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ORIGINAL ARTICLE

A Study on T Regulatory Cells in Patients with Respiratory Allergies in Zagazig University Allergy and Immunology Unit

*[1]Ahmed A. Shaheen, [2]Manal M. Al-Amen, [2]Maha Atfy, [2]Hoda Fathy, [1]Shereen A. Baioumy**
[1]Medical Microbiology and Immunology and [2]Clinical Pathology Departments, Faculty of Medicine, Zagazig University, Egypt

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Autologous serum skin test;
Allergic rhinitis;
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T regulatory cells

ABSTRACT

Background: *A positive autologous serum skin test (ASST) reflects the presence of anti-FcεRI and/or anti-IgE autoantibodies and used to show autoimmunity in cases with respiratory allergies. Impaired CD4+CD25^{high} FoxP3+ T regulatory cell activity can cause autoimmune diseases and allergy.* ***Objective:*** *To investigate the association between respiratory allergies and CD4+CD25^{high} FoxP3+ T regulatory T cell count and re-evaluate the significance of ASST reactivity in respiratory allergy patients and healthy controls.* ***Methodology:*** *16 patients with persistent asthma, 14 seasonal allergic rhinitis patients and 30 control subjects were included. ASST and measurement of FoxP3+ regulatory T cell count was performed in all subjects.* ***Results:*** *ASST was positive in 18/30 (60%) respiratory allergic patients and none of control subjects. FoxP3+Treg cell count was lower in respiratory allergic patients than control subjects with negative correlation with disease severity.* ***Conclusion:*** *Respiratory allergic patients have lower level of FoxP3+ regulatory T cell than normal individuals. A positive ASST response found in respiratory diseases patients suggests that it may occur as a result of some inflammatory events during the diseases process and could be related to disease activity.*

INTRODUCTION

Allergic rhinitis is very common in patients with asthma, with a reported prevalence of up to 100% in those with allergic asthma, suggesting the concept of "one airway, one disease". [1]

About 235-300 million people worldwide suffer from respiratory allergy. It continues to prevail, especially among children, and by 2025, this number is expected to increase by 100 million [2].

Regulatory mechanisms are necessary to maintain peripheral tolerance by the immune system. Allergy is one of the immune tolerance-related disorders due to a failure of the regulatory network[3]. The most important cells involved in the immune system regulation are CD4+CD25^{high} regulatory T cells (Tregs). Naturally occurring Tregs, express the forkhead transcription factor (FoxP3) which is a crucial factor for the development of Tregs in the thymus and is needed to maintain the suppressive activity of mature peripheral Tregs[4]. It has been shown that anomalies in the function or number of Tregs or absence of FoxP3 can be a primary cause of autoimmune disease, inflammatory disorders in humans, allergy and high immunoglobulin E (IgE) levels[5].

Corresponding Author:
Shereen Atef Baioumy
Microbiology & Immunology Department, Faculty of Medicine, Zagazig University
Email: drshereenatef@yahoo.com; Tel: +201282564234

In 1993, an autoimmune aetiology of allergy was suggested by Hide et al.[6] They identified the presence of IgG anti-high-affinity IgE receptor (FcεRI) and anti-IgE autoantibodies capable of activating mast cell and basophil degranulation[7].

In clinical practice, a positive autologous serum skin test (ASST) has been used as a potential for the measurement of functional autoantibodies to FcεRI, and it is assumed to be suggestive of an autoimmune pathogenesis of allergies.[7]

It is also noted that skin reactivity to ASST is not necessarily due to IgG autoantibodies against IgE and/ or FcεRI and may be due to the existence of non-immunoglobulin vasoactive histamine releasing factors and it has been observed in subjects with allergic respiratory diseases and healthy controls. ASST identifies subsets of patients exhibiting autoreactivity rather than establishing autoimmunity[8].

In this study, we aimed to investigate the association between respiratory allergies and CD4+CD25^{high} FoxP3+ T regulatory cell count and re-evaluate the significance of ASST reactivity in respiratory allergy patients and healthy controls.

METHODOLOGY

Subjects:

This study was carried out between 2014 and 2016 in the allergy and immunology unit, faculty of medicine, Zagazig university. Thirty patients with respiratory

allergy (26 females & 4 males), Their ages ranged from 19-50 with mean±SD (30.8 ± 8.5). Sixten patients with persistent bronchial asthma, 14 patients with seasonal allergic rhinitis and 30 healthy nonatopic controls (16 females and 14 males) and their ages ranged from 18-52 with mean±SD (28 ± 9.8). The control group had no history of atopy (atopic respiratory disease, atopic dermatitis or food allergy), urticaria, nonatopic respiratory disease, autoimmune diseases, pregnancy or any drug allergy and/or intolerance.

Antihistamines were withdrawn from anyone using them at least 1 week before skin testing was performed. Patients were included if they had documented asthma symptoms for at least one year. Also, patients with a documented history of seasonal allergic rhinitis for at least 2 years prior to the study were included. Patients with rhinitis and asthma were diagnosed as having allergic rhinitis/allergic bronchial asthma on the basis of clinical history, blood eosinophil count, serum total IgE concentration, pulmonary function tests and skin-prick and/or intradermal tests. The Global Initiative for Asthma (GINA)[9] workshop report and the Allergic Rhinitis and Its Impact On Asthma (ARIA) [10] document were considered as diagnostic criteria for allergic rhinitis/allergic bronchial asthma.

After obtaining written informed consent, all patients and controls underwent the following.

Intradermal skin tests:

The skin was disinfected with 70% ethyl alcohol and allowed to dry. The test sites were marked and allocated at least 3cm apart to avoid overlapping of reactions and influence of strong reactions on slightly positive or negative tests.

Hypersensitivity intradermal skin test:

It was performed using a panel for common aeroallergens including house dust, smoke, wool, cotton, mixed fungi, mixed pollens and hay dust. Positive and negative controls were histamine and physiological saline, respectively. Skin test was performed on the volar surface of the forearm leaving an area about 5cm from the wrist and 3cm from the elbow. Sterile disposable insulin syringes were filled with 0.05 ml of 1/1000 dilution of extracts. Each syringe had one extract. The needle of the syringe was applied at an angle of 45˚ to the forearm, and each diluted extract was injected intradermally. After 15 minutes, the tested sites were inspected and fully developed wheal and/or erythema reaction were recorded. If no result was observed after 15 minutes, the sites were inspected after 30 minutes. A mean wheal diameter of 3 mm or greater than that of obtained with control solution was considered as positive.

Autologous serum skin test (ASST):

Venous blood was collected in a sterile glass tubes and allowed to clot at room pemperature for 30 min. Serum was separated by centrifugation at 2500 rpm for 15 min. 0.05 ml autologous serum was injected intradermally into the patients forearm to perform ASST. Epidermal histamine 0.01 mg/ml and intradermal 0.9% sterile saline were used as positive and negative controls, respectively. The diameter of a wheal was calculated as the mean of the two longest perpendicular wheal diameters. ASST was considered positive when a serum-induced wheal had a diameter 3 mm greater than that of negative control surrounded by erythema at 30 min. We considered red or pink oedema as a positive response.

Immunophenotyping by flow cytometry:

Monoclonal antibodies that were used in this study were fluorescein isothiocyanate (FITC) conjugated: CD4, The phycoerythrin (PE) conjugated: CD25 and allophycocyanin (APC) conjugated:FoxP3. All antibodies from BD Bioscience. Measurements were performed on a FACS Calibur.

Sample processing: Two ml of venous blood samples were taken from every patient and control subjects under complete aseptic conditions and were collected into vacutainer tube with K_3EDTA and was mixed by gently inverting the tube several times. They were processed within 24 hours of collection, being preserved at refrigeration temperature (2-8)^{o}C. 100 µl whole blood were transferred into one tube for each sample (5 ml polypropylene tube) and the samples were labeled accordingly. 10µl of each antibody (CD4FITC and CD25PE) were added onto the blood sample. The samples were vortexed and incubated for 20 min in dark at room temperature. One ml 1x BD FACS Lysing Solution (10x solution diluted in distilled H_2O) was added in each sample then vortexed briefly and incubated for 10 minutes in the dark at room temperature. The tubes were centrifuged at 2000 rpm for 5 min at room temperature. Then the supernatant was discarded. 1 ml of PBS as a washing solution were added to each tube and mixed thoroughly. Then the tubes were centrifuged at 2000 rpm for 5 minutes and the supernatant was discarded. Cells were suspended in 500 µl PBS to be ready for acquiring data by flow cytometer. Acquization and analysis were carried out of each sample using CELLQuest software (BD Bioscience).

For staining intracellular antigens for flow Cytometry, Samples were centrifuged at 1200-1500 rpm at room temperature for 5 minutes and then the supernatant was discarded. Without washing, 1 mL of 1X permeabilization buffer were added to each tube and were incubated for 5 minutes then were centrifuged at 1200-1500 rpm at room temperature for 5 minutes then the supernatant was discarded. 10µl of anti-human Foxp-3APC conjugated were added for detection of intracellular antigen to cells and incubated in the dark at room temperature for at least 30 minutes. Washing steps were done twice. The samples were centrifuged at 2000 rpm at room temperature for 5 minutes then the supernatant was discarded. The stained cells were

resuspended in 200 µl PBS to be ready for acquisition by Flow Cytometry.

Interpretation: Gating was done on lymphocytes using SSC and FSC gating strategies. Percentage of positive cells among gated lymphocytes was defined for each marker. T regulatory cells were defined by positive co-expression of CD4+CD25^{high}. To detect the Foxp-3 a secondry gate was done on CD4+CD25^{high} positive cells.

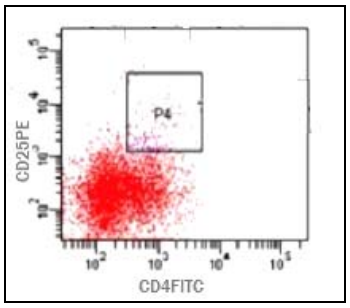

Fig. 1: Shows how to identify the T-reg by gating on CD4+CD25^{high} positive cells.

Statistical analysis

Statistical analysis was performed with spss for Windows (Statistics Products Solutions Services (SPSS) 16.0 Inc., Chicago, IL, USA). The Shapiro-Wilk test was employed for quantitative variables to confirm data distribution. Statistical analysis was performed using the Chi-square test (χ^2), Chi-square for trend (Extended Mantel-Haenszel test, χ^2 for trend). Patient groups were compared using parametric tests (student`s t-test,T) or nonparametric tests (Mann–Whitney U-test, MW) following standard statistical criteria. Correlation was assessed by calculating Pearson`s rank (r) correlation coefficient. P-values <0.05 were regarded as significant.

RESULTS

There was a significant association between respiratory allergy and female gender, also heritance was significantly associated with respiratory allergy.

ASST test positivity was significantly associated with respiratory allergy group (60% of cases are positive and none of the control group were positive (table 1).

Out of 30 allergic patients, 16 suffered from bronchial asthma, 14 suffered from allergic rhinitis. Severe disease represented 25% of asthma patients and 35% of rhinitis cases (table 2).

There was a significant association between ASST positivity and respiratory allergy severity (table3).

The percentage FoxP3+T reg. cells was significantly higher in control group than cases group. In case group, minimum value was 0.7 and maximum value was 8, 25th and 75th percentiles were 0.7 & 3 respectively and median value was 3. In control group, minimum value was 0.5 and maximum value was 29, 25th and 75th percentiles were 4.0 & 14.3 respectively and median value was 7.(table 4).

There was a significant negative correlation between of respiratory allergy severity with the percentage of FoxP3+T reg. cells (r=-0.6, p<0.001) (figure 2).

Table 1: Characteristics of studied groups

Variables	*Case group (n=30)*	*Control group (n=30)*	*Test of sig.*	*P*
Age:				
X ± SD	30.8 ± 8.5	28 ± 9.8	t: 2.8	0.07
Range	19 – 50	18 - 52		
Sex:				
Male	4 13.3%	14 46.7%	X^2: 7.5	**0.006***
Female	26 86.7%	16 53.3%		
Residence:				
Rural	17 56.7%	23 76.7%	X^2:2.7	0.1
Urban	13 43.3%	7 23.3%		
Occupation:				
Not working	17 56.7%	18 60.1%		
Employee	4 13.3%	4 13.3%	X^2:0.01	0.9
Professional	7 23.3%	4 13.3%		
Worker	2 6.7%	4 13.3%		
Smoking:				
Yes	0 0.0%	0 0.0%	X^2:0.0	1
No	30 100%	30 100%		
Heritance:				
Yes	25 83.3%	10 33.3%	X^2:15.1	**<0.001***
No	5 16.7%	20 66.7%		
ASST:				
Positive	18 60%	0 0.0%	X^2:25.7	**<0.001***
Negative	12 40%	30 100%		

"t" Student t test X^2 Chisquare test * Highly Significant

Table 2: Severity of respiratory allergies of studied cases

Severity	*Bronchial asthma (n=16)*		*Allergic rhinitis (n=14)*	
	No	**%**	**No**	**%**
Mild persistent	6	37.5	3	21.4
Moderate persistent	6	37.5	6	24.9
Severe persistent	4	25.0	5	35.7

Table 3: Association between ASST results and respiratory allergy

Severity	*ASST positive*		*ASST negative*		X^2 for trend	*P*
	No	**%**	**No**	**%**		
Mild persistent	3	16.7	6	50.0		
Moderate persistent	7	38.9	5	41.7	4.5	**0.03***
Severe persistent	8	44.4	1	8.3		
Total	18	100	12	100		

* Highly Significant

Table 4: Percentage of T reg. cells among studied groups

CD4+CD25+FOXP3+T reg. cells %	*Case group (n=30)*	*Control group (n=30)*	**MW**	*P*
X ± SD	2.6 ± 1.6	9.6 ± 6.9		
Median	3	7	174.5	**<0.001***
Range	0.7 - 8	0.5 - 19		

* Highly Significant

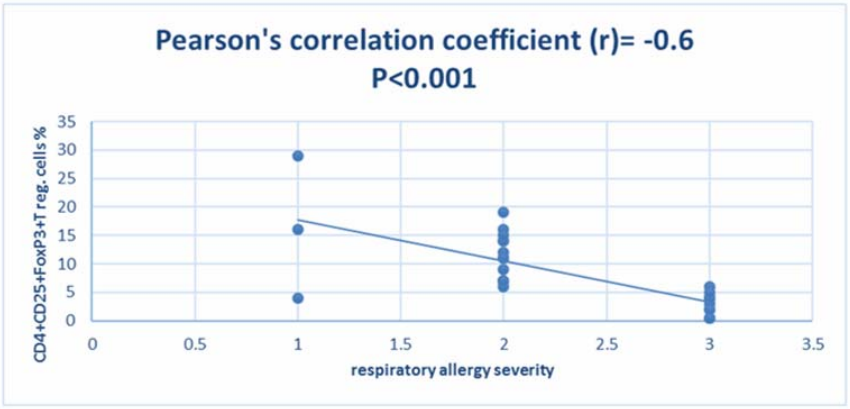

Fig. 2: Correlation of respiratory allergy severity with CD4+CD25+FoxP3+T reg. cells %

DISCUSSION

Because of FoxP3 is located on the X-chromosome, data analyses included females and males[11]. In this study, there was a significant increase in number of female patients more than male subjects. These results are consistent with Yuen et al,[12] where M/F (male/ female) ratio were (46%/54%). Also, Comi AL et al.,[13] stated that a clear female preponderance was found (65%) in agreement with other clinical studies on asthmatic patients. However Wei et al.,[14] found that AR was higher in male than female with M/F ratio (57.3%/42.7%). These differences may be due to occupational factors, hormonal changes or genetic susceptibility.

On studying the clinical characteristics of studied subjects, there was high statistical significant difference between study groups as regarding heritance. Heritance was highest in allergic patients and least in control subjects. This was in accordance with Abdel-Gawad et al.,[15] who found that family history of atopy was positive in asthma patients more than control subjects.

In this study, majority of allergic patients cases were significantly allergic to mixed pollens. Also Bauchau et al.,[16] found that pollen was the most common allergen among allergic rhinitis patients consulting primary care. However Yuen et al.,[12] found that house dust mite was the most common allergen causing positive skin test among clinically suspected

allergic rhinitis patients. This difference may be explained by change climate, geography and life style.

In this study, ASST was positive in 18/30 (60%) patients and negative in all control subjects. This was in accordance with , Comi AL et al.,[13] who found that ASST was positive in 29/55 (53%) patients and negative in all control subjects. Also, Kurt E et al.,[17]found positive ASST response in 8/43(19%) respiratory allergic patients and negative in all control subjects. In contrast to these findings, Mari[18] observed that ASST was positive not only in 47% of the adults and 84% of the children with allergy-like respiratory symptoms, but also in 40% of adult patients with respiratory allergy and in 45% of healthy adult subjects. This striking difference might be because of differences in ASST interpretation and serum samples preparation.

In this study, there was high statistical significant difference between study groups as regarding CD4+CD25+ FoxP3+ cells %. Control subjects had significantly the higher values than allergic patients. This was in accordance with Stelmaszczyk-Emmel et al.,[19] who found that The percentage of Tregs in samples from allergic patients was significantly decreased in comparison to healthy controls [median (25percentile; 75 percentile): 2.3 (1.63; 3.58) vs. 4.6 (3.69; 5.68), respectively, p = 0.003].

In allergic patients, There was statistical significant negative correlation of FoxP3 T reg cells % with disease severity. This was in accordance with Keying X. et al.,[20] who found that the CD4+CD25+Treg ratio in PBMCs of patients with asthma in exacerbation and persistent groups was lower than that in remission and normal control groups ($P<0.05$). Although the CD4+CD25+ Treg ratio in remission group was also lower than that in normal control group, there was no significant difference between them ($P>0.05$). As compared with persistent group, exacerbation group had lower CD4+CD25+Treg ratio ($P<0.05$). Recently, different studies have started to highlight the link between allergy and defective immunoregulation; however, the results are inconsistent. Lee et al.[21] and Xu et al.[22] described a reduction of the Tregs compartment in patients with allergy. Zhang et al.[23] found a significant decrease of Tregs only in acute exacerbation of asthma in comparison with chronic stable disease Nevertheless, other scientists state that the frequency of Tregs does not differ between allergic and nonallergic populations. The reasons given for this discrepancy remain speculative, but may be related to the clinical form of the disease, age of the patient, exposure to allergens and the implemented therapy.

CONCLUSION

Until a reliable method becomes available, the ASST could represent a valid diagnostic approach to all allergy/allergy-like conditions. Cutaneous and respiratory symptoms may possibly reflect the corresponding activation of mast cells in different tissues. In the general population, ASST positivity may define a risk factor for reactive healthy subjects, prone to allergy/allergy-like diseases. Follow-up studies are needed to define the role of ASST in atopic individuals.

Taken together, using several types of analyses and linking all data obtained, we have provided evidence that CD4+CD25^{high} FoxP3+ Tregs display substantial deficiencies in atopic patients as compared with healthy individuals. Although Treg deficiency is not the only abnormality of immune regulation in atopy, better quantitative and qualitative characterization of Tregs in allergy is needed because the decrease in the number and function of these cells may lead to downregulation of T cell tolerance and exacerbation of the disease.

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