ISSN 1735-1383

Iran. J. Immunol. March 2008, 5 (1), 57-63

Tahereh Mousavi, Alireza Salek Moghadam, Reza Falak

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Article Type: Research

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Immunotherapy of Chenopodium Album Induced Asthma by Intranasal Administration of CpG Oligodeoxynucleotides in BALB/c Mice

Tahereh Mousavi*, Alireza Salek Moghadam, Reza Falak

Department of Immunology, Iran University of Medical Sciences, Tehran, Iran

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ABSTRACT

Background: There are many therapeutic methods for allergic conditions. CpG oligonucleotides play a critical role in immunity via the augmentation of Th1 and suppression of Th2 responses. **Objective**: In the present study we aimed to estimate the effectiveness of intranasal administration of CpG ODN plus Chenopodium album allergen in allergic asthma compared with the administration of allergen alone and to find out how CpG ODN therapy is useful in the treatment of allergen induced asthma. **Methods:** BALB/c Mice were intraperitoneally and intranasally sensitized with allergenic extract precipitated on aluminum hydroxide. Therapy with CpG/Ag was performed intranasally. After antigenic challenge, a number of Immunologic variables such as serum IgE and IgG, systemic and local IL-10 and IFN-γ were studied in splenocytes, and lung tissue culture supernatants, respectively. **Results:** Our study indicated that intranasal administration of CpG/Ag had significant increases in both systemic and local levels of IL-10 and IFN-γ ($p \leq 0.001$), but showed no significant effect on the levels of IgE, IgG2a, and IgG1 in serum ($p = 0.06$). This study demonstrated that CpG ODN has therapeutic effects not only on splenocytes but also on nasal lymphocytes to produce IFN-γ as a Th1 cytokine, and IL-10 as a regulatory cytokine. **Conclusion:** According to these data from the mouse model, we conclude that intranasal administration of CpG motifs before allergen exposure may be useful for the control of allergic asthma. Therefore, further investigations on humans using CpG motifs are recommended in order to modulate the allergic effects of Chenopodium album as well as other regional allergens.

Keywords: Asthma, CpG ODN, Chenopodium album

INTRODUCTION

Inhalation of allergens in susceptible subjects evokes immune and inflammatory responses

*Corresponding author: Dr Tahereh Mousavi, Department of Immunology, Iran University of Medical Sciences, Tehran, Iran. Tel: (+) 98 912 2150042, Fax: (+) 98 21 88058719, e-mail: mousavi36@yahoo.com

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in the airway, resulting in bronchial asthma (1). Allergic diseases are candidate for immunotherapy, due to their nature in which antigen-specific Th2 cells induce most allergic manifestations (2). CpG motifs recognized via Toll like receptor-9 (TLR-9) are frequently found in bacteria (3-5). Synthetic Oligodeoxynucleotids containing CpG motifs (CpG ODNs) mimic the activity of bacterial DNA and have been used to explore the nature of CpG motifs (6). For the use of CpG motifs as adjuvants in the antigen-specific Th1 responses, CpG ODNs are often co- administered with the antigen (7). Moreover, it has been shown that CPG administration in previously sensitized subjects can reverse inflammatory responses (8).
In the present study, we report the experimental application of CpG ODNs for the treatment of bronchial asthma induced by Chenopodium album (Ch.a.), a very common allergenic plant in Iran. Induction of experimental asthma using Ch.a. allergen was priviously reported from our laboratory (9). Anti-inflammatory activity of CpG motifs is accompanied by the improvement of airway hyperresponsiveness and the induction of IFN-γ suggesting the possibility of Th1 and T regulatory involvment (10, 11). Based on these findings, we used a mixture of Ch.a. allergen and CpG motifs in order to study its effectiveness in comparison to Ag specific immunotherapy. Indeed, we hypothesised that CpG ODN immunotherapy may induce regulatory and Th1 responses in Ch.a. induced asthma in mice. There are many reports from other countries investigating different allergens. For example, human clinical trials on ragweed allergic rhinitis using CpG ODNs conjugated to the principal allergenic moiety of ragweed (Amb a 1) are carried out in the United States (12-14). Furthermore, based on other reports (8, 15), mucosal administration of allergen in immunotherapy has a number of potential advantages over traditional intradermal administration and may be the key to successful implementation of immunotherapy. Besides eliminating the need for injections (an important determinant of compliance, especially in children), mucosal administration has lower costs and may reduce the risk of anaphylactic responses. In addition, studies on CpG ODN as an immunostimulatory adjuvant have shown that it confers more effective immune responses when administered mucosally rather than parenterally (8, 13). Therefore, in this study we select the mucosal route of administration as an effective means of Ch.a./Ag delivery and compare its potential with Ag specific immunotherapy in sensitized mice and sham controls as well.

MATERIALS AND METHODS

CpG ODNs. The ODNs were obtained from InvivoGen, US. The CpG ODN (1826) consisted of 20 bases containing two CpG motifs (TCCATGACGTTCCTGACGTT). ODNs were in Phosphorothioate form synthesized and purified by the company using HPLC.
Antigen allergenic extract was prepared from Ch.a. pollen according to previously reported procedures (16).
Animals and Experimental Design. Fifteen female BALB/c mice at 4 to 6 weeks of age were obtained from Pasture Institute (Tehran, Iran) and were randomly divided to three groups of test and two control groups, each consisting of 5 animals. Immunization was carried out according to our previous study (9). Briefly, all animals with the exception of the sham controls were sensitized intraperitoneally with 50 µg of Ch.a. extract precipitated with 4 mg of aluminum hydroxide (alum) in 200 µl of phosphate-buffered saline (PBS) on days 1 and 7; followed by two times allergen exposure using 2% Ag solu-

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tion in PBS on days 14 and 16. All initially sensitized mice received three times immunotherapy on days 19, 26, and 33. Mucosal immunotherapy consisted of trans-nasal administration of 10 µg CpG ODN with Ch.a. (50 µg) in test group, antigen alone (50 µg in PBS) in antigen control mice, and PBS in sham controls. Later, all of the mice in the three groups underwent repeated allergen exposure to Ch.a. allergen (2% solution in PBS for 30 min) on days 40 and 47. Blood samples were obtained from all of the mice two days after the final antigen challenge; sera were separated and stored at -20 °C for antibody detection. Spleens and lungs were excised after animal sacrifice. Single-cell suspensions were prepared from the spleens. Lung tissues were cut to small sections (1 mm^3). Lung sections and splenocytes were cultured in separate micro plates and culture supernatants were then collected and stored at -80 °C for cytokine assays.

Cell Culture Condition. Splenocytes were plated in 24-well dishes using 5 x 10^6 cells per ml in RPMI-1640 complete medium with 10% (vol/vol) FCS, 50 mM 2-mercaptoethanol, 100 units/ml penicillin G/streptomycin. Lung tissues were also cultured in 24-well micro plates under the same conditions. All culture reagents were purchased from Sigma (US). The cells and tissues were then stimulated with 50 µg of allergen and incubated at 37°C for 72 hours in a 5% CO2 atmosphere. Following 72 hours incubation, supernatants were harvested and stored at -80º C for cytokine analysis. The experiments were performed in triplicate, twice.

Antibody Assay. ELISA assay was used to detect specific IgG antibodies and total IgE. All reagents were purchased from Sigma, US and Becton Dickinson, Oxnard, CA and experiments were carried out according to manufacturer's instructions

Cytokine Assay. IL-10 and IFN-γ concentrations in splenocytes and lung tissue culture supernatants were determined using ELISA (PharMingen San Diego, CA) method. Assay was performed according to the manufacturer's recommendations. The detection limits for IFN-γ and IL-10 were 78 pg/ml for and 19 pg/ml respectively.

Statistical Analysis. Data were expressed as mean ± standard error of the mean. For in vivo experiments, each group consisted of four or five mice. Each experiment was repeated at least twice. Student's t test was used in the analysis of the results.

RESULTS

Results of IL-10 and IFN-γ assays in splenocytes and lung tissue culture supernatants are presented in Figure 1 and 2, respectively. Following exposure to the allergen, CpG/Ag treated mice induced significantly higher levels of systemic IL-10 and IFN-γ compared to untreated mice in sham controls and specific Ag treated mice in the positive group ($p\leq 0.001$). However, these effects were not shown in the absence of CpG motifs, as observed in Ag-treated mice which failed to induce significant changes in systemic IL-10 ($p=0.06$). Furthermore, there was no significant rise in local IFN-γ levels of test versus Ag treated group ($p=0.06$), but IL-10 induced in the lung tissue culture of CpG/Ag treated mice was significantly higher than those of the two control groups ($p\leq 0.001$).

The results of total serum IgE concentration and specific IgG subclasses are shown in Figure 3. The ratio of the OD values of IgG2a to IgG1 has been reported to demonstrate the shift of IgG subclass from IgG1 to IgG2a. The data in this study indicates that the differences in antibody concentrations between CpG/Ag, Ag alone treated and the untreated mice were not statistically significant ($p=0.06$) (Figure 4).

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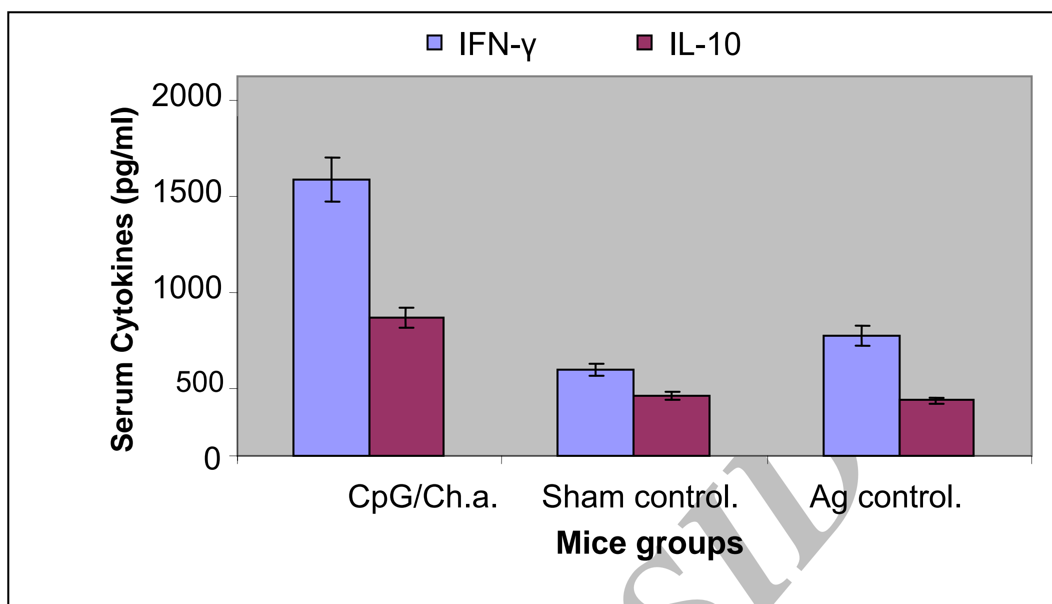

Figure 1. IFN-γ and IL-10 concentrations in splenocytes culture supernatants measured by ELISA. Sensitized test mice, sham and Ag control groups received CpG/Ch.a., PBS and Ch.a. alone for therapy, respectively.

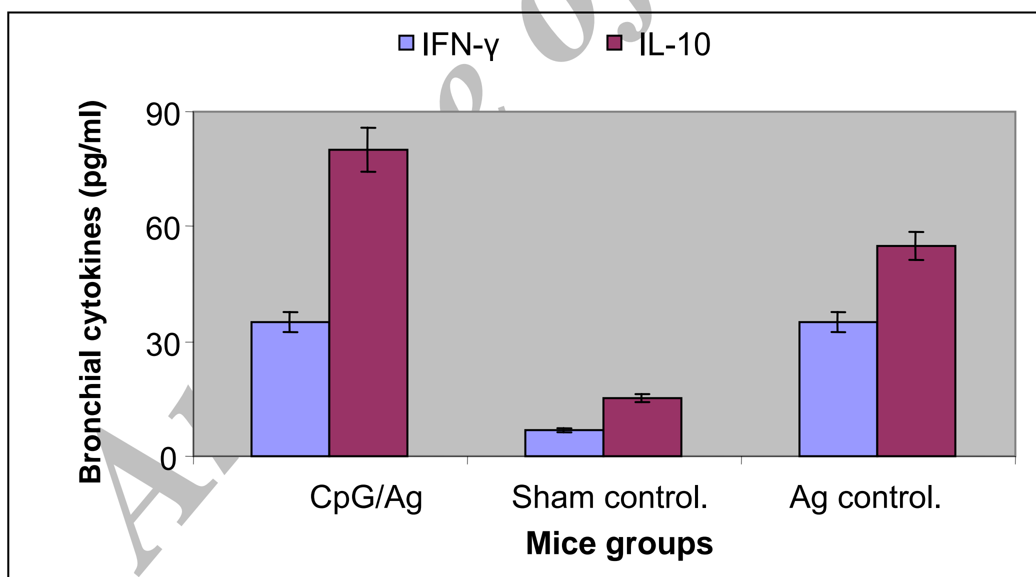

Figure 2. IFN-γ and IL-10 concentrations in lung tissue culture supernatants are measured by ELISA. Sensitized test mice, sham and Ag control groups received CpG/Ch.a., PBS and Ch.a. alone, respectively.

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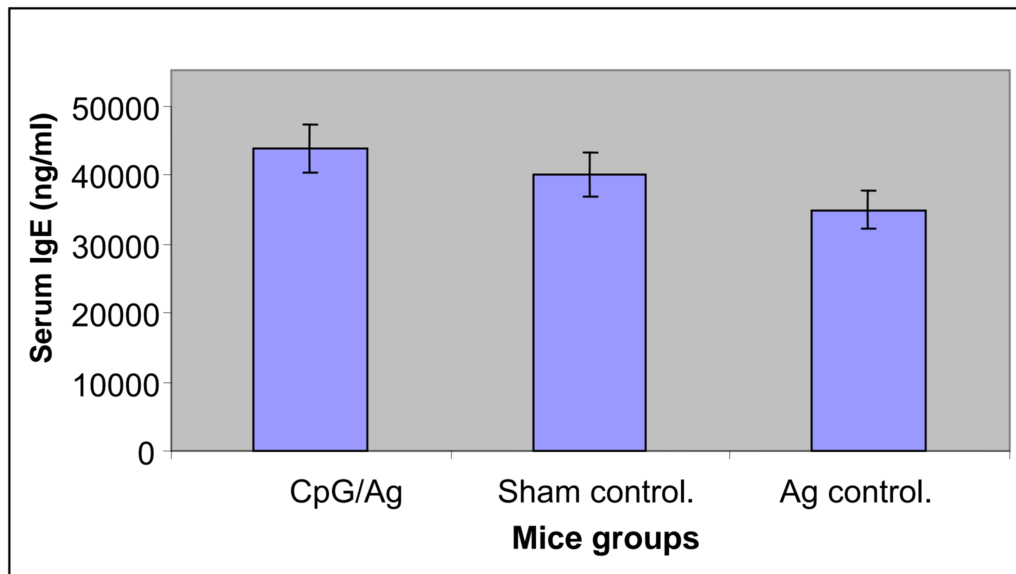

Figure 3. Total serum IgE concentrations are measured by ElISA in sensitized test mice, sham and Ag control groups receiving CpG/Ch.a., PBS, and Ch.a. alone, respectively. No significant changes are shown in test mice compared to either control groups (p=0.06). Similarly, there was no improvement in IgE levels after Ag specific therapy versus PBS treated control mice (p=0.06).

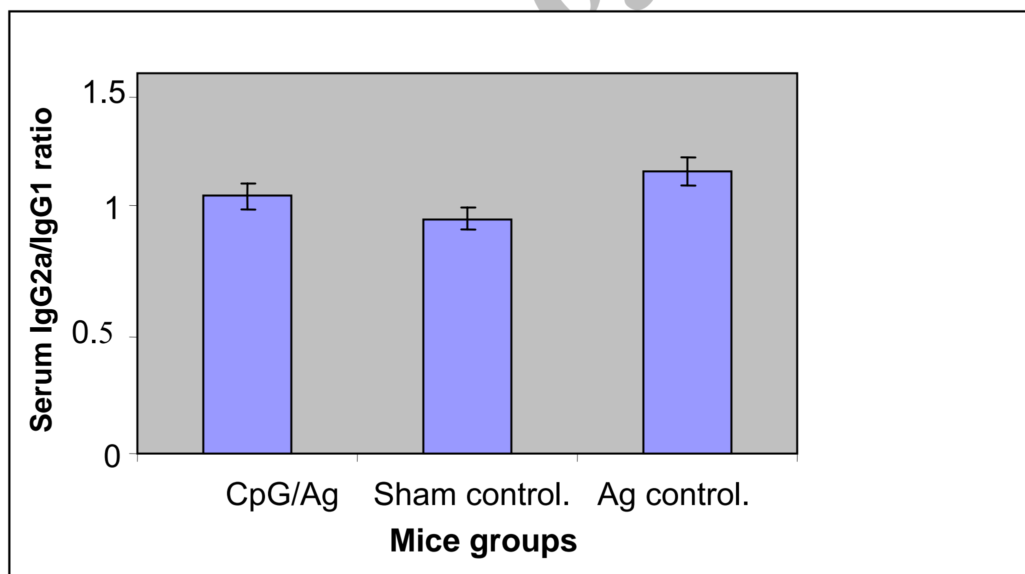

Figure 4. Serum IgG2a and IgG1 assayed by ElISA and presented as OD ratios. Sensitized test mice, sham and Ag control groups received CpG/Ag, PBS, and Ch.a. alone, respectively. No significant diffrence in IgG2a/IgG1 OD ratios was noticed in test and control groups (p=0.06).

DISCUSSION

In this study, we compared the antiallergic effects of the mucosal co-administration of CpG/Ch.a. with Ag immunotherapy in a mouse model of asthma. Based on the experi-

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ments, we detected IFN-γ induction as a marker for Th1 activity and IL-10 increase as a marker for regulatory responses. However, we noticed that CpG ODN effect on IL-10 was more than its effect on IFN-γ. CpG ODN enhanced both the systemic and the local increase in IL-10 whereas IFN-γ was only increased systemically in response to CpG therapy. In order to elevate the effects of cytokines on antibody production, we measured total IgE and specific IgG subclasses as markers of allergy. In contrast to some other reports (17, 18), we detected no significant reduction in IgE and IgG1 levels in CpG treated mice compared with controls. We suggest that this failure could be attributed to short and insufficient period of time between the immunotherapy and blood sampling. However, the lack of reduction of IgE is not surprising as it was previously shown by Mo on an animal model of allergic rhinitis (19), and by Inoue on atopic dermatitis (20). Suzuki (21) suggests that mucosal immunotherapy seems to be a successful alternative to the traditional systemic routes. In our study, we found that during intranasal administration addition of CpG motifs to antigen makes it more potent than using Ag alone regarding cytokine deviations from Th2 to Th1 and regulatory like responses. Indeed, mucosal administration of CpG ODN is able to raise the local production of IL-10 in the lung; while this effect was not seen in local concentrations of IFN-γ. Accordingly, since T-reg like responses seem to be effective in immunomodulation of allergic manifestations, we suggest that IL-10 induction in mice receiving CpG/Ch.a. intranasally could be an important factor in suppression of inflammatory responses. However, in addition to cytokines, further studies are required on clinical symptoms to confirm this suggestion.

In contrast to Vipul who found that levels of IL-10 in the airways were suppressed in CpG-treated vs. untreated mice (15), we found a significant increase in systemic and local production of IL-10 after CpG/Ch.a. therapy compared to the controls. Therefore, we suppose that a possible mechanism for the effects of CpG ODN is regulation of IL-10. Although IL-10 is described as a Th2 cytokine (22), its role in atopic asthma is complicated. In addition to its local effects on airways, IL-10 has been reported to have the systemic anti-inflammatory properties, suppressing both Th1 and Th2 cytokine responses, and induction of anergy or tolerance when applied to $CD4^+$ T lymphocytes or dendritic cells (23, 24). In this study we have demonstrated that CpG ODNs strongly induce IL-10 from splenocytes and lung immune cells. We suggest that enhancement of IL-10 may partially mediate the protection induced by CpG ODNs against Ch.a. allergy. One possibility for the source of IL-10 is the regulatory T cells ($CD25^+CD4^+$, T-reg cells), which seem to play an important role in maintaining immunological tolerance (25, 26). Induction of T-reg cells might explain the antigen-specific effects of CpG ODNs in atopic inflammation. Thus, depending on systemic and mucosal expression, IL-10 can lead to beneficial consequences in asthma. Because of many advantages for nasal immunotherapy, such as low risk of shock and also convenience, we recommend to perform more investigations on CpG applications in human asthma. Based on the results obtained on Ch.a., we suggest that the administration of CpG motifs with different allergens can increase the therapeutic effects of allergens in specific immunotherapy.

In conclusion, we suggest that mucosal co-administration of CpG ODNs with Ch.a. allergen may reverse airway inflammation through the induction of Th1 and regulatory cytokines. Our results support the need for more studies on application of CpG ODN in the immunotherapy of asthma induced by Ch.a. in humans. Moreover, based on the CpG ODN potentials to increase the effectiveness of Ch.a., specific immunotherapy, we conclude that these finding could be applied for other allergens as well.

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ACKNOWLEDGEMENTS

We like to thank Dr. Soheyla Ajdari, Dr. Nader Tajik, Parivash Danesh and Mehdi hejazi for their kindly helps. This study has funded by Iran University of Medical Sciences, grant number: 577.

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