Environmental Exposure to Endotoxin and Decreased Risk of Childhood Atopy

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ABSTRACT
The hygiene hypothesis implies, in immunological terms, that microbial stimulation in early life skews the immune system towards the development of a T helper type 1 (Th1)-type lymphocyte population, and away from a T helper type 2 (Th2)-type development that is associated with atopy. The hygiene hypothesis originally theorized that increased infections protected against atopy. This theory has evolved since harmful infections may not be as critical as exposure to microbial burden because exposure to microbes can occur in the absence of infections. Despite substantial public hygiene measures in modern metropolitan communities, an atopy-protective effect of endotoxin in these metropolitan environments still occurs. This article is a review of clinical and experimental studies concerning the protective effect of endotoxin exposure on the susceptibility to atopic responses. The discussion includes: (1) factors influencing household endotoxin levels; (2) recent developments in the potential use of endotoxin for the prevention of atopy and asthma; (3) association of endotoxin and pets with atopic sensitization and diseases; (4) animal studies on the protective effects of timing of endotoxin exposure on atopy and asthma and (5) animal studies on the protective effects of endotoxin dose on atopy and asthma.

KEY WORDS
asthma, atopy, endotoxin, hygiene hypothesis, metropolitan environment

INTRODUCTION
The prevalence of atopic diseases in children is increasing steadily in high-income countries. Changes in environmental and life style factors probably play an important role in this phenomenon. However, the underlying mechanisms for this phenomenon remain unknown. The so-called “hygiene hypothesis” proposes that limited exposure to microbes during early childhood causes an insufficient stimulation of T helper type 1 (Th1) cells, which is unable to counterbalance an expansion of T helper type 2 (Th2) cells and subsequently causes a predisposition to atopy. This theory has recently attracted much scientific interest.1,2

The hygiene hypothesis originally theorized that an increased past history of infections protected against atopy. Over time, this theory has evolved in part due to evidence that harmful infections may not be as critical as exposure to microbial burden. This stems from the fact that exposure to microbes can occur in the absence of infections,1,2 since viable and non-viable components of microorganisms are distributed in varying concentrations in domestic environments. The present review will concentrate on epidemiological and experimental studies that examine the protective effect of endotoxin exposure on the susceptibility to atopic responses.

ENDOTOXIN AND INNATE IMMUNITY
Endotoxin, a lipopolysaccharide (LPS), comprises the majority of the outer layer of the outer cell membrane in all gram-negative bacteria. Its potent immune stimulatory capacity is largely attributed to the lipid A moiety, which is highly conserved across bacterial species. Endotoxin and other bacterial products, such...
as peptidoglycan, lipoteichoic acid, outer-membrane lipoproteins, flagellin, and DNA cytosine-phosphate-guanine (CpG) motifs, activate innate immunity, which is closely linked to host defenses and secondary adaptive immune responses. This process is mediated through Th1-type immune development. The potential of inducers of Th1-type cytokines including interleukin (IL)-12 and interferon (IFN)-γ like endotoxin and other bacterial components that abrogate Th2-mediated responses before its onset has been established.1,3,4

Members of the Toll-like receptor (TLR) family are essential components in this immune process.5 Ten TLRs (TLR1-10) have been identified in mammals and the current paradigm dictates that individual TLRs have distinct ligands. For example, (1) TLR4 is a receptor for endotoxin, (2) TLR2 controls cellular responsiveness to a variety of bacterial cell-wall components that include peptidoglycan, lipoteichoic acid, and outer-membrane lipoproteins, (3) TLR5 mediates bacterial flagellin-induced cell activation and (4) TLR 9 acts with DNA CpG-motifs at the cell surface. Members of the TLR family have some common structural features including an extracellular domain consisting of a signal peptide, multiple leucine-rich repeats, and a cysteine-rich domain, followed by a transmembrane region and cytoplasmic Toll/IL-1-receptor (TIR) domain. In general, the signaling pathway used by TIR domain-containing proteins includes MyD 88, IL-1 receptor-associate kinase (IRAK), and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6).

ENDOTOXIN AND THE DOMESTIC ENVIRONMENT

Endotoxins are ubiquitous in our environment. The exposure levels in our homes are below those found in the occupational environment. However, the literature contains reports to investigate the convenient measurable nature of endotoxin in home environments and illness associated with exposures. In contrast, bacterial products other than endotoxin in the domestic environment have not been widely reported. It should be noted that coexistence of high levels of endotoxin and other bacterial components in dust samples might raise fascinating possibilities of synergy with regard to immune modulatory, atopy-protective, or proinflammatory effects.

FACTORS INFLUENCING METROPOLITAN ENDOTOXIN LEVELS

Some studies have shown the presence of higher amounts of endotoxin in farm and rural homes compared with metropolitan sites.6,7 Recently, Roy et al.7 measured endotoxin and bacterial DNA levels in dust samples from four different settings: farm barns and farm homes in the United States, rural homes in India, and urban homes in Denver. Endotoxin and bacterial DNA levels in dust samples differed significantly by setting, with highest levels in farm barn samples and lowest levels in urban homes (Fig. 1). A correlation between bacterial by-products with a trend towards lower levels in urban homes is consistent with the hygiene hypothesis and indicates that endotoxin may be a good marker of bacterial load in environments where dust samples are collected.

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House dust endotoxin levels in metropolitan homes probably vary greatly. However, there is insufficient information available to explain how environmental and lifestyle factors influence endotoxin levels in house dust from metropolitan homes. Gereda et al.\(^8\) showed that homes with dogs and cats in metropolitan Denver had significantly higher levels of house dust endotoxin, and Fel d 1 and Can f 1 levels had a positive correlative trend with house dust endotoxin levels. In contrast, there was a negative correlation between household Fel d 1 and endotoxin levels in homes without dogs and cats. Therefore, factors that influence allergen and endotoxin levels in house dust were different in homes without animals. They also reported several home conditions not associated with endotoxin levels in house dust. These conditions included type of building, season of dust sample collection, home dampness, number of home inhabitants, environmental tobacco smoke, cleanliness, rats, hamsters, birds, and cockroach. Heinrich et al.\(^9\) supported the correlation between higher endotoxin levels in house dust from metropolitan homes with cats or dogs but not other animals.

Rabinovitch et al.\(^{10}\) measured personal exposures to endotoxin in schoolchildren with asthma using portable monitors that collect respirable particles, which were less than 10 μm in aerometric diameter. Personal endotoxin exposures were associated with the number of household inhabitants and the presence of pets. Alternatively, other studies found a lack of association between endotoxin and pets.

Aside from the home, more recent studies have highlighted contamination of endotoxin in metropolitan public child-care facilities (i.e., daycare centers, preschools, kindergartens, and elementary schools), where children spend a lot of their time. Rullo et al.\(^{11}\) assessed endotoxin levels in house dust from metropolitan public child-care facilities in Sao Paulo, and showed that levels of endotoxin in daycare centers and preschools were three times higher than in elementary schools. Consequently, daycare centers and preschools may be important sources of exposure to endotoxin in urban areas. Based on the hygiene hypothesis, this finding provides an explanation for a previous study\(^{12}\) that showed children, who attended daycare within the first six months of life, were less likely to have asthma and recurrent wheezing in later childhood.

Since endotoxin can exacerbate allergy and asthma symptoms, studies have been conducted to determine if frequent cleaning reduces endotoxin exposure. Unfortunately, various cleaning techniques do not appear to significantly reduce endotoxin exposure. For example, reported cleaning frequency of metropolitan homes or appearance of tidiness was not associated with lower endotoxin levels.\(^9\) Dairy industrial cleaning practices in child-care facilities and schools produced no difference in endotoxin levels in the morning compared with after cleaning in the late afternoon.\(^{12}\) On the other hand, less active forms of environmental control can lower detectable endotoxin. Use of both a dehumidifier\(^{13}\) as well as air conditioning in Denver homes was associated with lower endotoxin levels.\(^9\) However, these studies did not determine if reduced endotoxin levels were relevant to the prevalence of atopic sensitization or symptoms.

**Fig. 2** Correlations between concentrations of house-dust endotoxin and proportions of interferon-γ-producing CD4 and CD8 T lymphocytes (Ref 15).

**REVIEW OF RECENT DEVELOPMENT IN THE AREA OF EPIDEMIOLOGY**

**URBAN STUDIES**

Public hygiene measures are well implemented in modern metropolitan communities. However, an atopy-protective effect of endotoxin in these clear metropolitan environments still occurs.\(^{1,14}\) Gereda et
al. compared concentrations of endotoxin in house dust with allergen sensitization in infants at a high risk for developing asthma. The homes of allergen-sensitized infants contained lower concentrations of house dust endotoxin than those of non-sensitized infants (mean 468 vs. 1,035 EU/ml). Increased house dust endotoxin concentrations correlated with increased populations of IFN-γ-producing CD4 T cells (Fig. 2). Such concentrations did not correlate with populations of cells that produced IL-4, 5, or 13. This study may provide the first direct in vivo evidence that indoor endotoxin exposure early in life may protect against allergen sensitization by enhancing Th1-type immunity. Gehring et al. showed that in the cross-sectional study of 740 children from eastern Germany, between five and ten years of age, endotoxin levels were inversely associated with sensitization to one or more allergens. This is the first study to demonstrate an association of higher endotoxin exposure from house dust with a lower prevalence of atopic sensitization in children. On the other hand, Litonjua et al. investigated the longitudinal effects of exposure to house dust endotoxin on wheezing in 226 young children younger than five years over a four-year period. Exposure to high concentrations of house dust endotoxin greater than the median level (81.3 EU/mg dust) was associated with an increased risk for wheezing over the period of observation, but this risk rapidly decreased over time.

The results of the study by Bottcher et al. showed that lower levels of atopic diseases at two years of age were found in the population from Estonia where endotoxin exposures were higher than in the comparison population from Sweden. However, the study has some weakness. For example, only eight atopic children were studied from Estonia, and different sampling methods were used in the two populations. Therefore, more studies of comparable populations, including regional birth-cohort studies and international comparative studies, are necessary. In addition, other indoor factors, such as pollutants, allergens, toxins and social factors should also be investigated to clarify a possible causal effect of the levels of endotoxin.

### FARMING STUDIES

The collaborative Allergy and Endotoxin Study Team (ALEX) reported on the relationship between children’s mattress levels of endotoxin at school age and the occurrence of asthma and allergies. Braun-Fahrlander, a member of ALEX, reviewed epidemiological studies examining the effect of bacterial exposure on atopy and asthma in rural populations, as follows. Greater endotoxin exposure was associated with less allergen sensitization, hay fever, atopic asthma, and atopic wheeze in a dose-dependent manner. This effect was observed in addition to and independent of the effect of first-year exposure to farm characteristics and was not restricted to farming households. It was equally strong in children from non-farming homes, indicating that even lower levels of endotoxin, as encountered in non-farming environments, may favorably influence the risk of atopic diseases in childhood. The association between mattress endotoxin was also examined in relation to the cytokine production profile of peripheral blood leukocytes after activation of the immune system by stimulation with endotoxin and staphylococcal enterotoxin B. Greater endotoxin exposure was associated with reduced production of TNF-α, IL-10, IL-12 and IFN-γ, suggesting a general suppression of the capacity for cytokine production in response to activation of the innate immune system. Reduced responsiveness upon repeated stimulation with endotoxin is a phenomenon previously described in the literature as endotoxin tolerance.
group population, the blood cells of farmers’ children expressed higher amounts of CD14 and TLR2, which are innate immune receptors for bacterial products that include endotoxin. An increased expression of CD14 as well as TLR2 was also observed in human leukocytes after treatment with endotoxin in vitro, which suggests that the differences found in vivo between farmers’ and non-farmers’ children mirror different degrees of exposure to such bacteria or bacterial components in the environment.

Unfortunately, there is still no proof to support the premise that specifically endotoxin, of all the possible microbiological exposures experienced by farming children, was responsible for the protection afforded against atopic sensitization. As in the occupational setting, it could be that endotoxin is just one of a complicated microbiological milieu responsible for the immune response differences and that endotoxin is just a convenient, measurable marker of the environmental exposures experienced in these communities.

ASSOCIATION OF ENDOTOXIN AND PETS WITH ATOPIC DISEASES

The triad of endotoxin exposure, household pets, and atopic diseases / asthma in childhood has been extensively studied in recent years. A number of studies suggested that early pet exposure protected against atopy and asthma development. Many surveys showed a positive correlation between endotoxin and household pets. These results consistently supported an association between endotoxin and pets with a reduced risk for atopy and asthma development; however, no surveys demonstrate a clear relationship.

Litonjua et al. showed that exposure to cat allergen and the presence of a dog in the home, but not necessarily household endotoxin, were both associated with a decreased risk for wheezing. The negative association between exposure to dogs and cat allergen and wheezing appeared to be independent of the effects of endotoxin and suggested that separate mechanisms might mediate the effects of house dust endotoxin exposure and pet exposure on the developing immune system. Bolte et al. indicated that endotoxin had the potential to promote the development of Th1 cells, whereas cat allergen was associated with increased proportions of Th2 cells and both Th1- and Th2-like CD8+ cells.

Some epidemiological issues are relevant to interpreting the inverse correlation between pet exposure and a subsequent risk of atopic sensitization and asthma. In a birth cohort study of 4,089 two-month-old infants, pet ownership was lower in families with a parental history of atopic diseases. In another birth cohort study, cat allergen exposure, as monitored by levels found in the parental mattress, was lower in families with atopic mothers. Thus, parents with atopic diseases were likely to avoid owning pets and, therefore, exposure to pets. Consequently, children, who had pets, were less likely to be atopic on the basis of genetic predisposition. On the other hand, Celldon et al. performed a birth cohort study of 505 infants who were two to three months of age and whose parents had a history of atopy, and reported that maternal history of asthma was not associated with exposure to a cat or dog in their household. These unexpected results suggested that some of the variables (i.e., parent-reported history of asthma, allergic rhinitis, or eczema) might not be related to cat or dog allergy. The choice of variables to measure pet allergy in parents might also lead to different assessments of the correlation between endotoxin exposure, household pets and subsequent risk of atopic sensitization and diseases.

ANIMAL MODELS FOR PARADOXICAL ENDOXIN EFFECTS

Endotoxin is a potent proinflammatory substance that activates various cells in the respiratory tract, including epithelial cells, endothelial cells, polymorphonuclear leukocytes, macrophages, and mast cells after inhalation. As a result, inhaled endotoxin produces inflammatory responses, remodeling in the respiratory system, and hyperresponsiveness in the airway. On the other hand, as described above, endotoxin exposure in early childhood may paradoxically protect children from the subsequent development of atopy and asthma in later life. Experimental animal studies also suggest that the relationship between endotoxin and atopic immune responses and diseases may be complicated. Many parameters that include dose, timing of exposure, environmental cofactors, and genetics are probably relevant to this reduced risk of increased susceptibility to atopic responses.

PROTECTIVE EFFECTS OF TIMING OF ENDOXIN EXPOSURE ON ATOPY AND ASTHMA

In an IgE-sensitized rat model, Piebald-Virol-Glaxo rats were exposed to a single dose of inhaled endotoxin 1 day prior to sensitization and 1, 2, 4, 6, 8, and 10 days after sensitization to ovalbumin (OVA). On day 11, animals were exposed to aerosolized 1% OVA, and 24 h later inflammatory cell influx and microvascular leakage into bronchoalveolar lavage fluid (BALF) were measured. Pretreatment with inhaled endotoxin 1 day before and up to 4 days after OVA sensitization protected against the development of OVA-specific IgE and eosinophil and neutrophil influx into BALF. However, endotoxin exposure 6, 8, or 10 days after OVA sensitization further exacerbated the OVA-induced cellular influx and increased plasma leakage with no effect on OVA-specific IgE levels (Table 1). In addition, OVA-induced airway hyperresponsiveness to methacholine was abolished in an animal group pretreated with inhaled endotoxin 1 day before
Fig. 3 Effect of lipopolysaccharide (LPS, 20 μg/animal) administered subcutaneously 18 h or just (#) before an intravenous injection of Sephadex beads on an eosinophil count in the BALF from Sephadex-treated rats (upper panel). Effect of LPS (20 μg/animal) administered subcutaneously 18 h before an inhalation of LPS (30 μg/ml, 30 min) on a neutrophil count in the BALF from LPS-exposed rats (lower panel).

* P < 0.01 vs. saline-pretreated and saline-challenged animals (sham control). Results are expressed as means ± SEM (n = 5–6).

NS: not significant.

OVA sensitization. This study demonstrated that exposure to endotoxin modified the development of atopic sensitization and inflammation in vivo.

In an IgE-sensitized mouse model,45 Balb/c mice were systemically sensitized with OVA on days 1 and 14 and challenged with inhaled OVA on days 34 to 36. Serum OVA-specific IgE level, OVA-specific Th2 cytokine production by splenic mononuclear cells in vitro, and differential cell counts in BALF were assessed. Intraperitoneal administration of endotoxin before OVA sensitization reduced OVA-specific IgE, production of Th2 cytokines (IL-4 and IL-5) and extent of airway eosinophilia, as compared to sham pretreatment in OVA-sensitized mice. Nasal administration of endotoxin to sensitized mice before OVA challenge induced IFN-γ production by peribronchial lymph node cells in vitro, which was associated with reduced BALF eosinophilia. Interestingly, inhibitory effects of endotoxin on allergen sensitization and eosinophilic accumulation in BALF were inhibited by administration of anti-IL-12 antibodies before endotoxin exposure. These results indicated that systemic and local applications of endotoxin modulated systemic and local Th1/Th2 immune responses, probably in an IL-12-dependent mode. Most rodent models for atopic asthma consistently show that early exposure to endotoxin prevented atopic diseases and later exposures increased diseases.

Tulic et al.44 showed that co-administration of allergen with endotoxin modified both the acute and late-phase airway responses to the allergen, including an earlier acute change in lung function and a dose-dependent inhibition of late-phase responses to the allergen. Piebal-Viro-Glaxo rats were sensitized with OVA and 11 days later challenged with 1% OVA in the presence and absence of endotoxin given in the same nebulizer. Presence of endotoxin in the nebulizer during OVA challenge decreased the time-to-peak response for airway resistance and the late-phase airway hyperresponsiveness to methacholine, cellular influx into BALF and airway microvascular leakage at 24 h.

Eosinophilic infiltration into BALF of Sephadex (dextran) bead-treated animals are elicited via Th2 cytokines IL-4-, IL-5-, and IL-13-, eotaxin-, VCAM-1-, and ICAM-1-mediated pathways,45-47 indicating that a fundamental mechanism exists between eosinophilic infiltration into the lungs of Sephadex bead-treated and allergen-exposed animals. We used Sephadex bead-treated Wistar rats and showed that eosinophilic infiltration into BALF was remarkably reduced by endotoxin administered subcutaneously (20 μg/animal) before 18 h Sephadex bead administration. However, the same dose of endotoxin administered subcutaneously just before Sephadex bead treatment had no inhibitory effect on the airway response (Fig. 3, unpublished data). On the other hand, the same dose of endotoxin administered subcutaneously at the two different time-points had no effect on neutrophilic infiltration into BALF induced by nebulized endotoxin (30 μg/ml, 30 min) in Wistar rats (Fig. 3). The potential protective effects of endotoxin in the two-phase rat model of asthma44 and Sephadex-induced lung eosinophilia appear to be mediated principally via enhancement of Th1 function, thus providing feedback inhibition of atopy-associated Th2 immunity.3

Gerhold et al.48 investigated whether repeated exposure of infant mice to inhaled endotoxin inhibited subsequent allergen-induced immune and inflammatory responses. Infant Balb/c mice were pre-exposed to nebulized endotoxin three times weekly for the first four weeks of life before systemic sensitization...
with OVA (days 1–14) and repeated airway challenge (days 28–30) with OVA. Pre-exposed endotoxin before OVA sensitization failed to prevent production of OVA-specific IgE, Th2-mediated immune responses including IL-4 and IL-5 production by splenic mononuclear cells in vitro, and eosinophilic infiltration into BALF in OVA-sensitized mice. This study appears to be in conflict with previous epidemiological and animal studies supporting the hygiene hypothesis. Langenkamp et al. have recently shown that endotoxin-induced IL-12 production by dendritic cells was transient and exhausted dendritic cells subsequently preferentially induce Th2 or non-polarized T-cell responses, which may provide an explanation for these unexpected findings.

PROTECTIVE EFFECTS ENDOTOXIN DOSE ON ATOPY AND ASTHMA

Lower doses of endotoxin prime macrophages to release TNF-α and IL-12, while higher doses prime toxic radical productions (i.e., nitric oxide and its metabolites). Therefore, Th1-promoting immune responses may occur at lower doses. Eisenbarth et al. reported that signaling of low doses of endotoxin through TLR4 was necessary to induce Th2 responses to inhaled antigens in a mouse model of allergic sensitization. The mechanism by which endotoxin signaling caused atopic sensitization involved the activation of antigen-containing dendritic cells. In contrast, inhalation of high doses of endotoxin with allergen produced Th1 responses. These studies suggested that the dose of endotoxin determined the type of inflammatory response and provided a potential mechanistic explanation for epidemiological data on endotoxin exposure and asthma prevalence.

CONCLUSION

The hygiene hypothesis implies that microbial stimulation in early life skews the immune system towards the development of a Th1-type lymphocyte population and away from a Th2-type development that is associated with atopy. While epidemiological support for this theory is increasing, a cause-and-effect relationship between endotoxin exposure and a lower prevalence of atopy and asthma has not been proven; perhaps endotoxin is a marker for other microbial exposures or environmental or lifestyle factors that are actually preventing disease onset. Therefore, further prospective studies in different locales are necessary to determine if endotoxin is actually responsible for the atopy- and asthma-protective effect.

Since atopy is caused by both environmental and genetic factors, studies are needed to identify genetic variants that affect endotoxin sensitivity. The interaction of CD14 and endotoxin may represent the first documented gene and environmental interaction in the development of atopy and asthma. Variants in the CD 14 gene are associated with atopy in different populations all over the world. The polymorphisms of TLR4 gene were associated with a modified response to endotoxin, but the functional relationship requires clarification. Prospective cohort studies that measure the genotypes of CD14 and TLR4 genes and environmental exposures at different time-points in early life will enable us to better understand the interaction of the environmental and genetic factors. Microbe-derived immune modulatory therapies are currently undergoing clinical trials. The key parameters of timing, dosage, environmental co-factors, and genetics of endotoxin should be determined in order to provide effective and safe endotoxin-derived interventions for atopic diseases and asthma.

The difference in the route of endotoxin exposure has not been discussed in the present review, although the route of the gut is already considered an important route of exposure.

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