

Enhanced Serum Neutrophil Chemotactic Activity was Noted in Both Early and Late Asthmatic Responses During Lysine-Aspirin Bronchoprovocation Test in ASA-Sensitive Asthmatic Patients

To investigate the pathogenic mechanism of late asthmatic response in comparison to early asthmatic response, changes of serum neutrophil chemotactic activity (NCA) using the Boyden chamber method and histamine level using the automated fluorometric analyzer were observed in 13 aspirin (ASA)-sensitive asthma subjects (group I: 7 early responders and group II: 6 dual responders) during lysine aspirin bronchoprovocation test (L-ASA BPT). Sera were collected before, and 30 min and 240 min after L-ASA BPT. Serum NCA increased significantly after 30 min ($p=0.02$) and decreased significantly at 240 min ($p=0.02$) in group I, while serum NCA of group II increased significantly at 30 min ($p=0.04$), tending to increase further up to 240 min with no statistical significance. NCA at 240 min in group II subjects was significantly higher than baseline NCA ($p=0.02$). The serum NCAs collected before and 240 min were significantly higher in group II than in group I ($p<0.05$, respectively). There were no significant changes in serum histamine levels during L-ASA BPT in both groups. NCA derived from mast cell may contribute to the development of early asthmatic response induced by L-ASA inhalation. There may be a possible involvement of NCA derived from mononuclear cells during late asthmatic response.

Key Words : Asthma; Neutrophils; Chemotaxis; Leukocyte; Bronchial Provocation Tests

Sun-Sin Kim, Hae-Sim Park,
Ho-Joo Yoon*, Young-Mok Lee,
Soo-Keol Lee¹, Dong-Ho Nahm

Department of Allergy & Clinical Immunology, Ajou University School of Medicine, Suwon; Department of Internal Medicine, Hanyang University School of Medicine*, Seoul; Department of Internal Medicine, Dong-A University School of Medicine¹, Busan, Korea

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Address for correspondence

Hae-Sim Park, M.D.
Department of Allergy and Clinical Immunology,
Ajou University School of Medicine, San-5,
Wonchondong, Paldalgu, Suwon 442-749, Korea
Tel : +82.31-219-5196, Fax : +82.31-219-5154
E-mail: hspark@madang.ajou.ac.kr

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INTRODUCTION

Aspirin (ASA) and other non-steroidal anti-inflammatory agents (NSAIDs) cause bronchoconstriction in 10 to 20% of adult asthmatic patients (1, 2). A recent investigation demonstrated that the prevalence of ASA-sensitive asthma among patients with non-atopic asthma was 36.1% based upon lysine aspirin bronchoprovocation test (L-ASA BPT) results (3).

The mechanism of ASA-induced bronchoconstriction has not yet been fully defined. However, it is widely accepted that ASA and other NSAIDs block the cyclo-oxygenase pathway and that this may cause arachidonate substrates to be diverted to the 5-lipoxygenase pathways. Products of this pathway can induce chemotaxis via leukotriene B₄ and are potent bronchoconstrictors and secretagogues (4). A recent review (5) emphasized the importance of cysteinyl leukotriene (cys-LT) overproductions in the mechanism of ASA-induced bronchoconstriction.

The L-ASA BPT has become an alternative diagnostic test for detecting ASA sensitivity in asthmatic patients, and is used routinely in Europe and other parts of the world (6, 7). L-ASA inhalation could induce late asthmatic responses as well as early

asthmatic responses (8).

Although there has been some evidence indicating mast cell activation after L-ASA inhalation (9, 10), there has been no report on indicating cellular activation profiles during late asthmatic response. To investigate the possible involvement of mast cell and neutrophil in ASA-induced asthma, we compared changes of serum NCA and histamine levels during L-ASA BPT between early and late asthmatic responders.

MATERIALS AND METHODS

Subjects

The 13 ASA-sensitive asthma patients confirmed by positive L-ASA BPT were classified into two groups: the group I included seven patients showing isolated early asthmatic responses and the group II included six dual asthmatic responders. The clinical characteristics of the study subjects were summarized in Table 1. Atopy was defined on the basis of skin prick test results showing more than 2+ responses: two or more common inhalant allergens such as *Dermatophagoides farinae*, alder, oak,

Table 1. Clinical characteristics of the study subjects

	Patient No.	Sex/Age (yr)	Duration of asthma (yr)	Rhinitis	Nasal Polyp	PC ₂₀ (mg/mL)
Group I	1	F/27	<1	Present	Absent	2.5
	2	M/46	20	Absent	-	0.62
	3	F/67	<1	Present	Present	ND
	4	M/37	3	Present	Present	1.25
	5	F/57	10	Present	-	0.31
	6	F/45	20	Present	-	0.31
	7	M/26	5	Absent	Present	0.31
Group II	1	F/40	7	Present	Present	5.0
	2	M/21	4	Present	Present	1.25
	3	M/30	10	Present	Present	0.075
	4	M/34	3	Present	Present	0.62
	5	F/35	15	Present	Absent	1.25
	6	F/33	2	-	-	25
	7	F/27	6	Present	Present-	0.31

Group I; Early response; Group II; Dual response; ND; Not done.

rye grass, mugwort, ragweed and *Aspergillus* spp. (Bencard Allergy Service, Brentford, Middlesex, U.K.). Most of them were treated with inhaled corticosteroid with short acting β_2 -agonist inhalers as needed.

No subject was investigated within 4 weeks of an upper respiratory tract infection or an exacerbation of asthma. All the subjects gave their written informed consent. This study was approved by the Ajou University Hospital, Suwon, Korea.

Bronchoprovocation test

Airway hyperresponsiveness to methacholine and L-ASA BPT were performed according to the method described previously (8). All medications, including theophylline, bronchodilators, and steroids, were stopped 48 hr before the procedure. Pulmonary function was measured with a spirometer, the FEV₁ and maximum mid-expiratory flow (MMEF), before and during provocation. Normal saline was inhaled as a placebo solution. The patients were asked to breathe the nebulized aerosol up to their vital capacity 10 times. L-ASA (Young-Jin Co. Seoul, Korea), as a powder containing 1,800 mg of L-ASA with 200 mg of glycine, was made up freshly on each challenge day with 10 mL of normal saline to produce a L-ASA solution containing 180 mg/mL. The L-ASA solutions were delivered by a DeVilbiss 646 nebulizer (DeVilbiss Co., Somerset, Penn., U.S.A.) and connected to a compressed air source (5 L/min). The challenges with placebo were performed seven days before the L-ASA BPT. If there was less than a 10% fluctuation of FEV₁ with placebo inhalation, L-ASA BPT from 11.25 up to 180 mg/mL was performed to determine the provocative dose of L-ASA, which induced more than a 20% fall of FEV₁. All provocations were carried out at the same time of the day. The FEV₁ and MMEF were measured frequently during the first hour, and then hourly for 8 hr. Late asthmatic responses were noted from 240 min and 420 min after L-ASA inhalation.

Measurement of serum neutrophil chemotactic activity

Sera were collected from group I and group II subjects before, 30 min and 240 min after the L-ASA bronchial challenges. Serum NCA was measured using the Boyden chamber method as described in our previous reports (11, 12). Neutrophils were obtained from heparinized whole blood of AB-type normal volunteers by sedimentation in 6% dextran dextrose solution followed by centrifugation on Ficoll-Hypaque solution (specific gravity of 1.077) and the supernatants were aspirated and frozen at -20°C. Cells containing more than 95% neutrophils were suspended in Hank's balanced salt solution (HBSS) with 0.4% of bovine serum albumin (BSA) at the concentration of 1×10^6 cells/mL. Polycarbonate filter with 5- μ m pore size (Osmonics, Livermore, CA, U.S.A.) was topped over chemotaxin followed by distribution of neutrophil suspension. The chamber was incubated for 90 min at 37°C in a humidified incubator containing 5% CO₂.

Thereafter, the filter was removed, fixed in 100% methanol, and subsequently stained with Diff Quick stain solution. The number of neutrophils, which migrated through the filter, was determined microscopically at 40 \times objectives. Five fields were counted per well and the experiments were conducted in quadruplicate. The results were expressed as the mean of the number of migrated neutrophils per field in each sample. Opsonized serum was used in the positive control and HBSS with BSA in the negative control.

Measurement of serum histamine level

Serum was diluted 1/10 with distilled water and was analyzed immediately. Serum histamine was measured using Astoria™ Histamine Analyzer 300 system (Astoria-Pacific international, U.S.A.) with the automated fluorometric procedure as described by Siraganian (13). The chemistry was separated into two phases: extraction step to eliminate interference from var-

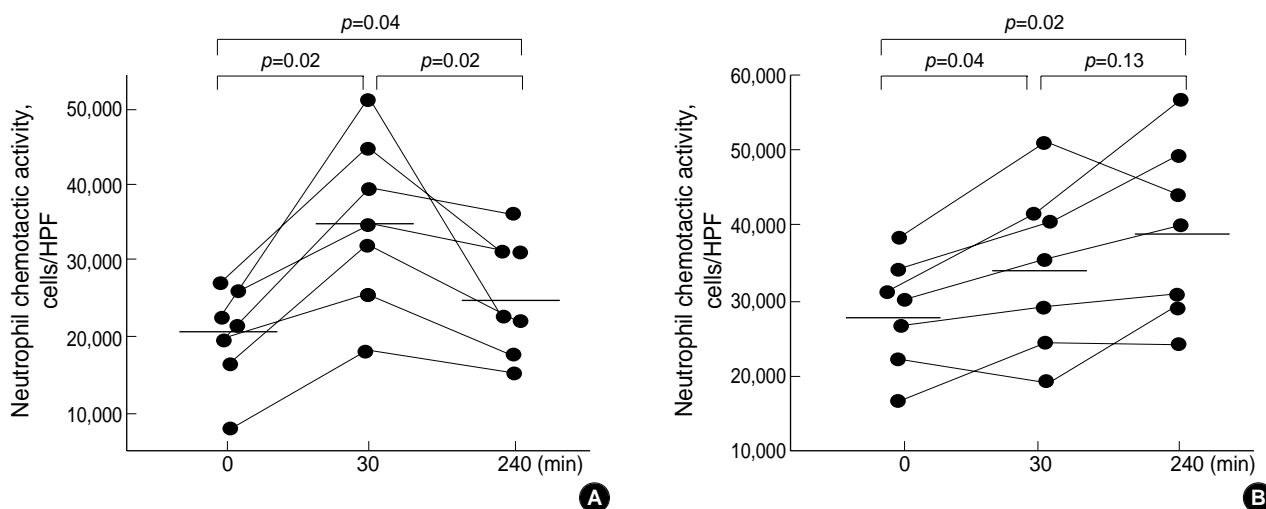


Fig. 1. Changes of serum neutrophil chemotactic activity during L-ASA bronchoprovocation test in early asthmatic responders (A) and dual asthmatic responders (B) of ASA-sensitive asthmatic patients. Horizontal bars indicate median values.

ious histamine-related compounds, including Spermine, N-acetylhistamine, Histidine and Serotonin; and in condensation step, histamine was reduced with the O-phthalaldehyde reagent at an alkaline pH 12.4-12.7. The reaction reached maximum fluorescence after 4 min and then acidified to produce a stable fluorescent complex. The fluorescence was determined using a fluorometer.

Statistical analysis

The Wilcoxon-signed rank tests, and Mann-Whitney U-test were applied using SPSS version 7.0 (Chicago, IL, U.S.A.) to evaluate the statistical differences between the data. A p -value of 0.05 or less was regarded as significant.

RESULTS

Changes of serum neutrophil chemotactic activity during the L-ASA BPT

Sera from groups I and II were collected at 0 (before the challenge test), 30 and 240 min after the L-ASA BPT. Fig. 1 shows the changes of serum NCA in group I and group II subjects. All the subjects of group I showed similar changing patterns: serum NCA significantly increased ($p=0.02$) and peaked at 30 min after the L-ASA inhalation, and thereafter decreased at 240 min ($p=0.02$). When the changes of serum NCA were compared in six subjects with dual asthmatic response (group II), there were significant increases of serum NCA at 30 min ($p=0.04$) after L-ASA inhalation, and they tended to increase further up to 240 min, but no statistical significance was reached ($p=0.13$). However, NCA collected at 240 min was significantly higher than that collected before the challenges in group

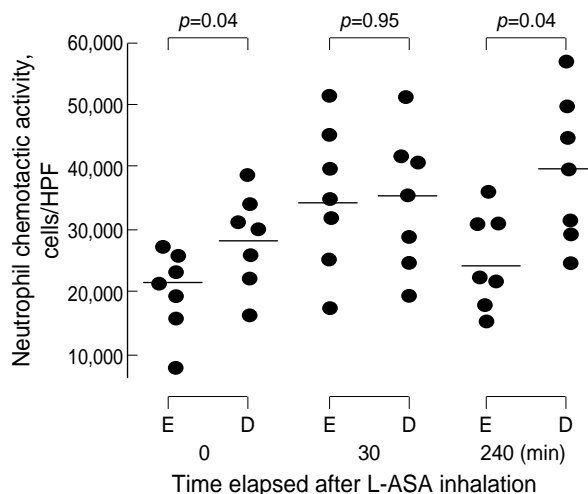


Fig. 2. Comparison of serum neutrophil chemotactic activity during L-ASA bronchoprovocation test between early (E) and dual (D) asthmatic responders of ASA-sensitive asthmatic patients. Horizontal bars indicate median values.

II patients ($p=0.02$).

Comparison of serum NCA between group I and group II

Fig. 2 demonstrates serum NCA at each time point between groups II and I. Serum NCAs collected before and those at 240 min were significantly higher in group II than in group I ($p<0.05$, respectively), but no statistical significance was noted in 30 min-samples ($p>0.05$).

Changes of serum histamine level during L-ASA BPT in both groups

Fig. 3 shows the changes of serum histamine level during

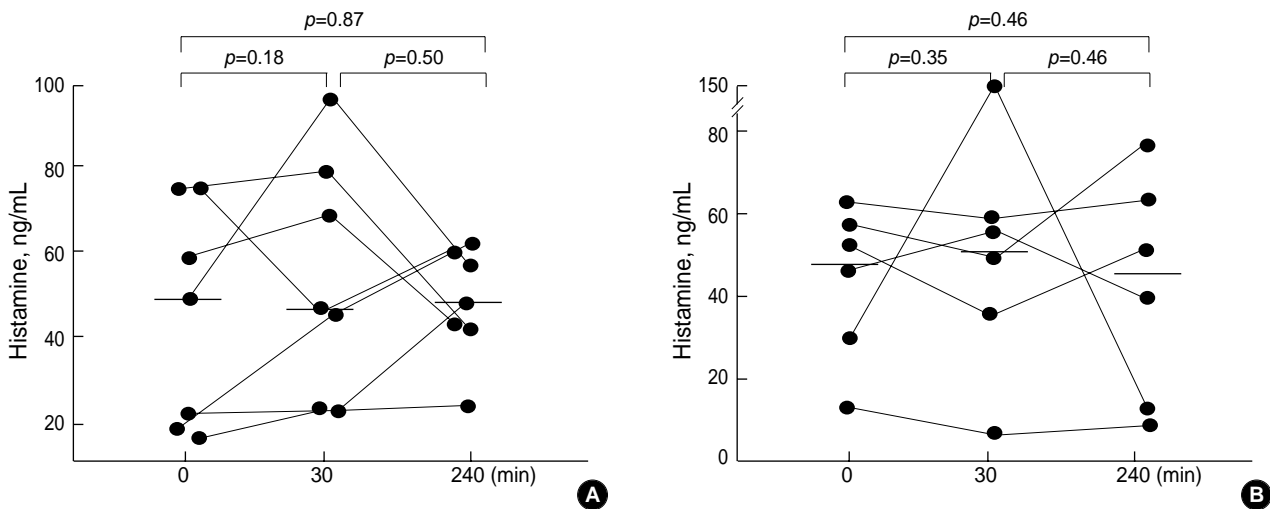


Fig. 3. Changes of serum histamine level during L-ASA bronchoprovocation test in early (A) and dual asthmatic responders (B) of ASA-sensitive asthmatic patients. Horizontal bars indicate median values.

Table 2. Effect of anti-IL-8 on serum neutrophil chemotactic activity (cells/HPF) which was collected during lysine-ASA bronchoprovocation test

Patients	Before anti IL-8 treatment			After anti-IL-8 treatment		
	Baseline	30 min after L-ASA BPT	240 min after L-ASA BPT	Baseline	30 min after L-ASA BPT	240 min after L-ASA BPT
A	22,229	26,325	17,365	17,621	23,680	18,389
B	13,056	17,194	14,250	12,416	16,512	12,245
C	26,410	32,597	36,535	26,539	31,488	35,968
D	25,130	30,762	36,394	25,984	32,597	36,309

A, B: Early asthmatic responders; C, D: Dual asthmatic responders.

L-ASA BPT in group I and group II. There were no significant changes in histamine level in both group I and group II during L-ASA BPT ($p > 0.05$, respectively). There were no significant correlations between serum NCA and histamine level at each time point (data not shown).

Effect of anti-IL-8 antibody in serum neutrophil chemotactic activity

In order to evaluate the effect of anti-IL-8 antibody on serum NCA, sera from two subjects of group I, which had been collected at 30 min after the L-ASA challenges, and sera from two subjects of group II, which were collected at 240 min after L-ASA BPT, were used. Each serum was pre-incubated with an equal volume of anti-IL-8 antibody, and then NCA was measured as described in the above. Pre-incubation of anti-IL-8 antibody (10 μ g, R&D, Minn., U.S.A.) did not affect the enhanced NCA in either group I or group II as shown in Table 2.

DISCUSSION

Although the pathogenic mechanism of ASA-induced asthma

still remains to be further clarified, there has been some evidence indicating mast cell activation after L-ASA inhalations (9, 10). Increased levels of tryptase, histamine, and cysteinyl-leukotrienes after nasal challenge of L-ASA were noted in nasal lavage fluid of ASA-sensitive asthma and significant increases of urinary PGD₂ and 9 α 11 β -PGF₂ were noted after L-ASA BPT. One report showed increased NCA after oral ASA challenges (14). In this study, we compared changes of serum NCA between early and dual asthmatic responders after the L-ASA BPT. The presence of NCA has been demonstrated in sera of individuals with asthma after provocation with allergens (15, 16) or non-specific stimuli such as exercise (17). The origin of these activities is still elusive, although some indirect data would indicate mast cells as the origin in the case of heat-stable activity, and monocyte/macrophage for the heat-labile one (15, 16), since the time course for the occurrence of serum heat-labile activity is clearly different from that of the heat-stable one. The latter activity showed a quick rise coinciding with or preceding early reduction of FEV₁, whereas heat-labile activity was demonstrated following a second peak, which coincided with the late asthmatic response (17). In this study, L-ASA itself did not have NCA. Group I subjects had a peak of NCA at 30 min after inhalation challenge, followed

by a significant decrease in activity at 240 min. In this study, on the basis of the time course, we speculated that NCA, found during early asthmatic responses induced by L-ASA inhalation, may have originated from mast cells, but further studies are needed to characterize NCA released after L-ASA inhalation. These findings could support the evidence of mast cell activation occurring in early asthmatic response after L-ASA inhalation reported by Fisher et al. (13).

In this study, enhanced NCA was also noted during late asthmatic response induced by L-ASA inhalation. In group II subjects, NCA collected at 240 min was significantly higher than the baseline value and, NCA of group II subjects at 240 min was significantly higher than that of group I subjects. Based upon the time sequence, enhanced NCA at 240 min after L-ASA inhalation found in this study may have been derived from mononuclear cells.

Recently, several studies supporting evidence of neutrophil involvement in the pathogenesis of bronchial asthma have been reported. Inhalation of grain dust was found to induce increased neutrophil infiltration and enhanced serum NCA to grain dust (10). In vitro studies revealed the release of substances presenting NCA by bronchial epithelial cells in response to grain dust (18). In the case of toluene diisocyanate (TDI)-induced asthma, neutrophilia in bronchoalveolar lavage fluid had been noted in subjects with late asthmatic reactions induced by isocyanate challenge test (19) and myeloperoxidase (MPO) level representing neutrophil activation was increased after TDI bronchial challenges with concordant increase of IL-8 in the induced sputum of TDI-induced asthma (20). In patients with severe asthma and those with a history of status asthmaticus, neutrophil activation was noted in airway mucosa (21, 22). Not only as mere terminal effector cells, neutrophils can also affect other cells by releasing various cytokines (23, 24). In this study, serum NCA was increased during early asthmatic responses and increased further during late asthmatic response. We also observed the effect of anti-IL-8 on NCA, as IL-8 is the key chemokine to induce neutrophil activation and migration. In this study, pre-incubation of anti-IL-8 antibody did not affect NCAs in both early and late asthmatic responders. Further studies will be needed to clarify the role of neutrophil during ASA-induced asthmatic responses.

Histamine has been known as one of the major chemical mediators from mast cells/basophils and not from other inflammatory cells. In this study, to confirm the role of mast cell/basophil activation during late asthmatic responses as well as early asthmatic responses after L-ASA inhalation, we compared serum histamine level between early and dual asthmatic responses. There were no significant changes in histamine level in either groups. Although the serum histamine level may be used to represent an indirect marker of basophil activation, it has very low sensitivity since circulating histamine is too remote from the target tissues of asthmatic airway. These findings suggest that the possibility of basophil activation during late asthmatic responses is very low.

In conclusion, NCA derived from mast cell may contribute to the development of early asthmatic response induced by L-ASA inhalation. There may be a possible involvement of enhanced NCA derived from mononuclear cells during late asthmatic response induced by L-ASA inhalation.

REFERENCES

- McDonald JR, Mathison DA, Stevenson DD. *Aspirin intolerance asthma, detection by oral challenge. J Allergy Clin Immunol* 1972; 50: 198-207.
- Srector SL, Wangaard CH, Farr RS. *Aspirin and concomitant idiosyncrasies in adult asthmatic patient. J Allergy Clin Immunol* 1979; 64: 500-6.
- Park HS. *Aspirin-sensitive asthma: recent advances in management. BioDrugs* 2000; 13: 29-33.
- Stevenson DD, Lewis RA. *Proposed mechanisms of aspirin sensitivity reactions. J Allergy Clin Immunol* 1987; 80: 788-90.
- Szczeklik A, Stevenson DD. *Aspirin-induced asthma: advances in pathogenesis and management. J Allergy Clin Immunol* 1999; 104: 5-13.
- Melillo G, Podovano A, Cocco G, Masi C. *Dosimeter inhalation test with lysine acetylsalicylate for the detection of aspirin-induced asthma. Ann Allergy* 1993; 71: 61-5.
- Dahlen B, Zetterstrom O. *Comparison of bronchial and per oral provocation with aspirin in aspirin-sensitive asthmatics. Eur Respir J* 1990; 3: 527-34.
- Park HS. *Early and late onset asthmatic responses following lysine-aspirin inhalation in aspirin-sensitive asthmatic patients. Clin Exp Allergy* 1995; 25: 38-40.
- O'Sullivan S, Dahlen B, Dahlen SE, Kumlin M. *Increased urinary excretion of the prostaglandin D₂ metabolite 9 α , 11 β -prostaglandin F₂ after aspirin challenge supports mast cell activation in aspirin-induced airway obstruction. J Allergy Clin Immunol* 1996; 98: 421-32.
- Fischer AR, Rosenberg MA, Lilly CM, Callery JC, Rubin P, Cohn J, White MV, Igarashi Y, Kaliner MA, Drazen JM, Israel E. *Direct evidence for a role of the mast cell in the nasal response to aspirin in aspirin-sensitive asthma. J Allergy Clin Immunol* 1994; 94: 1046-56.
- Park HS, Jung KS. *Enhanced neutrophil chemotactic activity after bronchial challenge in subjects with grain dust-induced asthma. Ann Allergy Asthma Immunol* 1998; 80: 257-62.
- Park HS, Jung KS, Hwang SC, Nahm DH, Yim HE. *Neutrophil infiltration and release of IL-8 in airway mucosa from subjects with grain dust induced occupational asthma. Clin Exp Allergy* 1998; 28: 724-30.
- Siraganian RP. *Refinements in the automated fluorometric histamine analysis system. J Immunol Methods* 1975; 7: 283-90.
- Atkins PC, Norman M, Weiner H, Zweiman B. *Release of neutrophil chemotactic activity during immediate hypersensitivity reactions in humans. Ann Intern Med* 1977; 86: 415-8.
- Nagy L, Lee TH, Kay AB. *Neutrophil chemotactic factor in antigen-induced late asthmatic reactions. N Engl J Med* 1982; 306: 497-501.
- Venge P, Dahl R, Hakansson L. *Heat-labile neutrophil chemotactic activity in subjects with asthma after allergen inhalation: relation to*

- the late asthmatic reaction and effects of asthma medication. J Allergy Clin Immunol* 1987; 80: 679-88.
17. Wisnewski AV, Redlich CA. *Recent developments in diisocyanate asthma. Curr Opin Allergy Clin Immunol* 2001; 1: 169-75.
 18. Wisnewski AV, Srivastava R, Herrick C, Xu L, Lemus R, Cain H, Magoski NM, Karol MH, Bottomly K, Redlich CA. *Identification of human lung and skin protein conjugated with hexamethylene diisocyanate in vitro and in vivo. Am J Respir Crit Care Med* 2000; 162: 5-6.
 19. Lange RW, Day BW, Lemus R, Tyurin VA, Kagan VE, Karol MH. *Intracellular S-glutathionyl adducts in murine lung and human bronchoepithelial cells after exposure to diisocyanatotoluene. Chem Res Toxicol* 1999; 12: 931-6.
 20. Wisnewski AV, Lemus R, Karol MH, Redlich CA. *Isocyanate-conjugated human lung epithelial cell proteins: a link between exposure and asthma? J Allergy Clin Immunol* 1999; 104: 341-7.
 21. Lamblin C, Gosset P, Tillie-Leblond I, Saulnier F, Marquette CH, Wallaert B, Tonnel AB. *Bronchial neutrophilia in patients with non-infectious status asthmaticus. Am J Respir Crit Care Med* 1998; 157: 394-402.
 22. Gibson PG, Norzila MZ, Fakes K, Simpson J, Henry RL. *Pattern of airway inflammation and its determinants in children with acute severe asthma. Pediatr Pulmonol* 1999; 28: 261-70.
 23. Lummus ZL, Alam R, Bernstein JA, Bernstein DI. *Diisocyanate antigen-enhanced production of monocyte chemoattractant protein-1, IL-8, and tumor necrosis factor- α by peripheral mononuclear cells of workers with occupational asthma. J Allergy Clin Immunol* 1998; 102: 265-74.
 24. Strieter RM, Lukacs NW, Standiford TJ, Kunkel SL. *Cytokines and lung inflammation: mechanisms of neutrophil recruitment to the lung. Thorax* 1993; 48: 765-9.
 25. Fujisawa T, Kato Y, Atsuta J, Terada A, Iguchi K, Kamiya H, Yamada H, Nakajima T, Miyamasu M, Hirai K. *Chemokine production by the BEAS-2B human bronchial epithelial cells: differential regulation of eotaxin, IL-8, and RANTES by T_H2- and T_H1-derived cytokines. J Allergy Clin Immunol* 2000; 105: 126-33.