Environmental conditions, immunologic phenotypes, atopy, and asthma: New evidence of how the hygiene hypothesis operates in Latin America

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Background: It has been proposed that improved hygiene and reduced experience of infections in childhood influences the development of allergic diseases. The mechanisms by which the hygiene operates are not well established but are underpinned by two apparently incompatible immunologic paradigms, the balance of T_H1 versus T_H2 cytokines and IL-10–mediated regulation of T_H2 cytokines.

Objective: This study defined immunologic phenotypes with the use of latent class analysis and investigated their associations with environmental factors, markers of allergy and asthma, in a Latin American population.

Methods: We studied 1127 children living in urban Brazil. Data on wheeze and environmental exposures were collected with standardized questionnaires. Atopy was measured by specific IgE in serum and skin prick test reactivity to aeroallergens. Cytokines were measured in culture after the stimulation of peripheral blood leukocytes with mitogen. Infections with pathogens were assessed by serology and stool examinations. Children were classified as having high or low burden of infection. Latent class analysis was used to identify immune phenotypes on the basis of cytokine production. Logistic regression was used to evaluate the adjusted effects of environment and burden of infection on the immunologic phenotypes and the effect of the phenotypes on atopy and asthma.

Results: Three phenotypes were identified, labeled underresponsive, intermediate, and responsive. Children of more educated mothers, living in improved environmental conditions, and with a low burden of infection were significantly more likely to have the responsive phenotype. The responsive phenotype was significantly associated with an increased prevalence of atopy but not asthma.

Conclusion: Our findings contribute to a better understanding of the immune mechanisms by which the hygiene hypothesis operates in urban Latin America. (J Allergy Clin Immunol 2013;131:1064-8.)

Key words: *LCA, environment, infections, immune phenotypes, children, hygiene hypothesis, SCAALA*

The increase of allergic diseases in the industrialized world and more recently in lower and middle income countries has been explained by a decline in infections during childhood, the so-called "hygiene hypothesis."[1,2] The first proposed immunologic explanation underpinning the hygiene hypothesis was that bacterial and viral infections during early life shift the balance of the maturing immune system toward T_H1, away from proallergic T_H2 responses, and that a reduction in microbial burden leads to weaker T_H1 responses, thus weakening the control of T_H2 responses that cause allergy. Subsequent research pointed out that this failed to explain the low prevalence of allergy in populations with T_H2-skewed parasitic helminth infections[2,3] and the increase in T_H1-related autoimmune diseases.[1-3] An alternative immunologic explanation was put forward, postulating that both parasitic and microbial infections lead to an increase in anti-inflammatory cytokines, such as IL-10, that downregulate both T_H1 and T_H2 responses, causing a decrease in allergy and autoimmune diseases.[1,3] Here, we investigate these two apparently incompatible immunologic paradigms in a Latin American population, which is undergoing rapid changes, including urbanization, migration, economic development, and adoption of a westernized lifestyle. Investment in improvements in water supply, sanitation, waste collection, and other hygienic measures have occurred in many Latin American countries over recent years,[4,5] raising the possibility that these important interventions may have had unexpected consequences for the development of allergic diseases, which are reaching epidemic levels in the region.[6,7] The context of Latin America, therefore, is ideal for the study of this dynamic and environment-dependent immunologic process.

We have previously demonstrated in this group of children living in urban Brazil that (1) those with a high burden of current or past infections had a decreased risk of atopy[8] and (2) the proportion of children producing mitogen-induced T_H1 and T_H2 cytokines by peripheral blood leukocytes was lower among those with poor living conditions and that this suppressive effect was stronger in children producing IL-10.[9] In the present study, we define immune phenotypes according to patterns of cytokine production and analyze the associations between these phenotypes

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Abbreviations used
BMI: Body mass index
HAV: Hepatitis A virus
LCA: Latent class analysis
OR: Odds ratio
sIgE: Specific IgE
SPT: Skin prick test
Treg: Regulatory T cell

and environmental exposures and markers of infection and also their effects on atopy and asthma symptoms.

METHODS

Study population and design

This study was conducted in the city of Salvador in Northeastern Brazil that has a population of 2.5 million (see Table E1 in this article's Online Repository at www.jacionline.org). The design of this study has been reported in details elsewhere.[8,10-12] In short, the study population included children living in poor neighborhoods of a Brazilian city that were recruited before 3 years of age in a previous study that measured the effect of a citywide sanitation program on childhood diarrhea.[13] At baseline, data on demographic characteristics and social variables as well as on the home environment and stool samples were collected. In 2005, stool and blood samples were obtained and the International Study of Asthma and Allergies in Childhood Phase II questionnaire was administered for 1445 of these children. Ethical approval for the study was obtained from the Brazilian National Ethical Committee, and written informed consent was obtained from the legal guardian of each child.

Paired stool samples were collected and analyzed for parasites from each child at each of the 2 sampling times. Exposure to *Toxoplasma gondii*, *Helicobacter pylori,* herpes simplex virus, varicella-zoster virus, and Epstein-Barr virus were determined by measurement of specific IgG in sera with the use of commercially available immunoassays (Diamedix, Miami, Fla). Exposure to hepatitis A virus (HAV) was determined by the presence of anti-HAV IgG antibodies with the use of kits from Adaltis (Toronto, Ontario, Canada). The effect of markers of infection was analyzed by stratifying into light burden (presence of positive tests for 0 to 3 different infection markers) and heavy burden (4 to 8 markers), consistent with a previous study.[8]

Whole blood culture and measurement of cytokines

We collected venous blood into heparinized tubes and cultured the blood at a dilution of 1:4 in RPMI medium (Gibco, Auckland, New Zealand) that contained 10 mmol/L glutamine (Sigma-Aldrich, St. Louis, Mo) and 100 μg/mL gentamicin (Sigma-Aldrich). The cells were cultured within 6 hours of collection and were maintained in a humidified environment of 5% CO_2 at 37°C for 24 hours for detection of IL-10 and for 5 days for the detection of IL-13, IL-5, and IFN-γ in the presence of pokeweed mitogen (Sigma-Aldrich; 2.5 μg/mL) or media alone. We measured the production of T_H2 (IL-5 and IL-13), T_H1 (IFN-γ), and regulatory T cell (Treg; IL-10) cytokines in whole blood culture supernatant fluids with the use of commercially available antibody pairs and recombinant cytokine standards (BD Pharmingen, San Diego, Calif) by sandwich ELISA according to the manufacturer's instructions. Cytokine concentrations were determined by interpolation of standard curves. Responders were defined as those children with cytokine concentrations above the lower detection limits.[9,11]

Atopy and asthma

Skin prick tests (SPTs) were done on the right forearm of each child with the use of extracts (ALK-Abelló, São Paulo, Brazil) of *Dermatophagoides pteronyssinus*, *Blomia tropicalis*, *Blattella germanica*, *Periplaneta americana*, fungi, and cat and dog epithelia. Saline and 10 mg/mL histamine solution were used as negative and positive controls, respectively. Reactions were read after 15 minutes, and a mean wheal size of at least 3 mm greater than the negative control was considered positive.

Determination of specific IgE (sIgE) serum concentration was done for *D pteronyssinus*, *B tropicalis*, *B germanica*, *P americana*, with the use of the Immunocap assay (Phadia Diagnostics AB, Uppsala Sweden). Children with >0.70 KU/mL sIgE for any of the allergens tested were considered positive.[8]

Children were classified as having current wheeze with the use of questionnaire data (wheezing in the past 12 months) and were considered to have asthma if parents reported wheezing in the previous 12 months plus at least one of the following: (1) previous diagnosis of asthma, (2) wheezing with exercise, (3) ≥4 episodes of wheezing, or (4) waking up at night because of wheezing. We defined asthma phenotypes by stratifying the population into 4 groups: healthy (sIgE negative and no asthma symptoms), atopic (sIgE positive for at least 1 allergen) asthmatic, nonatopic (sIgE negative) asthmatic, and atopic nonasthmatic.

Statistical analyses

Cytokine production in whole blood cultured in the presence of a mitogen stimulus, to measure maximum cytokine production, was dichotomized into responders and nonresponders with the use of the lowest detection level for each cytokine. The detection limits of the cytokine assay were defined with 4-parameter logistic curves. Associations among cytokine production, sex, and age group were evaluated with the χ^2 test. We identified a set of mutually exclusive latent classes of children on the basis of their responses to the binary indicators of cytokine production with the use of latent class analysis (LCA).[14] LCA yields a definition of classes on the basis of the probability of specific characteristics of the population according to association patterns across categorical variables and has been used, for example, to identify disease phenotypes.[15-17] In this study, we used LCA to empirically classify children into distinct immunologic phenotypes. Groups of children were identified according to similar patterns of cytokine production. The association among the categorical indicators is evaluated with LCA to provide estimates of the conditional probabilities of observed cytokine responses given the immunologic phenotype (or the latent class). These probabilities are used to characterize the latent classes in the same way that factor loadings are used to characterize latent factors in factorial analysis or principal components analysis. The classes (immunologic phenotypes) defined through LCA were used as outcomes to investigate the role of environmental and social characteristics and infection markers on them, and adjusted effects were estimated with multinomial logistic regression, which is an extension of logistic regression when the categorical dependent outcome has more than 2 unordered levels and in which 1 level is used as reference. The effect of the different phenotypes on atopic markers and asthma symptoms was also evaluated, and adjusted effects were estimated with binary or multinomial logistic regression. LCA was performed with Mplus version 5 software,[18] and the other statistical analysis were done with Stata version 10.0 (Stata Corporation, College Station, Tex).

RESULTS

A total of 1127 children with complete data were included in this analysis, 52.7% were boys, 42.6% were older than 7 years, and the mean body mass index (BMI; calculated as weight divided by height; kg/m^2) was 15.6 (2.1). Response rates for each cytokine after mitogen stimulation were 86.2% for IFN-γ, 69.6% for IL-5, 77.0% for IL-13, and 85.4% for IL-10 (see Table E1 in this article's Online Repository at www.jacionline.org). No age or sex differences were observed in response rates (data not shown). With the use of LCA, we identified 3 distinct immune phenotypes that we have referred to as underresponsive, intermediate, and responsive (Table I). The *P* value for G-Lo-Mendell-Rubin likelihood ratio test was <.01, indicating that a model with 3 classes fitted the data better than a model with 2 classes. The entropy of the final model was 0.79. No differences were observed between children classified into the 3 immunologic phenotypes with respect to age, sex, or BMI (data not shown).