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Effect of Immunotherapy on Basophil Activation Induced by Allergens in Patients with Atopic Dermatitis

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ABSTRACT

Background: Subcutaneous allergen-specific immunotherapy (IT) is a proved, highly effective treatment for respiratory IgE-mediated diseases. However, few studies have explored the immunological mechanisms of IT in patients with atopic dermatitis.

Objective: To explore the immune response of atopic dermatitis patients receiving house dust mite (HDM) immunotherapy, according to humoral response and basophil activation.

Material and method: An open label study was done which assessed the severity of atopic dermatitis with SCORAD in 20 patients (10 with immunotherapy and 10 without it) every three months during two years. Serum samples were taken before the follow up, and at the first and second year of study to analyze CD63 basophil expression, total IgE levels and specific IgE and IgG4 to *Der p* and *Der f*. Ten patients with allergic rhinitis receiving IT and 5 non-allergic subjects were used as controls.

Results: CD63 expression after basophil stimulation with *Der p* was higher in atopic dermatitis patients than in rhinitis and non-allergic subjects. After the first and second year of treatment, CD63 expression was lower in atopic dermatitis active group than in the atopic dermatitis control group. We observed a correlation between SCORAD, IgG4 and CD63 expression.

Conclusion: In patients with atopic dermatitis, basophil activation test could be a biomarker of clinical response and basophil modulation can result in a better clinical control.

Key words: atopy, allergy, dermatitis, eczema, basophil, immunotherapy.

Efecto de la inmunoterapia en la activación de basófilos inducida por alergenos en pacientes con dermatitis atópica

RESUMEN

Antecedentes: la inmunoterapia subcutánea con alergenos ha demostrado ser sumamente efectiva para el tratamiento de las enfermedades respiratorias mediadas por IgE. Sin embargo, pocos estudios exploran los mecanismos inmunológicos de la inmunoterapia en pacientes con dermatitis atópica.

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Objetivo: explorar la respuesta inmunológica en pacientes con dermatitis atópica que reciben inmunoterapia con ácaros de acuerdo con la inmunidad humoral y la activación de basófilos.

Material y método: estudio abierto en el que se evaluó la severidad de la dermatitis con el índice SCORAD en 20 pacientes (10 con inmunoterapia y 10 sin inmunoterapia) cada tres meses durante dos años. Las muestras de suero se tomaron previo al inicio del estudio y al primer y segundo año de seguimiento para evaluar la expresión de CD63 en basófilos, concentraciones de IgE total, IgE e IgG4 específica para *Der p* y *Der f*. Diez pacientes con rinitis alérgica y cinco controles no alérgicos se incluyeron en el estudio como controles.

Resultados: la expresión de CD63 en los basófilos después de la estimulación con *Der p* fue más alta en los pacientes con dermatitis que en los pacientes con rinitis y en los sujetos no alérgicos. Luego del primer y segundo año de tratamiento, la expresión de CD63 fue menor en el grupo de pacientes con dermatitis que recibieron inmunoterapia en comparación con los tres grupos control. Observamos una correlación entre el SCORAD, IgG4 y la expresión de CD63.

Conclusión: en pacientes con dermatitis, la prueba de activación de basófilos podría usarse como biomarcador de respuesta clínica; asimismo, la modulación de esta célula puede llevar a un mejor control clínico.

Palabras clave: atopia, alergia, dermatitis, eccema, basófilos, inmunoterapia.

BACKGROUND

Atopic dermatitis is an inflammatory skin disease characterized by pruritus, eczema and family history of atopy. A better understanding of the pathophysiological mechanisms of this disease has led to propose different phenotypes according to the underlying mechanisms. Around 30% to 40% of patients have defects in the production or function of those proteins that make the natural moisturizing factor like filaggrin,[1] besides that, Th1 and IgE response against self-proteins have been implicated in the severity of the disease in 10% to 50% of patients.[2,3] The diversity of pathophysiological mechanisms and the intervention of genetic and environmental factors make atopic dermatitis a complex disease that must be addressed integrally; however, for most of these processes there aren't yet specific therapeutic approaches. Similar to allergic respiratory diseases, exposure to allergens and Th2 hypersensitivity seems to be important at the beginning and during the exacerbations of atopic dermatitis, and sensitization is present in almost 80% of the patients.[4] Avoidance measures, especially to mite's allergens, are difficult and generally not effective, while pharmacological treatment only controls acute symptoms.[5] At present, immunotherapy represents the only therapy for allergic diseases targeting sensitization itself. Several studies have shown that immunotherapy could be an option in patients with atopic dermatitis and one meta-analysis supports these results;[6-8] however, there are fewer studies evaluating the underling immunological mechanisms.[9,10] The aim of this study was to evaluate the effect of immunotherapy on basophil activation in patients with dermatitis. To achieve the goal we use flow

cytometry techniques and evaluate basophil activation by fluorochrome-labeled antibodies using anti-CD63 as a marked of basophil activation.

MATERIAL AND METHOD

Patients and study design

An open label study was done with four arms: Atopic dermatitis patients with immunotherapy and basic pharmacologic treatment (active group), atopic dermatitis patients only with pharmacologic treatment (AD control group), allergic rhinitis patients with immunotherapy (rhinitis group), and subjects without allergic diseases or IgE sensitization to mites (non-allergic group). Pharmacologic treatment was: oral antihistamines, emollients, topical steroids (hydrocortisone or betamethasone), topical tacrolimus and oral steroids. Skin management was administrated in staggered steps according the severity of symptoms,[11] and the use of oral steroids, as the potency of topical steroids and their frequency of use, were carefully recorded.

Subjects were recruited from the Allergy Unit of University of Antioquia (Medellin, Colombia) in 2010, from February to May. Ethic committee from University of Antioquia approved the protocol and informed consent was obtained from all subjects or their parents. The number of patients in groups with dermatitis was determined according to the patients who met the selection criteria during the period of recruitment and the availability of reagents for conducting experiments. The minimal number of patients per group (n=5) was taking as reference previous studies evaluating the reproducibility of basophils activation test in other allergic diseases. We selected patients over five years of age with clinical history of atopic dermatitis for more than two years, and IgE sensitization to *Dermatophagoides farinae* (*Der f*) and *Dermatophagoides pteronyssinus* (*Der p*) and Scoring of Atopic Dermatitis (SCORAD)[11] over 15 points at the beginning without significant improvement of symptoms during the last six months before immunotherapy administration. Patients with diagnostic of persistent moderate/severe allergic rhinitis (ARIA guideline)[12] for more than two years, were included as control group. Other sensitizations (cat dander, dog dander, pollen grains and fungi) were evaluated by skin test in all patients. Patients using immune suppressors like cyclosporine or biological agents in the last three months, and patients with systemic diseases that contraindicated the use of immunotherapy were excluded.[13]

Patients with dermatitis were diagnosed by an allergist, according to Hanifin y Rajka criteria.[14] The severity was assessed with SCORAD scale at the baseline and each three months during the follow up. Patients with atopic dermatitis were randomly (with Excel program for Windows) matched between active and control group in a location ratio of 1:1 according to severity, age and the pattern of sensitization (mono [only mites] and poly-sensitization [more than two unrelated allergen sources]). The use of concomitant medications, as emollients and topical and systemic drugs, was permitted in both groups according to the clinical evolution of each patient and was regularly registered. As we reported in a previous study,[15] we classified as "good control" those patients with no skin exacerbations for at least six months, and reduction >40% in topical steroid use and SCORAD *versus* baseline. "Regular control" classification applied to patients with less than 2 skin exacerbations in the last six months, reduction over 20% in topical steroids and 40% in SCORAD. "Poor control" was used when it didn't meet any of the above. Laboratory techs were blinded to the samples they received.

Total IgE and specific IgE and IgG4 for *Der p* and *Der f*

Serum samples were collected at baseline and at the first and second year of follow up. As we

described before,[15] serum levels of *Der f* and *Der p* specific IgE were measured using a flouroenzyme immunoassay (Phadia ImmunoCap System, Uppsala, Sweden). Sera yielding specific IgE levels above 100IU/mL were preliminarily diluted (1:5) to maintain the test within the dynamic range. IgG4 was measured using ELISA technique as we previously reported.[15]

Basophil activation test (BAT)

For BAT, 8 mL venous blood was drawn into acid-citrate-dextrose tubes and stored at 4°C (less than 6 hours) before immunotherapy and at the first and second year of follow up. Basophil activation test with 1ng/mL of *Der p* extract stimulation was carried out within 6 hours of blood sampling for all samples and were incubated with allergen at 37°C for 30 minutes. Flow cytometric studies were done using standard techniques as previously published.[16] Anti-IgE-FITC was used as basophil select-marker and Anti-CD63-PE was used as to evaluate basophil activation. Data were analyzed using a FAC-Scan flow cytometer and CellQuest software (Becton Dickinson, New Jersey, USA). Before stimulation with allergen extract, basal CD63 expression was measured. Seven days before blood collection, patients suspended antihistamines and any other drug that could interfere with the test.

Immunotherapy

Subcutaneous immunotherapy with depigmented polymerized mites extract (0.5mL *Der f*/*Der p*, 50DPP, Laboratorios Leti (Madrid, España) or 0.5mL *Der f*/*Der p*, 10.000 UT, Inmunotek (Madrid, España) was administrated monthly. Mite allergen extracts were administered in two refracted doses of 0.2 and 0.3 mL at build up phase, and in single 0.5 mL dose in maintenance phase.

Statistical analysis

Statistical analysis was performed with IBM SPSS statistics 21.0 for Windows. Total IgE, specific IgE and IgG4, and CD63 expression were compared between both AD groups using the Mann-Whitney test, and the Kruskal-Wallis test when compared the four groups. Calculated values were expressed as means and standard deviations of the mean, and medians and interquartile percentile 25 and 75 (IQ_{25-75}). The non-parametric tests were used after performance of the Shapiro-Wilks test to compare the normality of the samples; $p < 0.05$ was considered statistically significant. For evaluating the correlation between SCORAD, BAT, serum-specific IgE and IgG4, we used the Spearman test.

RESULTS

Significant reduction in SCORAD and pharmacotherapy in active group

The baseline characteristics of patients are described in Table 1. After six months, active group had a significant improvement over atopic dermatitis control group in SCORAD ($p = 0.04$). When we evaluated separately the 3 SCORAD parameters, the greatest reduction was observed in body surface area affected, followed by patient's subjective assessment and intensity of symptoms in the first and second year.

After one year of follow up, a significant reduction in the frequency of topical steroids and tacrolimus was presented in the active group ($p = 0.02$). Table 2. Active group also required less oral steroid cycles than atopic dermatitis control group ($p < 0.01$, 4 patients in immunotherapy and 6 in atopic dermatitis control group). In the second year, 7 patients with immunotherapy (IT) and 4 in control group (CG) had "good control" classification; 3 IT and 4 CG, "regular control",

Table 1. Population characteristics

Baseline characteristics	Atopic dermatitis		Rhinitis	Healthy group
	With IT (%)	Without IT (%)	With IT (%)	Without IT (%)
Patients number	10 (100)	10 (100)	10 (100)	5 (100)
Age	8 (5-22)	8 (5-20)	9 (7-16)	12 (10-20)
Gender (female)	6 (60)	5 (50)	5 (50)	3 (60)
Atopic dermatitis	10 (100)	10 (100)	0	0
Asthma/rhinitis	8 (80)	8 (80)	10 (100)*	0
Mono-sensitization	5 (50)	5 (50)	7 (70)	0
Poly-sensitization	5 (50)	5 (50)	3 (30)	0
SCORAD (points)	33 (23-37)	33 (25-36)	N/A	N/A

* In rhinitis group four patients had asthma.
SCORAD: Scoring Atopic Dermatitis; N/A: not apply.

Table 2. Clinical response between atopic dermatitis groups

SCORAD	Control group	Active group	*p*
Basal	33 (25-36)	33 (23-37)	>0.05
1st year	31 (23-36)	19 (12-23)	0.03
2nd year	29 (22-35)	18 (12-22)	0.03
Topical immune suppressors	100%	100%	>0.05
1st year	80% (50-100)	60% (20-85)	0.02
2nd year	74% (54-94)	40% (10-63)	0.01
Oral steroids*	14	13	>0.05
1st year	10	6	<0.01
2nd year	8	5	<0.01

Severity was evaluated with SCORAD (Scoring Atopic Dermatitis) reduction in use of topical after one and two years compared with basal.
* Number of patients who received at least one cycle of oral steroid in the last year.

and 0 IT and 2 CG "poor control". None of the patients with immunotherapy presented systemic reactions.

Total IgE and specific IgE and IgG4 levels

There were no significant changes in total IgE and specific IgE for *Der p* and *Der f* between groups during the follow up, but concentration in atopic dermatitis groups (Table 3) was significantly higher than in rhinitis (data not shown). In non-allergic group, total IgE was less than 100KU/L and specific IgE for *Der p* and *Der f* was < 0.1 KU_A/L during the whole study. Specific IgG4 levels for *Der p* and *Der f* were significantly increased in the active group during follow up over the other three groups. In the rhinitis group, increase in IgG4 was observed after the first and second year with IT but it was not statistically significant (data no shown). No differences were found between immunoglobulin levels and groups according to age, sex or sensitization pattern (mono, poly-sensitization).

Basophil activation test

The CD63 expression before stimulation with *Der p* was less than 15% for all groups without significant difference between them. Both groups with atopic dermatitis had a similar frequency of basophil activation before treatment (% CD63 expression: atopic dermatitis and IT 66% *vs* atopic dermatitis without IT 63%), and it was higher than in the group with rhinitis (43%) and in the non-allergic one (10%) (Figure 1). Atopic dermatitis patients with immunotherapy showed a significant reduction in CD63 positive basophils after *Der p* stimulation when compared with atopic dermatitis control group at first (% CD63 expression: 51% *vs* 61% $p = 0.01$) and second year (% CD63 expression: 49% *vs* 61%

Revista Alergia México
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Table 3. Immunoglobulin levels in atopic dermatitis groups

Time	Control group	Active group	*p*
Total IgE	894 kU/L (461-5,450)	984 kU/L (431-5,600)	>0.05
1st year	878 kU/L (301-6,345)	893 kU/L (304-5,668)	>0.05
2nd year	904 kU/L (461-5,450)	924 kU/L (331-5,451)	>0.05
IgE *Der p*	127 kU_A/L (82-137)	130 kU_A/L (69-148)	>0.05
1st year	127 kU_A/L (88-140)	120 kU_A/L (55-149)	>0.05
2nd year	132 kU_A/L (67-156)	122 kU_A/L (50-154)	>0.05
IgE *Der f*	120 kU_A/L (68-127)	119 kU_A/L (62-121)	>0.05
1st year	110 kU_A/L (59-141)	116 kU_A/L (59-129)	>0.05
2nd year	128 kU_A/L (76-131)	120 kU_A/L (57-144)	>0.05
IgG4 *Der p*	0.612 mcg/mL (0.484-0.718)	0.624 mcg/mL (0.567-0.712)	>0.05
1st year	0.604 mcg/mL (0.503-0.712)	0.768 mcg/mL (0.633-0.901)	<0.01
2nd year	0.625 mcg/mL (0.514-0.716)	0.789 mcg/mL (0.645-0.890)	<0.01
IgG4 *Der f*	0.612 mcg/mL (0.563-0.711)	0.624 mcg/mL (0.547-0.700)	>0.05
1st year	0.599 mcg/mL (0.560-0.699)	0.760 mcg/mL (0.600-0.914)	<0.01
2nd year	0.621 mcg/mL (0. 510-0.718)	0.783 mcg/mL (0.627-0.850)	<0.01

$p = 0.01$). At the first and second year, rhinitis group had a significant reduction in BAT compared with basal (first year: 37% $p = 0.01$, second year: 29% $p = 0.01$). The final percentage of the reduction was a little higher, but not statistically significant, in active group compared with the rhinitis group (% reduction [baseline - second year]: active group 17% *vs* rhinitis group 14%), and the final proportion of reduction was a little higher in rhinitis group, but it was not statistically significant (% proportion reduction [second year reduction x 100%/baseline]: active group 25.7% *vs* rhinitis group 32.5%). In non-allergic group BAT was always less than 12%. We observed a significant direct correlation between reduction in SCORAD and BAT after first ($r = 0.535$, $p = 0.01$) and second year ($r = 0.617$, $p < 0.01$) of follow up (Figure 2).

Respiratory symptoms

Patients in the active group with asthma and/or rhinitis, and those in the rhinitis group, presented a significant reduction in respiratory symptoms after four to six months compared with atopic dermatitis control group according to Asthma Control Test (ACT) and the ESPRINT-28 questionnaire for rhinitis (data no shown).

DISCUSSION

Atopic dermatitis typically begins in early infancy and usually has a good prognostic with remission or with a significant reduction of severity. However, in a group of patients atopic dermatitis persists, having a severe impact in the quality of life and more risk of side effects due to requirement of immunosuppressive therapies (oral steroids, cyclosporine, etc.).[17]

The principal aim of this study was to evaluate the immunological changes between atopic dermatitis patients with IT, and due to the small sample size, we must be careful to evaluate the effectiveness. Nevertheless, similar to a previous study published by us,[15] we observed that atopic dermatitis patients with immunotherapy presented an important reduction of symptoms, affected surface body area, and use of topical immunosupressors and oral steroids after six months of treatment compared with a control group with only pharmacotherapy. Many other studies have

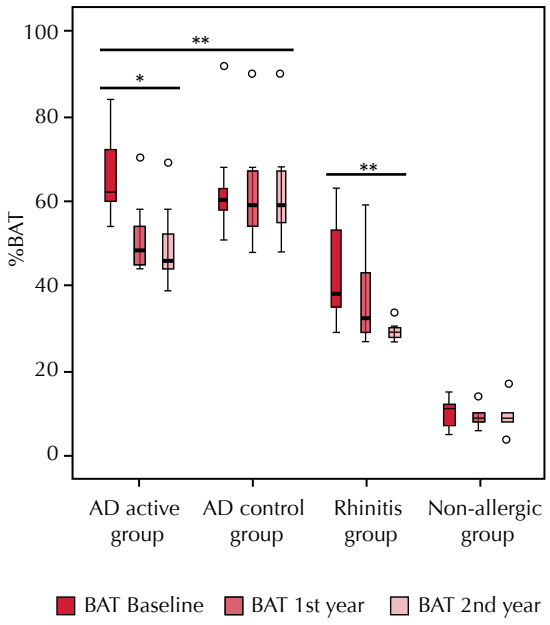

Figure 1. Basophil activation test (BAT) in patients with atopic dermatitis and immunotherapy (AD IT), atopic dermatitis without immunotherapy (AD without IT), rhinitis and immunotherapy and non-allergic group. Median and IQ_{25-75}: active group (baseline 62 IQ 60-72, 1st year 49 IQ_{45-54}, 2nd year 46 IQ_{44-52}), AD control group (baseline 60 IQ 58-63, 1st year 59 IQ_{54-67}, 2nd year 60 IQ_{55-67}), rhinitis group (baseline 39 IQ_{35-53}, 1st year 33 IQ_{29-43}, 2nd year 29 IQ_{28-30}), non-allergic group (baseline 11 IQ_{7-12}, 1st year 9 IQ_{8-10}, 2nd year 10 IQ_{8-10}).

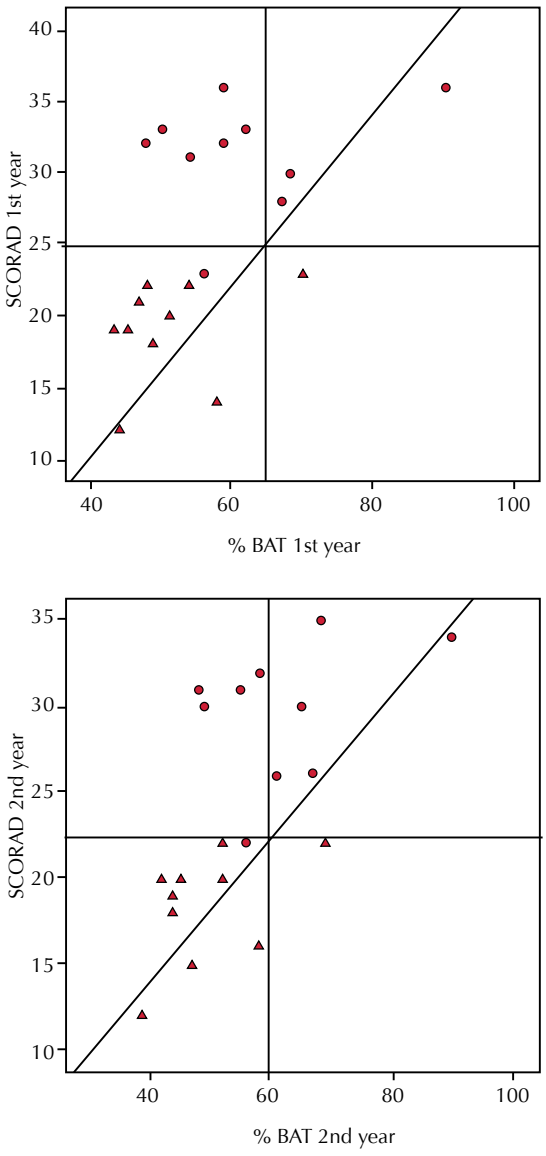

Figure 2. Correlation between SCORAD and BAT after 1st (r 0.535, $p = 0.01$) and 2nd (r 0.617, $p < 0.01$) year. Group with IT (triangles) has less basophil activation and SCORAD than patients with only pharmacotherapy (circles).

reported similar results with different impact according to the severity of patients;[18-21] however, less research has been done focusing on the immunological mechanisms of this clinical effect. Bussman, et al. found a significant reduction in mites specific IgE and increased in IgG4 for *Der p* 1 and *Der p* 2.[18] Similar results were observed by Novak et al., but other studies have no found changes in the level of immunoglobulins and contradictory results have also been observed with other biomarkers.[20,22-24] The heterogeneity found in the immunological markers may be due to study design (sampling time, number of patients), but also to differences in environmental exposure, sensitization pattern, dermatitis phenotypes and genetic background. We observed a significant increase in mites-specific IgG4, but not a significant change in specific and total IgE in patients with immunotherapy compared

to control group before and after twelve and twenty-four months of treatment. The lack of changes in the levels of total IgE and specific IgE in our population may be because in the tropical environment there is a very high and constant exposure throughout the year to mites[25] and a high segment of the population has a history of intermittent exposure to helmints like *Ascaris lumbricoides* who share cross reactivity with mites as we previously reported.[26,27] Similar to our previous study,[15] the mono-sensitized group receiving immunotherapy had a tendency to higher increase in mite-specific IgG4, remarking that some variances in the immune response may be due to the pattern of sensitization.

The basophil activation test (BAT) complements skin tests and specific IgE determination in the diagnostic of immediate-type reactions to allergens such as aeroallergens, hymenoptera venom, latex, foodstuffs, and drugs.[16,28-30] For several years, BAT has been tested as a potential marker of clinical response to immunotherapy, especially with hymenoptera venom,[31] and recently for the follow up of patients with food or drug allergy with desensitization protocols.[32,33] However, no single universal protocol for BAT is available.[34] To our knowledge, this is the first study in patients with atopic dermatitis that evaluated the response to immunotherapy with basophil activation test. We observed that after a year with *Der f/Der p* immunotherapy, activation of basophils with *Der p* stimulation, was lower than in control groups and this reduction was more significant at twenty-four months. We also observed a clear correlation between SCORAD, BAT and specific IgG4 concentration, suggesting that the BAT and IgG4 levels can be used as biomarkers to monitor the efficacy of immunotherapy in patients with dermatitis. We did not evaluate the BAT by stimulation with *Der f*; however, due to the high cross-reactivity between *Der p* and *Der f*, we suppose that we would have found similar results.

We use a group of patients with only rhinitis to compare basophil activation among patients with allergic respiratory and skin disease. Our hypothesis is that due to the aggressive environment of the skin in patients with atopic dermatitis, exposure to allergens and other irritants is increased in quantity and variety compared with patients with only rhinitis. For that reason, in atopic dermatitis, basophils are more stimulated and have an increased sensitivity to react to various stimuli, which probably leads to develop more FcR1-exilon receptors in their membranes, thereby facilitating activation. This hypothesis is supported by the lower activation of basophils found by us among patients with rhinitis, and by the results of Weisse et al.,[35] who observed that environmental factors like tobacco smoke and volatile organic compound are associated with changes in numbers of circulating eosinophils and basophils progenitors at first year of age and early life skin manifestations, so the recruitment and differentiation of eosinophils and basophils in response to environmental triggers may play a role in the development and severity of atopic dermatitis. Despite the higher basophil activation in patients with atopic dermatitis over rhinitis patients after stimulation with allergens, this do not appear to increase the risk of systemic reactions after immunotherapy. Kim et al.[36] reported that systemic reactions were observed in 4 of 15 patients (26.7%) with atopic dermatitis during rush immunotherapy and 4 of 18 patients (22.2%) with asthma ($p > 0.05$); however, Novak et al.[22] reported systemic adverse reactions such as flare-ups of eczematous and urticarial lesions, symptoms of rhinitis, pruritus, transient headache, and asthma in only 8% of actively treated patients and 10.7% in placebo treated patients. We recently published a safety study of immunotherapy with depigmented extracts in allergic diseases including 101 patients with atopic dermatitis with a media of 10 injections for patient (more than 1,000 injections in total)

and none had a systemic reaction,[37] suggesting that other factors such as the type of extract used and severe lower respiratory symptoms have more influence as risk factors of systemic reactions than dermatitis condition.

The major limitation of this study is that it is open label, making it difficult to evaluate the significance of clinical improvement. The small number of subjects in each group further limits the significance of clinical results. Nevertheless, the principal aim of this study was to evaluated basophil activation in atopic dermatitis patients after IT, and this outcome is little or unaffected for open label design; all patients who received immunotherapy had a significant reduction in BAT compared to control groups and with baseline values, suggesting an immunological changed in common. Since most patients with AD also had an allergic respiratory disease, we can't be totally sure that BAT inhibition is a marker of IT mechanism of action in AD treatment because it could be just as well an indication of its efficacy to treat allergic rhinitis and/or asthma as other authors have shown before.[16] However, the moderate correlation between BAT inhibition and SCORAD reduction suggests that at least part of this association may reflect an effect of immunotherapy in skin. In fact, this correlation was also present between the two atopic dermatitis patients without rhinitis who received immunotherapy, but not between the two atopic dermatitis patients without respiratory diseases in the control group.

CONCLUSION

Specific allergen immunotherapy may have a significant impact on clinical improvement in atopic dermatitis by changing the activation threshold of basophils and promoting IgG4 elevation, but more information is needed before recommending these methods for routine clinical evaluation.

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