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Cytokine Responses during Exacerbation Compared with Stable Phase in Asthmatic Children

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SUMMARY Bronchial asthma is a chronic inflammatory disorder of the airways. Balancing in Th1 and Th2 response is a target in the treatment. Recent studies show that interleukin-10 (IL-10) has an important role in the regulation of Th2 and allergic responses and its amount was found to decrease in asthmatic patients. This study was to focus on cytokine responses, including interferon-gamma (IFN- γ), IL-4 and IL-10 in asthmatic children during acute exacerbation compared to stable period. Peripheral blood mononuclear cells (PBMCs) from fourteen asthmatic children during exacerbation and stable phase were stimulated with phytohemagglutinin (PHA) and mite allergen (*Der p*) for 72 hours. Levels of IFN-γ, IL-4 and IL-10 in cell culture supernatants were measured using enzyme-linked immunosorbent assay. The median level of IL-10 in PBMCs stimulated with PHA was significantly lower in acute asthma exacerbation compared with stable phase (464 *vs*. 859.5 pg/ml, $p = 0.03$). However, there was no difference in the level of IL-10 in PBMCs stimulated with *Der p*. The level of IFN-γ and IL-4 were not different between exacerbation and stable phase both in PHA and *Der p*-stimulated PBMCs. The decrease of IL-10 production in asthmatic children during acute exacerbation may emphasize the role of IL-10 in immune regulation in allergic disease.

Bronchial asthma is a chronic inflammatory disorder of the airways. T lymphocytes are important for the initiation and maintenance of the allergic inflammatory response, particularly T helper 2 (Th2) cells. Balancing in T helper 1 and T helper 2 response is the target of treatment.[1] However, therapies targeting Th2 cytokine both in mouse models and clinical trials have generally met with only partial success.[1]

It is well established that interleukin-10 (IL-10) have an important role in the regulation of Th2 and allergic responses.[2-4] There is an inverse association between IL-10 levels and the severity of asthma and allergic disease. IL-10 in bronchoalveolar fluid from asthmatic patients was lower than healthy control. The levels of supernatant IL-10 in asthmatic PBMCs treated with lipopolysaccharide was lower than in healthy individuals.[5-7] Furthermore, IL-10 secreting regulatory T cells were also lower in allergic patients compared with healthy individuals.[8]

During allergic inflammatory responses after exposure to allergen, Th2 cells are activated, thus producing IL-4, IL-5 and IL-13. In contrast, Th1 cells producing interferon-gamma (IFN-γ) have been

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proved to inhibit allergic disorders. However, recently claimed regulatory T cells producing IL-10 and transforming growth factor-beta (TGF-β) were found to regulate both Th1 and Th2 response.

A previous study showed that there was a decrease in suppressive function of CD4+CD25+ cells, a marker of regulatory T cells, in hay fever patients during in season than out of season.[9] Furthermore, CD4+ CD25+ cells were increased in atopic asthma during exacerbation, but the suppressive function of these cells was not different between exacerbation and stable phases. IL-10 level was not evaluated in the study.[10]

The aim of this study was to assess the changes in cytokines response of PBMCs to mitogen and allergen including IFN-γ, IL-4 and IL-10 in asthmatic children during acute asthma exacerbation compared to stable phase.

MATERIALS AND METHODS

Subjects

Fourteen asthmatic children with acute asthma exacerbation were enrolled into this study. All patients were house dust mite (*Dermatophagoides pteronyssinus*, *Der p*)-sensitized, defined by having a positive skin prick test. Patients were excluded if they received systemic steroids within 4 weeks before enrollment or if they were on immunotherapy. The patients were treated with the same doses of inhaled corticosteroids between the two episodes, i.e. exacerbation and stable episodes.

The diagnosis, clinical asthma severity and severity of exacerbation were made according to the Global Initiative for Asthma (GINA) guidelines for children 2006.[11] The demographic data included age, sex, clinical asthma severity, severity of asthma exacerbation and family history of atopy. The study was approved by the Human Research Ethic Committee of the Faculty of Medicine Ramathibodi Hospital. Informed consent was obtained from parents or guardians before the commencement of the study.

Evaluation of cytokine responses

Peripheral blood was obtained from each subject in two episodes, i.e. when they had asthma exacerbation prior to receiving systemic steroids, and at 6-8 weeks later during stable phase.

Peripheral blood mononuclear cells (PBMCs) were separated by Histopaque-1077 density-gradient centrifugation (Sigma-Aldrich, INC., St. Louis, MO, USA). Freshly isolated PBMCs were cultured at a concentration of 1 x 10^6 cells/ml in the presence of mite (*Dermatophagoides pteronyssinus*, *Der p*, ALK, Horsholm, Denmark) 10 μg/ml each and phytohaemagglutinin (PHA, Sigma-Aldrich, INC., St. Louis, MO, USA) 10 μg/ml each for 72 hours. Tests including medium control were done in duplicates. Supernatants were collected and stored at -70°C until further analysis. Concentrations of IFN-γ, IL-4 and IL-10 were measured with ELISA kits specific to each cytokine (R&D systems, Minneapolis, MN, USA). The minimum detectable concentration was < 8 pg/ml for IFN-γ, < 10 pg/ml for IL-4 and < 3.9 pg/ml for IL-10.

Statistical analysis

Sample size was calculated by accepting type I error (α) of 0.05 and β = 0.2. Mean ± S.D. or median ± interquartile range (IQR) was applied to analyze the demographic data and cytokine levels. The Wilcoxon Signed ranks test was applied to analyze the data comparing cytokine levels at exacerbation and at stable phase with the same stimulus. To analyze data between cytokine levels at exacerbation and at stable phase with different stimuli, the Mann-Whitney-U-test was used. Pearson's correlation was applied to analyze correlation between cytokine levels and asthma severity, severity of exacerbation, asthma score, diameter of positive skin prick test to *Der p*. Differences were considered to be significant at the p value < 0.05.

RESULTS

Clinical characteristics of patients

A total of 14 asthmatic children: 2 mild intermittent, 6 mild persistent and 6 moderate persistent participated in the study with mean age of 9.7 years old. The mean age of onset of asthma was 4.3 years with mean duration of asthma for 58.9 months. The mean size of skin prick test to mite (*Der p*) was 6.6 mm. The demographic data of patients are summarized in Table 1.

Table 1 Clinical characteristics of patients

Clinical characteristics of patients	N = 14
Age: mean (S.D.), years	9.7 (3.1)
Male/female: no.	5 / 9
Asthma severity: Mild intermittent: no. (%)	2 (14.3)
Mild persistent: no. (%)	6 (42.9)
Moderate persistent: no. (%)	6 (42.9)
Age onset of asthma: mean (S.D.), years	4.3 (3.7)
Duration of asthma: mean (S.D.), months	58.9 (44.4)
Size of skin prick test to mite (*Der p*): mean (S.D.), mm.	6.6 (3.9)

Average time of onset of asthma exacerbation was 8.5 hours before obtaining blood specimen. Severity of asthma exacerbation was mild in 9 patients (64.3%), and was moderate in 5 patients (35.7%).

Cytokine production by activated PBMC

The median level of IL-10 in PHA-stimulated PBMCs was significantly lower in acute asthma exacerbation compared to levels in stable phase (464 ± 628.25 *vs.* 859.5 ± 796 pg/ml, p = 0.03). However, there was no significant difference in level of IL-10 in *Der p*-stimulated PBMCs (Fig. 1A). The median levels of IFN-γ and IL-4 were not significantly different between acute asthma exacerbation and stable phase both in PHA and *Der p* stimulated PBMCs (Fig. 1B). The cytokine ratios (IL-4/IFN-γ, IL-10/IL-4, IL-10/IFN-γ) for all stimuli were not significantly different between exacerbation and stable phase.

The median level of IL-10 in moderate persistent asthma patients was lower than mild persistent asthma patients with PHA-stimulated PBMCs (737 ± 691.25 *vs*. 1,375 ± 870.75 pg/ml, p = 0.055) and with *Der p*-stimulated PBMCs (73 ± 216.73 *vs*. 268 ± 205.18 pg/ml, p = 0.17). However, there was no statistical significance (Fig. 2). Change in IL-10 level between exacerbation and stable phase, both in mild persistent and moderate persistent asthmatic patients, were not significantly different both with PHA-stimulated PBMCs (502.5 pg/ml *vs.* 108 pg/ml, p = 0.055; Fig. 3A) and with *Der p*-stimulated PBMCs (143.99 pg/ml *vs*. -21.94 pg/ml, p = 0.109; Fig. 3B).

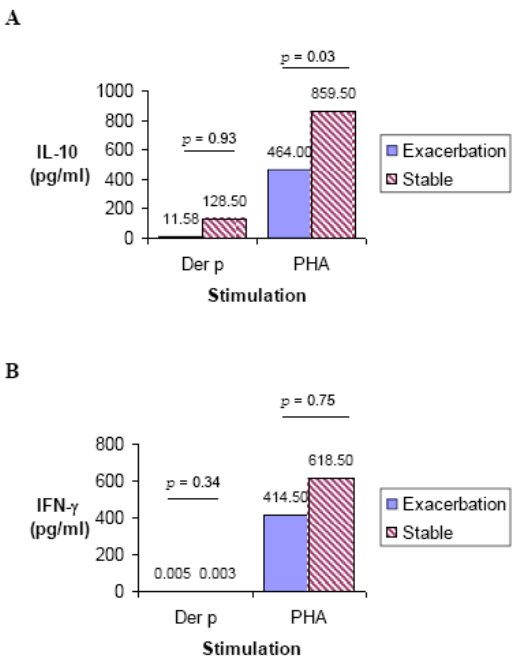

Fig. 1 Cytokine production from PBMC cultures stimulated with *Der p* or PHA for 72 hours. The concentration of IL-10 (A) and IFN-γ (B) were assayed with ELISA kits. PBMCs were from asthmatic children at acute exacerbation and stable phase.
*Data are median.

There were no significant correlations between IL-10 levels in PHA-stimulated PBMCs and duration of asthma exacerbation (r = 0.06, p = 0.83), asthma score (r = 0.21, p = 0.46), diameter of posi-

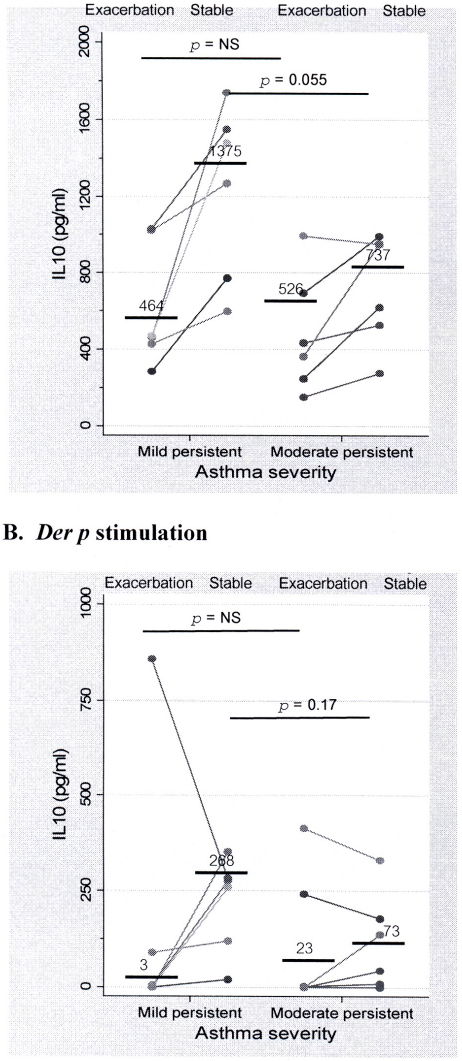

Fig. 2 IL-10 production from PBMC cultures in patients with mild persistent and moderate persistent asthma (in exaerbation and stable phase). The concentrations of IL-10 stimulated by PHA (A), and by *Der p* (B) were assayed with ELISA kits.
*Data are median, NS = not significant

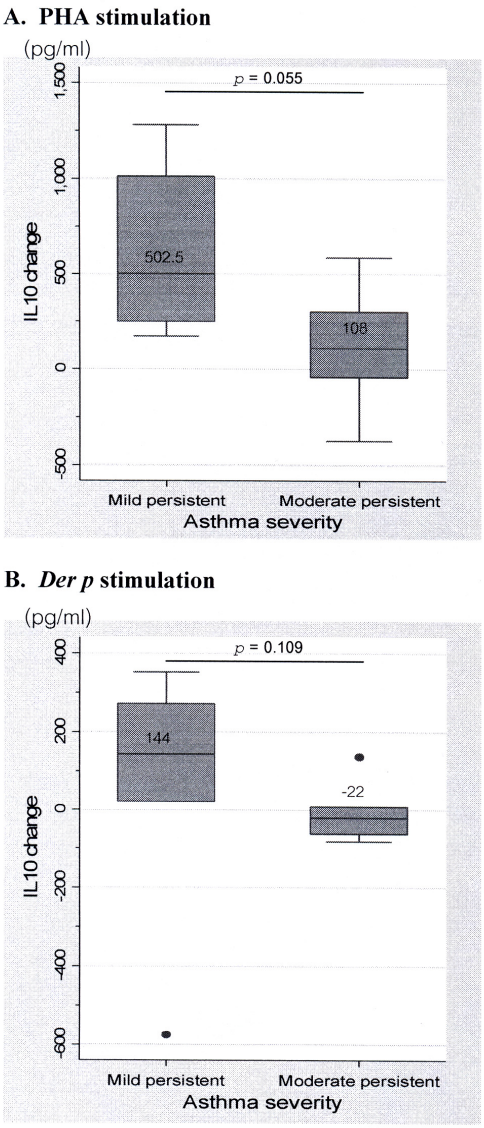

Fig. 3 The change in IL-10 levels between exacerbation and stable phase in mild persistent and moderate persistent asthmatic patients stimulated by PHA (A), and by Der p (B).
*Data are median.

tive skin prick test to *Der p* (r = 0.31, $p = 0.28$) and also among with other cytokine levels (IFN-γ, IL-4).

DISCUSSION

Immune responses generated by cytokines are essential for pathogenesis of asthma. To determine the role of T lymphocyte-derived cytokines during asthma exacerbation, we examined the changes in cytokine responses, including IFN-γ, IL-4 and IL-10 in asthmatic children during exacerbation compared with stable phase.

We have demonstrated that IL-10 level in PHA-stimulated PBMCs was significantly lower during acute asthma exacerbation compared with stable phase which may reflect the defect in IL-10

productive function of T lymphocytes. However, Shi *et al*.[10] found an increase in the number of CD4+CD25+ cells in asthma exacerbation. This result reflects that CD4+CD25+ may not represent IL-10 producing cells but may be the marker of activated T cells.[12-15] Further studies towards specific markers of IL-10 producing cells may be useful. IL-10 levels in PHA stimulated cells was higher than in *Der p*-treated ones could be explained by the fact that PHA is a potent stimulator for cytokines production by lymphocytes but *Der p* may stimulate only the production of IL-4 and IL-5 of lymphocytes which in turn downregulate the IL-10 production.[16]

We also showed that IL-10 level both in PHA and *Der p*-stimulated cells were lower in moderate persistent asthma compared with in mild persistent asthma (borderline significant). The finding was supported by the similar results in a previous report by Matsumoto *et al*.[17] which found a decrease in the percentage of IL-10+ cells in patients with severe unstable asthma compared to mild asthma. Moreover, Noma *et al.*[16] found that IL-10 production in patients with active asthma was higher after PHA stimulation than in patients who were in remission. However, Lee *et al*.[18] have demonstrated that there was an increase in IL-10 mRNA expression in moderate to severe asthmatic patients compared with mild asthma. The authors speculated that the finding was associated with persistent antigen stimulation and with frequent airway inflammation. However, it might have been related to the higher dosage of steroid use among patients with relatively severe disease.[19] In our study, we had excluded the patients who received systemic steroid within 4 weeks before the study and patients who were treated with the same doses of inhaled corticosteroids between exacerbation and stable phase.

In this study, IL-4 and IFN-γ in PHA-stimulated PBMCs, and IFN-γ in *Der p*-stimulated PBMCs levels were not significantly different between exacerbation and stable phase. In addition, the cytokine ratios for all stimulations were not significantly different between exacerbation and stable phase. A previous report in cytokine production from PBMC-stimulated with PMA and ionomycin showed that IL-4 level was not significantly different among exacerbation and stable episodes, but IFN-γ production was lower in acute exacerbation phase.[20] The difference in results could be explained from difference in cell stimuli and activating times between studies.

We also showed that IL-4 level in *Der p*-stimulated cells was below the level of detection. Babara *et al.*[21] also could not detect IL-4 level in PBMC cultured 6 days with HDM extract and PPD stimulation. This may be explained by the duration and dose of antigen stimulation.

In conclusion, we have demonstrated the significant decrease of IL-10 production in asthmatic children during acute exacerbation. This may emphasize the role of IL-10 in immune regulation in asthma and other allergic disease. Further studies in IL-10 producing cells and treatment to enhance IL-10 responses might be useful in novel strategy of prevention and treatment of asthma.

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