Allergy 2005: 60: 774–779

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ALLERGY
DOI: 10.1111/j.1398-9995.2005.00784.x

Original article

Effects of allergen inhalation and oral glucocorticoid on serum soluble CTLA-4 in allergic asthmatics

Background: The serum soluble cytotoxic T lymphocyte associated antigen-4 (sCTLA-4) concentration is significantly elevated in patients with asthma, and sCTLA-4 concentration correlate with the severity of asthma. The aim of the present study was to investigate effects of allergen inhalation and oral glucocorticoid on concentration of serum sCTLA-4 in patients with allergic asthma.
Methods: Allergen inhalation challenge was conducted in allergic asthmatics with isolated early asthma response and those with dual asthma response. In a randomized, double-blind, placebo-controlled, parallel group fashion, prednisolone or placebo was give orally once a day for 2 weeks. Venous blood samples were collected before and after allergen inhalation or prednisolone administration for obtaining sera. The serum sCTLA-4 concentrations were determined using enzyme-linked immunosorbent assay.
Results: The serum sCTLA-4 concentrations in the dual responder group increased from 29.0 (14.5–43.7) μg/l [median (25–75 percentiles)] before allergen inhalation to 44.0 (24.3–61.3) μg/l 24 h after allergen inhalation. In the isolated early responders, there were no significant increase in serum sCTLA-4 concentrations after allergen inhalation compared with baseline levels. There was a significant decrease in serum sCTLA-4 concentrations after 2 weeks of glucocorticoid therapy [22.0 (15.5–31.0) μg/l] compared with baseline values [37.0 (19.5–53.0) μg/l], whereas there was no significant difference in the placebo group.
Conclusion: This study has demonstrated that serum sCTLA-4 concentrations increased after allergen inhalation in sensitized asthmatic subjects, and that serum sCTLA-4 concentrations were downregulated by prednisolone therapy.

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Key words: allergen; asthma; glucocorticoid; serum, soluble CTLA-4.

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Accepted for publication 4 October 2004

Bronchial asthma is characterized by reversible airflow obstruction, airway mucosal inflammation, and airway hyperresponsiveness (AHR). The role of inflammation in the development of symptomatic asthma and AHR has been emphasized in the past decade, and asthma treatment has moved from bronchodilator to anti-inflammatory drugs (1). The mechanisms by which inflammation is controlled are therefore of considerable interest. The allergic response to common environmental allergens has been regarded as an important mechanism in the development of airway inflammation of patients with asthma. Allergen inhalation challenge in sensitized subjects typically induces an immediate bronchoconstriction (the early asthmatic response), which is maximized within 30 min and resolves between 1 and 3 h (2). A proportion of subjects will proceed to develop a second, delayed bronchoconstriction (the late asthmatic response), which is associated with prolonged AHR and pronounced airway eosinophilia (2–4). As airway allergen challenge is associated with eosinophilic inflammation and AHR, this procedure is useful as a model to study mechanisms of airway inflammation in asthma.

In the treatment of patients with asthma, the clinical value of glucocorticoids is well documented (5). Early studies demonstrated that glucocorticoids caused an improvement in forced expiratory volume in 1 s (FEV_1) and forced volume capacity (FVC), and these drugs are now routine used for the treatment of asthma. Treatment with glucocorticoids have also been shown to improve AHR and reduce inflammatory cells in the airways, including mast cells, eosinophils, and lymphocyte (6, 7). Because asthma is now recognized to be a disease of inflammation, this reduction in inflammatory cells is believed to be a key glucocorticoid effect.

Cytotoxic T lymphocyte associated antigen-4 (CTLA-4) was initially described as a classical type I glycoprotein on the surface of activated T cells (8). The CTLA-4 plays an important role in the down-regulation of T-cell response, in T-cell homeostasis and in maintenance of peripheral tolerance. Recently, alternative

transcripts of CTLA-4 and a native, soluble form of CTLA-4 (sCTLA-4) have been reported in human serum (9). The sCTLA-4 is a functional molecule with B7 binding activities. In a more recent study, we have shown that serum sCTLA-4 concentrations in both atopic asthmatics and nonatopic asthmatics were all higher than that in normal controls (10). The aim of the present study was to examine the effects of inhaled allergen and oral glucocorticoid on the changes in serum concentrations of sCTLA-4 in allergic patients with asthma.

Materials and methods

Subjects

The study protocol was approved by the Ethics Committee of Guangxi Medical University, P. R. China, and all subjects provided written consent. A total of 40 nonsmoking patients, who met the criteria for a diagnosis of asthma as defined by the NHLBI/WHO Workshop on the Global Strategy for Asthma (GINA guidelines) (11), were enrolled in this study. At the screening visit, each subject underwent a medical history, physical examination, allergen skin prick test, and pulmonary function testing including spirometry to determine FEV_1, FVC, reversibility of FEV_1 to β_2-agonist, or/and a provocative concentration of methacholine producing a 20% fall in FEV_1 (PC_{20}-Mch). Each patient had positive skin prick test responses to house dust mite (HDM) or/and other aeroallergens, but none was receiving immunotherapy or glucocorticoid therapy. In the preliminary detection, sCTLA-4 was detected in serum samples from all asthmatics studied.

Fifteen mild allergic asthmatics (eight dual responders and seven isolated early responders) were recruited in allergen inhalation study (Table 1). Subjects were classified as dual responders if they developed both early- and late-phase asthmatic responses as defined by a $>15\%$ drop in FEV_1 from baseline, or as isolated early responders if they developed only an early fall in FEV_1 $>15\%$ from baseline. Asthmatics were stable at the time of study, requiring only intermittent use of inhaled β_2-agonists, with baseline FEV_1 values $>70\%$ predicted.

Twenty-five allergic asthmatics with baseline FEV_1 values of 56.6–109.0% [mean ± standard error of mean (SEM), 82.6 ± 3.2%] of predicted values were included in oral glucocorticoid study (Table 2).

Allergen inhalation study

Allergen inhalation challenge was conducted as described by O'Byrne et al. (12). The allergen producing the largest skin-wheal diameter was diluted in 0.9% saline for inhalation. In this study, inhaled allergen used for all asthmatics was HDM. The concentration of HDM extract for inhalation was determined from a formula described by Cockcroft et al. (13), using the results from the skin test and the PC_{20}-Mch values. The starting concentration of HDM extract for inhalation was two doubling concentrations below that predicted to cause a 20% early decrease in FEV_1, and doubling concentrations of HDM were given until a 15% early decrease in FEV_1 was reached. The FEV_1 was measured at 10 min and then again at 20, 30, 45, 60, 90, and 120 min after HDM inhalation, and then on an hourly basis until 7 h after HDM inhalation. The early asthmatic response was taken to be the largest decrease in FEV_1 within 2 h after HDM inhalation, and the late asthmatic response was taken to be the largest decrease in FEV_1 between 3 and 7 h after HDM inhalation. At the time right before HDM inhalation and 24 h thereafter, the venous blood was withdrawn for obtaining sera.

Table 1. Characteristics of mild allergic asthmatics included in allergen inhalation study

				Max % fall in FEV_1	
Subject	Age (years)	Sex	HDM dilution	EAR	LAR
Dual responders					
1	36	F	1 : 128	21.2	32.3
2	35	M	1 : 1024	23.6	17.5
3	31	M	1 : 64	18.9	27.6
4	28	F	1 : 512	24.4	21.4
5	18	M	1 : 256	23.7	18.6
6	22	F	1 : 256	30.1	22.3
7	25	M	1 : 1024	19.8	28.3
8	18	F	1 : 512	20.3	27.2
Mean ± SEM	26.6 ± 2.5			22.8 ± 1.3	24.4 ± 1.8
Early responders					
1	29	F	1 : 64	21.2	4.4
2	40	F	1 : 8	29.3	5.7
3	21	M	1 : 8	18.2	11.2
4	39	F	1 : 32	20.2	6.8
5	18	M	1 : 16	26.2	−2.8
6	31	M	1 : 64	25.0	8.9
7	17	F	1 : 128	20.5	5.0
Mean ± SEM	27.9 ± 3.6			22.9 ± 1.5	5.6 ± 1.7

EAR, early asthmatic response; LAR, late asthmatic response; HDM, house dust mite.

Oral glucocorticoid study

This section of the study was performed in a randomized, double-blind, placebo-controlled, parallel group fashion. Placebo or prednisolone in a dose of 30 mg was given orally once a day, 30 min after breakfast, for 2 weeks. At the time of entry day and at the last day of the study, venous blood sample were collected for obtaining sera. Patients with asthma were asked to record their clinical symptoms on a standard chart consisting of checklists such as dyspnea, wheezing, chest tightness, cough, sputum, and peak flow in the morning and at 17:00 hours to assess if there was any major asthma attack during the study.

Detection of sCTLA-4

A previous described sandwich enzyme-linked immunosorbent assay (10) was used for detection of sCTLA-4 in serum. For this purpose, wells of a 96-well microtiter plate were coated with 1.0 μg/ml anti-CTLA-4 mAb (clone BNI3; PharMingen, San Diego, CA). After blocking, 100-μl samples in buffer solution were added to each wells and the plates were incubated for 2 h at room temperature. After washing, 100-μl polyclonal goat anti-CTLA-4 Ab (1.0 μg/ml, R & D System, Minneapolis, MN) was applied and the plates were incubated for 2 h at room temperature. Following the addition of antigoat, IgG conjugated HRP (1 : 10 000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), the color was developed using 3,3′,5,5′-tetramethyl-benzidine substrate for 15 min at room temperature, and the reaction was terminated with 2 N H_2SO_4. The optical density (OD) was read at 450 nm. A standard curve was generated with the use of a dilution series of a commercially available CTLA-4Ig fusion protein (Ancell, Bayport, MN). Each

Table 2. Characteristics of allergic asthmatics included in glucocorticoid study

Subject	Age (years)	Sex	%FVC	$\%FEV_1$
Glucocorticoid group				
1	46	M	106.9	109.0
2	40	M	90.6	87.6
3	34	F	97.4	97.3
4	33	F	79.1	75.3
5	20	M	79.2	76.9
6	18	F	104.5	107.3
7	25	F	67.4	65.4
8	18	M	106.1	101.2
9	26	F	57.1	56.8
10	25	M	86.4	88.5
11	23	F	79.2	69.8
12	37	F	69.6	73.3
13	48	M	77.7	78.5
Mean ± SEM	30.2 ± 2.8		84.7 ± 4.4	83.6 ± 4.6
Placebo group				
1	32	F	86.4	83.7
2	55	M	74.6	76.4
3	44	M	66.1	64.4
4	23	F	103.8	98.4
5	21	M	102.0	108.2
6	27	M	97.4	97.0
7	43	M	92.3	89.4
8	32	F	57.7	59.4
9	34	F	72.0	71.2
10	29	M	83.8	81.5
11	19	F	60.3	56.6
12	20	M	88.4	92.1
Mean ± SEM	31.6 ± 3.2		82.1 ± 4.7	81.5 ± 4.3

FEV_1, forced expiratory volume in 1 s; FVC, forced volume capacity.

sample was run in duplicate and was corrected for background by subtraction of OD obtained when the sample was incubated in wells coated with an isotype-matched mAb of irrelevant specificity (anti-rat CD4). Intrassay coefficient of variation was 3.2% ($n = 16$), and the interassay coefficient of variation was 10.6% ($n = 18$).

Statistics

Data of age and $\%FEV_1$ are presented as mean ± SEM; Data of serum sCTLA-4 concentrations are presented as medians (25–75 percentiles). Nonparametric tests were used to analyze data variables as these variables were not normally distributed. Statistic differences between before and after allergen inhalation or glucocorticoid administration were determined by a Mann–Whitney *U*-test. Comparison between baseline values of the two study protocols were made using a Wilcoxon signed-rank test. *P*-values of <0.05 were considered significant.

Results

Effects of allergen challenge on serum sCTLA-4 concentrations

The mean maximal percent fall in FEV_1 during the early asthmatic response was 22.8 ± 1.3% in the dual responders and 22.9 ± 1.5% in the isolated early responders (Table 1). The maximal percent fall in FEV_1 during the late asthmatic response was 22.4 ± 1.8% in the dual responders and 5.6 ± 1.7% in the isolated early responders. Before HDM inhalation, the serum sCTLA-4 concentrations in dual responders [29.0 (14.5–43.7)μg/l] and those in isolated early responders [24.0 (14.0–45.0)μg/l] showed no significant differences ($P = 0.817$) (Fig. 1). The serum sCTLA-4 concentrations in the dual responder group increased from baseline levels to 44.0 (24.3–61.3)μg/l 24 h after HDM inhalation ($P = 0.025$). In the isolated early responders, there were no significant increase in serum sCTLA-4 concentrations after HDM inhalation [22.0 (19.0–40.0)μg/l] compared with baseline levels ($P = 0.667$).

Effects of prednisolone on serum sCTLA-4 concentrations

Randomization resulted in 13 patients with asthma receiving prednisolone and 12 patients receiving placebo treatment. All subjects completed the study, and none reported side effects during the treatment. There were no significant differences between glucocorticoid and placebo group in age, %FVC, and $\%FEV_1$ before treatment (Table 2). Before treatment, glucocorticoid group [37.0 (19.5–53.0)μg/l] and placebo group [31.0 (17.0–53.5)μg/l] showed no significant differences in serum sCTLA-4 concentrations ($P = 0.849$) (Fig. 2). There was a significant decrease in serum sCTLA-4 concentrations after

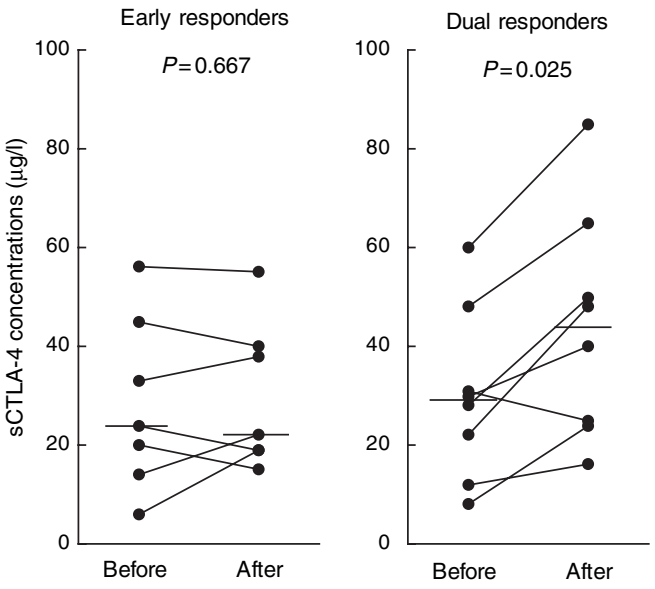

Figure 1. Effects of allergen inhalation on changes in serum soluble cytotoxic T lymphocyte associated antigen-4 (sCTLA-4) concentrations. Serum sCTLA-4 concentrations before and after allergen inhalation in isolated early (A) and dual (B) responders of allergic asthmatics were determined using enzyme-linked immunosorbent assay. There was a significant increase in sCTLA-4 concentrations after allergen inhalation in the dual responder group, but not in the isolated early responder group. Distributions and medians are shown, and values of *P* were calculated using Wilcoxon signed-rank test.

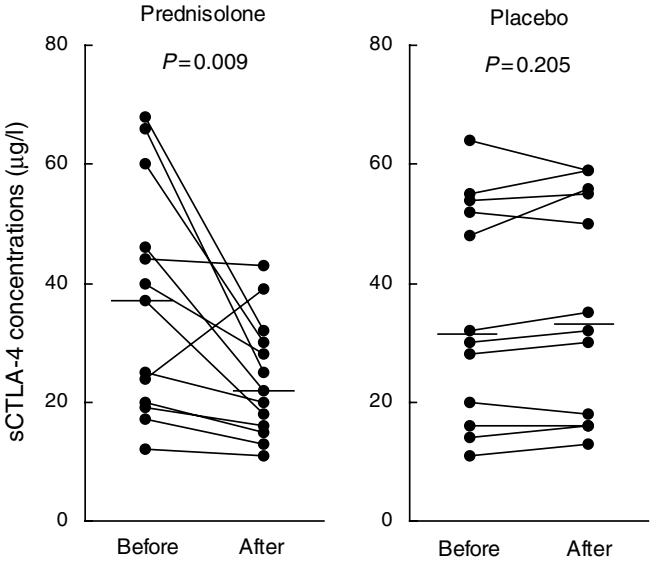

Figure 2. Effects of oral glucocorticoid on changes in serum soluble cytotoxic T lymphocyte associated antigen-4 (sCTLA-4) concentrations. Serum sCTLA-4 concentrations before and after oral prednisolone (A) and placebo (B) treatments in allergic asthmatics were determined using enzyme-linked immunosorbent assay. There was a significant drop in sCTLA-4 concentrations in prednisolone treatment group, but not in placebo control group. Distributions and medians are shown, and values of *P* were calculated using Wilcoxon signed-rank test.

2 weeks of prednisolone therapy [22.0 (15.5–31.0)μg/l] compared with baseline values ($P = 0.009$), whereas there was no significant difference in the placebo group [33.5 (16.5–55.8)μg/l; $P = 0.205$].

Discussion

Bronchial asthma is characterized by acute and chronic airway inflammation. Allergen exposure in susceptible individuals results in the recruitment and activation of inflammatory cells, which leads to bronchoconstriction and further perpetuation of the inflammatory response (1). Central to this process is the T lymphocyte (14). It has been established that full activation of T lymphocytes requires two signals (15, 16). The first signal occurs following the interaction of polymorphic T-cell receptors with the major histocompatibility complex peptide complex on potent antigen-presenting cells. Complete T-cell activation and differentiation also requires a second, noncognate set of interactions, the so-called costimulatory stimulus. The best-studied costimulatory stimulus involves the CD28/B7 family of molecules. CD28 is a cell-surface glycoprotein homodimer expressed on the majority of functional T cells. Both CD28 and the homologous family member CTLA-4 bind to the same B7 family of coreceptors, of which there are at least two members, CD80 (B7-1) and CD86 (B7-2) (17). These B7 molecules are differentially expressed on distinct tissues, have different requirements for induction, and exist in multiple forms with varying affinities for the CD28 and CTLA-4 receptors (18, 19).

Previous studies have shown that signals mediated through the CD28 and CTLA-4 receptors are required for the processes of T-cell recruitment to the airway and T-cell differentiation, demonstrating that these receptors are important regulatory components in the development of allergic airway inflammation (20). It has also been reported that blockade of T-cell costimulation by administration of CTLA-4Ig fusion protein, that prevents B7 from 12 binding either CD28 or CTLA-4 ameliorates the asthmatic manifestations in murine models (21). The CTLA-4Ig might be an effective alternative therapy for established allergic asthma, especially in situations of mild disease (22).

Recently, alternative transcripts of CTLA-4 and a native sCTLA-4 have been reported in human serum (9). The functional molecule sCTLA-4 exhibits B7 binding activities, and might play an important role in immune regulation by binding with the B7 molecules, thus, interfering with the binding of CD28 or/and CTLA-4. *In vivo* experiments with mutant lacking the cytoplasmic domain of CTLA-4 indicated that its inhibitory effects are mediated in part through the ability of the extracellular domain to compete for ligands (23). Recombinant sCTLA-4 at high concentrations *in vitro* could inhibit mixed leukocyte reactions and cellular proliferation (24), probably by interference with CD28 signaling. sCTLA-4 might also interfere with CTLA-4-B7 interactions and block negative signals transmitted via surface-bound CTLA-4. In a more recent study (10), we have demonstrated that sCTLA-4 was elevated in the sera of patients with asthma. The concentration of serum sCTLA-4 in asthmatic patients was inversely correlated with $\%FEV_1$ and %PEF, and was positively correlated with arterial carbon dioxide tension. These findings suggest a potentially important role for sCTLA-4 in the pathogenesis of asthma. Our previous results also showed that the serum sCTLA-4 level was positive correlated with numbers of lymphocytes, but not with those of neutrophils, eosinophils or monocytes. These results suggest that sCTLA-4 detected in asthmatic serum might derive from lymphocytes. In the present study, we have extended the above findings and demonstrated that the serum sCTLA-4 concentrations in the dual responder group of allergic asthmatics increased significantly after HDM inhalation. The high levels of CTLA-4 in sera from dual responders of asthmatics after HDM inhalation may play a protective role via interference with CD28-B7 interaction or, alternatively, contribute to asthma attack via interference with the CD28-B7 interaction. Actually, it has been demonstrated in human that significant associations between *CTLA-4* polymorphisms and serum IgE levels, allergy, asthma, and reduced lung function, but not AHR, suggesting that a major contribution of *CTLA-4* polymorphisms is to atopy and atopic asthma. All

associated alleles were opposite those alleles associated with autoimmune diseases, providing suggestive evidence for a genetic control site for Th1-mediated *vs* Th2-mediated immunity within the *CTLA-4* gene. These data suggest a role for *CTLA-4* polymorphisms in determining the Th1/Th2 balance and identify CTLA-4 signaling as a potential therapeutic target in atopic diseases, including asthma (25).

As glucocorticoids can be lympholytic and although lymphocyte counts are not abnormally low, there could be a higher turnover in those people on glucocorticoids. It would be interesting to investigate the effects of glucocorticoids on the concentrations of sCTLA-4. In the present double-blind, placebo-controlled, parallel group study, we investigated the effect of oral prednisolone on the serum sCTLA-4 concentrations in patients with allergic asthma. Our data showed that there was a significant decrease in serum sCTLA-4 concentrations after 2 weeks of prednisolone therapy compared with baseline values, whereas there was no significant difference in the placebo group. It has been known that glucocorticoids are effective in reducing airway inflammation through multiple mechanisms, including the modulation of cytokine and chemokine production in lymphocytes and endothelial cells (5–7). Glucocorticoids bind to specific receptors in the cytosol, are translocated to the nucleus, and bind to specific regulatory sequences of target genes (26). Glucocorticoids have been shown to act directly on T cells, suppressing mRNA expression for interleukin-2 and interferon-γ (27). By regulating cytokine gene expression (27), glucocorticoids may critically modulate effector functions of T cells. Our present data suggest that glucocorticoids exert their therapeutic effects in the treatment of asthma by reducing serum sCTLA-4 concentrations.

In summary, this study has demonstrated, for the first time, increases in serum sCTLA-4 concentrations after allergen inhalation in sensitized asthmatic subjects. We have also demonstrated that serum sCTLA-4 concentrations were downregulated by 2 weeks of 30 mg/day prednisolone therapy. It may explain in part the beneficial effects of glucocorticoids in the treatment of allergic asthma.

Acknowledgments

This study was supported in part by research grant No. 30260041 from National Natural Science Foundation of China, in part by research grant No. 200260 from the Ministry of Education, P. R. China, and in part by research grant No. 200203 from the Bureau of Health, Guangxi Zhuang Autonomous Zone, P. R. China.

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