INTRACELLULAR CYTOKINE PRODUCTION IN CHILDREN WITH ATOPIC DERMATITIS: EFFECT OF LEVOCETIRIZINE TREATMENT

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Objective The aim of this study was to evaluate intracellular cytokine production in peripheral blood of children with atopic dermatitis (AD) before and following 2 weeks of treatment with levocetirizine.

Patients and Methods Ten Kuwaiti children having mild to moderate AD were enrolled in the study. Following levocetirizine treatment, the clinical symptoms and flow cytometric analysis of T cell intracellular cytokines were evaluated.

Results The drug-treated children exhibited a reduction in percentages of eosinophil count ($p < 0.05$) as well as the major clinical symptoms, itching/scratching ($p < 0.05$) and the subsequent bleeding of lesions ($p < 0.01$); however the total symptom score was not significantly changed. Levocetirizine treatment was also associated with a reduction in IL-5 and IL-13 along with an increase in IL-10 expression on CD4+ T cells ($p < 0.05$). Trends for correlation of change in cytokine expression with clinical symptom score after treatment were noted, but failed to attain statistical significance.

Conclusions An immunoregulatory function for leveocetirizine is suggested by the present study, however such a claim may be more definitively made in future studies if trends established here prove consistent with larger sample number.

Key words: Levocetirizine ▪ Atopic dermatitis ▪ Children ▪ Cytokines

Introduction

Atopic dermatitis (AD) represents a pruritic chronic inflammatory skin disease with a complex background, triggered by genetic and environmental factors which begins pri-

marily in early childhood (1, 2, 3). The pathophysiology of AD is the product of a complex interaction between various susceptibility genes, host environments, defects in skin barrier function, and immunologic responses (4). Activation of T lymphocytes, dendritic cells (DCs), macrophages, keratinocytes, mast cells, and eosinophils are characteristic of AD skin inflammatory responses (5).

Recent investigations have been focused on T-cell contribution to the abnormal regulation of the immune response in atopic diseases. Studies on T-cell clones support the concept that activation of a subpopulation of CD4+T helper cells leads to the release of various cytokines important for the pathogenesis (6). Upon encountering antigen-carrying dendritic cells in secondary lymphoid organs, naïve T-lymphocytes proliferate and differentiate into two subgroups (Th 1 and Th 2) based on their cytokine production profiles: Th1 (mainly producing IFN- γ) and Th2 (secreting IL-4, IL-5, and IL-13) (7). The onset of acute AD is strongly associated with the production of IL-4 and IL-13, levels of which are significantly higher in AD individuals compared with control subjects (8). Mediating isotype switching to IgE synthesis and upregulating expression of adhesion molecules on endothelial cells, IL- 4 and IL-13 are implicated in the initial phase of tissue inflammation. IL-5, which is a Th2-associated cytokine involved in eosinophil development and survival, is also a hallmark cytokine produced by T cells at high levels in AD (8). Peripheral blood levels of IFN-γ are lower in atopic patients relative to Th2 cytokines (9). The Th2-dominated atopic state is also characterized by increased IgE levels, and mast-cell degranulation on exposure to antigen (10). Moreover, a recently published report demonstrated a preferential apoptosis of circulating Th1 cells in AD, which also may contribute to Th2 predominance in AD patients (11).

There is now considerable evidence, from both in vitro and in vivo studies, that several H1 antihistamines possess anti-allergic/anti-inflammatory effects, some of which may be dependent on H1-receptor blockade while others are receptor independent (12). Understanding of the molecular mechanisms by which H1-antihistamines interact with histamine H1-receptors has increased greatly during the past decades and it is now known that they do not block these receptors. Rather, all H1-antihistamines in use function as inverse agonists. They have a preferential affinity for the inactive state of the histamine H1-receptors. They stabilize the receptors in this conformation, with a shift in equilibrium toward the inactive state and consequent down-regulation of acute and chronic allergic inflammation (13, 14). Down-regulation of NF-κB, which acts as a potent transcription factor in initiating inflammation, may represent a possible mechanism for H1-antihistamines to inhibit inflammatory cell accumulation (15). Low concentrations of the histamine H1 antihistamines cetirizine and azelastine have been demonstrated to down-regulate NF-κB expression in parallel to inhibition of pro-inflammatory cytokines (16).

Levocetirizine (Xyzal®) is the active R-enantiomer of the racemate cetirizine. It is highly selective for the human histamine H1-receptor, with which it has twice the binding affinity of cetirizine (17) and is a potent antihistamine, as demonstrated by inhibition of histamine-induced weal and flare reactions (18) and in clinical studies (19). Studies of its pharmacology suggest that it modulates the profile of inflammatory mediators, including cytokines, growth factors, proteinases, and antiproteinases produced by eosinophils in vitro (20); however, the effect of therapeutic levels in vivo is still under investigation.

The aim of this study was to investigate the immunoregulatory properties of levocetirizine and its effectiveness in reducing

any of the clinical symptoms in treatment of children with atopic dermatitis.

Patients and Methods

Patients

The study included 10 children; 6 girls and 4 boys (9-14 years old: mean age 12.36 ± 0.9), having mild -moderate chronic atopic dermatitis, since early childhood. All of them had frequent relapses and poor response to the treatment. The diagnosis of atopic dermatitis was based on a positive personal and family history of atopy, typical eczematous skin lesions in that age group. Atopy was confirmed by increased level of total serum IgE and specific IgE to one or more inhalant and /or food allergens. Disease severity was assessed by a physician based on skin condition and impaired quality of life, expressed as sleep disturbance and poor school performance due to itching during disease relapse experienced in the last 6 weeks. It was expressed as clinical symptom scores as shown in Table 1.

Only children with mild or moderate symptoms were included in the study, all patients had TCSS 4-8 (range 4-12). An age and sex matched healthy group of children

Table 1 *The severity of the disease expressed as clinical symptom score in Atopic dermatitis patients*
Tabela 1 *Klinički simptomi/znaci izraženi brojčano u odnosu na težinu bolesti kod djece sa atopijskim dermatitisom*

Clinical symptoms/ Klinički simptomi	Score/Skor		
	1 (Mild/Blaga forma)	2 (Moderate/Srednja forma)	3 (Severe/Teška forma)
Skin dryness & thickening/ Suha i zadebljala koža	Area of dry skin without significant skin thickening/ Područja sa suhom kožom bez značajnih zadebljanja zahvaćene kože	General skin dryness and localized thickening/Suhoća kože u cijelosti uz zadebljanja kože na pojedinim ekcematoznim lezijama	Skin dryness with widespread skin thickening/Veoma suha koža u cijelosti sa zadebljanjima kože na mnogobrojnim ekcematoznim lezijama
Skin itching & scratching/Svrbež i izgrebanost kože	Itching without significant scratching/ Blagi svrbež bez izraženog jačeg grebanja kože	Frequent itching with localized excoriation/ Često svrbež sa lokalizovanim ogrebotinama kože	Incessant itching/ scratching with loss of skin integrity/ Stalni svrbež i grebanje sa većim oštećenjem kože
Bleeding of scratched areas/Krvarenje iz razgrebane i oštećene kože	No bleeding/Bez znakova krvarenja iz svrbežom zahvaćene kože	Bleeding from a few lesions/Neznatno krvarenje iz zahvaćenih kožnih promjena	Most of the lesions bleed and/or have dark pigmentation/ Veći dio ostećene kože krvari i/ili je tamno pigmentiran
Quality of life expressed as night sleep and school performance disturbance/Kvalitet života promijenjen poremećajem u spavanju i dnevnim aktivnostima (školske obaveze)	Not affected/Nema promjena u kvaliteti života	Affected occasionally/ Povremeno zahvaćen	Frequently affected/ Često negativno promijenjen

with negative family history of atopy and low total IgE (< 140 IU/ml) served as a control group. The patients who used topical corticosteroids within the previous 4 weeks, or antihistamines within the previous week were excluded. Skin emollients have been allowed to be used during the trial. Blood samples were collected before treatment with levocetirizine (5 mg/day) and following two weeks of the treatment. Levocetirizine was taken regularly, regardless of signs and symptoms of disease. Informed consent was obtained from parents of each child included in the study and all phases of the study were undertaken in compliance with Ministry of Health regulations for studies involving human subjects.

Intracellular cytokines analysis

Peripheral blood mononuclear cells (PBMC) were separated by Ficoll-paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. The cells were washed and suspended in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD) at density of 1×10^6 cells/ml. 200 μl cultures of PBMC in 96-well plates were incubated under humidified conditions for 24 hours at 370 C. The cells were stimulated in the presence of 50 ng/ml of phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO), 1 ng/ml of ionomycin (Sigma) and 2 mM monensin (Sigma). Following CD3 and CD8 cell surface staining for 15 min, cells were fixed for 15 min using a Fix and Per cell permeabilization kit (Caltag Laboratories, Burlingame, CA) and permeabilized with the same kit and stained intracellularly for 30 min at room temperature with monoclonal antibodies to IL-4, IL-5, IL-10, IL-13, TNF-α and IFN-y provided by BD Pharmingen. After washing, cells were analyzed by flow cytometry. Three parameter analyses were undertaken on FC-500 (Beckman Coulter Corporation, Hialeah, FL, USA). Negative isotype controls were used to verify the staining specificity of the antibodies used. In the analysis of the cytokine production we refer to the CD3+/CD8- population as the CD4+ population.

Statistical analysis

To compare the results before and after treatment, paired student t-test, and Mann–Whitney U-test were applied. A P-value of <0.05 was considered statistically significant. The P values of significant differences are indicated in figure and tables. Correlations between variables were performed using Spearman rank correlation test.

Results

Table 2 shows the median and interquartile ranges (IR) for CD4+ and CD8+ T cells expressing IL-4, IL-5, IL-10, IL-13, TNF- α, and IFN-γ in patients and control groups. Atopic dermatitis patients at baseline showed a significant increase in the percentages of CD4+IL-4+ and CD4+IL-5+ cells compared to healthy controls (median: 3.32, IR: 4.2-2.8) versus (median: 2.08, IR: 2.24-1.44; $p < 0.01$) and (median: 2.45, IR: 2.80-2.14) versus (median: 1.23, 1.6-1.03; $p < 0.01$) respectively, on the other hand a significant decrease in the percentages of CD4+IFN-γ+ cells was observed (median: 5.9, IR: 8.1-4.1) versus (median: 11.5, IR: 13.4-8.8; $p < 0.05$). The percentages of both CD4+IL-13+ and CD8+IL13+ cells were significantly increased at baseline patients compared to controls (median: 5.04, IR: 9.7-4.1) versus (median: 2.36, IR: 3.3-1.24; $p < 0.01$) and (median: 0.84, IR: 1.1-0.8) versus (median: 0.71, IR: 0.94-0.39; $p < 0.05$) respectively.

Table 2 *Median and interquartile ranges (IR) of the frequency of eosinophils, neutrophils, CD4+ and CD8+ T cells expressing cytokines in atopic dermatitis at baseline, following levocetirizine treatment and control children*

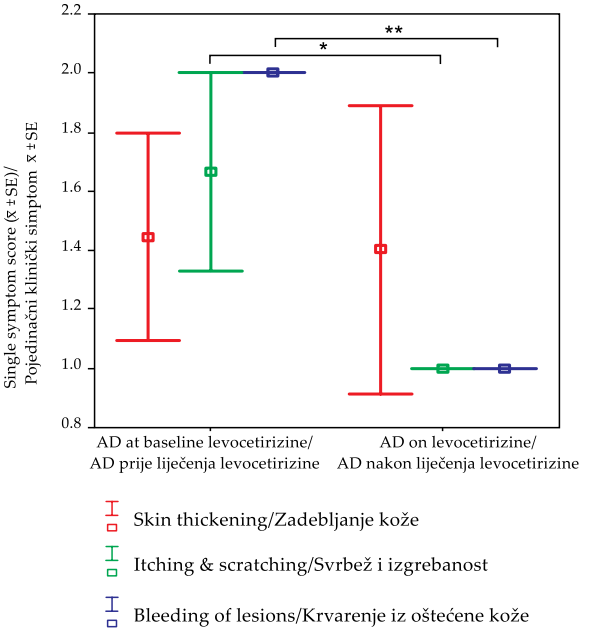
Tabela 2 *Vrijednosti eozinofilnih, neutrofilnih i CD4+ i CD8+ T stanica i njihovih citokina (Mediana i IR) prije i nakon terapije levocetirizinom kod oboljele i zdrave djece*

	Atopic dermatitis at baseline/Atopijski dermatitis prije liječenja		Atopic dermatitis on Levocetirizine /Atopijski dermatitis nakon liječenja Levocetirizinom		Control/Kontrola	
	Median	IR (75-25)	Median	IR (75-25)	Median	IR (75-25)
Eosinophils/ Eozinofilne stanice	8.60[A]	11.65-4.25	2.0[C]	4.25-1.85	2.2	4.25-0.83
Neutrophils/ Neotrofilne stanice	48	51-44	51.1	56.3-43.2	48	79-30
IL-4						
CD4+	3.32[B]	4.20-2.80	4.20-2.80	2.75-3.90	2.08	2.24-1.44
CD8+	0.94	1.18-0.32	1.18-0.32	0.78-0.31	0.57	0.93-0.32
IL-5						
CD4+	2.45[B]	2.80-2.14	0 .00[C]	2.10-1.54	2.10-1.54	1.60-1.03
CD8+	0.47	0.75-0.39	0.53	0.86-0.32	0.86-0.32	0.42-0.26
IL10						
CD4+	2.59	2.73-1.06	3.51[C]	4.05-2.90	2.72	2.91-2.14
CD8+	0.90	1.20-0.32	0.57	0.98-0.32	0.90	1.23-0.33
IL-13						
CD4+	5.04[B]	9.70-4.10	3.40[C]	5.0-2.60	2.36	3.30-1.24
CD8+	0.84[A]	1.10-0.80	0.47[C]	0.8-0.37	0.71	0.94-0.39
TNF-α						
CD4+	6.10	9.80-5.20	10.45	12.48-7.45	5.43	7.20-3.30
CD8+	4.30	5.70-3.60	7.55	11.93-2.10	3.60	5.90-3.10
IFN-γ						
CD4+	5.90[A]	8.10-4.10	6.70	8.50-5.60	11.5	13.40-8.80
CD8+	4.75	7.73-3.88	5.30	5.90-2.75	4.0	6.12-2.25

[A] $p < 0.05$, [B] $p < 0.01$ versus healthy control/u odnosu na zdravu djecu; [C] $p < 0.05$ versus baseline/u odnosu na vrijednosti prije tarapije

Levocetirizine treatment reduced the percentages of eosinophils but not neutrophils in AD (median: 2.0, IR: 4.25-1.85) versus baseline (median: 8.6, IR: 11.65-4.25; $p < 0.05$) (Table 2).

The analysis of single symptoms score showed a decrease in itching/scratching ($p < 0.05$) as well as the subsequent bleeding of lesions ($p < 0.01$) following levocetirizine, however the total symptoms score was not significantly changed (Figure 1)



AD = Atopic dermatitis/Atopijski dermatitis

Figure 1 *Error bars of the single symptom score before and after treatment with levocetirizinom in Atopic dermatitis children*
Slika 1 *Pojedinačni klinički simptomi i znaci prije i nakon liječenja levocetirizinom kod djece sa atopijskim dermatitisom*

A significant decrease was observed in the percentage of CD4+ T cells expressing IL-5 in the levocetirizine treated group compared to baseline levels (median: 2.0, IR: 2.1-1.54) versus (median 2.45, IR: 2.8-2.14; $p<0.05$). CD4+ and CD8+ T cells expressing IL-13 were significantly decreased versus baseline levels (median: 3.4, IR: 5.0-2.6) versus (median: 5.04, IR: 9.7-4.1; $p < 0.05$) and (median: 0.47, IR: 0.8-0.37) versus (median: 0.84, IR: 1.10-0.8; $p < 0.05$) respectively. In addition, CD4+ T cells expressing IL-10 were significantly increased (median 3.51, IR: 4.05-2.9) versus baseline (median: 2.59, IR: 2.73-1.06; $p < 0.05$) (Table 2). The total symptom score did not show any correlation with T cell cytokine production in AD patients at baseline or following levocetirizine treatment. On the other hand, skin itching/scratching correlated directly with increased percentages of CD4+IL-5+ T cells at baseline ($r = 0.943$) (Table 3). The improvement in clinical symptoms did not correlate with reduced lymphocyte expression of IL-5 and IL-13 or upregulation of IL-10 after Levocetirizine treatment.

Discussion

In this study we analyzed the frequency of intracellular-cytokine-producing T cells in peripheral blood of AD children. We found a significant increase in IL-4 and IL-5 but a significant decrease in IFN-γ in CD4+ T cells; on the other hand IL-13 was increased in both CD4+ and CD8+ T cells (Table 2). Earlier studies have shown similar results such as reduced IFN-γ production after in vitro stimulation of PBMCs from patients with

Table 3 *Relationships between clinical symptom score and frequency of CD4+ cells expressing IL-5 in peripheral blood of atopic dermatitis patients at baseline*
Tabela 3 *Vrijednosti - CD4+ IL-5 T stanica u perifernoj krvi i njihov odnos sa kliničkim simptomima prije započete terapije*

	Skin thickening/Zadebljanje zahvaćene kože	Skin itching & scratching/Svrbež i grebanje	Bleeding of the lesions/Krvarenje iz oštećene kože
At baseline CD4+ IL5/ Prije liječenja CD4 + IL-5	-0.544	0.943[A]	-0.272

[A] $p < 0.05$

AD (21-23). Patients with AD are char-acterized by enhanced IgE production (24, 25). It has been shown that the class switch of IgE and production of IgE in B cells is regulated by cytokines produced by T cells (26, 27), particularly IL-4, IL-13 and Il-10 (28). In contrast, IFN-γ inhibits IgE synthesis. Therefore enhanced IgE synthesis in patients with AD may be caused by up-regulation of Th2 cytokine-producing cells, especially those that produce IL-4, and IgE synthesis may be suppressed by Th1 cytokine-producing cells, especially IFN-γ-producing cells.

The mechanism underlying reduced IFN-γ production in AD is not clear and needs further investigation. A possible explanation could be that activated T cells with skin-homing properties, which express high levels of IFN-γ, predominantly undergo apoptosis in the circulation, skewing the immune response to surviving Th2 cells (29). There is also a possibility that steroid therapy may contribute to a reduced ability to produce IFN-γ as was reported by Jung et al. (30), however, our patients had not received steroid therapy for 4 weeks before the study.

Recent studies focused on the functional heterogeneity of CD8+ T cell as they can secrete Th2 cytokines and help B cells with antibody production (31). These cells appear to participate in the induction of high IgE levels, although the exact role is still controversial (26). A similar pattern of cytokine production in CD4+ as in CD8+ cells has been found in asthmatic patients (27) and in AD patients (31). In our study, only IL-13 expression by the CD8 subpopulation was higher at baseline compared to the control, and this was minor compared with the cytokine expression by CD4 subpopulation (Table 2).

Our study investigated in vivo effects of levocetirizine on lymphocyte expression of intracellular cytokines which play a critical role in pediatric AD. Levocetirizine was shown to reduce IL-5 and IL-13 in CD4+ cells, IL-13 in CD8+ cells and increase IL-10 in CD4+ cells. IL-5 is capable of both recruiting and activating eosinophils, resulting in degranulation and secretion of granule proteins (32) which deposit in AD skin lesions (33). The increased percentages of CD4+IL-5+ T cells at baseline correlated directly with skin itching/scratching ($r = 0.943$, $p < 0.05$) in all patients (Table 3). IL-13 may also be critical in regulating inflammatory and immune responses, such as inducing CD23 expression on B cells; enhancing CD72, surface immunoglobulin M (IgM), and class II major histocompatibility complex (MHC) antigen expression; and inducing germline IgE heavy chain gene transcription in B cells (34). Akdis et al. has demonstrated that Cutaneous lymphocyte-associated Ag (CLA)+ T cells in AD spontaneously secrete IL-5 and IL-13, and functionally prolong eosinophil survival and induce IgE synthesis (35). Reduced expression of cytokines may reduce the recruitment of inflammatory cells into the skin. Also several cytokines such as IL-2, IL-4 or IL-15 prolong T-cell survival (36); in their absence activated lymphocytes may undergo apoptosis.

IL-10 is known to regulate various effector immune responses (37). A recent investigation found that mildly affected AD subjects have higher frequency of CD4+IL-10+ T cells than severely affected subjects suggesting that a process of active suppression could occur during the natural course of the disease and this argues in favor of the role of IL-10 in the control of AD (38). In our study, children on levocetirizine exhibited elevated levels of IL-10 relative to baseline levels. To our knowledge, this is the first study that reports this effect. This trend is in favor of the role of levocetirizine in control of atopic inflammation, however this needs to be further investigated in studies with a larger sample number.

The single symptoms score analysis showed that levocetirizine had reduced major clinical symptoms (Fig. 1); however the total symptom score was not changed. The reduction of cytokines on levocetirizine was not associated with improvement in clinical symptoms, which may be due to the small sample size in this study. Our results suggest that levocetirizine has pharmacologic potential beyond H1 histamin-receptor inhibition. The data trends noted in the present study serve as a guide for further investigation of the immunoregulatory properties of this drug in future investigations.

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Sažetak

EFEKT LEVOCETIRIZINA NA PRODUKCIJU INTRACELULARNIH CITOKINA U DJECE SA ATOPIJSKIM DERMATITISOM

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Cilj Istraživanje je poduzeto sa ciljem da se ustanovi produkcija intracelularnih citokina u perifernoj krvi kod djece sa atopijskim dermatitisom prije i dvije nedjelje poslije tretmana sa Levocetirizinom.

Pacijenti i metode U studiju je uključeno desetero kuvajtske djece oboljele od blaže i umjereno teške forme atopijskog dermatitisa. Nakon tretmana Levocetirizinom evaluirani su klinički efekt i sekrecija intracelularnih citokina koristeći flow citometrijsku metodu.

Rezultati Utvrđeno je ublaženje glavnih kliničkih simptoma: svrbeža kože ($p < 0.05$) i pojave krvarenja iz kožnih lezija izazvanog mehaničkom provokacijom ($p < 0.01$), kao i smanjenje broja eosinofila u perifernoj krvi ($p < 0.05$). Međutim, ukupni klinički skor nije signifikantno promijenjen. Nakon terapije Levotirizinom ustanovljeno je smanjenje produkcije IL-5 i IL-13, dok je ekspresija CD4 T stanica i IL-10 povećana ($p < 0.05$). U ovom istraživanju nije ustanovljena statistički signifikantna korelacija u produkciji citokina i promjene u ukupnom kliničkom skoru.

Zaključak Dobijeni rezultati sugerišu imunoregulatorni potencijal nove generacije antihistaminika. Za ovakvu tvrdnju potrebne su daljne slične studije na većem uzorku djece.

Ključne riječi: Levocetirizine ▪ Atopijski dermatitis ▪ Citokini

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