreover, costimulatory molecule expression levels were similar on BMDDCs activated with HDEs or purified TLR ligands.

Correlations between HDE endotoxin levels and bioactivities

Consistent with other studies, we found the mean endotoxin content of house dust samples obtained from homes with pets ($n=7$) was more than twice that for house dust samples obtained from homes without pets ($n=8$) [60]. In addition, while mean IL-6 responses were similar, HDEs from homes with pets elicited IL-12p40 responses that were 60% stronger than those of HDEs from pet-free homes. In further analyses, correlations between HDE endotoxin levels and BMDDC cytokine-inducing capacities were assessed [60]. Considered separately, HDEs from homes with and without pet exposures had correlation coefficients (r values) above 0.5, but they were not statistically significant by Z testing. However, while r values were not strengthened, correlations between endotoxin levels and IL-6 ($r=0.523$; $P=0.044$) and IL-12p40 ($r=0.573$; $P=0.024$) inducing activities did reach statistical significance when all HDEs were considered together. These experimental findings support the following assertions: (1) compared to pet-free homes, HDEs derived from pet-exposure homes have increased levels of endotoxin; (2) HDE bioactivities correlate loosely but significantly with their endotoxin content; and (3) endotoxin is not the only immunostimulatory molecule contained within HDEs.

The role of TLRs in HDE responsiveness

To further evaluate the contribution of TLR4 in mediating the bioactivities of HDEs, HDE-induced wild-type (WT) and TLR4 knockout (ko) BMDDC responses were compared [60]. Ten HDEs found to have the greatest bioactivity were selected for these studies. Compared to WT BMDDCs, TLR4 ko BMDDCs demonstrated a marked reduction in HDE-induced cytokine production and costimulatory molecule expression, but residual responsiveness remained. These experiments confirmed that while playing a role, TLR4 was not the only cellular receptor contributing to HDE responsiveness.

In additional experiments, WT, TLR2 ko, and TLR9 ko BMDDCs were cultured with HDEs, and cytokine production and costimulatory molecule expression profiles were again compared [60]. While HDE-stimulated TLR2 ko BMDDCs produced less IL-6 than WT BMDDCs, IL-12p40 production and costimulatory molecule expression were preserved. In contrast, HDE-stimulated TLR9 ko BMDDCs were found to produce less IL-6 and IL-12p40 than WT BMDDCs. Furthermore, while TLR4 ko BMDDCs displayed a greater deficit, HDE-activated

TLR9 ko BMDDCs expressed lower levels of costimulatory molecules than WT BMDDCs. These findings support the view that in addition to TLR4, both TLR2 and TLR9 contributed to the HDE-mediated BMDDC responses.

Experimental findings presented thus far suggested that TLR-signaling pathways play an important role in mediating HDE-induced BMDDC responses. Nonetheless, these results did not exclude the possibility that HDEs might also activate BMDDCs by completely TLR-independent pathways. Therefore, given that MyD88 plays a critical role in signaling through all TLRs except TLR3 [63, 64], a final series of experiments compared cytokine production and costimulatory molecule up regulation by HDE-activated WT and MyD88 ko BMDDCs [60]. In these studies, HDE-stimulated MyD88 ko BMDDCs were found to produce only trace amounts of IL-6 and IL-12p40, and only a slight increase in costimulatory molecule expression was observed. These results established that TLR signaling pathways play a critical role in BMDDC activation by HDEs.

The adjuvant activities of HDEs

In order to assess the adjuvant activities of HDEs, mice were intranasally (i.n.) immunized with ovalbumin (OVA) alone or with 21 μ l of HDE (100 mg/ml; concentration prior to filtration) on three occasions, at weekly intervals [59]. Additional groups of control mice were i.n.-immunized with OVA and Pam-3-Cys, LPS, or ISS, according to the same vaccination schedule. While adjuvant potential varied, mice i.n.-immunized with OVA and HDE had far more robust adaptive responses than mice i.n.-immunized with OVA alone, establishing that HDEs have adjuvant activities in the airways. Furthermore, HDEs ($n=10$) were consistently found to act as Th2-biasing adjuvants, as they induced strong allergen-specific IgE and Th2 polarized cytokine responses but weak IgG2a and IFN γ responses. In fact, most HDEs studied were more potent Th2 adjuvants than Pam-3-Cys or low-dose LPS, both of which have previously been found to be Th2 adjuvants [1, 59]. Moreover, the adjuvant activities of HDEs were dependent on MyD88 [59], further suggesting their dependence on signaling through TLRs. In addition to developing Th2-biased adaptive responses, mice immunized with OVA and HDE developed Th2-biased airway hypersensitivities, as reflected in their eosinophil-rich airway inflammatory response and increased bronchial responsiveness to methacholine after i.n. OVA challenge [59]. These results challenge the commonly held belief that microbial products in general, and TLR ligands in particular, inherently favor development of Th1-biased responses to allergens.

The tolerogenic activities of HDEs

Experiments just discussed might be construed to suggest that many, if not all, living environments intrinsically promote the development of Th2-biased airway hypersensitivities. However, in these studies, mice were airway-exposed to the immunostimulatory elements within HDEs at weekly intervals and at levels likely to be in great excess of daily physiological exposures. In contrast, individuals are thought to inhale air laced with low concentrations of immunostimulatory elements on a continuous basis [65]. Therefore, additional experiments were designed to better model real-world exposures. In these investigations, mice received three weekly i.n. OVA immunizations, as in previously described experiments, while low-dose HDE (1/7 weekly dose; 3 μ l) was i.n.-delivered daily, beginning 1 week before the first and ending with the last dose of OVA, weekly with OVA (as in the previous experiments), or both [59].

Daily i.n. HDE delivery had little adjuvant effect on OVA specific responses. More importantly, daily airway HDE exposures prevented mice concurrently receiving weekly i.n. OVA and HDE (adjuvant dose) from developing both Th2-biased adaptive responses and experimental asthma [59]. Additional unpublished studies determined whether i.n. daily HDE/weekly OVA delivery induced long-lasting allergen tolerance. In these studies, mice received a series of weekly i.n. OVA vaccinations either alone or with weekly adjuvant doses (21 μ l) or daily low doses (3 μ l) of HDE, as just described. Approximately 1 month after the last dose of OVA, all mice were OVA-sensitized by weekly i.n. OVA/adjuvant dose HDE delivery (three doses). Mice receiving i.n. OVA and daily HDE during primary immunization were found to be highly resistant to Th2 sensitization, while mice in other primary immunization groups (OVA alone or weekly OVA/adjuvant dose HDE) were not. Finally, it should be noted that HDE adjuvant and tolerogenic activities just described could be replicated with LPS [59].

Recognizing that immunostimulatory molecules are ubiquitous in inspired air but that levels vary widely [65], these experimental results suggest a new paradigm by which ambient exposures might modulate airway immunity and allergic risk during the first years of life. According to this model, basal levels of daily exposure to endotoxin and other immunostimulatory materials present in ambient air are generally not sufficient to provide airway adjuvant activity but rather serve to attenuate innate responsiveness to these molecules. However, episodic exposures to ambient air laced with high concentrations of immunostimulatory molecules can provide sufficient adjuvant activity

to induce a breakdown in allergen tolerance if prior 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.

Conclusions

Both epidemiological and laboratory investigations discussed in this paper strongly suggest that living environments have the potential to impact significantly on allergic risk. Nonetheless, understanding of the molecular variables and mechanisms responsible is far from complete. Studies discussed in the first half of the review demonstrate a correlation between farm, animal, unpasteurized milk and/or endotoxin exposures during childhood and a reduced incidence of allergic manifestations. In contrast, increased levels of ambient endotoxin exposure appear to increase the risk of non-atopic wheezing. However, as reviewed, many of these epidemiological trends have been inconsistently reported or have been found to be relatively weak. Moreover, these studies provide little insight as to the mechanisms by which living environments influence allergic risk.

Laboratory investigations described in the second half of this paper offer an alternative approach to characterizing how living environments modulate host immunity in general and allergic risk in particular. Early studies demonstrated the TLRs play a central role in sensing and responding to allergen nonspecific immunostimulatory molecules contained within HDEs and ubiquitous in living environments [60]. Additional i.n. vaccination studies revealed that weekly airway exposures to adjuvant doses of HDEs induced Th2-biased airway hypersensitivities to coadministered allergen, while daily HDE exposures promoted development of long-lived allergen tolerance [59]. The implication of these observations is that the primary immunological consequence of airway exposures to allergen-nonspecific immunostimulants present in living environments is either to promote the development of Th2-biased hypersensitivities or allergen tolerance but not to drive the development of Th1-biased responses to allergens.

In additional studies, we found that even the innate airway response to bolus HDE exposure (neutrophilic inflammation and cytokine release) is inhibited by pretreatment of mice with a week of daily i.n. low-dose HDE delivery [59]. The phenomenon of reduced responsiveness with repetitive exposure has previously been described with LPS tolerance and can be induced by other TLR ligands as

well [66–68]. Moreover, in unpublished studies, we found that daily i.n. HDE delivery increased local expression of mRNAs for molecules thought to mediate LPS tolerance (IL-10, STAT3, IRAKM, SHIP) [66, 68–70]. These observations may explain why human lungs remain uninflamed despite continuous inhalation of the pro-inflammatory molecules contained in HDEs [71]. Moreover, these findings suggest that mechanisms associated with LPS tolerance (innate immunity) could also play an important role in the physiological development of allergen-specific tolerance by non-atopic infants and toddlers, a focus of ongoing investigations in our laboratory.

If regular and adequate TLR stimulation drives the development of immunological and clinical tolerance to allergens ubiquitous in living environments by mechanisms associated with LPS tolerance, then exposure levels for individual molecules could prove far less important than the sum total of all ambient immunostimulatory molecule exposures in determining a child's allergic risk. This consideration may help to explain why epidemiological studies have yet to identify a specific molecule for which ambient exposure levels strongly and consistently correlate with relative allergic risk. Another implication of this view is that bioassays of HDE immunostimulatory activity may be highly predictive of the allergic risk associated with living environments. We are currently testing this hypothesis in ongoing investigations.

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