

ORIGINAL ARTICLE

Toll-like receptor 4 expression in obese asthmatic children with allergic inflammation

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ABSTRACT

Background. Asthma is characterized by chronic inflammation of airways and obesity by persistent systemic inflammation. Exposure to lipopolysaccharides (LPS) may induce the development and severity of asthma. Toll like-receptor 4 (TLR4) recognizes LPS and can direct the response of T-helper cells. Level of expression of TLR4 in patients with asthma and obesity is currently unknown.

Methods. We conducted a pilot study that included patients with asthma-obesity, asthma, obesity and those who were apparently healthy. Using immunocytochemistry, we determined the level of expression of TLR4 with specific anti-TLR4 monoclonal antibody, blocking Fc receptors. With a BX-40 Olympus microscope, 100 cells were evaluated per slide. Differences in the number and degree of positive cells were established by Kruskal-Wallis test and Dunn post hoc analysis.

Results. TLR4 expression in the cells of the asthmatic group of patients was significantly greater than in the healthy group of patients and higher than any of the other two groups. Conversely, the obese group expressed less TLR4 than the healthy group and any of the other two groups ($p < 0.001$). The asthma-obese group showed no significant difference with respect to controls. Additionally, we observed significant differences among the three groups of patients ($p < 0.05$).

Conclusions. TLR4 expression was different in the three groups of patients. The highest level in the asthmatic patient may be explained by the high sensitivity to LPS or to other TLR4 ligands. This is the first study to show the level of expression in obese patients with asthma.

Key Words: asthma, obesity, Toll-like receptor 4, inflammation.

INTRODUCTION

Obesity and asthma are chronic diseases whose prevalence has been increasing worldwide in recent decades, a phenomenon that has been proven in longitudinal studies. WHO has called these diseases “epidemics of the new century.” It is estimated that >300 million people suffer from asthma. In the last century, asthma showed a significant increase until the early 1990s when it began to plateau, which

continues until today.¹ Through the International Study of Asthma and Allergies in Childhood (ISAAC),² it has been possible to standardize and compare its frequency, up to the last report of 2009 where it was 6.8-21.7% in the group of school-age children and 5.1-22% in adolescents.³ In Mexico City there is a frequency of 3.6-6.8% in school-age children and between 3.9 and 10% in adolescents.⁴ The mortality rate in Mexico is 14.5 deaths/100,000 population (~4000 deaths per year), one of the highest in America.⁵

Obesity is among the main diseases in adolescents. Excess weight (overweight/obesity) in this group is about 30%.⁶ Its prevalence has more than doubled in the last 20 years without showing signs of decline. The highest percentage increase has been in the childhood population as well as the presentation of co-morbidities and complications.⁷ In Mexico, the National Health Survey (ENSANUT, 2000) found that the rate of obesity was 6.8 to 10% in females and of 9.2-11.8% in males in the age group of 12-17 years. Taking into account the metropolitan area, obesity and overweight increase the figures to 28% in males and 30.1% in females.⁸

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Some longitudinal studies indicate that obesity precedes asthma and that the relative risk of incidence of asthma is directly proportional to the increase in obesity.⁹ It would appear that asthma predisposes obesity and that obesity worsens asthma.¹⁰ In both conditions there is inflammation.¹¹ Toll-like receptors (TLR) are involved in the inflammatory response so it is necessary to know how their expression is modified in children with asthma and obesity.

Asthma is characterized by chronic inflammation of the airways, with the continued and increased presence of T helper type 2 lymphocytes (Th2), eosinophils and neutrophils.¹¹ The mechanism involved in generating the Th2 response, inherent to inhaled antigens, is unknown. Epidemiological evidence suggests that exposure to lipopolysaccharide (LPS) can influence the development and severity of asthma. However, the mechanism by which LPS influences asthma pathogenesis remains undefined, although it is known that signaling through TLRs is required to direct the response generated by T helper cells. It is reported that in murine models of allergic sensitization, low levels of inhaled LPS generate signaling through Toll-like receptor 4 (TLR4) and that it is necessary to induce Th2 responses to inhaled antigens. The mechanism by which LPS signals a Th2 response involves activation of dendritic cells.¹² In contrast to low levels, inhalation of high levels of LPS with antigens causes Th1 responses. These studies have suggested that the level of exposure to LPS can determine the type of inflammatory response generated and provide a possible explanation for epidemiological exposure to endotoxin and prevalence of asthma.¹³ However, we know that LPS is not the only TLR4 ligand and that, in addition, there may be endogenous ligands and environmental pollutants that can participate in the inflammatory response that occurs in asthma and obesity. Due to the importance of the participation of TLR4 in the recognition of LPS and other ligands, the aim of this study was to determine the level of expression of TLR4 in patients with inflammatory response in asthma and obesity.

SUBJECTS AND METHODS

We conducted a pilot study that included a group of 10 asthmatic school-age patients of both genders with obesity (asthma-obese) who regularly attended the service of Allergy and Immunology and the Obesity

Clinic of the Hospital Infantil de México Federico Gómez. As the controls, we included 10 obese children without any respiratory problem, 10 non-obese children with asthma and 10 healthy children with no history of obesity or asthma.

Clinical criteria for the diagnosis of intermittent or mild persistent allergic asthma were presence of cough, dyspnea and wheezing of 3 months duration or at least two episodes of bronchospasm in the past 12 months and a positive skin test for one or more aeroallergen. Controls were healthy school children of the same age range and without any associated disease or asthma or obesity.

None of the children included in the study had immune system disorders, rheumatic diseases, chronic degenerative diseases or metabolic disorders other from obesity and none had an infectious process during the 30 days prior to the study. None used topical or systemic steroids during the past 8 weeks.

Purification of Peripheral Blood Mononuclear Cells and Preparation of Slides

A sample of 5 mL peripheral blood was taken in a vacutainer tube with EDTA as anticoagulant. Mononuclear cells were obtained by density gradient separation using Lymphoprep™ (Axis-Shield PoC AS, Oslo, Norway). Previously, a 1:2 dilution of blood with saline in a 15 mL tube (Falcon sterile) was performed and centrifuged at 1750 rpm for 30 min. The band of mononuclear cells was then extracted. They were washed twice with saline and centrifuged at 1500 rpm for 10 min. Cells were stained with trypan blue and counted in a Neubauer chamber (Neubauer-improved, depth of chamber 0.1 mm, Marienfeld, Germany) under light microscopy (10X). We performed the calculation for the number of total cells in 1 mL and with the value obtained the number of cells was adjusted to 10,000 cells/μL and placed in 1.5 mL tubes (Eppendorf sterile, 2 mL). One mL of 1X phosphate buffered solution (PBS 1X, pH 7.2) was added to proceed to place the spots on the slides (frosted slides 25 × 75 mm, Madesa of Mexico).

Slides were dried at room temperature and labeled. Cells were fixed with 4% formaldehyde for 20 min at 4°C and then washed with 1X PBS for 5 min to remove formaldehyde residue. Finally, the slides were allowed to dry at room temperature and stored for subsequent immunostaining.

Immunocytochemistry

Immunostaining was done with the prepared cell slides. Antigen retrieval was performed with sodium citrate (pH 6, 0.01 M) for 20 min at 90°C in a water bath. There were two washes with PBS 1X. Endogenous peroxidase activity was eliminated with methanol and hydrogen peroxide 3% for 15 min (twice) and blockage of the Fc receptors was performed with normal pig serum at 2% for 2 h in a humidity chamber and ambient temperature. Subsequently, avidin/biotin was added and incubated for 15 min each and then were washed three times for 5 min. Afterwards, we proceeded to incubate with the antibodies anti-TLR4 or isotype control 1:500 (mouse monoclonal anti-human TLR4 and normal mouse IgG, Santa Cruz Biotechnology, Santa Cruz, CA) in the humidity chamber overnight at room temperature.

After incubation, five washes were performed for 8 min to remove residue from each of the antibodies, then incubated with the secondary antibody conjugated to biotin for 30 min at room temperature in a humidity chamber. After this time, a wash with PBS 1X for 5 min was performed, streptavidin conjugated to horseradish peroxidase was added (HRP, Universal LSAB + Kit/HRP, DAKO Cytomation, Glostrup, Denmark) for 30 min. Finally, the color was generated by adding the substrate chromogen diaminobenzidine (DAB) during the time required for each antibody with regard to the isotype control. The reaction was stopped with water and was counterstained with hematoxylin. Samples were dehydrated with distilled water, ethanol 70%, 90% to 100% and xylene in subsequent baths of 5 min each. The preparations were covered with resin and allowed to dry at room temperature.

Quantification of Immunostained Positive Cells

Slides were analyzed on an Olympus BX-40 microscope and positive cells (brown) were classified according to the degree of color intensity; 100 cells were counted on each slide.

Statistical Analysis

A database was developed and the information was processed using a statistical analysis program (Sigma Plot and Sigma Software Stad). Evaluation of the difference in the number and degree of positive cells of the immunocytochemical reactions was performed using Kruskal-Wallis and Dunn post hoc tests.

RESULTS

TLR4 Expression Levels

We evaluated the level of expression of TLR4 taking into account the percentage of positive cells for each group and the expression level: low (1), moderate (2) and high (3); 100 cells were analyzed for each sample of patients or controls. We found that mononuclear cells in the healthy group showed high expression of TLR4 (45%), whereas only 22% and 28% showed low and moderate expression, respectively, and 5% of the cells did not express the receptor. The obese group showed a high expression in <20% of the cells and moderate and low expression in 55% and 22% of the cells, respectively. The asthmatic group showed high expression of TLR4 in 60% of the cells and moderate and low expression in 22% and 10%, respectively, observing in this group the highest expression. In the obese-asthmatic group of subjects the greatest expression was 40% of the cells and moderate and low expression was shown in <40% and 5%, respectively. It was notable that ~20% of the cells did not express the receptor (Figure 1).

Statistical analysis was performed and it was found that the median of the expression in mononuclear cells of the asthmatic group was significantly higher than the healthy group (3 and 1, respectively) and greater than any of the other groups (3 and 2) (Figure 2). With the Kruskal-

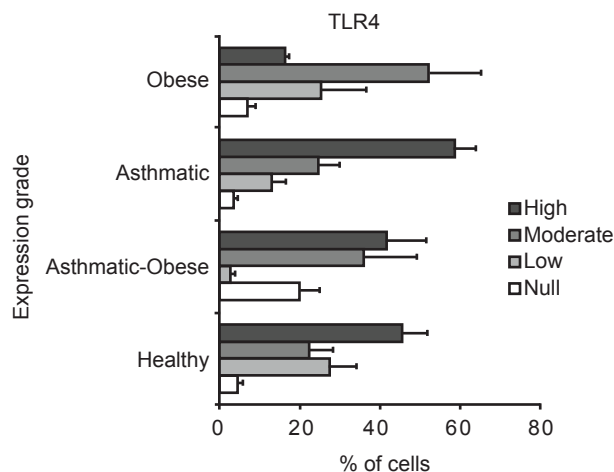


Figure 1. Degree of expression of TLR4 in peripheral blood mononuclear cells. We determined the level of expression of TLR4 in 100 cells from each patient or healthy control of the different groups studied and were rated as high (3), moderate (2), low (1) or no expression (0).

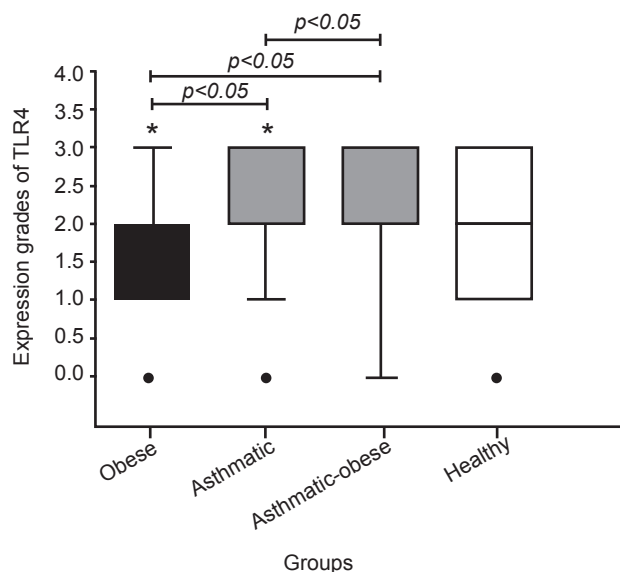


Figure 2. Statistical analysis of the results of the expression of TLR4. Medians and ranges of variation in the expression of TLR4 in the different groups included in the study are shown: obese (1), asthmatics (2), asthmatic-obese (3) and controls (4). * $p < 0.001$, statistically significant difference compared to the healthy control group obtained by Kruskal-Wallis one-way ANOVA. Analysis of the difference between groups was performed with the Dunn post hoc method with statistical significance $p < 0.05$.

Wallis analysis of variance it was found that the obese group expressed less TLR4 than the healthy group and the group of asthmatic subjects showed a higher expression than the control group. These differences were statistically significant ($p < 0.001$). However, the asthmatic-obese group showed no significant difference from the healthy group. We also analyzed the difference in the expression of TLR4 between study groups using the Dunn post hoc test and found significant differences between the obese group and the asthmatic group, between the obese group and the asthmatic-obese group, and between the group of asthmatic subjects and asthmatic-obese subjects ($p < 0.05$).

DISCUSSION

The innate immune response to LPS can modulate adaptive immune responses to allergens. Recent studies with mouse models reported that sensitization to aeroallergens with low doses of LPS (0.1 mg) induced a Th2 response in asthma phenotypes, presenting typical symptoms of asthma such as bronchial hyperresponsiveness and eosinophilic

inflammation. In contrast, allergen sensitization with high doses of LPS (10 mg) did not produce asthma symptoms. These findings suggest that exposure levels of the airways to LPS induce different forms of asthma, which can be differentiated in phenotypes 1 and 2 for different levels of LPS, high and low, respectively.¹⁴

The fact that obesity can aggravate asthma can be partly explained by the mechanical effects of obesity on the respiratory system. Obesity causes a decrease in tidal volume and residual functional capacity. These changes have resulted in a reduction in the stretch of smooth muscle and, thus, the ability to respond to physiological stress. Smooth muscle inherently has a cycle of excitation and contraction, but these cycles are shorter in obese subjects and are associated with decreased functional capacity, resulting in a conversion of the rapid cycles of actin-myosin to slower cycles.¹⁵ However, the exact dose-effect relationship between the quantity and/or body fat distribution and changes in respiratory mechanics are still unknown.

The effect of the inflammatory response generated during obesity in asthma is unknown. An association has been reported between obesity and asthma and various inflammatory markers such as tumor necrosis factor alpha (TNF- α), cytokines such as IL-6 and IL-1 β and C-reactive protein. It has been shown that IL-6 and TNF- α are expressed in adipocytes and correlate directly with total body fat. Moreover, levels of TNF- α are also increased in asthma and are related to the production of IL-4 and IL-5 (Th2 cytokines) by the bronchial epithelium and IL-6 and IL-1 β (Th1-type cytokines).¹¹ These cytokines are produced in response to stimulation of Toll-like receptors, among which is TLR4.¹⁶ TLR4 has been associated with the inflammatory response in asthma by sensitization that can be generated by LPS of gram negative bacteria.

In this study we observed that the expression of TLR4 was significantly higher in the group of patients with asthma with respect to the healthy group and the other two groups (obese and asthmatic-obese), which may probably explain the high sensitization of asthmatic patients to LPS or other TLR4 ligands. The obese group presented a decreased expression of TLR4, which may explain the expression found in the asthma-obese group, which did not present significant difference from the healthy group. Even among the three groups of patients, differences were statistically significant.

The relationship between asthma and LPS is only documented in the airway and the present study was performed with peripheral blood. This was done because it was considered important to determine the expression at systemic level rather than local level because apart from patients with asthma, we included obese patients who are known to experience systemic inflammation as well as obese-asthma patients.

This is the first study to determine the level of expression of TLR4 in obese-asthmatic patients. As noted, in the obese group, TLR4 is reduced and in the asthmatic group it is increased significantly compared to the healthy group, showing that these conditions affect differentially the expression of this receptor and thus probably the inflammatory response. However, additional studies are needed to determine the relevance of these findings in the pathophysiology of asthma and obesity.

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