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Co-Administration of Chenopodium Album Allergens and CpG Oligodeoxynucleotides Effects on Peripheral Blood Mononuclear Cells of Patients with Allergic Rhinitis Treated with Intranasal Corticosteroids and Antihistamines

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ABSTRACT

Allergic Rhinitis (AR) is one of the most common chronic diseases in the developed countries. This study was performed to investigate the effect of CpG-ODN in alteration of T-helper (Th)1/Th2 balance of patients with AR treated with intranasal corticosteroids (INCs) and antihistamines. Peripheral blood mononuclear cells (PBMCs) of 20 patients with AR were isolated before and after 45 days therapy.

Cytokine production (IL-4, IL-10, IL-13, IFN- γ) and specific Ch.a IgE in response to CpG co-administration of natural chenopodium album (CpG/Ch.a) or recombinant Ch.a (CpG/rCh.a) allergen were investigated in supernatants of cultured PBMCs using ELISA. Intracellular IL-10 was also assessed in CD4⁺ cells using flow cytometry. Significant increase in production of IFN- γ and IL-10 and decrease in production of IL-4 were found in supernatants of cultured PBMCs activated with CPG/ch.a and CPG/rch.a. of both CpG/Ch.a and CpG/rCh.a compared to allergens alone, before and after therapy.

After therapy, IFN- γ production with CpG/Ch.a was significantly increased in comparison with before (237 *vs.* 44 pg/ml, $p=0.001$). IFN- γ and IL-10 production with CpG/rCh.a was significantly increased after therapy compared to before (407.6 *vs.* 109 pg/ml, $p=0.01$ for IFN- γ ; 171.7 *vs.* 52.6 pg/ml, $p=0.008$ for IL-10), whilst IL-4 was significantly decreased (2.1 *vs.* 5.8 pg/ml, $p=0.02$). Intracellular IL-10 expression was also significantly increased in response to either CpG/Ch.a or CpG/rCh.a that showed intracellular assay could be more sensitive than ELISA. Also, treatment with intranasal corticosteroids and antihistamines could enhance this CpG effect, *in vitro*.

Keywords: Allergic Rhinitis; CpG-ODN; Chenopodium album; Cytokines; Specific IgE

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INTRODUCTION

Allergic rhinitis (AR), an inflammatory disorder of the mucosa in the upper airways, is a common disease which is estimated that affect 10-40% of general population, particularly in the developed countries.¹⁻³ The exact prevalence of AR in Iran is unknown, but a prevalence of 23.5% was estimated in school-aged children in Tehran.⁴

Allergic diseases such as AR result from T-helper (Th)2-dominant immune imbalance due to overproduction of Th2 type cytokines [particularly interleukin (IL)-4 and IL-5] and decrease in Th1 cytokine production [such as interferon (IFN)- γ and IL-12].⁵⁻⁹ Therefore, one aim of treating AR with immunomodulators would be to try to shift the dominance of Th2 to Th1 type response. Numerous studies proposed a new strategy for biasing the immune response toward Th1 in allergic diseases using toll-like receptor (TLR) ligands (particularly TLR9) in combination with allergens.¹⁰⁻¹³ TLR9 has been found in peripheral blood mononuclear cells (PBMCs) like monocytes/ macrophages, B cells as well as in dendritic cells.^{14,15}

bacterial cytosine phosphorothioate guanosine (CpG) DNA and synthetic oligodeoxynucleotides containing unmethylated CpG motifs (CpG-ODN) bind to TLR9.¹⁶⁻¹⁸ These components enhance the production of IFN- γ , IL-6 and IL-12 and reduce the production of IL-3, IL-4, IL-5 as well as IgE synthesis by PBMCs.¹⁹⁻²³ It has been reported that co-administration of CpG-ODN and allergens could attenuate AR.^{24,25} Moreover conjugated forms of CpG-ODN and allergens were more effective to induce the production of a wide variety of cytokines from multiple cell types.^{26,27} However, there is not enough evidence on the effects of CpG-ODN alone or in combination with recombinant allergens on human subjects. In addition, our previous animal experiments indicated that intranasal co-administration of CpG and chenopodium album (Ch.a) allergen significantly increased both systemic and local levels of IL-10 and IFN- γ .^{28,29} We also reported that CpG had no significant effect on the levels of serum IgE, IgG2a, and IgG1 in asthma animal models,²⁸ while IgG1/IgG2a ratio was increased after co-administration of CpG and Ch.a allergen.²⁹

In this study, we sought to define comprehensively the pattern of immune reactivity of PBMCs of AR

patients which were co-cultured with CpG-ODN and a homemade natural Ch.a extract or recombinant chenopodium album (rCh.a) allergen. This study was performed to evaluate the potential effects of CpG-ODN in the immune deviation from Th2 to Th1 responses. For this, several cytokines such as IFN- γ , IL-4, IL-13, IL-10 and Ch.a specific IgE were measured in culture supernatants of PBMCs of patients. Moreover, in order to assess the synergistic effects of intranasal corticosteroids (INCs) and antihistamines, as regular therapy for AR patients, with CpG we measured aforementioned cytokine profiles of supernatants of PBMCs after therapy.

PATIENTS AND METHODS

Patients

Twenty patients (7 male and 13 female), with mean age of 29 ± 6 years, who were allergic to Ch.a [mean diameter weal of 7.7 ± 6.2 mm in skin prick test (SPT)] were enrolled in the study. The inclusion criteria were: patients with age range of more than 18 years, presence of rhinitis symptoms, history of rhinitis ≥ 1 year and positive SPT to Ch.a allergen (tested with Ch.a extract, defined as a 3 mm weal greater than saline as negative control and histamine chloride (10 mg/ml) as positive control).

All participants had been free of medications 3 months prior to the study. The study was approved by the local Ethics Committee. Written informed consent was obtained from all subjects before any intervention. INCs and antihistamines prescribed for all patients. Blood samples were collected before starting therapy at first visit and 45 days after starting therapy.

Materials

Ch.a natural allergen was extracted and purified from whole Ch.a pollen, prepared in our laboratory according to previously reported procedures.³⁰ rCh.a allergen (AY082337 pub med, Che a 2) was prepared in Immunobiochemistry Lab, Immunology Research Center, Avicenna Research Institute, Mashhad University of Medical Sciences, Mashhad, Iran. ODN 2006 a CpG oligonucleotide type B- human TLR9 ligand [5'- tcg tcg ttt tgt cgt ttt gtc gtt -3' (24 mer)], and ODN 2006 Control [5'-tgctgcttttgtgcttttgtgcttt-3'(24mer)] contained GpC dinucleotides as a negative control was purchased from InvivoGen, San Diego, USA.

Peripheral Blood Mononuclear Cell Isolation and Culture

Ten milliliters (ml) of venipuncture blood from subjects were collected into falcon tubes containing EDTA (EthyleneDiamineTetraacetic Acid) or heparin (sample for flow cytometry) from each individual. PBMCs were immediately isolated using Ficoll-Paque (Fresenius Kabi Noge As for Axis-Shield Poc As, Oslo, Norway) gradient centrifugation. Washed cells were cultured in (at 37°C and with 5% CO₂) a 24-well plate (JET Biofil®, Canada) in RPMI 1640 containing 10% fetal calf serum (FCS), 50 µg/ml penicillin, 50 µg/ml streptomycin, 0.3 mg/mL L-glutamine, at a density of 1×10⁶ cells per well. PBMCs were cultured with the optimal concentration of CpG-ODN co-administration with natural Ch.a (CpG/Ch.a) or rCh.a (CpG/rCh.a). Also, cells were co-cultured with no-CpG no-natural Ch.a (ODN/Ch.a) or no-rCh.a (ODN/rCh.a) as control.

The optimal concentrations and culture durations were obtained from a pilot study using different reagents concentrations (5, 10, 25, 50 µg/ml for allergens, 2.5, 5, 10, 20 µg/ml for CpG-ODN and ODN as control) and culture periods (for 24, 72 and 96 h). Methyl Tetrazolium Bromide (MTT) assay³¹ was performed for determination of nontoxic dose of allergens.

The levels of secretory cytokines IFN-γ, IL-4, IL-10, IL-13 and Ch.a specific IgE were measured in culture supernatants, respectively, at each time point. Results of the pilot study were shown that the best reagents concentrations for natural Ch.a, rCh.a, CpG-ODN and ODN control were 50, 10, 5 and 5 µg/ml, respectively. Also, the best time period for culture of PBMCs was 96 h.

Cytokines and Ch.a Specific IgE Measurement

The supernatants were harvested 96 h after culture and stored at -80°C until the measurement of cytokine levels. ELISA sets for human IFN-γ, IL-4, IL-10, and IL-13 with sensitivity 2 pg/ml, purchased from U-CyTech biosciences (Utrecht, The Netherlands) were used for cytokine assay.

Also, Ch.a specific IgE was measured using ELISA kit from r-biopharm (Darmstadt, Germany) with sensitivity of 0.35 IU/ml.

Intracellular IL-10 Assay with Flow Cytometry

PBMCs (0.5×10⁶) were cultured for 12h with aforementioned reagents. Cells were harvested and washed. Intracellular IL-10 was assessed in CD4+ cells. For CD4+ staining, 10 µg FITC-conjugated mouse IgG1 anti-human IL-10 monoclonal antibody (mAb) (IQ Product, Groningen, The Netherlands) was added and incubated for 20 minute. Then Phorpol 12-myristate 13-acetate (PMA) (IQ Product, Groningen, The Netherlands) (10 µg/mL), ionomycin (IQ Product) (10 µg/mL) and monensin (IQ Product) (10 µg/mL) were added to stimulate the expression of the imprinted IL-10 pattern (5 h cells cultured, in 5% CO₂). After fixation, cells were washed and were then resuspended in 1.5 ml permeabilization solution. For intracellular IL-10 staining, 10 µg PE-conjugated mouse IgG1 anti-human mAb (IQ Product) was added, tubes were mixed well by vortexing and incubated for 20 min at 4°C in the dark. Resuspended cells were centrifuged at 200g with 1.5 ml permeabilization solution for 5 min, resuspended again and analyzed with a flow cytometer (Partec, Münster, Germany)

Statistics

Statistical analysis was performed using SPSS 16 software package (San Diego, CA). The effect of CpG-ODN on alteration of cytokine profiles was compared using Wilcoxon Signed Ranks Test. Comparisons of the results before and after treatments were also done using Wilcoxon Signed Ranks Test. *P* values of <0.05 were considered statistically significant.

RESULTS

Effect of CpG/Ch.a on Cytokine Profiles

The levels of IFN-γ, IL-4, IL-10 and IL-13 secreted in culture supernatants by CpG/Ch.a -stimulated PBMCs are shown in table 1. The stimulated cells with CpG/Ch.a secreted significantly higher levels of IFN-γ (Figure 1) and IL-10 (Figure 2) compared to the cells stimulated with Ch.a alone, while a significant reduction of IL-4 was found in CpG/Ch.a -stimulated cells (Figure 3). Similarly, these significant changes of cytokine production before therapy were achieved after therapy as well. Among these cytokines, the level of IFN-γ was significantly increased after in comparison with the level of this cytokine before therapy (Table 1).

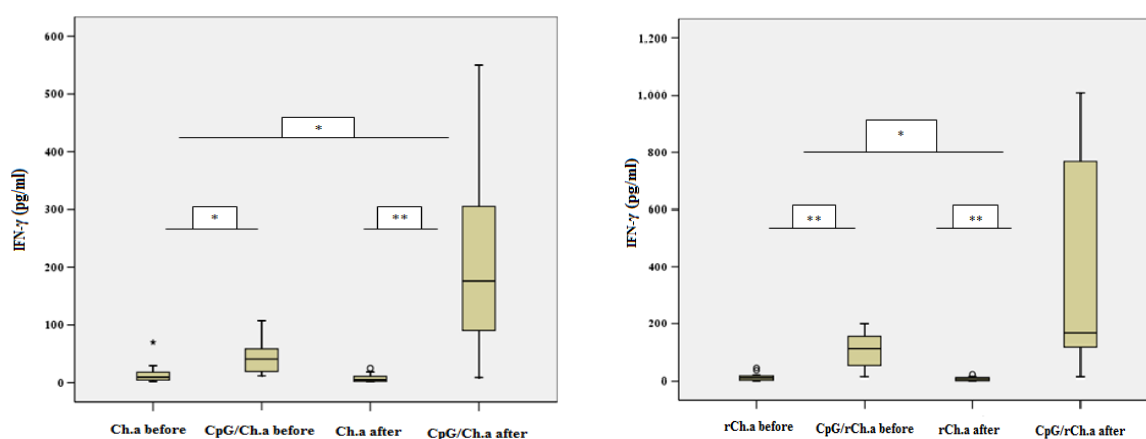


Figure 1. CpG/Ch.a or CpG/rCh.a induced a Th1-like (IFN- γ) response in PBMCs cultures from patients with Ch.a allergy after 96h of culture before and after therapy of patients by INCs and antihistamines. Statistical significance comparing Ch.a or rCh.a with CpG/Ch.a or CpG/rCh.a stimulation was calculated by using Wilcoxon. * $P < 0.05$; ** $P < 0.005$.

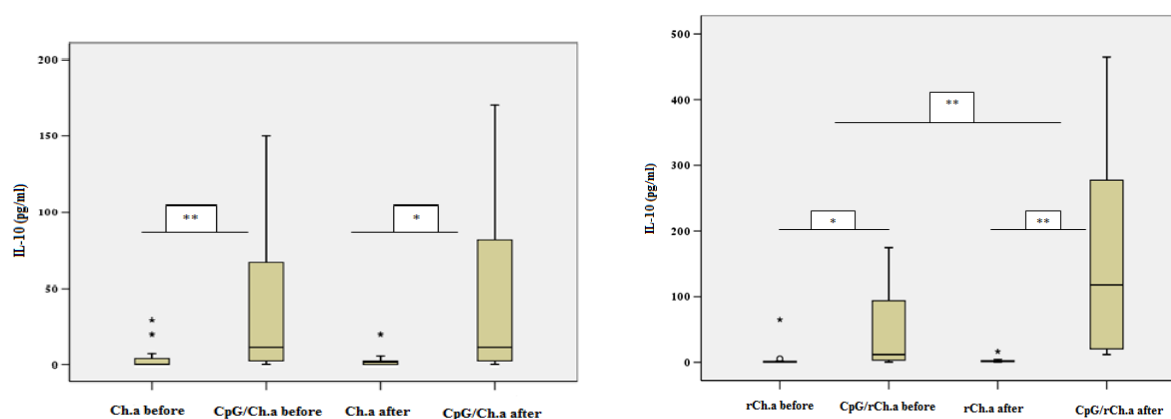


Figure 2. CpG/Ch.a or CpG/rCh.a induced immunoregulatory (IL-10) response in PBMCs cultures from patients with Ch.a allergy after 96h of culture before and after therapy of patients with INCs and antihistamines. Statistical significance comparing CC or RC with CpG/Ch.a or CpG/rCh.a stimulation was calculated using Wilcoxon. * $P < 0.05$; ** $P < 0.005$.

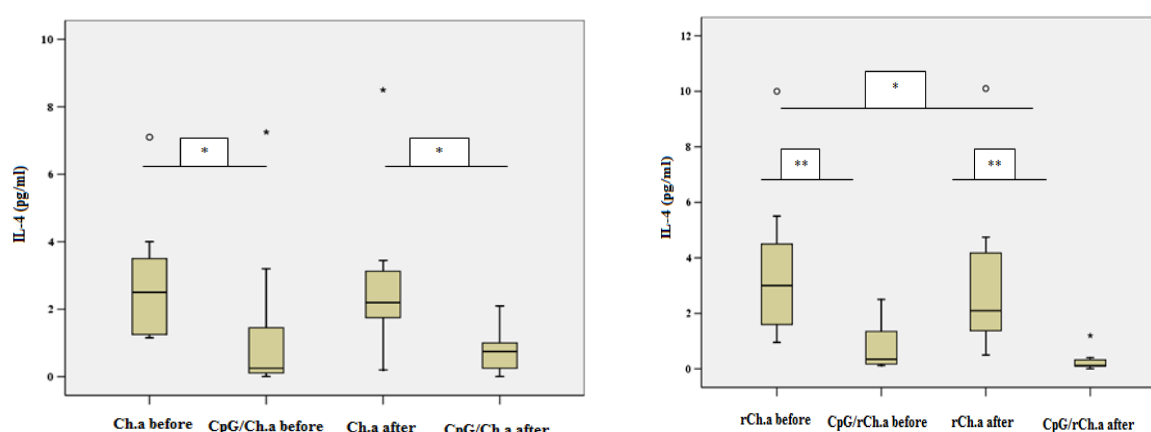


Figure 3. CpG/Ch.a or CpG/rCh.a inhibited a Th2-like (IL-4) response in PBMCs cultures from patients with Ch.a allergy after 96 h of culture before and after therapy of patients with INCs and antihistamines. Statistical significance comparing CC or RC with CpG/Ch.a or CpG/rCh.a stimulation was calculated using Wilcoxon. * $P < 0.05$; ** $P < 0.005$.

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Table 1. Cytokine profiles of CpG/Ch.a or CpG/rCh.a stimulated PBMCs in comparison with Ch.a or rCh.a stimulated cells alone from patients with AR before and after treatment with INCs and antihistamines.

Cytokine (pg/ml)	Stimulant	Before therapy	After therapy	P-value	Stimulant	Before therapy	After therapy	P-value
IFN-γ	Ch.a	16 \pm 1.5	8.4 \pm 0.7	0.2	rCh.a	14 \pm 1.5	7.8 \pm 1.5	0.3
	CpG/Ch.a	44 \pm 3.8	237 \pm 21.9	0.01	CpG/rCh.a	109 \pm 6.5	407.6 \pm 38	0.04
	<i>P-value</i>	0.01	0.004		<i>P-value</i>	0.003	0.003	
IL-4	Ch.a	8.2 \pm 0.8	6.5 \pm 0.7	0.1	rCh.a	12.4 \pm 0.6	8.7 \pm 0.7	0.09
	CpG/Ch.a	4.1 \pm 0.1	2 \pm 0.1	0.7	CpG/rCh.a	5.8 \pm 0.3	2.1 \pm 0.5	0.02
	<i>P-value</i>	0.04	0.004		<i>P-value</i>	P=0.01	P=0.003	
IL-13	Ch.a	5.7 \pm 0.6	4.2 \pm 0.9	0.7	rCh.a	7.7 \pm 2.1	6.7 \pm 2.6	0.95
	CpG/Ch.a	4.1 \pm 0.8	3.4 \pm 0.8	0.4	CpG/rCh.a	7 \pm 3.1	5.7 \pm 2.2	0.4
	<i>P-value</i>	0.85	0.3		<i>P-value</i>	0.95	0.95	
IL-10	Ch.a	5.3 \pm 0.9	3.1 \pm 0.8	0.8	rCh.a	6.7 \pm 0.9	5.5 \pm 0.8	0.4
	CpG/Ch.a	42.2 \pm 6.2	47 \pm 8.6	0.06	CpG/rCh.a	52.6 \pm 5.6	171.7 \pm 11.8	0.008
	<i>P-value</i>	0.003	0.01		<i>P-value</i>	0.003	0.003	

Natural Chenopodium album (Ch.a), ODN co-administration of natural Ch.a (CpG/Ch.a), recombinant Ch.a (rCh.a), ODN co-administration of recombinant Ch.a (CpG/rCh.a),

Values are mean \pm standard deviation (SD)

*P< 0.05 CpG/Ch.a and CpG/rCh.a compared with Ch.a, or rCh.a alone

Table 2. Intracellular IL-10 expression in CD4⁺ cells and Ch.a specific IgE secretion by CpG/Ch.a or CpG/rCh.a stimulated PBMCs compare with stimulated Ch.a or rCh.a cells from patients with AR before and after treatment with INCs and antihistamines.

Variable	Stimulant	Before therapy	After therapy	P- value	Stimulant	Before therapy	After therapy	P- value
Intracellular IL-10 (%)	Ch.a	1.4 \pm 0.9	3.4 \pm 0.6	0.1	rCh.a	2.7 \pm 0.8	3.7 \pm 0.8	0.2
	CpG/Ch.a	5.1 \pm 2.8	8 \pm 2.1	0.04	CpG/rCh.a	6.7 \pm 3.7	9.3 \pm 2	0.04
	<i>P-value</i>	0.04	0.04		<i>P-value</i>	0.04	0.04	
Specific Ch.a IgE (IU/ml)	Ch.a	10.1 \pm 0.3	8.2 \pm 0.2	0.3	rCh.a	13.3 \pm 3.1	9.6 \pm 0.5	0.2
	CpG/Ch.a	6.7 \pm 0.6	0.7 \pm 0.05	0.02	CpG/rCh.a	4.7 \pm 0.9	0.5 \pm 0.06	0.01
	<i>P-value</i>	0.01	0.01		<i>P-value</i>	0.02	0.01	

Natural Chenopodium album (Ch.a), ODN co-administration of natural Ch.a (CpG/Ch.a), recombinant Ch.a (rCh.a), ODN co-administration of recombinant Ch.a (CpG/rCh.a),

Values are mean \pm standard deviation (SD)

*P< 0.05 CpG/Ch.a and CpG/rCh.a compared with Ch.a, or rCh.a alone

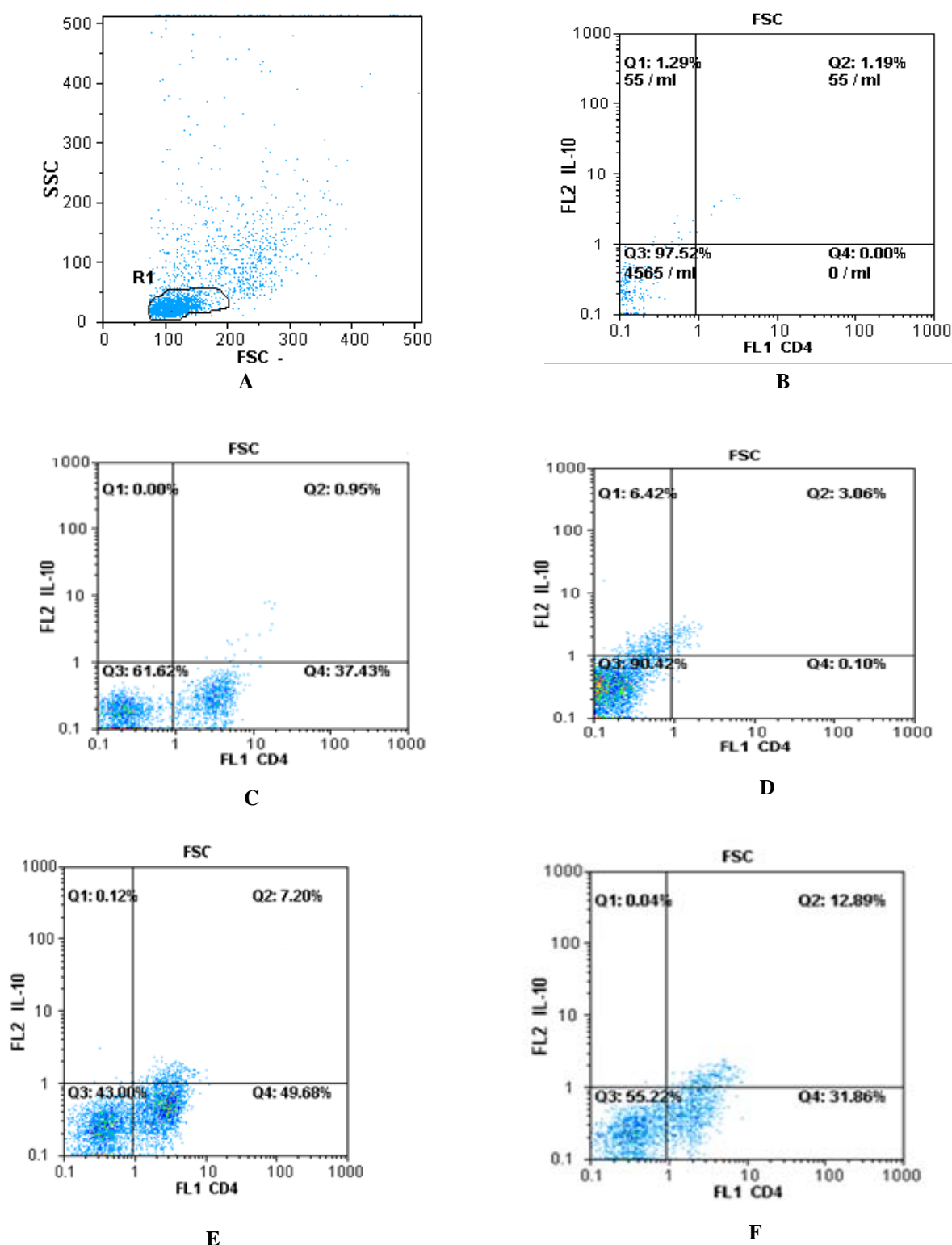


Figure 4. Flow cytometer results of PBMCs were stimulated with 10 μ g/ml PMA and 10 μ g/ml ionomycin in the presence of 10 μ g/ml monensin for 12 h in 5% CO₂. Samples were stained with CD4 FITC and intracellular IL-10-conjugated mouse IgG1 anti-human mAb. (A) selected PBMCs (B) Negative control (C) CD4⁺ cells (D) IL-10⁺ cells (E) Two-color CD4⁺ IL-10⁺ cells stimulated with CpG/Ch.a (F) Two-color CD4⁺ IL-10⁺ cells stimulated with CpG/rCh.a.

Effect of CpG/rCh.a on Cytokine Profiles

Stimulation of PBMCs with CpG/rCh.a resulted in a significant reduction of Th2 cytokine (IL-4) and increases in the levels of Th1 cytokine (IFN- γ) and IL-10 (Table 1). Comparisons of the results before and after therapy of patients showed that production of IFN- γ and IL-10 were significantly increased after therapy (Figures 1 and 2), while IL-4 production was significantly decreased (Figure 3). Moreover, the effects of recombinant form of allergen (rCh.a) in combination with CpG, was significantly vigorous than natural Ch.a allergen to increase IFN- γ and IL-10 and to decrease IL-4 levels.

Effect of CpG/Ch.a or CpG/rCh.a on Ch.a Specific IgE

Before therapy of patients, both CpG/Ch.a and CpG/rCh.a exhibited significantly lower levels of Ch.a specific IgE than natural Ch.a or rCh.a alone. This inhibitory effect was significantly shown after therapy (Table 2).

Effect of CpG/Ch.a or CpG/rCh.a on Intracellular IL-10 Expression

A significant rise in expression of IL-10 in CD4⁺ cells was detected upon stimulation of PBMCs with both CpG/Ch.a and CpG/rCh.a compared to stimulation with each allergen alone. This inducing effect on PBMCs was the same before and after therapy of patients (Table 2, Figure 4).

DISCUSSION

Allergic rhinitis is an inflammatory disorder with Th2-dominant immune imbalance. Designing a therapeutic strategy to shift the immune response toward Th1 is as of interest of special several researchers during last decade.¹⁰⁻¹³ In this study, we assayed cytokine profiles and Ch.a specific IgE to investigate the immunostimulatory and immunomodulatory properties of CpG-ODN co-administration with allergens on PBMCs of patients with AR. Moreover, we evaluated the effects of INCs and antihistamines as the drugs for the therapy of AR patients on PBMCs that stimulated with CpG-ODN co-cultured natural or rCh.a.

It has been reported that in animal models, co-administration or conjugation of CpG with allergens increased production of IFN- γ and IL-10 cytokines, and

decreases IL-4, IL-5 and IL-13.^{27,28,32-38} These studies indicated an enhanced production of Th1 cytokine decreased production of Th2 cytokine and increased production of the regulatory cytokine IL-10 *in vivo*.

Marshall et al.²⁶ demonstrated that a mixture of CpG and Der p 1 allergen enhanced IFN- γ production, while decreased production of IL-4 in cultures of PBMCs from individuals with dust mite allergy. Moreover, it was reported that IFN- γ production by PBMCs of both subjects, allergic to birch and grass pollen, and control subjects in response to CpG were detected.^{26, 38-39} In agreement with these studies, the results of our study showed that PBMCs treated with CpG/Ch.a or CpG/rCh.a *in vitro*, enhanced IFN- γ and IL-10 and decreased IL-4. Moreover, both above stimulants induced the suppression of Ch.a specific IgE. This effect could be the result of downmodulatory effect of CpG on IgE production.⁴⁰ Indeed, the inhibitory effect of CpG may be mediated in a Th1 cytokine-independent pathway, possibly by direct activation of effector B cells. It has been described that plasmacytoid dendritic cells and B cells are the primary cell types expressing TLR9. These cells are known to respond directly to CpG stimulation that regulates antibody class switching leading to IgE decreases.^{32,41,42} In addition, IL-10 is also a potent suppressor of both total and allergen-specific IgE.⁴³ Therefore, both cytokine dependent and independent pathways may mediate the suppression of Ch.a specific IgE production with PBMCs, which was shown in our study.

In this study, we showed an increase of secreted IL-10 in supernatants of PBMCs treated only with CpG/Ch.a, whereas, the intracellular assessment of this cytokine indicated that both CpG/Ch.a and CpG/rCh.a induced IL-10 expression in CD4 positive PBMCs. Therefore, intracellular cytokine assay seems to be more sensitive for evaluation of CpG effects on cytokine expression.

We also found that CpG/rCh.a was significantly more potent to increase the levels of IFN- γ and IL-10 and to decrease IL-4 in stimulated PBMCs compared to CpG/Ch.a. Thus, it could be suggested that co-administration of recombinant allergen with CpG is more effective to shift Th2 to Th1 responses.

Altogether, this study suggests that co-administration of CpG/Ch.a or CpG/rCh.a is effective *in vitro* in suppression of Th2 and stimulation of Th1 cytokine production. Interestingly, such effect on

cytokine production was significantly enhanced after therapy. Although, we have not investigated the synergic effects of CpG therapy and regular AR therapy *in vivo*, the results of this study suggest that PBMCs from AR patients could respond more efficiently to CpG *in vitro*, after a period of treatment with INCs and antihistamines. Hence, it could be suggested that the use of CpG and this drug regiment may have a synergistic effect on PBMCs of AR patients. However, both pharmacotherapy and immunotherapy with CpG are recommended in further studies to determine the effectiveness of these combination therapies.

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