Infant home endotoxin is associated with reduced allergen-stimulated lymphocyte proliferation and IL-13 production in childhood

Joseph H. Abraham, ScD,[a,b,c] Patricia W. Finn, MD,[d] Donald K. Milton, MD,[b,c] Louise M. Ryan, PhD,[e] David L. Perkins, MD,[d] and Diane R. Gold, MD[b,c] *Boston, Mass*

Background: Infant endotoxin exposure has been proposed as a factor that might protect against allergy and the early childhood immune responses that increase the risk of IgE production to allergens.
Objective: Using a prospective study design, we tested the hypothesis that early-life endotoxin exposure is associated with allergen- and mitogen-induced cytokine production and proliferative responses of PBMCs isolated from infants with a parental history of physician-diagnosed asthma or allergy.
Methods: We assessed household dust endotoxin at age 2 to 3 months and PBMC proliferative and cytokine responses to cockroach allergen (Bla g 2), dust mite allergen (Der f 1), cat allergen (Fel d 1), and the nonspecific mitogen PHA at age 2 to 3 years.
Results: We found that increased endotoxin levels were associated with decreased IL-13 levels in response to cockroach, dust mite, and cat allergens, but not mitogen stimulation. Endotoxin levels were not correlated with allergen- or mitogen-induced IFN-γ, TNF-α, or IL-10. Increased endotoxin levels were associated with decreased lymphocyte proliferation after cockroach allergen stimulation. An inverse, although nonsignificant, association was also found between endotoxin and proliferation to the other tested stimuli.
Conclusion: Increased early-life exposure to household endotoxin was associated with reduced allergen-induced production of the T_H2 cytokine IL-13 and reduced lymphoproliferative responses at age 2 to 3 years in children at risk for allergy and asthma. Early-life endotoxin-related reduction of IL-13 production might represent one pathway through which increased endotoxin decreases the risk of allergic disease and allergy in later childhood. (J Allergy Clin Immunol 2005;116:431-7.)

Key words: *Endotoxin, lymphocyte proliferation, cytokine, childhood, allergy*

Abbreviations used
EU: Endotoxin units
OR: Odds ratio
SI: Stimulation index
TLR4: Toll-like receptor 4

Childhood allergic diseases, such as asthma and hay fever, are increasing in prevalence, cause chronic ill health, and are a substantial public health concern in developed countries.[1,2] Measurable childhood sensitization to inhaled allergens occurs primarily after the age of 3 years,[3] but the immunologic underpinnings of allergic disease and airway inflammation likely develop far earlier in life.[4,5] Manifestation of an allergic phenotype likely results from the complex interplay of genetic, developmental, and environmental influences. Adaptive immune processes, including activation of helper T lymphocytes and subsequent B-lymphocyte activation with IgE isotype switching, underlie the process of allergic sensitization in individuals genetically predisposed to development of allergic responses.[6] Although still controversial,[7,8] there is mounting evidence from animal models[9-13] and the epidemiologic literature[14,15] suggesting that exposure to endotoxin, a potent activator of innate immunity, might influence subsequent adaptive immune responses to allergen. Furthermore, these studies suggest that the timing and dose of endotoxin exposure influence the nature of the immune response, leading to the hypothesis that early life might be a crucial time window during which endotoxin might reduce the risk of allergy through its influence on innate immunity and downstream T-cell and B-cell regulation of cytokine and IgE expression.

The immunologic pathway linking endotoxin exposure to adaptive immunity and evidence for its effects on the development of allergic diseases have recently been reviewed.[7,16,17] Briefly, endotoxin is biologically active LPS, a primary component of the outer cell membrane of gram-negative bacteria.[18,19] Even minute amounts of endotoxin provoke innate immune responses *in vitro* and *in vivo*.[20] The nature of that response, which is only partially understood, might depend on the developmental

From the Departments of [a]Epidemiology, [b]Environmental Health, and [e]Biostatistics, Harvard School of Public Health, and [c]The Channing Laboratory, Department of Medicine, and [d]the Department of Medicine, Brigham and Women's Hospital and Harvard Medical School.
Disclosure of potential conflict of interest: None disclosed.
Supported by NIEHS R01 ES-07036; NIEHS 2P30ES00002; NIH/NHLBI HL07427-23; and AI/EHS35786.
Received for publication December 10, 2004; revised March 28, 2005; accepted for publication May 9, 2005.
Available online July 5, 2005.
Reprint requests: Diane R. Gold, MD, Channing Laboratory, 181 Longwood Ave, Boston, MA 02115. E-mail: diane.gold@channing.harvard.edu
0091-6749/$30.00

doi:10.1016/j.jaci.2005.05.015

stage of the organism (eg, infant or adult) and on the sequence and mode of exposure to endotoxin and allergen. Inhaled endotoxin activates the innate immune system by binding transmembrane toll-like receptors (TLRs) expressed on macrophages and dendritic cells[21] through IL-12 signaling.[22] Given the right timing of exposure and genotype, these endotoxin-activated antigen-presenting cells might stimulate production of IFN-γ and other T_H1 cytokines.[23] Through production of IFN-γ by T_H1-biased lymphocytes or through alternative pathways, endotoxin might downregulate T_H2 cytokine (including IL-13) secretion, IgE production, and consequent allergic disease. Although studied extensively in animal models, few prospective data are available on the effects of home LPS exposure on cytokine production in young children.

Using a prospective birth cohort with a parental history of allergy or asthma, we explored the hypothesis that early-life endotoxin exposure alters immune system responsiveness by examining the relationship between house dust endotoxin and PBMC responses to allergen stimulation. Specifically, we examined associations between house dust endotoxin levels measured 2 to 3 months after the child's birth and allergen- and mitogen-induced cytokine and proliferative responses of mononuclear cells isolated from peripheral blood sampled at age 2 to 3 years. Previously, we had found that increased endotoxin levels in infancy were associated with decreased risk of eczema in the first year of life, suggesting that endotoxin might have a protective effect against allergic disease and the biologic pathways influencing the risk of allergy.[24] We hypothesized that endotoxin levels would be positively correlated with levels of IFN-γ, a T_H1 cytokine, and inversely associated with levels of the T_H2 cytokine IL-13, which can mediate isotype switching to IgE.[25] We further hypothesized that endotoxin levels would be associated with decreased proliferative responses after allergen stimulation.

METHODS

Description of cohort

The Epidemiology of Home Allergens and Asthma study is a longitudinal birth cohort study of environmental predictors of allergy and asthma development. A description of the recruitment of study participants and study protocol has been previously published.[26] In brief, 505 children from 499 families with a parental history of asthma or allergy were enrolled in a birth cohort study designed to examine the effects of allergen exposure in early life on the development of asthma. The Brigham and Women's Hospital Human Research Committee approved the study. Informed consent was obtained from the parents for blood collection and longitudinal follow-up. Mothers in the greater Boston metropolitan area delivered at a large Boston hospital were screened with the following questions: (1) Have you ever had asthma, hay fever, or allergies? (2) Has the biologic father of your child ever had asthma, hay fever, or allergies? Mothers responding yes to either question were asked to complete a screening questionnaire. Families were not approached if the index child was premature, had a major congenital anomaly, or was in the neonatal intensive care unit or if the mother was less than 18 years old or could not speak English or Spanish. Informed consent was obtained from the parents for blood collection and longitudinal follow-up. The Brigham and Women's Hospital Institutional Review Board approved the study protocol.

Environmental sampling and endotoxin measurements

The home sampling protocol has been described previously.[27] A trained research assistant visited participants' homes within 2 to 3 months of the child's birth. During these visits, conducted between 1994 and 1996, detailed demographic, socioeconomic, parental disease history, and home characteristics questionnaires were completed, and standardized dust sampling was conducted in various sites within the home, including the family room. Dust samples to be used for endotoxin assays were stored desiccated at −20°C until extraction.

Endotoxin activity of dust samples was determined by using the kinetic Limulus amebocyte lysate assay with resistant-parallel-line estimation, as previously described.[28-30] The Limulus amebocyte lysate was supplied by BioWhittaker (Walkersville, Md). Reference standard endotoxin was obtained from the United States Pharmacopoeia, Inc (Rockville, Md), and control standard endotoxin was supplied by Associates of Cape Cod (Woods Hole, Mass). Results were reported in endotoxin units (EU) per milligram of dust adjusted to account for lot-to-lot variation in Limulus amebocyte lysate sensitivity to house dust endotoxin and referenced to the reference standard endotoxin EC5 and EC6 (US Pharmacopoeia, Inc; 1 ng of EC5 and EC6 = 10 EU).[30] Because of the prioritization schema for assaying dust samples, the availability of endotoxin measurements was conditional on there being sufficient dust to first assay for home allergens and fungi. As such, for 19 of the 115 subjects with biomarker outcome data, family room dust endotoxin levels were unavailable.

PBMC responses

At 2 to 3 years of age, blood sampling and analysis was conducted in a subgroup of the study participants (n = 115). As previously described, selection of this subgroup was based on the home allergen levels measured during the initial home visit.[31,32] The goal in choosing these subjects was to maximize variability in early-life exposure to the allergens with which their cells were to be stimulated.

PBMCs were isolated from this blood sample by using Ficoll-Hypaque centrifugation.[33] Fresh cells were incubated in media; media containing either 30 μg/mL cockroach allergen (Bla g 2), 30 μg/mL house dust mite allergen (Der f 1), or 1000 U/mL cat allergen (Fel d 1); or media plus 10 μg/mL PHA. Optimal stimulant concentrations used for the assay were determined in a prior dose-response analysis.[31]

At 24 and 60 hours after the initiation of stimulation, supernatants were harvested, and cytokine concentrations were quantified by means of ELISA (Endogen, Woburn, Mass). On the basis of prior optimization for detection of cytokine levels, IL-10 and TNF-α were measured in the 24-hour samples, and IFN-γ and IL-13 were measured in the 60-hour samples. The lower limits of detection for cytokine assays were as follows: IFN-γ, less than 2 pg/mL; IL-13, less than 7 pg/mL; IL-10, less than 3 pg/mL; and TNF-α, less than 5 pg/mL. Because supernatant quantities were limited, cytokine assays were prioritized, with IFN-γ levels being measured first. As a result, fewer subjects have observations of IL-13, IL-10, and TNF-α levels.[32]

After incubation of PBMCs with allergen or mitogen for 72 hours, 1 μCi of tritiated thymidine was added to each well. After incubation for an additional 8 hours, the cells were harvested, and tritiated thymidine uptake was determined by means of β-counting.