ORIGINAL ARTICLE

Early Exposure to Unhygienic Conditions and Infections is Associated With Expression of Different Toll-Like Receptors

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Abstract

Background: We have previously shown a lower prevalence of atopy in children living in foster homes than in children living with their parents.
Objectives: In this study, we explored the associations between atopy and expression of Toll-like receptors (TLRs) 2, 4, 7 and 9 in the same groups of children.
Material and Methods: We enrolled all the atopic children living in foster homes in Lodz, Poland and carefully selected, on the basis of age, sex, sensitization profile, clinical manifestation of allergy, and treatment, a similar number of nonatopic children living in foster homes, and a similar number of both atopic and nonatopic children living with their parents. Expression of TLRs 2, 4, 7 and 9 was analyzed in all children.
Results: Expression of TLR2 in foster care children was significantly higher in nonatopic children than atopic children (*P*=.047), while that of TLR7 and TLR9 was significantly higher in atopic children than in nonatopic children. Additionally, expression of TLR9 in nonatopic children in foster care was significantly lower than in nonatopic children living with their parents (*P*=.003). We also found that both groups of nonatopic children had a greater number of features characteristic of foster home children (poor living conditions in the first year of life) than atopic children.
Conclusion: Our results may suggest that alternative mechanisms might underlie the in vivo regulation of the expression of different TLRs involved in the development of atopy.

Key words: Toll-like receptors. Atopy. Children.

Resumen

Antecedentes: Observamos previamente una menor prevalencia de atopia en niños residentes en casas de acogida de menores, con respecto a niños que viven con sus padres.
Objetivos: En este estudio, exploramos las asociaciones entre la atopia y la expresión de los receptores de tipo Toll 2, 4, 7 y 9 en el mismo grupo de niños.
Material y Métodos: Reclutamos a todos los niños atópicos, residentes en casas de acogida de menores en Lodz, Polonia, seleccionados cuidadosamente, en base a edad, sexo, perfil de sensibilización, manifestaciones clínicas de la alergia, y tratamiento, y un número similar de no atópicos residentes en casas de acogida. Asimismo, un número similar tanto de pacientes atópicos como no atópicos de niños residentes con sus padres. Se analizó la expresión de receptores de tipo Toll 2, 4, 7 y 9 en todos los niños.
Resultados: La expresión de TLR2 en los niños residentes en casas de acogida fue significativamente mayor en los niños no atópicos que en los atópicos (*P*=,047), mientras que el TLR7 y TLR9 fue significativamente mayor en niños atópicos que en no atópicos. Además, la expresión de TLR9 era significativamente menor en los niños no atópicos, de las casas de acogida, que en los niños no atópicos que residían con sus padres (*P*=,003). También encontramos que los dos grupos de niños no atópicos presentaban mayor número de características de las casas de acogida (condiciones de vida pobres en el primer año de vida) que los niños atópicos.
Conclusión: Nuestros resultados podrían sugerir mecanismos alternativos de la regulación in vivo de la expresión de diferentes TLR implicados en el desarrollo de la atopia.

Palabras clave: Receptores tipo Toll. Atopia. Niños.

Introduction

Toll-like receptors (TLRs) and their ligands provide an important molecular interface for cross-talk between microbes and mammalian immunity, as was evidenced by the results of a relatively recent study [1]. TLR expression has also been proposed as a potential marker of exposure to various microbial products [2,3]. Although many nonmicrobial regulatory mechanisms of TLR-mediated immune response have been described, the upregulation of TLR2 and TLR4 in response to lipopolysaccharide (LPS) exposure and the environmental or pharmacological modulation of TLR9-mediated response to specific ligands seem to be the most interesting regulatory mechanisms involved in atopy development [4].

We have previously shown that children living in foster homes have a lower prevalence of atopy than children living with their parents (11.3% vs 25.9%, respectively) [5]. In Poland, children living in foster homes are usually from large families with a low economic status and where parent unemployment and alcoholism are major problems. Before entering foster care, these children grow up among numerous siblings in small, overcrowded flats, usually in old buildings with poor hygiene conditions and often without access to running water. They are particularly prone to contact with various infectious factors. We found that the extreme differences between children living in foster homes and children living with their parents in the first year of life, when accumulated, independently decreased the risk of atopy [5]. Additionally, the risk of atopy was lower in children who were older when they were placed in foster care. We hypothesized that extremely poor living conditions in early childhood predisposed children to strong, chronic TLR stimulation, which would favorably influence the development of immunological tolerance.

On the basis of these observations, in the present study, we aimed to explore the associations between atopy and expression of TLRs 2, 4, 7 and 9 in the same group of children [5].

Methods

Study Population

This study is a part of a survey originally conducted in a community-based cohort of 915 children to compare the prevalence of atopy and allergic disease in 2 distinct populations of children with different life experiences and different risk and protective factors for the development of atopy. We studied children living in community foster homes in Lodz, Poland and children living at home with their birth parents. These children were recruited through an insurance agency. Detailed characteristics of these populations have been described elsewhere [5]. In the present study, the children were carefully selected according to age, sex, sensitization profile, clinical manifestation of allergy, and corresponding treatment. We studied 4 groups including all the atopic children living in foster homes in Lodz and similar numbers of nonatopic children living in foster homes, atopic children living with their parents, and nonatopic children living with their parents. All the children lived in the same geographical region. Exclusion criteria included symptoms of viral, bacterial, or fungal infection in the 3 weeks before blood sampling (September 2007); presence of parasitic infection diagnosed on the basis of positive results for parasite eggs and *Giardia lamblia* cysts in 3 stool samples; and the presence of serum-specific immunoglobulin (Ig) M to *Toxocara canis*, *Toxoplasma gondi*, or *Ascaris lumbricoides*, determined by enzyme-linked immunosorbent assay. Additional variables that might have influenced the results of the study (such as the use of systemic corticosteroids in the month before the first visit and allergen immunotherapy) were also applied as exclusion criteria.

Study Variables

The age of foster home placement (measured in y) and cumulative features characteristic of foster home populations were calculated for each child and included in the analysis. These characteristic features were a lower-than-average birth weight, a lower-than-average Apgar score, premature birth, absence of breastfeeding, a larger-than-average Bacillus Calmette-Guérin vaccine scar, fewer-than-average antibiotic courses, a large family (>2 children), older siblings, a single parent–/no parent–family, parent alcoholism, parent unemployment, a home built before 1940, a small home (<3 rooms including the kitchen, living room, and bedroom), no central heating, and tobacco smoke exposure. The minimum feature was 1 and the maximum, 15. The study was approved by the medical ethics committee of the Medical University of Lodz, and written informed consent for participation in the study was obtained from all the children, foster home authorities, and parents or guardians. All the children were given the option of not undergoing testing.

Laboratory Measurements

Blood samples were taken from each participant. Peripheral blood mononuclear cells were separated from 9 mL heparinized blood by Histopaque 1077 density gradient centrifugation (Sigma-Aldrich, Highland, Illinois, USA). Surface expressions of CD14FITC (Becton Dickinson, San Jose, California, USA), TLR2, TLR4 (eBioscience, San Diego, California, USA), and intracellular expression of TLR7-ATTO 488 (Imgenex San Diego, California, USA) and TLR9 (eBioscience) were determined using a FACSCalibur flow cytometer (Becton Dickinson) and analyzed using CellQuest software (Becton Dickinson). $CD14^+$ cells only were used for the analysis of TLRs.

Statistical Analyses

The Mann-Whitney test was used to compare between-group differences and the Spearman test to analyze linear correlations between study variables. All statistical analyses were performed using SPSS 11.5 (SPSS Inc., Chicago, Illinois, USA) and STATISTICA for Windows release 6.0 software (StatSoft Inc., Tulsa, Oklahoma, USA). Statistical significance was set at $P<.05$.

Results

Eighty-one children completed the study (see Table 1 for characteristics). Seven children were withdrawn because of current parasitic infection. We compared TLR expression between atopic and nonatopic children within and between groups (Figure, Table 2), and found statistically significant differences between those with atopy and those without in terms of TLR2, TLR7, and TLR9 expression. TLR2 levels in children from foster care homes and TLR9 levels in children living with their parents were significantly higher in nonatopic children than in atopic children. TLR7 levels, in contrast, were higher in atopic than in nonatopic children in both groups and TLR9 levels were significantly higher in atopic than in nonatopic children in foster care homes. Finally, TLR9 levels in nonatopic children from foster care homes were significantly lower than in nonatopic children living with their parents. We also found a greater accumulation of features characteristic of foster children in both the nonatopic groups than the atopic groups. Finally, foster care children had a significantly higher number of these features than children living with their parents (Figure, Table 2).

Correlations

We found a significant negative correlation between foster care placement age and TLR9 expression (r=-0.65, P<.001) (Figure), with TLR9 levels decreasing with increased age. This correlation was also significant in subgroups of atopic

Table 1. Characteristics of the Study Population[a]

	Children in Foster Care		Children Living With Their Parents	
	Atopic (n=20)	Nonatopic (n=21)	Atopic (n=18)	Nonatopic (n=22)
Demographic and anthropometric data				
Age, y				
<6	3 (15)	3 (14.3)	2 (11.1)	4 (18.2)
6-10	5 (25)	5 (23.8)	6 (33.3)	5 (22.7)
11-14	10 (50)	10 (47.6)	9 (50)	11 (50)
15-18	2 (10)	3 (14.3)	1 (5.5)	2 (9.1)
Female sex, %	50	52.4	50	50
Body mass index,[b] mean (SD)	18.5 (2.1)	17.9 (1.9)	18.2 (3.1)	18.4 (2.3)
Foster care placement age, median (IQR range), y	2.8 (1.1-5.2)	4.2 (2.1-7.8)	NA	NA
Atopic status				
Children with a positive SPT result				
Dermatophagoides pteronyssinus	11 (55)	NA	10 (55.5)	NA
Dermatophagoides farinae	11 (55)	NA	10 (55.5)	NA
Timothy grass	5 (25)	NA	5 (27.7)	NA
Rye	5 (25)	NA	5 (27.7)	NA
Birch	3 (15)	NA	2 (11.1)	NA
Alder	2 (10)	NA	1 (5.5)	NA
Hazel	1 (5)	NA	1 (5.5)	NA
Cat	1 (5)	NA	NA	NA
Allergic disease				
Asthma	7 (35)	0	6 (33.3)	0
Allergic rhinitis	9 (45)	0	9 (50)	0
Atopic dermatitis	5 (25)	0	3 (16.6)	0
Treatment				
Inhaled corticosteroids[c]	3 (15)	0	3 (16.6)	0
LTRA add-on therapy	3 (15)	0	2 (11.1)	0
Antihistamines	10 (50)	0	10 (55.5)	0
Local corticosteroids	4 (20)	0	2 (11.1)	0

Abbreviations: IQR, interquartile range; LTRA: leucotriene receptor antagonists; NA, data not applicable; SPT, skin prick test.
[a] Data are given as number of children (percentage) unless otherwise indicated.
[b] Calculated as weight in kg divided by height in m^2.
[f] >400 mcg of budesonide or equivalent per day.

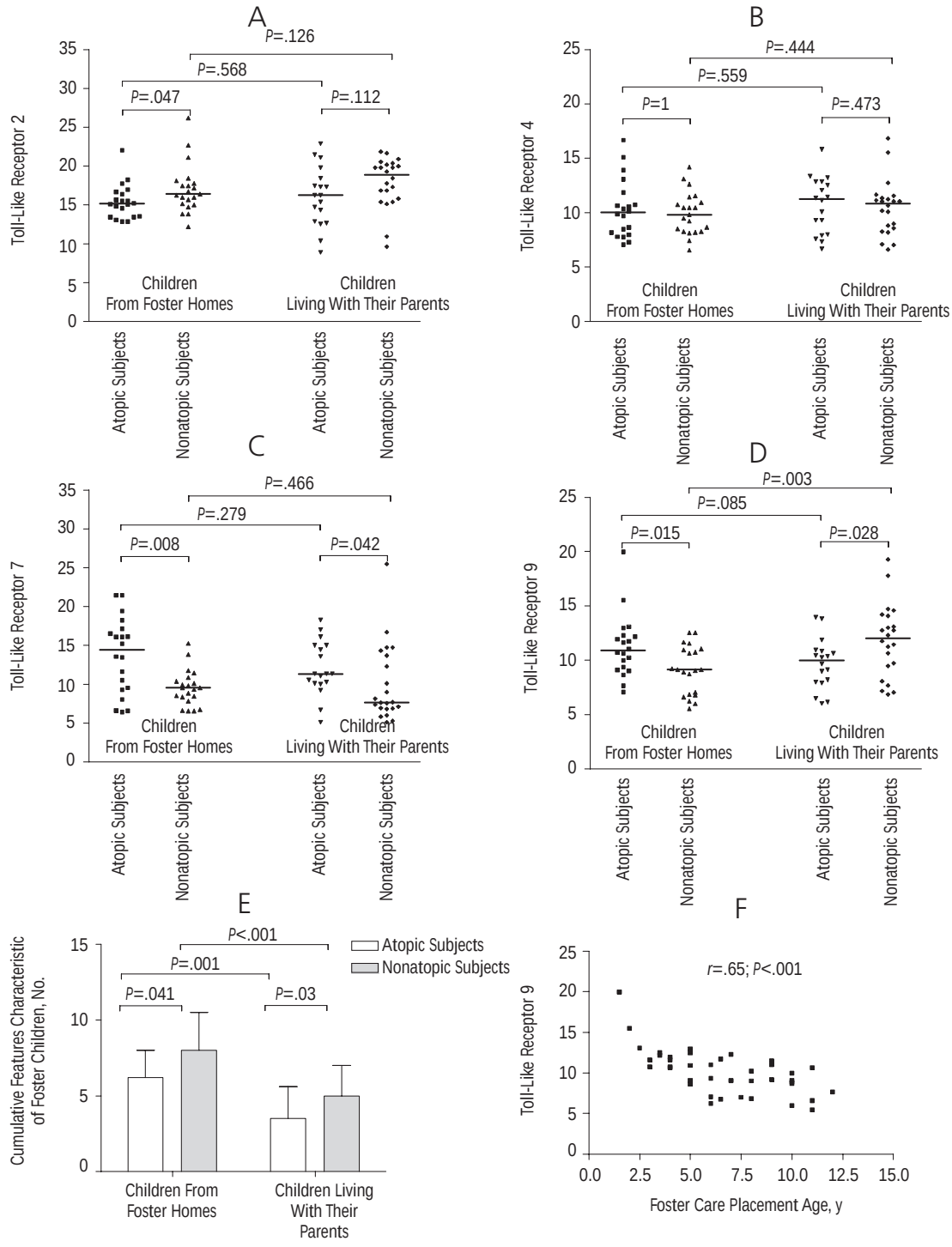

Figure. A, B, C, D, mean fluorescence intensity of Toll-like receptors in CD14+ cells. E, cumulative features characteristic of foster home children. F, correlation between foster care placement age and TLR9 expression.

Table 2. Mean Fluorescence Intensity of Toll-like Receptors (TLRs) 2, 4, 7 and 9 in CD14+ Cells and Cumulative Features Characteristic of Foster Care Children in Atopic and Nonatopic Children from Foster Homes and Atopic and Nonatopic Children Living With Their Parents[a]

	Children in Foster Care		Children Living With Their Parents	
	Atopic (n=20)	Nonatopic (n=21)	Atopic (n=18)	Nonatopic (n=22)
TLR2	15.2 (13.5-16.6)	16.4 (15.1-17.8)	16.3 (12.9-18.4)	18.9 (15.8-20.2)
TLR4	9.7 (8.0-10.8)	9.6 (8.3-10.8)	11.4 (9.3-12.9)	11.0 (8.6-11.6)
TLR7	14.4 (9.4-16.9)	9.6 (8.4-10.4)	11.3 (10.1-15.0)	7.7 (6.9-13.7)
TLR9	10.9 (9.3-12.2)	9.1 (6.9-10.9)	10.0 (8.0-10.8)	12.0 (9.5-14.1)
Cumulative features characteristic of foster children,[b] mean (SD)	6.2 (1.8)	8 (2.5)	3.5 (2.1)	5 (2.0)

[a] Data are given as median values (interquartile range) unless otherwise specified.
[b] Including a mininum of 1 and a maximum of 15 of the following: a lower-than-average birth weight, a lower-than-average Apgar score, premature birth, absence of breastfeeding, a larger-than-average Bacillus Calmette-Guérin vaccine scar, fewer-than-average antibiotic courses, a large family (>2 children), older siblings, a single parent–/no parent–family, parent alcoholism, parent unemployment, a home built before 1940, a small home (<3 rooms including the kitchen, living room, and bedroom), no central heating, and tobacco smoke exposure.

(r=-0.62, *P*=.003) and nonatopic children in foster care (r=-0.58, *P*=.006). There was no correlation between the age of foster care placement and expression of TLR2 (r=0.15, P=.76), TLR4 (r=0.18, P=.65) or TLR7 (r=-0.21, P=.096) in the group as a whole or in subgroups of atopic and nonatopic children in foster care (data not shown).

Discussion

We found that TLR2 expression was higher in nonatopic than in atopic children in foster care and that TLR9 expression was higher in nonatopic than in atopic children living with their parents. These differences might be the result of higher microbial exposure in early childhood in nonatopic subjects, since the cumulative features characteristic of foster home children in the first year of life were significantly higher in both the nonatopic groups than in the atopic groups. The above results seem to correlate with previous observations of associations between atopy development and TLR upregulation in response to bacterial stimulation [4,6]. Nonetheless, the higher levels of TLR7 in both groups of atopic children and of TLR9 in foster care children with atopy cannot be explained by current microbial exposure, nor can the higher expression of TLR7 and TLR9 in atopic subjects be explained by atopy since IgE-dependent activation plays a crucial role in suppressing TLR9-dependent responses in dendritic cells [7]. These results could be explained by the downregulation of TLR expression induced by persistent stimulation. This phenomenon has been previously observed in response to commensal bacteria in intestinal epithelial cells (IECs) [8]. Specific response to pathogen-associated molecular patterns leads to proinflammatory gene expression in many different cell types. However, in healthy organisms, an inflammatory response from intestinal epithelial cells to commensal bacteria and their components is not observed. It has been found that IECs limit dysregulated LPS signaling by downregulating the expression of MD-2 (a critical component of TLR4 signaling) and TLR4. In the light of this study, it is possible that low TLR expression might reflect a natural response to persistent bacterial stimulation. We did not observe any differences between the study groups in terms of TLR2, TLR4, or TLR7 expression. There were clear differences, however, in TLR9 expression, suggesting that TLR9 might be more sensitive to stimulation, and that regulation of its expression may have pivotal characteristics. While receptor expression is upregulated by slight stimulation, it is downregulated by strong, chronic stimulation. We speculate that such a strong stimulation (extremely poor living conditions) might have occurred in early childhood (before foster care placement) and given rise to immunological and clinical consequences that have persisted until now. The significant negative correlation between foster care placement age and TLR9 expression provide additional support for this hypothesis.

This study has several limitations. Because we examined peripheral blood monocytes, we cannot exclude the possibility that differences in TLR expression between atopic and nonatopic subjects may only reflect proportions of distinct subtypes of dendritic cells as a consequence of atopy itself. This is not very likely, however, since the differences were only observed in mean fluorescence intensity and not in the percentage of TLR-positive cells. Also, dendritic cells account for less than 1% of peripheral blood mononuclear cells. We were unable to evaluate direct associations between environmental features, TLR expression, and the development of atopy due to the retrospective nature of our study. Our results suggest that alternative mechanisms might underlie the in vivo regulation of the expression of different TLRs in the development of atopy. This study presents an alternative view of the hygiene hypothesis. However, more indepth studies on dendritic cells in atopic and nonatopic children are required.

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