Co-Administration of CpG Oligonucleotides and Chenopodium album Extract
Reverse IgG2a/IgG1 Ratios and Increase IFN-Gamma and IL-10 Productions in
a Murine Model of Asthma

Tahereh Mousavi 1, Alireza Salek Moghadam1, Reza Falak1, and Majid Tebyanian2

1 Immunology Department, Iran University of Medical Sciences, Iran
2 Razi Institute, Karaj, Iran

ABSTRACT

Asthma is a disorder of increasing severity and prevalence. Recent knowledge about the
pathogenesis of asthma emphasizes its inflammatory nature. CpG oligonucleotides are a class
of compounds containing motifs based on the cytosine-guanine dinucleotides (CpG-ODNs).
These motifs are suppressed in mammalian DNA. They induce inflammation in mammals
characterized by the induction of T helper type 1 and regulatory responses.

In this paper, the effect of CpG DNA co-administration with a homemade Chenopodium album (Ch.a) extract in a murine model of asthma is reported for the first time. Balb/C mice were sensitized using Ch.a. pollen allergenic extract plus CpG-ODNs intraperitoneally and were challenged with aerosolized allergen. Results measured included IL-10 and IFN-gamma cytokines as well as IgG subclasses. For this, splenocytes from mice treated with CpG/Ag or Ag alone, were cultured in the presence of antigen.

The results showed that CpG ODN administered at the time of Ch.a sensitization, effectively increased cytokines and IgG2a/IgG1 ratios compared with those in mice treated with antigen or with PBS alone (P ≤ 0.001). Our experiments revealed that Ch.a. sensitization decreased IgG2a/IgG1 compared with non-sensitized mice (P ≤ 0.001), while CpG ODN/Ch.a reversed this ratio, indicating CpG potentials towards IgG2a subclass switching.

We conclude that Co-administration of Ch.a. allergen and CpG ODN prevents the
development of TH2-mediated response probably through the IL-10 regulatory effects.
Thus, these components could be used with the other allergens in order to induce the
prevention of inflammatory conditions. We suggest further studies are necessary to identify
the potential effects of CpG-ODNs administration in conjunction with other antigens
prepared from the regional allergens in Iran. Taken together, we suppose that the results
obtained in this study in animal models may be useful in human trials conducted by other
investigators.

Key words: Asthma; CpG motifs; Chenopodium album

Corresponding Author: Tahereh Mousavi, PhD;
Immunology department, Iran University of medical sciences, Shahid
Hemmat highway, 14496, Tehran Iran.

Tel: (+98 21) 8805 8652, Fax: (+98 21) 8805 8719,
E-mail: mousavi36@yahoo.com
INTRODUCTION

An allergic asthma response is characterized by activation of T-helper type 2 (Th2) lymphocytes. These responses play a major role in the pathogenesis of allergic inflammation in asthma. Steroids as the current widely used drugs, are effective only in minimizing the manifestations of inflammation, however it does not cure the disease. At present, immunotherapy seems to be the best therapy in all allergic conditions.

On the other hand, decreased exposure to bacteria may be partially responsible for the increased incidence, severity, and mortality due to allergic diseases such as asthma, atopic dermatitis, and rhinitis in the developed countries. This hypothesis is supported by evidence that bacterial infections or products can inhibit the development of allergic disorders in experimental animal models and clinical studies. Bacterial and synthetic DNAs containing CpG dinucleotides in specific sequence contexts activate the vertebrate immune system via Toll-like receptor 9 (TLR9). Upon activation by CpG DNA, TLR9 initiates signaling pathways leading to systemic expression of Th1-like cytokines (e.g. IL-12 and IFN-γ) and chemokines in both mice and humans. Thus, the potent T helper 1 immune response produced by CpG activation of the innate immune system supports the broad therapeutic application of CpG DNA against cancer, infectious diseases, asthma, and allergies and as adjuvant in immunotherapy.

Many investigators report that CpG DNAs alone or in conjunction with allergen decrease production of IL-4, IL-5, IgG1 and IgE in mouse models of allergic asthma. The application of CpG motifs as the preventive agent in asthma has been studied by many investigators using antigens with different sources. However, there are no published reports studying CpG effects in allergic conditions using the regional homemade allergens in Iran. Thus, in the present study we aimed to evaluate the prophylactic potentials of CpG ODN in response to a common regional allergenic plant named Chenopodium album (Ch.a). Here, in order to evaluate the potential effects of CpG-ODN in the immune deviation from Th2 to Th1 responses, we measured a number of immunological parameters such as IFN-γ, IL-10 and IgG subclasses in CpG-treated and control mice. In this study we evaluated serum IgG2a and IgG1 levels in mice treated with CpG motifs as the preventive agents. Furthermore, according to other studies which reported that regulatory responses can be activated in response to CpG motifs and leading to suppression of Th2 activity, we measured IL-10 levels as a regulatory parameter in CpG treated mice as well as in controls.

MATERIALS AND METHODS

CpG Oligonucleotides
CpG-ODN (ODN 1826G): 5' – tcc atg acg ttc ctg acg tt-3', containing unmethylated CpG oligodeoxynucleotides was purchased from in vivo (Gen, USA).

Antigen
The antigen used in this study is a crude allergenic extract from Chenopodium album pollen prepared in our laboratory according to previously reported procedures. The potential allergenic role of this plant to induce asthma in mice has previously been reported.

Animals
Fifteen 4- to 6-weeks-old BALB/c mice, obtained from Pasteur Institute of Iran, were randomly divided in three groups, 5 in each (test, positive and negative controls). The animals belonging to test group were considered to study the preventive potency of CpG co-administration with Ag at the time of the sensitization. The mice in group 2 received Ag alone and considered as the Ag sensitized positive controls. The negative control mice in group 3 received only PBS instead of antigen or CpG/Ag as the non-sensitized and non-challenged controls. The experimental program is represented in brief in figure 1.

Ag-Sensitization and Ag-Challenge of Mice
Mice were given two i.p. injections on days 0 and 7 for sensitization step (10 µg CpG plus 50 µg Ag in PBS per mouse for test group, 50 µg Ag in PBS/mouse for positive controls and PBS alone for negative controls. All mice in groups 1 and 2 were intranasally challenged by inhalation on days 14 and 21 with the 1% antigen solution in PBS for 30 minutes inhalation. PBS was used for sensitization and challenging steps in third group of mice (negative controls). All mice were then killed by CO₂ inhalation 72 h after the last challenge.
Cell Culture
Spleens were excised after killing the mice and single-cell suspensions were prepared. Splenocytes were plated in 24-well dishes using 5 x 10⁶ cells per ml in RPMI complete medium with 10% (vol/vol) FCS, and 100 units/ml penicillin G/streptomycin. All culture reagents were purchased from Sigma Company. The cells with the exception of those from negative controls were then stimulated with 50 µg of antigen and incubated at 37°C for 72 h in a 5% CO2 atmosphere. Following 72-h incubation, supernatants were harvested and stored at -80°C for cytokine analysis. The experiments were performed twice in triplicate.

Cytokine Assay
IFN-γ and IL-10 levels in culture supernatants were measured by ELISA using BD Bioscience reagents (San Diego, CA) according to manufacturer’s instruction. Detection limits were 78 pg/ml for IFN-γ and 19 pg/ml for IL-10.

Antibody Detection
Levels of the anti-Ch.a. IgG2a and IgG1 antibodies were determined in mice sera prepared from blood immediately before killing the mice. ELISA was conducted for IgG assays. Briefly, plates (Maxisorp Nunc-Immuno; Nalge Nunc, Roskilde, Denmark) were coated with allergen (5 µg/ml) in a coating solution (14·3 mg Na₂CO₃, 10·3 mg NaHCO₃, 0·02% NaN₃, pH 9·6), incubated at 4°C overnight and then blocked with 10% FBS in PBS for 60 min at 37°C. Serum samples were applied in dilution of 1:10. After incubating the plates for 2 hr at 37°C, biotin-conjugated anti-mouse IgG1 (A85-1; Sigma) and IgG2a (R19-15; Sigma) were added for detection of specific antibodies. After washing, plates were incubated at room temperature for 30 min with StreptAB kit (Dako, Carpinteria, CA). To detect bound antibodies, the OPD substrate (Sigma) was added. The reaction was stopped by the addition of 50 µl of a 16% solution of sulphuric acid. The optical density (OD) was read at 490 nm.

Data Analysis
Statistical analysis was performed using analysis of variance (ANOVA). All three groups were compared using Student’s t-test. Results were expressed as the mean ±S.E.M. All comparisons were two tailed and the statistical significances were shown as p<0.001.

RESULTS
Th1 Cytokine Production
Cultured splenocytes from, CpG/Ag-sensitized and Ag challenged mice secreted higher levels of IFN-γ.
compared to two control groups (p ≤ 0.001), suggesting that T helper 1 cells were mainly activated (Figure 2). When the Ag-sensitized and Ag-challenged mice (antigen controls) splenocytes were cultured, no significant increase in IFN-γ secretion was observed (p=0.07). However, IFN-γ levels in test group were higher than those in negative controls. Altogether in this study, there were significant differences among the IFN-γ levels secreted by splenocytes from three groups. The results obtained from IFN-γ measurements in test and control mice are represented in figure 2.

**Regulatory Cytokine Induction**

Mice that received injections of CpG/Ag in sensitization phase showed a high and significant rise in IL-10 levels compared with two other control groups (p ≤ 0.001) (Figure 3), suggesting the potential role of CpG motifs to induce regulatory cell responses. A significant increase in IL-10 levels was also detected in Ag control mice that were sensitized and challenged by Ag, compared to PBS treated mice (p ≤ 0.001). The results of IL-10 levels in three groups are presented in figure 3.

**Ag-Specific IgG**

Mice immunized with CpG/Ag presented higher IgG2a/IgG1 ratio than did those in the positive and negative controls (p ≤ 0.001).

Interestingly, in contrast to cytokines, IgG2a/IgG1 ratios in Ag sensitized mice were lower than those in PBS controls. Indeed, in comparison with PBS treated mice, Ag sensitized mice secreted more IgG1 antibody than IgG2a subclass while in CpG/Ag treated mice antibody subclasses tended to switch from IgG1 to IgG2a. The comparison of serum specific IgG subclasses is demonstrated in figure 4.

**DISCUSSION**

The main goal of this investigation was to verify the potential role of CpG-ODNs concerning its potency to prevent the development of Ch.a induced allergic asthma in BALB/c mice. Because of its abundance in the most parts of Iran, Ch.a was chosen for this study. Furthermore, a crude allergenic extract from Ch.a pollens was previously prepared and applied to develop allergic asthma in mice in our laboratory. In spite of several studies reported from all over the world on the preventive and therapeutic effects of CpG motifs as the potent immunomodulatory agents, we still have no published data from Iran on the CpG-ODNs and allergic inflammations. Collectively, like many studies using different antigens, our study showed that the CpG-ODNs administration in conjunction with Ch.a at the time of animal sensitization, induce the significant increase in IFN-γ and IL-10 cytokines, as well as elevation in IgG2a versus IgG1 antibodies.
Accordingly, these experimental changes demonstrated a Th1-like response leading to prevent development of asthma.

Based on the facilities available in our laboratory, the parameters measured in this study are IFN-γ as the Th1-like response, IL-10 a regulatory cytokine and IgG2a/IgG1 ratios as the Th1/Th2 deviation markers. Here, we demonstrate the statistically significant differences regarding the above mentioned parameters between the test and control mice. Indeed, in consistency with others, our study showed that the Ch.a induced asthma can be modulated towards the Th1 responses when CpG-ODNs are co-administered with the antigen at the time of sensitization. Furthermore, we found a significant elevation in IL-10 levels in test group indicating that, IL-10 seems to have a regulatory effect leading to IFN-γ induction and Th2 inhibition.

Although the pathologic characteristics of asthma such as eosinophilia were not considered in our study due to some limitations, based on the knowledge on immunopathogenesis of asthma we may conclude that increased IFN-γ and IL-10 in response to CpG-ODN can prevent the development of inflammatory responses in asthma.

On the subject of Th2 responses, there are different reports regarding IL-4, IL-5, IgE, or IgG subclasses and the relative influence of Th1 versus Th2 cytokines in vivo. Accordingly, in our experiments we found a significant increase in IgG2a/ IgG1 ratios in CpG-treated mice compared to controls. Moreover we found that this ratio was reversed in Ag-sensitized mice compared with the negative controls.

Because determination of the dose-response profile was not aimed in our study, based on the different dosages used by several investigators regarding the Ag and CpG, we concluded that the amount of 50 µg per mouse for crude antigenic extract and 10 µ per mouse for CpG motifs would be suitable.

According to other reports on different and mostly recombinant antigens, we here report that co-administration of a crude allergenic extract such as Ch.a pollen with a CpG motif has the potent influence to diverse immune responses from Th2 to Th1. However, consistent with other reports, this deviation was accompanied by significant elevation of IL-10.

Taken together, we suggest that the CpG/Ch.a co-administration preferentially primes the Th1 and regulatory immune response. However, the magnitude of the Th1 response, characterized by IFN-γ levels was much higher than IL-10. Moreover, according to several reports on protective effects of CpG motifs in response to many allergenic and infectious agents, our data in this study also showed that these effects might be partially due to regulatory responses characterized by IL-10. Indeed, the overall immune responses observed in CpG-ODN-treated mice in this study show consistency to those of other studies reported on other various antigens.

CpG motifs seem to be safe because endogenously produced cytokines by CpG-ODN are likely to follow a regulatory mechanism and thereby may cause less toxicity than exogenously administered cytokines. Thus we suggest that immunotherapy for different forms of allergens even crude extracts plus CpG motifs instead of Ag-specific desensitization may be a safe and convenient protocol in allergy immunotherapy. However, before the clinical trials, preclinical studies on therapeutic effects and safety of the CpG motifs in Ch.a. induced asthma as well as other antigens is required. Finally, we conclude that further investigations using other important allergens are needed to confirm and complete our findings on CpG applications as a safe vaccine adjuvant.

ACKNOWLEDGEMENTS

We wish to thank Dr. Soheila Ajdari for her gift of IgG assay reagents. This study was financially supported by Iran University of Medical Sciences.

REFERENCES


24. Hopfenspirger MT, Parr SK, Townley RG, Agrawal DK. Attenuation of the late allergic response by mycobacterial antigens is independent of IgE in a mouse model of asthma. Allergol Intern 2002; 51:21–32.


