

Cytokine Expression Before and After Aspirin Desensitization Therapy in Aspirin-Exacerbated Respiratory Disease

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Abstract—Aspirin exacerbated respiratory disease (AERD) is induced by acetylsalicylic acid (ASA) and/or nonsteroidal antiinflammatory drugs (NSAIDs). Effects of desensitization on many mediators have been examined previously, but few studies addressed the influence of desensitization on T lymphocytes and T lymphocyte-derived cytokines. This study was performed to examine peripheral blood lymphocyte (PBL) cytokine expression in aspirin-sensitive patients who have asthma before and after aspirin desensitization. In this study, the release of interleukin-2 (IL-2), interleukin-4 (IL-4), and interferon-gamma (IFN- γ) by CD4⁺ T lymphocytes prior to aspirin desensitization were also measured at intracellular levels, and expression of these cytokines after 1 month aspirin desensitization was evaluated. Twelve patients with AERD were included in the study. Two different control groups were formed, one consisted of 15 healthy people and second 12 aspirin tolerant asthmatic (ATA) patients using aspirin. A blood sample was collected prior to desensitization, and the tests were repeated by taking a second blood sample 1 month after the 4-day desensitization treatment. The proportion of lymphocytes secreting IFN- γ in the study group was 15.61 ± 4.40 % before desensitization and 15.08 ± 5.89 % after desensitization. The rate of IFN- γ secreting CD4⁺ T lymphocytes was 20.51 ± 4.41 % in the normal control group and 16.07 ± 5.7 % in the ATA group ($p=0.021$). The ratio of CD4⁺ T lymphocyte secreting IFN- γ was reduced in patients with AERD before desensitization compared to normal control group ($p=0.040$). The levels of IL-2, IL-4, and the subsets of lymphocyte were not different before and after desensitization compared to control groups.

KEY WORDS: desensitization; aspirin sensitivity; asthma; cytokines.

INTRODUCTION

Aspirin-exacerbated respiratory disease (AERD) also known as aspirin-sensitive asthma, includes rhinitis (with or without polyps), asthma, acetylsalicylic acid (ASA), and/or other nonsteroidal antiinflammatory drugs (NSAIDs) sensitivity [1]. The prevalence of intolerance reactions caused by ASA and other NSAIDs in patients with asthma varies according to the method chosen; based

upon the self-reported statements of patients, 3 % of adults and 2 % of children experience intolerance reactions, while according to ASA provocation, the rates rise to 21 % in adult patients with asthma and 5 % among children [2].

In these cases, even some patients who do not use ASA medication suffer from severe asthma that is often difficult to keep under control, and nearly half of these patients are able to control their symptoms with systemic steroids [3]. Approximately, 14 % of the cases requiring mechanical ventilation in the intensive care unit due to severe asthma are patients with AERD, and intake of ASA was found to be the cause of attack in 8 % of these patients [4]. In addition to the treatment of chronic asthma, avoidance of the analgesics in patients with ASA reactions is the most common therapy, and ASA desensitization treatment may be applied by experts.

Cysteinyl-leukotrienes (Cys-LTs) are strong constrictor agents for airways [5–10]. The source of Cys-

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LTs is mast cells and eosinophils in bronchial mucosa biopsies. The most important functions of these agents, in addition to increasing cell permeability, are to enhance the migration of eosinophils in the region of inflammation, to increase mucus secretion, and to cause hypertrophy and hyperplasia in bronchial smooth muscle [5–7]. They are generated from arachidonic acid in the cell wall via the cyclooxygenase (COX) enzyme. The COX enzyme has two isoforms, COX-1 and COX-2. While COX-1 is released continuously from cells at basal levels, COX-2 is released during inflammation. Proinflammatory prostanoids such as PGD₂ and PGF-2 α and antiinflammatory prostanoids such as PGE₂ are produced from airway cells through the COX-1 enzyme [11, 12].

LTC₄, LTD₄, and LTE₄ (referred to as Cys-LTs) are synthesized from arachidonic acid through the 5-lipoxygenase pathways; this pathway then becomes active after COX-1 inhibition, an effect that is disadvantageous to the patient. The reduction in protective prostanoids, especially PGE₂, occurs through COX-1 enzyme inhibition with the use of ASA and other NSAIDs. Drugs that inhibit the enzyme COX-2 do not decrease PGE₂, which is known to have protective functions [13, 14]. The severity of respiratory symptoms induced by ASA and NSAIDs is directly proportional to the strength of COX-1 enzyme inhibition of the drug. While drugs that strongly inhibit COX-1 enzyme may cause severe respiratory symptoms, poor inhibitors do not cause any symptoms, or only result in symptoms at high doses. Christie *et al.* showed increased levels of LTE₄ in the urine of patients with bronchospasm that developed after the use of ASA. In these patients, increased expression of Cys-LTs receptor leukocytes in the nasal mucosa was demonstrated [15]. In another study, the expression of LTC₄ synthase in leukocytes infiltrating airway mucosa was found to be high in patients with AERD [16]. This increase may be caused by polymorphisms formed in the promoter region of the gene encoding LTC₄ synthase. In a study conducted by Arm *et al.*, comparison of histamine inhalation and LTE₄ airway sensitivity in ASA-sensitive asthmatics revealed that the response to histamine in individuals with AERD was not different from healthy individuals. However, 13-fold higher airway hyper-responsiveness to LTE₄ was observed among individuals with AERD [17]. Elevated expression of Cys-LT and significantly increased sensitivity to these stimuli in the airway are both important in the pathogenesis of this condition.

Aspirin desensitization has now been accepted as a treatment modality that can reverse this process. Aspirin

may be administered daily at doses of 80–1,300 mg for desensitization [18–21]. Increasing doses of aspirin, starting from small doses to 400–650 mg doses, are applied over 2–3 days. Considerable progress has been reported in nasal symptoms, sense of smell, number of sinus infections and need for sinus surgery, asthma symptoms, and the number of hospitalizations and emergency visits for asthma following the aspirin desensitization [19–21].

The effects of the process of desensitization on many mediators have been studied by many researchers; however, few studies have examined T lymphocytes, and T lymphocyte-derived cytokines are affected by the process of desensitization. In this study, CD4+ T lymphocytes release of interleukin-2 (IL-2), interleukin-4 (IL-4), and interferon-gamma (IFN- γ) were measured at intracellular levels prior to and 1 month after aspirin desensitization.

Patient Selection and Follow-up

In our study, study group consisted of 12 patients with AERD, 15 healthy people without any respiratory or systemic disease formed the first control group, and 12 patients with ATA formed the second control group. AERD cases with uncontrolled asthma even with regular use of systemic steroids and who underwent recurrent paranasal sinus operation were included in the study. Cases with a history of severe anaphylactic reaction with ASA or other NSAIDs, cases with a history of severe cardiac, gastrointestinal tract, liver, and kidney disease that was severe enough to preclude the use of ASA, and cases with a history of upper respiratory tract infection a month before desensitization, pregnancy, those using B-receptor blockers, individuals with psychiatric disorders, and individuals with less than 70 % of the expected forced expiratory volume-1 (FEV1) value, and patients who were not willing to participate were excluded from this study. Normal control group included individuals without respiratory or systemic disease. The control group with asthma did not have ASA sensitivity who were matched with the study group in respect to asthma severity. A detailed history and physical examination were obtained from the patients in the study group 15 days prior to desensitization. Presence of sinusitis and/or nasal polyps and prior operation, asthma diagnosis, existing respiratory symptoms with the use of ASA, and/or NSAIDs were recorded during anamnesis. Pulmonary function tests (PFT) were performed in 12 patients who were thought to be AERD based on history. Patients with the value of FEV1 over 70 %, or over 70 % of their best value

according to pulmonary function tests, were included in the study. PFT was performed three times in each patient with variability under 0.2 LT. The use of all medications (long-acting inhaled bronchodilators, inhaled corticosteroids, short-acting bronchodilator as needed, theophylline, initiate antileukotriene if it is not used) was obtained from patients during the first appointment (15 days before desensitization). Methyl prednisolone was started 48 h before desensitization according to the patient's health status. If the patient was steroid-dependent, prednisolone was given at doses of 60 mg/day, and if the patient was not steroid-dependent, prednisolone was given at doses of 40 mg/day, and also proton pump inhibitors were started.

Aspirin Desensitization Protocol

Arterial blood pressure, FEV1, nasal symptoms (runny nose, sneezing, itching, and congestion), laryngopharyngeal signs (burning in the throat, hoarseness), headache, respiratory symptoms (shortness of breath, wheezing, and cough), skin symptoms (urticaria, itching), gastrointestinal symptoms (vomiting, abdominal pain), and eye symptoms (itching, tearing, and swelling) were recorded after each dose of placebo given three times at an interval of 3 h during the first day of desensitization. The same parameters were recorded after the administration of 25, 50, 75, and 100 mg of ASA, respectively, on the first day of desensitization. The same parameters were recorded after the administration of 150 and 300 mg of ASA on the second day. The same parameters were evaluated after the administration of 600 mg ASA on the last day of the desensitization process. H1 and H2 receptor antagonists were administered to patients who had complains of skin, nasal, and eye symptoms during the study. Additional proton pump inhibitors were administered to patients who had stomach complaints, and additional prednisolone and bronchodilators were administered to those who had laryngeal and respiratory symptoms. The percentage of CD4+ T lymphocytes expressing IFN- γ , IL-2, IL-4, and lymphocyte subsets were examined on the first day before the desensitization and at the end of first month after desensitization. The study was approved by the ethics committee of Faculty of Medicine at Osmangazi University (decision dated September 25, 2008), and informed consent was obtained from patients.

Measurement of Intracellular Cytokine Level of T Helper Cells in Whole Blood

Blood (1 ml) collected in heparinized syringe was mixed with 1 ml+2 mM L-glutamine RPMI. Phorbol 12-myristate 13-acetate, calcium ionophore, and brefeldin were

added to this mixture. The blood was incubated for 4–6 h at 37 °C in an incubator with 5 % CO₂. One hundred microliter of blood was inserted into each of three tubes. Two milliliter of the diluted PharmLyse (BD) was added to each tube and vortexed. Tubes were centrifuged for 5 min at 500×g after incubation for 10 min in the dark.

After discarding the supernatant, cells were washed with 2 ml staining buffer (BD). CD4 fluorescein isothiocyanate (FITC)/CD3 PerCp (Becton Dickinson) was added into the first tube, CD4FITC/CD3 PerCp (Becton Dickinson) was added into the second tube, and CD4PE/CD3PerCp (Becton Dickinson) was inserted into the third tube. Antibodies were added and incubated in the dark for 15 min. The antibody-labeled cells were washed with staining buffer, the supernatant was discarded, 500 ml of Cytofix/Cytoperm was added to the pellet, and the tubes were vortexed. Tubes were incubated at room temperature for 20 min in the dark. They were centrifuged for 5 min at 1,800 rpm, and the supernatant was discarded. Two milliliter of Perm/Wash solution was placed into tubes, which were vortexed and kept at room temperature for 10 min. Tubes were centrifuged for 5 min at 1,800 rpm. The supernatant was discarded, and IL-2 PE (Pharmingen) antibodies were added to the first tube, the second tube was supplemented with IL-4 PE (Pharmingen) antibodies, and IFN- γ FITC (Pharmingen) antibodies were added to the third tube and incubated for 30 min in the dark. The cells were washed once with 2 ml Perm/Wash and were evaluated by CellQuest program in the flow cytometry device [22]. Results were given as the percentage of cytokine release from CD4+ T lymphocytes.

Lymphocyte Subgroups

The blood samples taken into tubes with K3-EDTA were divided into four separate tubes. Tubes were marked as FITC/CD19PE CD3 (BD), CD3 FITC/CD4 PE (BD), CD3 FITC/CD8 PE (BD), and CD3 FITC/CD16 + 56 PE (BD). After adding 20 μ l of monoclonal antibodies to each tube, 100 μ l of blood was added. They were stored in the dark at room temperature for 20 min. Two milliliter of the diluted erythrocyte lysis solution was placed into each tube, and they were stored in the dark at room temperature for 10 min. After the supernatant was discarded following the centrifugation, the cells were washed with 2 ml phosphate buffer saline (PBS) twice. Each tube was resuspended in 0.5 ml PBS and evaluated in a flow cytometer.

Statistical Analysis

Standard forms were generated for recording the data of the cases, and the data was saved for computa-

tional data. Statistical analysis was performed using SPSS-win 11.0 software. Values are expressed as mean $\pm$ standard deviation. One-way ANOVA test and the Bonferroni's correction test were used to compare the values of the three groups. Wilcoxon t test was used to compare values before and after desensitization. $P < 0.05$ was considered statistically significant.

RESULTS

Three groups were included in our study as follows: group 1 with AERD patients (48.67 ± 10.2 years), group 2 with normal controls without respiratory or systemic disease (50.87 ± 7.33 years), and group 3 with ATA controls (49.83 ± 9.49 years). There was no difference between the study groups for gender distribution (Kolmogorov–Smirnov $Z = 0.437$, $S = 0.991$) (Table 1).

There was no difference in FEV1 values before and after desensitization (3.74 ± 1.06 and 3.82 ± 0.8 L, $p = 0.5$).

The proportion of lymphocytes secreting IFN- γ in the study group was 15.61 ± 4.40 % before desensitization and 15.08 ± 5.89 % after desensitization. The rate of CD4+ T lymphocytes secreting IFN- γ was 20.51 ± 4.41 % in the normal control group, and 16.07 ± 5.7 % in the asthmatic control group, respectively. There was a significant difference between the groups ($p = 0.021$). The ratio of CD4+ T lymphocyte secreting IFN- γ was reduced in patients with AERD before desensitization compared to normal control group ($p = 0.040$) (Table 2).

There was no difference between groups in respect to percentages of CD4+ T lymphocytes expressing IL-2 and IL-4 and also no difference in respect to CD3+CD4+ T lymphocytes and CD3+CD8+ T lymphocytes ($p > 0.05$). In the AERD group, the percentages of CD4+ T lymphocytes expressing IFN- γ , IL-2, and IL-4 were not changed significantly after 1 month of desensitization ($p > 0.05$).

DISCUSSION

Despite the fact that aspirin desensitization therapy is an effective therapeutic modality in selected cases with AERD, the mechanism is still unclear. Although several

studies regarding to Cys-LT1 receptors, leukotrienes and leukotriene antagonists have been conducted to elucidate this mechanism, there are only a limited number of studies investigating the effects of chronic aspirin desensitization on inflammatory markers. The period of chronic desensitization is stated as 2 weeks or more in patients with AERD [6]. Studies have revealed a significant reduction in synthesis of LTB4 by peripheral monocytes in subjects with AERD following aspirin desensitization [18, 23]. The reduction in the number of inflammatory cells presenting Cys-LT1 receptor is related to desensitization. Sousa *et al.* show that the increased number of cells that the Cys-LT1 receptor present and the reduction of receptor expression after aspirin desensitization may suggest an important mechanism of aspirin desensitization [24]. In the same study, it was shown that the number of Cys-LT receptor in leukocytes of nasal mucosa was decreased to normal levels after ASA desensitization [24]. In another study, there was a statistically significant decrease in LTE4 urinary excretion, levels of tromboxane-B2, and LTB4 in blood monocytes after 1,300 mg/day of ASA desensitization for 2 weeks [23]. In other studies, a 33-fold decrease in airway sensitivity to inhaled LTE4 was observed beginning on the first day of desensitization [17].

IFN- γ is a cytokine that controls both the natural and acquired immune response. The secretion of IFN- γ from the subset of CD4+ T lymphocytes inhibits the proliferation of Th2-type cells. In our study, percentages of CD4+ T lymphocytes express IFN- γ in patients with AERD was found to be decreased both before desensitization and after desensitization compared with normal control group. No statistically significant difference was seen in the ATA control group compared with the control group. These findings suggest that defective IFN- γ response may occur during the treatment of asthma and even after desensitization in patients with AERD, and this observation had not changed significantly after 1 month of ASA desensitization. Cho *et al.* demonstrated that the release of IL-4, IL-5, and IFN- γ from unstimulated CD8+ T cells from sputum is increased relative to CD4+ T cells in cases with asthma and is associated with disease severity. The secretion of cytokines in peripheral blood polarized toward IFN- γ was significantly higher in cases with asthma than in a control group [25]. In another study, the secretion of IFN- γ was significantly reduced in CD4+ T cells of atopic non-asthmatic patients [26]. In another study, increased secretion of IFN- γ due to increased cross inhibition of Th2-type cytokines in patients with asthma was reported [27]. In this study, conducted by Shome *et al.* including

Table 1. Baseline Demographics and Characteristics

	AERD	Control	ATA
Age	48.67 ± 10.2	50.87 ± 7.33	49.83 ± 9.49
Gender	5 M/7 F	6 M/9 F	3 M/9 F

Table 2. The Cytokine Expression in Patients With AERD After and Prior to Desensitization

	BD	AD	Control	ATA
CD4+IFN- γ + (%)	15.61 $\pm$ 4.40*	15.08 $\pm$ 5.89	20.51 $\pm$ 4.41	16.07 $\pm$ 5.7
CD4+IL-2+ (%)	41.50 $\pm$ 8.12	39.41 $\pm$ 8.83	43.42 $\pm$ 9.19	35.25 $\pm$ 11.72
CD4+IL-4+ (%)	3.91 $\pm$ 1.21	4.81 $\pm$ 1.63	4.75 $\pm$ 1.78	3.82 $\pm$ 1.42
CD3+CD4+ (%)	47.16 $\pm$ 10.30	46.93 $\pm$ 9.96	45.22 $\pm$ 8.03	45.82 $\pm$ 7.06
CD3+CD8+ (%)	28.30 $\pm$ 8.57	28.34 $\pm$ 8.08	28.77 $\pm$ 8.08	29.18 $\pm$ 8.37
CD4/CD8 ratio	1.80 $\pm$ 0.74	1.74 $\pm$ 0.66	1.71 $\pm$ 0.87	1.68 $\pm$ 0.67

BD before desensitization, AD after desensitization, Control healthy control group, ATA aspirin tolerant asthma

one patient with AERD, there was a statistically significant increase in the expression of IFN- γ before ASA desensitization in comparison to the value after desensitization [27]. Similarly, in our study, a significant reduction in the initial IFN- γ expression compared to the control group was observed. The researchers in the Shome *et al.* study stated that the rate of CD4+ T lymphocytes secreting IFN- γ increased after treatment with ASA desensitization, and pointed out that this increase could be due to the inhibition of PGE₂ [27]. In our study, no increase in the level of this cytokine was observed. Pietruszewska *et al.* found that IFN- γ secreted from Th1 lymphocytes in the polyp tissue was significantly lower in patients treated for recurrent polyps [28].

Continuous aspirin ingestion for 6 months after desensitization decreased IL-4 levels in sputum [29]. In our study, IL-4 levels in the normal control group were not statistically different compared with the asthmatic control group, and no statistically significant difference was detected before and after desensitization. Perhaps our study will result in showing that the definition of chronic desensitization, which is defined as the period of after 15 days, is an insufficient period of time in terms of cytokine levels. The primary mechanism mediating this advantageous property of aspirin has not been revealed, though it may be related to the inhibition of IL-4. Decreased secretion of CysLT1R and LT synthesis indicates successful aspirin desensitization [17, 24, 25]. Aspirin directly blocks IL-4 secretion by T cells. The study conducted by Cianforeni *et al.* showed that when a therapeutic dose of ASA was applied to CD4+ T cells obtained from human peripheral blood, a significant decrease in the release of RNA, which induces IL-4 secretion, was observed [30]. On the other hand, this did not alter the release of IL-2 and IFN- γ [30]. In this study, therapeutic levels of ASA significantly inhibited the expression of IL-4 gene in activated CD4+ T cells [30]. In our study, no statistically significant differences were detected when the rate of T lymphocytes secreting IL-2 were compared among the control groups. In addition, the

rate of IL-2 expression in the asthmatic control group was not statistically different compared to the normal control group. We found no difference when we compared the values before desensitization with the values after desensitization. Jung *et al.* demonstrated that IL-2 secretion was reduced in CD4+ T cells of atopic and non-asthmatic patients [26]. Katial *et al.* quantified IL-4 levels in the sputum of patients with AERD after desensitization with aspirin and treatment with daily aspirin for 6 months. The reevaluated IL-4 levels in sputum were distinctly decreased from baseline values. IL-4 increases cysteinyl-leukotriene 1 receptors and induces Th2-mediated inflammation through the elevated expression of signal transducer and activator of transcription 6 [29]. Even though the role of CD4+ T cells has been shown in the pathogenesis previously, we have little knowledge regarding the role of CD8+ T lymphocytes. The ratio of CD4/CD8 in patients with severe asthma was not significantly different compared with mild asthmatics and a healthy control group [31]. In our study, the ratio of CD4+/CD8+ T lymphocytes in the ASA-sensitive group was not statistically different compared with the normal control and ATA groups, and this ratio was not changed after desensitization. A study conducted by Bernstein *et al.* compared the percentage of lymphocytes secreting CD4+ T lymphocytes or CD8+ T type cytokines in nasal polyp tissue and in the peripheral blood [32]. The distribution of T cells in nasal polyps was different than peripheral blood T cell distribution, and it was thought that this could be related to the impact of irritants in the nasal tissue such as allergens, fungi, and bacteria. While CD8+ T cells formed the predominant population of T cells in nasal polyp tissue, CD4+ T lymphocytes were found to be significantly higher in peripheral blood. Therefore, the ratio of CD4+/CD8+ T lymphocytes in the blood was significantly higher. In our study, the CD4+/CD8+ T lymphocytes ratio in polyp tissue was not examined, but we observed that the ratio in the blood before desensitization was not different in comparison to both of the controls, and this ratio remained unchanged

after desensitization. A study conducted by Taylor *et al.* included an equal number of patients with ASA-sensitive and nonsensitive asthma, and the rate of CD4+ T cells in the blood of normal individuals was 51.3 %, while the rate was 51.1 % in the cases with AERD, 46.1 % in group with ATA [33]. No statistically significant difference between the groups was reported.

There are some limitations about the results of our study. First, the number of the patients in the study was relatively low. Second, cytokine expression was studied in PBLs, a comparison with cytokine expression in lymphocytes from bronchial lavage fluids might have been considered. Third, we presented the data supporting the hypothesis that ASA desensitization is effective in changing the important cytokine of disease activity in AERD patients as early as the first month of treatment. Assessment of long-term cytokine levels may give important information regarding the long-term effect of desensitization.

As a result, desensitization did not cause a significant change in the CD4+ T lymphocyte expression of IFN- γ , IL-2, and IL-4 in patients with AERD after 1 month of desensitization therapy. There is a need for further studies with other lymphocyte subsets in addition to CD4+ T cells to elucidate the mechanisms mediating the immunological effects of ASA desensitization in patients with AERD.

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