

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 atopy⁸ 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% CO₂ 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/m²) 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).

TABLE I. Proportions of children producing detectable levels of mitogen-induced cytokines by immunologic phenotype (underresponsive, intermediate, and responsive)

Cytokines*	Overall (n = 1127)	Underresponsive (n = 194)	Intermediate (n = 129)	Responsive (n = 804)
IFN- γ	86.2	7.0	93.2	98.5
IL-5	69.6	5.4	33.7	92.3
IL-13	77.0	25.4	36.5	100.0
IL-10	85.4	26.7	100.0	97.0

Values are percentages.

*Cytokine data are from mitogen-stimulated whole blood cultures.

Responsive was the phenotype characterized by generalized cytokine production above the detection limits, underresponsive phenotype was characterized by few persons who produced cytokines above the detection limits, and intermediate phenotype had a mixed pattern. The majority of children were classified as having a responsive immune phenotype (71.3%), whereas only 17.2% had an underresponsive and 11.5% had an intermediate phenotype (Table I). In the responsive group nearly all children produced the 4 measured cytokines, whereas in the intermediate group almost all children produced IFN- γ and IL-10 and approximately one-third produced IL-5 and IL-13, and in the underresponsive group a quarter of children produced IL-13 and IL-10 and <10% produced IFN- γ and IL-5. Geometric mean levels for each cytokine by immune phenotypes are presented in Fig 1. Table II shows a summary of the associations between hygiene-related environmental factors and markers of infection and the immune phenotypes for which significant associations were observed in the multinomial logistic analysis (using intermediate phenotype as reference as a proxy for a healthy immune response). In the comparison of responsive with intermediate phenotypes, 3 variables were significantly associated: high maternal education (adjusted odds ratio [OR], 1.61; 95% CI, 1.04-2.50), adequate street paving (adjusted OR, 2.16; 95% CI, 1.46-3.17), and light infection burden (adjusted OR, 1.65; 95% CI, 1.09-2.51). No variable was found to be significantly associated with the underresponsive phenotype compared with the intermediate group.

The prevalence of positive sIgE and positive SPT in the 3 immunologic phenotypes, respectively, was 27.8% and 34.5% in the underresponsive phenotype, 20.9% and 25.6% in the intermediate phenotype, and 32.9% and 40.4% in the responsive phenotype. Table III shows the associations between the 3 immune phenotypes and atopic markers. The chance of having a positive sIgE for at least 1 allergen (OR, 2.01; 95% CI, 1.32-3.07) or SPT positivity for at least 1 allergen (OR, 1.92; 95% CI, 1.22-3.02) was higher among the responsive group after controlling for age, sex, and BMI than the intermediate group. Although the frequency of atopic markers was also increased in the underresponsive group (compared with the intermediate group), no statistically significant associations were observed.

Further, we could not see evidence of significant associations between immune phenotypes and wheezing or asthma outcomes in this population (Table IV).

DISCUSSION

In the present analysis of a large sample of children living in poor urban neighborhoods in Latin America, we identified 3

distinct immunologic phenotypes on the basis of the production of key cytokines after *in vitro* stimulation of whole blood with mitogen. These were a phenotype characterized by the generalized low production of T_H1 (IFN- γ), T_H2 (IL-5, IL-13), and Treg (IL-10) cytokines and, hence, termed underresponsive, by a phenotype associated with the production of all 4 cytokines (responsive phenotype), and by a phenotype in which IFN- γ and IL-10 were present in most children but IL-5 and IL-13 (T_H2) were present in a minority (intermediate phenotype). In particular, the responsive immune phenotype was more frequent in children whose mothers were more highly educated and in children living in better environmental conditions and with lower burdens of infection. The responsive phenotype was associated with a greater chance of having both atopic markers (SPT and sIgE) but not asthma symptoms. We have observed previously in the same study population that a higher burden of infection was associated with a lower prevalence of atopic markers.⁸ The intermediate phenotype, with a relatively higher production of the T_H1 cytokine, IFN- γ , had the lowest prevalence of both atopic markers, whereas the phenotype associated with the production of all 4 cytokines (responsive) had a higher (and statistically significant) prevalence of atopy. This is consistent with the T_H1/T_H2 shift explanation of the hygiene hypothesis. Finally, the underresponsive phenotype (with relatively high IL-10 and low T_H1 and T_H2 cytokines) was not significantly associated with either atopic marker, which may be more consistent with the downregulatory explanation for the hygiene hypothesis. Our observations surprisingly provide evidence to support both paradigms that have been put forward to explain the development of allergy (the balance of T_H1 versus T_H2 cytokines and IL-10-mediated regulation of T_H2 cytokines).⁹ Our findings emphasize the complexity of the mechanisms involved in the allergic process in human populations.

Interestingly, when we evaluated the associations between immune phenotype and social (ie, high maternal educational level) and environmental (ie, living in environmentally improved areas) factors, such factors were more likely to be present in children with a responsive phenotype, providing support for an immunologic mechanism to underlie the link between exposures in early life and the development of the human immune response and allergy. Such environmental variables have been associated previously with a lower frequency of helminth and bacterial infections in this^{9,11,19,20} and other study²¹ populations. Helminths and microbial components have been shown to induce regulatory and suppressive immune responses.²² In addition, microorganisms or microbiota assembled in ecologic niches such as the intestine of a host may also have an important role to play in the modulation of the immune system.^{3,23} In the present study, we observed that a lower burden of infections with intestinal helminths (*Trichuris trichiura* but not *Ascaris lumbricoides*) or viral (HAV but not herpes virus infections), protozoal (*T gondii*), or bacterial (*H pylori*) pathogens was associated with a higher frequency of the responsive immune phenotype. These observations have particular significance, given that the responsive phenotype was associated with a greater frequency of atopic markers (positive SPT or sIgE).

In the present analysis, we did not observe a significant association between the 3 immune phenotypes and wheezing or different asthma phenotypes, indicating that, although these immune phenotypes are associated with atopy, they do not appear to affect asthma prevalence in this population. This may be because, although a high prevalence of asthma symptoms has

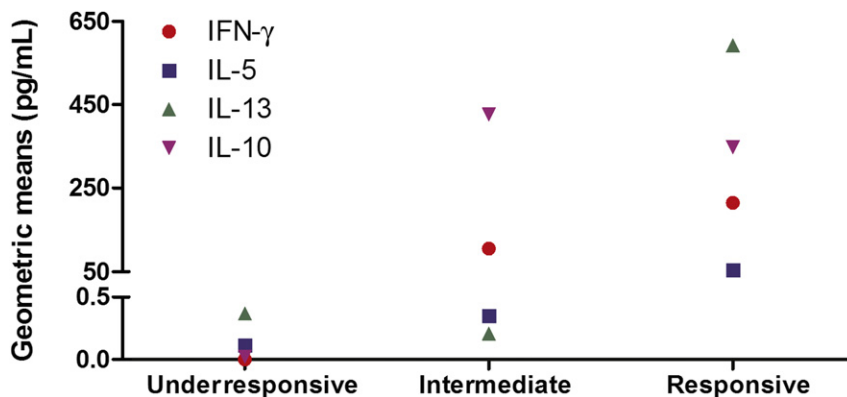


FIG 1. Geometric means for cytokine concentrations from mitogen-stimulated cultures by immunologic phenotype.

TABLE II. Associations between environmental exposures and infection markers and immunologic phenotypes

Variables	Underresponsive OR (95% CI)*	Intermediate†	Responsive OR (95% CI)*
High maternal education	1.16 (0.69-1.93)	1.0	1.61 (1.04-2.50)
Adequate street paving	1.55 (0.98-2.46)	1.0	2.16 (1.46-3.17)
Light burden of infections	0.75(0.46-1.21)	1.0	1.65 (1.09-2.51)

*OR was calculated with multinomial logistic regression in which the intermediate phenotype was used as reference and adjusted for child's sex, age, and nutritional status.

†Reference group.

been consistently observed in urban centers in Latin America,⁶ most cases are not atopic,²⁴⁻²⁷ which could lead us to not have sufficient power to see associations. Poor hygiene and living conditions have been shown to be important determinants of nonatopic asthma in urban populations in Latin America.^{6,26,27} To statistically address whether the inverse association between higher burden of infection and lower prevalence of atopy might be attributable to mitogen-induced cytokine production, we performed a mediation analysis within the latent variable framework. In this analysis, we evaluated whether there was an indirect effect of infection burden on atopy mediated by cytokine response. With the use of such an approach, we observed a significant indirect effect of infection burden (data not shown; $P < .01$) on atopy (defined as either SPT or by the presence of allergen sIgE) mediated by the cytokine response.

As mentioned before, the intermediate category was chosen as reference category because we consider it as a proxy of a healthy immune response. The use of a different reference category for the analysis did not materially affect our study conclusions.

Strengths of the study were the large sample size with immunologic, environmental, and clinical observations for the same population and the lack of systematic sampling bias for blood sampling and the phenotype to which they were ultimately assigned. The cross-sectional study design, in general, makes it difficult to define the direction of possible causal links. However, in the present case there is no ground for the investigated associations to occur in another direction. We defined the immune phenotypes with the use of LCA, a well-known classificatory

TABLE III. Associations between immunologic phenotypes and markers of atopy: positive allergen sIgE and positive SPT

Immunologic phenotypes	sIgE OR* (95% CI)	SPT OR* (95% CI)
Underresponsive	1.54 (0.94-2.54)	1.51 (0.89-2.57)
Intermediate	1.00	1.00
Responsive	2.01 (1.32-3.07)	1.92 (1.22-3.02)

*OR was adjusted for child's sex, age, and nutritional status.

analytic approach, equivalent to factorial and cluster analysis for categorical data, but better suited when such data are highly correlated. LCA assumes that there is a nonobservable factor that underlies the observed data. In the present analysis, LCA was used to define groups of children by patterns of cytokine production and allowed us to identify 3 distinct phenotypes. Despite being a widely used and powerful classificatory technique, as for any such technique, group formation is maximized for the intrinsic characteristics of the variables rather than any underlying biological mechanisms that may exist between them. However, we feel that our observations showing associations among environmental and social characteristics, infection markers, atopy, wheezing, and asthma, which are consistent with other observations in the literature, validate our analytic approach. A few associations related with the underresponsive group, although not significant, showed confidence limits closed to 1. Although this does not change the interpretation of the findings observed here, further studies with larger population are recommended.

In summary, we identified distinct immune phenotypes in children living in poor urban neighborhoods in Brazil. Environmental characteristics related to an improved environment and lower exposures to pathogens were associated with a responsive immune phenotype and a greater prevalence of atopy. This observation might explain how a failure to induce appropriate immune regulation early in life as a consequence of improved environment and lower exposures to pathogens may lead to a higher risk of atopy with possible inflammatory consequences later in life. The importance and priority of controlling infectious diseases in low- and middle-income countries is beyond dispute, but a better understanding of the mechanisms by which infectious pathogens may influence inflammatory responses could lead to the development of new strategies for the control of infectious diseases that do not reduce potentially beneficial immune regulatory mechanisms.

TABLE IV. Associations between immunologic phenotypes and wheezing and asthma outcomes

Immunologic phenotypes	Wheezing/asthma*			Asthma phenotypes†	
	Wheezing past 12 mo OR (95% CI)‡	Wheezing + symptoms past 12 mo OR (95% CI)‡	Asthma ever OR (95% CI)‡	Nonatopic wheezers (vs nonatopic non wheezers) OR (95% CI)‡	Atopic wheezers (vs atopic nonwheezers) OR (95% CI)‡
Underresponsive	1.17 (0.72-1.92)	1.54 (0.87-2.74)	1.38 (0.57-3.34)	1.02 (0.36-2.85)	3.61 (0.42-30.75)
Intermediate	1.00	1.00	1.00	1.00	1.00
Responsive	0.93 (0.61-1.41)	1.41 (0.86-2.32)	1.14 (0.53-2.45)	0.84 (0.36-2.00)	2.79 (0.37-21.26)

*Standard logistic regression.

†Multinomial logistic regression.

‡OR was adjusted for child's sex, age, and nutritional status.

M.L.B., L.C.R., N.M.A.-N., and P.J.C. designed the original study. N.M.A.-M., C.A.F., S.M.A.M., and M.L.B. supervised data collection. N.M.A.-N. and C.A.F. were responsible for the laboratory work. L.D.A. conducted the data analysis. C.A.F. wrote a first draft. All authors contributed to data interpretation and reviewed the manuscript.

Key messages

- Children with more highly educated mothers, living in improved environmental conditions, and with a low burden of infection were significantly more likely to have a generalized activated immune phenotype according to the production of T_H1, T_H2, and Treg cytokines.
- Generalized activated immune phenotype was associated with an increased prevalence of atopy but not asthma.
- Our results may contribute to a better understanding of the immune mechanisms that affect allergy occurrence in urban Latin America.

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TABLE E1. Observed cytokine response patterns in SCAALA-Salvador

Responses				Observed frequency	Latent class assignment
IFN- γ	IL-5	IL-13	IL-10		
-	-	-	-	42	1
-	+	-	-	0	NA
-	-	-	+	3	1
-	-	+	-	29	1
-	+	+	-	4	2
-	+	-	+	5	1
-	-	+	+	11	1
-	+	+	+	7	3
+	-	-	-	3	1
+	+	-	-	0	NA
+	-	-	+	1	1
+	-	+	-	50	2
+	+	+	-	29	2
+	+	-	+	6	3
+	-	+	+	56	3
+	+	+	+	475	3

Mitogen-induced cytokine responses are dichotomous, classified as values above (+) or below LDL (-) the lower detection limit. Latent class assignment: 1, underresponsive; 2, intermediate; 3, responsive.

NA, Not applied (patterns not observed in our data); SCAALA, Social Changes Asthma and Allergy in Latin America.