

Reduced expression of CD69 and adhesion molecules of T lymphocytes in asthmatic children receiving immunotherapy

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T lymphocytes play a fundamental role in the initiation and regulation of chronic inflammatory responses in patients with asthma. CD69 is an early marker of T-cell activation. The levels of intercellular adhesion molecule-1 (ICAM-1, CD54) and L-selectin have been reported to increase in patients with allergic diseases and asthma. The present study was therefore undertaken to investigate the expression of CD69, CD54, and L-selectin by T lymphocytes of children with asthma, before and after immunotherapy. Eighteen children newly diagnosed with asthma, 11 good and nine poor responders to immunotherapy, and 16 normal subjects, were enrolled in this study. The percentages of CD69⁺, CD54⁺, and CD62L⁺ cells in T lymphocytes were measured by using flow cytometry. The levels of CD69, CD54, and CD62L in serum and culture supernatants were determined by using enzyme-linked immunosorbent assay (ELISA). The expression of CD69 and CD54 on CD3⁺ T lymphocytes was significantly higher in children with asthma than in control patients. All the patient groups expressed (spontaneously and following stimulation with phorbol myristate acetate and ionomycin together with mite-extract proteins) greater amounts of CD69 and CD54 than did control subjects. With long-term immunotherapy, the percentages of CD69⁺ and CD54⁺ T lymphocytes were significantly lower in patients with a good response to immunotherapy. Our results also showed significantly lower serum L-selectin levels following immunotherapy. In conclusion, successful immunotherapy resulted in decreased expression and production of CD69 and CD54. These results may explain, in part, the clinical efficacy of immunotherapy.

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Asthma is a chronic inflammatory disease characterized by increased infiltration of various inflammatory cells into the airway (1,2). Recruitment of leucocytes from the peripheral blood into tissue requires a series of cell-adhesion molecule-mediated interactions between the surface of the vascular endothelium and leucocytes. Recent studies have demonstrated that several adhesion molecules play a critical role in the recruitment and migration of leucocytes to sites of inflammation in various diseases (3,4). Important adhesion molecules expressed on leucocytes or endothelial cells include intercellular adhesion molecule-1 (ICAM-1) and L-selectin. The levels of adhesion molecules have been

reported to increase in patients with allergic diseases (5–8). Higher levels of adhesion molecules in serum samples from atopic individuals may reflect the up-regulation of cell-surface ICAM-1 expression in allergic inflammation.

The human leucocyte membrane protein, CD69, is an early activation marker of response to inflammatory stimuli. CD69 expression serves as an ideal indicator of T-cell activation (9,10). Activated T lymphocytes have been detected in the peripheral blood of patients with mild-to-moderate asthma (11), and the number of activated T cells was correlated with the airflow obstruction, as assessed by peak flow measurements (12).

Immunotherapy has been widely used as an effective treatment for asthma (13). Therefore, immunotherapy might modulate the activation of lymphocytes and the expression of adhesion molecules in lymphocytes and endothelial cells. The aim of this study was therefore to investigate the changes of CD69, ICAM-1, and L-selectin expression in T lymphocytes from children with asthma, during and after immunotherapy.

Patients and methods

Patients

Thirty-eight children with moderately severe asthma were enrolled in this study between January 1996 and June 2000. The diagnosis and classification of clinical severity of asthma was performed according to the National Asthma Education and Prevention Program (14). All the patients were sensitive only to mites (*Dermatophagoides pteronyssinus* and *D. farinae*) in terms of having a positive history of exposure (asthma attack during house cleaning), a skin test response greater than 2+ (wheal > 10 mm, by intracutaneous route with mite extract at a concentration of 10^{-6} g/ml), and a positive mite radioallergosorbent test (RAST) result (CAP system; Pharmacia Diagnostics, Uppsala, Sweden). Routinely, a panel of 12 inhaled allergens, including mites, cat, dog, pollens, cockroach, and molds, was tested using RAST and/or CAP testing and skin tests.

The efficacy of immunotherapy was evaluated by comparing, from patients' records, the sum of the frequency of asthmatic attacks and the medication required (total doses of β_2 -agonists, theophylline, and steroids) for 1 year before and after immunotherapy. This symptom and medication grading was ranked according to a previously described method (15). The result was considered to be excellent if the reduction of both parameters was >90%, much improved for a 75–90% reduction, improved for a 50–75% reduction, slightly improved for a 25–50% reduction, and poor for a reduction of $\leq 25\%$ (15). In this study, good responders were those who showed an improvement of >75%, and poor responders were those who showed an improvement of $\leq 25\%$. For comparison of the immunologic difference of immunotherapy between good and poor responders, only those two groups were analysed in this study. The Human Research Committee of Chang Gung Children's Hospital approved this project. Informed consent to participate in this investigation was obtained

from each patient's parents or guardians prior to commencement of the study.

Twenty asthmatic children who had received immunotherapy with mite extract for longer than 2.5 years were designated as Group A. According to the effectiveness of immunotherapy, we divided the group A patients into good (group A1) and poor (group A2) responders. The age of the children and duration of immunotherapy (2.5–3.0 years) were similar in these two groups. Eighteen age-matched untreated mite-sensitive children with newly diagnosed asthma were constituted as the asthma group (group B). Sixteen age-matched healthy children without any allergic disease served as the control group (group C).

Immunotherapy started with subcutaneous injection of a 10-allergen unit (AU) extract of *D. pteronyssinus*. The dose of the weekly injection was increased gradually until a maximal tolerated dose (1000 AU) was reached (usually within 3 months), after which the dose was maintained and injected at 4-week intervals. The treated patients had been receiving immunotherapy for longer than 2.5 years. All children with asthma were receiving treatment with inhaled corticosteroids, short-acting β_2 -agonist, and theophylline.

The scores of clinical responses and the result of laboratory examinations were analyzed independently by the clinicians and the immunologists, respectively.

Methods

Separation and culture of peripheral blood mononuclear cells. Heparinized blood samples were collected from asthmatic children, newly diagnosed (group B) and after immunotherapy (group A), and normal controls (group C). None of the subjects had clinical manifestation of infection. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll–Hypaque density-gradient centrifugation. The cells at the interface layer were collected carefully and washed twice with phosphate-buffered saline (PBS). Cell viability was determined by using the Trypan blue (Sigma Chemical Co., St Louis, MO, USA) exclusion test. For the analysis of adhesion molecule gene expression, freshly isolated PBMCs were cultured, at a concentration of 2×10^6 cells/ml, in RPMI-1640 with 10% fetal calf serum (FCS) for 7 days. In order to provoke allergen-induced lymphocyte activation, 1 μ g/ml ionomycin (Sigma Chemical Co.) and 50 ng/ml phorbol myristate acetate (PMA; Sigma Chemical CO.) together with

Table 1. Cumulative medication dose and asthma attacks in patients from good- and poor-response groups, 1 year before and after immunotherapy

	Good response		Poor response	
	Pre-IT	Post-IT	Pre-IT	Post-IT
β_2 -agonist (mg/year)	926.5 $\pm$ 477.3	93.8 $\pm$ 26.5	875.0 $\pm$ 176.8	726.0 $\pm$ 210.7
TV (g/year)	12.4 $\pm$ 1.2	1.4 $\pm$ 0.2	12.9 $\pm$ 1.0	12.4 $\pm$ 1.7
Oral steroid (mg/year)	1,350.0 $\pm$ 141.4	37.5 $\pm$ 4.1	1,300.0 $\pm$ 70.7	1,102.5 $\pm$ 82.0
Inhaled steroid (mg/year)	125.0 $\pm$ 7.1	35.0 $\pm$ 7.1	117.5 $\pm$ 9.6	112.5 $\pm$ 8.6
AE (episodes/year)	13.0 $\pm$ 1.4	1.3 $\pm$ 1.1	11.9 $\pm$ 2.0	8.8 $\pm$ 1.1

Good response: the sum of the frequency of asthma and the amount of medications consumed (total doses of β_2 -agonist, theophylline and steroids). The result was considered to be good if the reduction of both parameters was $>75\%$.

Poor response: if the reduction of both parameters was $\leq 25\%$.

AE, asthma with acute exacerbation (attacks); IT, immunotherapy; TV, theophylline.

10 μ g/ml mite-extracted proteins (Allergon; Pharmacia) were added to the cultures. The supernatants from unstimulated cells were also analyzed as a comparison for spontaneous production. Supernatants were collected and stored at -20°C until required for further analysis.

Flow cytometric analysis. About 1×10^6 freshly isolated PBMCs or cells stimulated with PMA and ionomycin were suspended in 0.1 ml of PBS and then incubated at room temperature for 30 min with appropriate fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (mAbs) specific to CD3 (Becton-Dickinson, San Jose, CA, USA), and with phycoerythrin (PE)-conjugated mAbs specific to CD54, CD69 (Becton-Dickinson), or CD62L (Pharmingen, San Diego, CA, USA). After washing with PBS, cells were subjected to flow cytometry analysis (FACSCalibur; Becton-Dickinson), as previously described (16). A total of 10,000 cells were counted and the percentage of each marker-positive cell type was determined by using Cellquest software (Becton-Dickinson).

Measurement of adhesion molecules in serum or from activated PBMC cultures. The concentration of the secreted adhesion molecules – soluble (s) ICAM-1 and soluble (s) L-selectin – were determined from serum samples obtained from asthmatic children and culture supernatants from PBMCs, as described previously (17). ELISA analysis was performed according to the manufacturer's instructions supplied with each kit (R & D Systems, Minneapolis, MN, USA). The minimum detectable concentration was 0.35 ng/ml for sICAM-1 and 0.30 ng/ml for sL-selectin.

Statistical analysis

Statistically significant differences were determined by the unpaired Student's *t*-test. Values of

$p < 0.05$ were considered to be significant between the two groups.

Results

Subjects

Thirty-eight children with moderately severe asthma (26 males, 12 females; mean age $\pm$ SD: 10.8 ± 2.8 years), who participated in this study, were followed-up regularly at the Allergy Clinic, Department of Pediatrics, Chang Gung Children's Hospital. Of the 20 patients who received immunotherapy for 2.5–3.0 years, 11 (seven boys and four girls, mean age 10.3 ± 2.7 years) showed a good response to immunotherapy. In contrast, the other nine asthmatic patients (six boys and three girls, mean age 11.3 ± 2.4 years) showed a poor response (Table 1).

The expression of CD69/3⁺, CD54/3⁺, and CD62L/⁺ on lymphocytes, as detected by flow cytometry

The expression of CD69 on T lymphocytes in the newly diagnosed asthma patients (group B) was higher than in the normal control (group C) ($p < 0.05$, Fig. 1a). The expression of CD69 on lymphocytes was significantly lower after immunotherapy in patients of group A ($7.00 \pm 5.72\%$) compared to patients of group B ($10.30 \pm 5.52\%$) ($p < 0.05$). However, after immunotherapy, the expression of CD69 was still higher than in the non-atopic group (group C). With long-term immunotherapy, the expression of CD69 on T lymphocytes was significantly different between good responders and poor responders ($p < 0.05$). The expression of CD54 (ICAM-1) on T lymphocytes in newly diagnosed asthma patients (group B) was higher than in the controls (group C, $p < 0.01$) (Fig. 1b). After immunotherapy, the expression of CD54 on T lymphocytes was significantly different between good responders and poor responders ($p < 0.05$). There was no

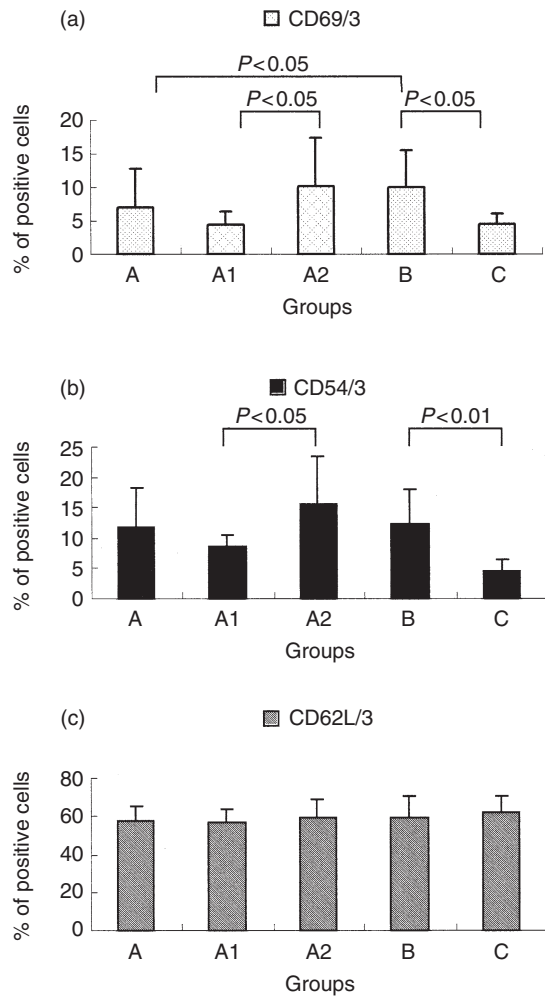


Fig. 1. The expression percentage of CD69/3 (a), CD54/3 (b), and CD62L/3 (c) peripheral blood mononuclear cells (PBMC) among various patient groups, as detected by flow cytometry. For the analysis of adhesion molecule gene expression, freshly isolated PBMCs were cultured at a concentration of 2×10^6 cells/ml for 7 days. Group A, asthmatic patients receiving immunotherapy. Group A1, patients of group A who had good responses to immunotherapy. Group A2, patients of group A who had poor responses to immunotherapy. Group B, newly diagnosed asthmatic patients without immunotherapy. Group C, healthy children without allergic disease. Percentage of positive cells: a total of 10,000 cells was counted and the percentage of each marker-positive cell was determined.

significant difference in the expression of CD62L (L-selectin) on T lymphocytes between asthma groups and the control group. A similar expression of CD62L on lymphocytes was observed in patients who did or did not receive immunotherapy ($p > 0.05$, Fig. 1c).

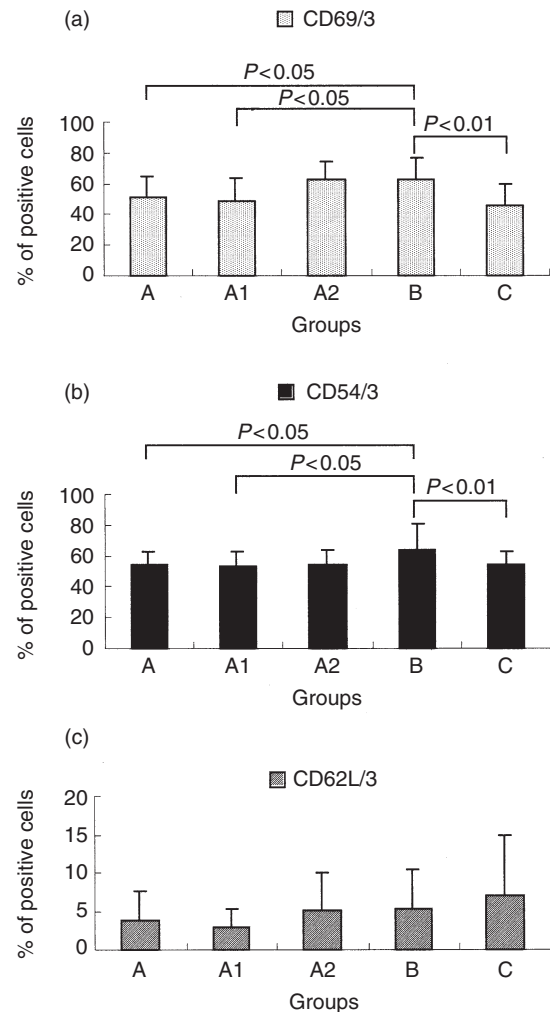


Fig. 2. The expression percentage of CD69/3 (a), CD54/3 (b), and CD62L/3 (c) peripheral blood mononuclear cells (PBMC) among the different groups of patients, after stimulation with allergen plus mitogen for 7 days. In order to provoke allergen-induced lymphocyte activation, 1 μ g/ml of ionomycin and 50 ng/ml of phorbol myristate acetate (PMA), together with 10 μ g/ml mite-extracted protein, were added to the cultures. Groups of patients were as described in the legend to Fig. 1.

The expression of CD69/3⁺, CD54/3⁺, and CD62L/3⁺ on T lymphocytes after stimulation for 7 days with PMA plus ionomycin and mite-extract protein

Mite-extract proteins were first used to stimulate allergen-induced activation of PBMC in different groups of patients. However, only a few cells survived in the cultures (data not shown). In order to enhance the cell activation, PMA and ionomycin were used as mitogens to stimulate allergen-activated lymphocytes. After stimulation with mitogen and mite-extract proteins, there was a significant difference in the expression of CD69/3⁺ on lymphocytes of asthmatic patients (group B) and controls (group C) ($p < 0.01$) (Fig. 2a). The

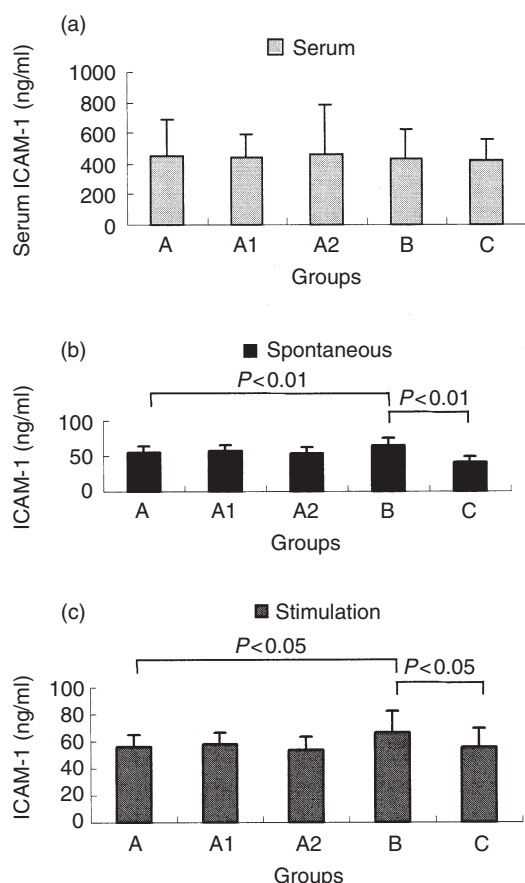


Fig. 3. Serum levels (a) and the production of intracellular adhesion molecule-1 (ICAM-1) in the supernatant of peripheral blood mononuclear cells with (b) or without (c) 7 days of stimulation. For comparison the supernatants from unstimulated cells were analyzed as spontaneous production (b). PBMC were activated with mitogen and allergen (c), as described in the legend to Fig. 2. Groups of patients were as described in the legend to Fig. 1.

level of CD69/3⁺ was lower in patients on immunotherapy than in those with newly diagnosed asthma. Furthermore, the expression of CD69/3⁺ on lymphocytes was significantly lower after immunotherapy in asthmatic children of the good response group (A1) than in those of the poor response group (A2) and before immunotherapy (group B) ($p < 0.01$). Similarly, the percentage of CD54⁺/3⁺ lymphocytes in children with asthma was higher than in the normal control group ($p < 0.05$). After immunotherapy, the percentage of CD54⁺/3⁺ lymphocytes was significantly lower in patients with a good response to immunotherapy (group A1) ($p < 0.05$) (Fig. 2b). There was no significant difference in the expression of CD62L between the asthma group and the control group. A similar expression of CD62L was detected in both good- and poor-response groups ($p > 0.05$) (Fig. 2c).

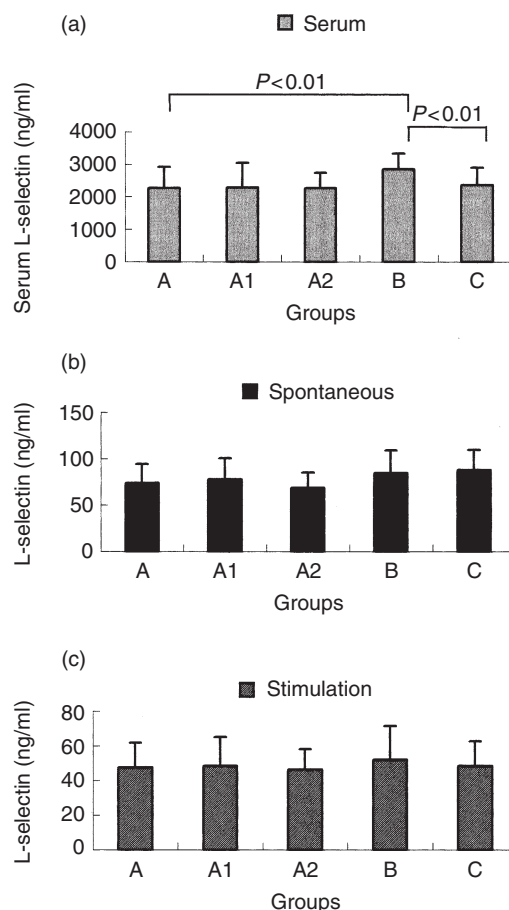


Fig. 4. Serum levels (a) and the production of L-selectin in the supernatant of peripheral blood mononuclear cells (PBMC) in the presence (b) or absence (c) of stimulation with phorbol myristate acetate (PMA) and ionomycin plus mite-extract protein, for 7 days among various groups. For comparison the supernatants from unstimulated cells were analyzed as spontaneous production (b). Activation of PBMC with mitogen and allergen (c) were as described as in the legend to Fig. 2. Groups of patients were as described in the legend to Fig. 1.

The serum levels and production of sICAM-1 and sL-selectin of PBMCs among various groups

Serum ICAM-1 levels were not significantly different between the asthma and control groups ($p > 0.05$) (Fig. 3a). The serum ICAM-1 level did not change after immunotherapy. The sICAM-1 production of PBMCs was significantly lower in the normal control group than the asthma group, in either the presence or absence of stimulation with PMA and ionomycin plus mite-extract protein ($p < 0.01$ and $p < 0.05$, respectively) (Fig. 3b, 3c). A significant difference was observed in the production of sICAM-1 from PBMCs of patients with and without immunotherapy ($p < 0.01$) (Fig. 3b). The serum level of sL-selectin in the group of patients with asthma was higher than in the control group ($p < 0.01$) (Fig. 4a). After immunotherapy, the serum level

of sL-selectin was significantly lower in patients of both good- and poor-response groups ($p < 0.01$). No significant difference was noted in the production of sICAM-1 from stimulated or unstimulated PBMC (Fig. 4b, 4c).

Discussion

Serum levels of sICAM-1 in persons with asthma have previously been demonstrated to be higher during acute exacerbations, and even during periods of stability, than those of normal controls (6,7,18,19). On the other hand, a decreased level of sICAM-1 can also be detected in children with moderately atopic asthma treated with corticosteroids (20). However, sICAM-1 levels were not significantly different between patients with asthma and control groups in the present study, with immunotherapy. This result was in contrast to the findings of increased sICAM-1 described by Montefort et al. (18). However, the statistical significance was attributed to a subgroup of asthmatics with a very high sICAM-1 response during acute asthmatic episodes. Conversely, lymphocytes from patients with asthma expressed higher levels of CD54 than those from healthy children, and the production of ICAM-1 from PBMCs in patients with asthma was higher than that in controls. A number of studies have shown that T lymphocytes might play a central role in asthma (21,22). CD69 expression serves as an ideal indicator of T-cell activation because it is the earliest molecule expressed during the activation of T cells (10). Hence, an increase in the expression of CD54 and CD69 reflects the ongoing inflammatory response at the inflamed site, and the detection of CD54 and CD69 molecules on lymphocytes could be useful in the investigation and monitoring of disease activity and inflammatory reactions in allergic disorders.

It is of clinical interest whether the expression of CD54 and CD69 can be modulated by treatment. With long-term immunotherapy, the percentage of spontaneous CD69⁺ and CD54⁺ T lymphocytes was significantly lower in patients with a good response to immunotherapy, but not in those with a poor response. Although only 0.1% of T cells are considered allergen-specific, continuous administration of allergen to patients receiving immunotherapy might anergize more T-cell activation *in vivo*. This suggests that immunotherapy can influence the expression of adhesion molecules and T-cell activation. As immunotherapy is an active form of treatment, unlike conventional drug therapy, it might modulate immune activation and inflammatory

events occurring in allergic diseases to decrease the expression of CD54 and CD69 in lymphocytes. The decreased expression of spontaneous CD69 and CD54 on lymphocytes after immunotherapy was considered to reflect the decrease in ongoing inflammatory response, thus resulting in symptomatic relief and reduction in the requirement for medication. Ohashi et al. (23) reported that the percentage decrease in sICAM-1 concentration was significantly correlated with the duration of immunotherapy and with the percentage of the decrease in symptom scores in allergic rhinitis. Our data further demonstrated lower expression of CD54 and CD69 after immunotherapy in asthmatic children with a good response. In contrast, neither CD54 nor CD69 showed a similar change after immunotherapy in those with a poor response. In the PBMC cultures with mite extract and mitogen, an higher level of CD69⁺ cells was observed in patients with a good response to immunotherapy, but not in patients with a poor response. The increased expression of this activation marker might be a result of the response of PBMC cells to cytokines produced from the allergen-activated T cells.

Our results also indicated a lower sL-selectin level following immunotherapy. According to the study of Monteseirin et al., decreased expression of L-selectin was demonstrated with an immunoglobulin E (IgE)-mediated mechanism when PBMC of asthmatic patients were cultured with anti-IgE antibody (24). The down-regulated serum L-selectin in this study might be attributed to similar mechanisms *in vivo*. In contrast, the percentage of CD62⁺ CD3⁺ cells was similar in asthmatic patients with or without immunotherapy and in controls. The elevated serum L-selectin level of patients might be contributed to from other inflammatory leucocytes, such as neutrophils (25), instead of T lymphocytes. Nevertheless, shedding or loss of L-selectin upon cell activation or migration through the endothelium to the inflammatory sites has been proposed (26). High concentrations of serum sL-selectin and a low percentage of CD62L⁺ peripheral lymphocytes were observed in patients with pulmonary sarcoidosis (27). Taken together, sL-selectin, as well as surface CD69 and CD54 molecules on T lymphocytes, could be useful markers for indicating the clinical activity of asthma patients receiving allergen immunotherapy.

With different allergens and duration of therapy, the efficacy of immunotherapy has been controversial (28,29). Several mechanisms have been proposed to contribute to the effec-

tiveness of immunotherapy, including the reduction in specific IgE antibody titer (30), the induction of IgG₄ blocking antibody (31), the modulation of T-cell activation (18), the decrease in T helper 2 (Th2) cytokines (30), and the alteration of cytokine profiles (32). Furthermore, our study suggests that the decreased expression of adhesion molecule on T lymphocytes and the number of activated T lymphocytes in asthmatic patients receiving treatment with immunotherapy may decrease the recruitment of the activated T lymphocytes into airway, resulting in a reduction of airway inflammation.

In conclusion, the findings of decreased expression of adhesion molecules and activated T cells in good responders and the reverse in poor responders may explain, in part, the clinical efficacy of immunotherapy.

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