

Substance P and β -endorphin-like immunoreactivity in lavage fluids of subjects with and without allergic asthma

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Six atopic subjects with grass pollen allergy and six nonallergic healthy volunteers were enrolled into this study. Substance P-like immunoreactivity (SP-LIR) and β -endorphin-like immunoreactivity (β E-LIR) were determined in bronchoalveolar lavage (BAL) and nasal lavage (NAL) fluids before and after allergen (grass pollen) provocation. A significant increase in the baseline concentration of SP-LIR and β E-LIR was seen in BAL of allergic subjects. In NAL of allergic subjects an increased baseline concentration of SP-LIR was found (β E-LIR not detectable). After allergen provocation there was a rise of SP-LIR and β E-LIR in BAL fluids of allergic subjects immediately after provocation. In NAL fluids of allergic subjects allergen challenge resulted in a rise of SP-LIR within 10 minutes. Allergen provocation did not influence SP-LIR and β E-LIR concentration in BAL and NAL in nonallergic controls. The demonstrated higher baseline levels of SP-LIR and β E-LIR as well as the increase after provocation in the BAL and NAL of allergic subjects but not in nonallergic controls support the hypothesis that these neuropeptides contribute to allergic reactions in airways of humans. (J ALLERGY CLIN IMMUNOL 1992;90:646-52.)

Key words: Neuropeptides, concentrations, BAL, NAL, allergy, rhinitis, asthma

One of the diagnostic features of bronchial asthma is airway hyperactivity to pharmacologic, environmental, and allergic stimuli. The mechanisms underlying this nonspecific airway hyperactivity, although subject to scientific and clinical investigations, are not yet established. From experimental and clinical studies it has been recognized that allergic bronchial asthma is a complex process not limited to an immediate mediator release by mast cells and basophils. Mast cells obviously are not involved in either the late response that follows allergen challenge or bronchial hyperactivity.¹ Experimental evidence exists that induced bronchial hyperactivity in animals is associated with airway inflammation.² Indeed, epithelial damage associated with airway inflammation is a characteristic feature in asthma and may be causally related to the accompanying airway hyperactivity.³⁻⁵

Abbreviations used

SP-LIR: Substance P-like immunoreactivity
 β E-LIR: β -endorphin-like immunoreactivity
BAL: Bronchoalveolar lavage
NAL: Nasal lavage
RIA: Radioimmunoassay
NEP: Neutral endopeptidase

A new but controversial hypothesis integrates the neuropeptides, cotransmitters of classic transmitters in central and peripheral tissues, to the processes of bronchial diseases.

Many neuropeptides have been identified in the human airways.⁶ Although the precise physiologic or pathophysiologic role of neuropeptides in airways remains uncertain, the potent effects of these peptides on various aspects of airway function suggest that they may be involved in controlling airway tone and secretion. Neural and neuropeptide control mechanisms might be affected by inflammation.⁷ Therefore it is possible that defective function of neuropeptide containing nerves might be involved in bronchial hyperactivity and asthma.⁸

A potent role for neuropeptides in asthma has been suggested recently, including vasoactive intestinal

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polypeptide, neurokinin A, calcitonin gene-related peptide, or substance P-like immunoreactivity (SP-LIR).^{7, 9} SP-LIR is the best evaluated neuropeptide. It is found in sensory nerve endings that are located in smooth muscle, in and around blood vessels, in glands, and in the lateral intercellular spaces of the surface epithelium. Release of SP-LIR from sensory nerves can be induced by capsaicin or by antidromic vagal stimulation. Physiologic stimuli including bradykinin, histamine, and nicotine have also been shown to induce the release of SP-LIR.¹⁰ Several effects have been demonstrated in vivo and in vitro. In accordance with the axon reflex hypothesis it was discussed that mechanical and chemical stimulation of sensory airway nerve endings cause antidromic release of SP-LIR from axon collaterals, leading to secretion, vasodilation, extravasation of plasma proteins, acute airway obstruction in whole animals, and contraction of airway smooth muscle in vitro,^{1, 11, 12-14} but few studies have examined its effects on airway function in humans.^{15, 16-18}

Other neuropeptides of the opioid family (leu-enkephalin, dynorphin) were also detected in the lung tissue and bronchoalveolar lavage (BAL) fluids of rats.¹⁹ However, opioids were mainly confined to diffuse granule-containing cells. The role of these neuropeptides in normal lung function and in pulmonary diseases is unknown.¹⁹ Bartho et al.²⁰ showed that the noncholinergic contraction of the guinea pig isolated main bronchi as a result of electrical stimulation were inhibited by a stable enkephalin peptide. This effect was abolished by naloxone. Naloxone alone induced no change in the evoked contraction of the bronchus, indicating that no endogenous opioid control is present in bronchi of untreated guinea pigs.²⁰

To get more information about the role of SP-LIR and opioids in the pathogenesis of allergic asthma, the baseline concentration of SP-LIR and β -endorphin-like immunoreactivity (β E-LIR) was determined in nasal lavage (NAL) and BAL fluids obtained from patients with grass pollen asthma/rhinitis and healthy nonallergic subjects. Furthermore, the effect of allergen provocation on SP-LIR and β E-LIR concentration was measured in BAL and NAL fluids of allergic patients in comparison with nonallergic healthy individuals.

MATERIAL AND METHODS

Subjects

The study protocol was approved by the local ethics committee. After giving written informed consent six healthy nonallergic subjects (volunteers: 3 women, 3 men; age range, 19 to 41 years) and six patients with grass pollen asthma/rhinitis (allergics: 2 women, 4 men; age range, 18 to 43 years) were enrolled into the study out of season. The

diagnosis was confirmed by history, skin prick test, and radioallergosorbent test.

The history of seasonal asthma lasted from 2 to 8 years. The following criteria for selection of allergic patients were used²¹:

1. Age 18 to 45 years
2. History of mild seasonal grass pollen asthma/rhinitis
3. Functional evidence of reversible obstructive airway disease by nonspecific (histamine) and specific (grass pollen allergen) bronchial provocation test
4. Normal pulmonary function for 4 weeks before examination without medication
5. FEV₁ immediately before lavage >80% of the predicted value
6. Total airway resistance <0.4 kPa/L/second.

No drugs were allowed during the study.

Bronchial provocation

The individual allergen provocation dose was determined by bronchial provocation with a drop of FEV₁ and rise of airway resistance of 20% and 100%, respectively.²¹ One week later, bronchoscopy with adequate provocation dose in the right middle lobe bronchus was performed with prechallenge and postchallenge BAL. BAL was repeated 8 hours and 24 hours later, and SP-LIR as well as β E-LIR were determined in the BAL fluids by radioimmunoassay. The premedication for the bronchoscopy consisted of the following drugs: 0.5 mg atropine and 0.1 mg fentanyl dihydrogen citrate. Commercially available grass pollen extracts from ALK-Scherax, Hamburg, Germany, were used (local provocation dose 10 to 2500 standardized quality units [SQU]).

Nasal provocation

Allergics and volunteers were challenged with grass pollen extract from ALK-Scherax in increasing concentration of 1000 SQU/ml, 10000 SQU/ml, or 100000 SQU/ml. Before the study the allergen provocation dose was determined for each individual by titration. The challenge material was sprayed onto the inferior turbinate area of both nostrils with a plastic hand-activated nebulizer (De Vilbiss 40; De Vilbiss Co., Somerset, Pa.). NAL was performed 10 or 20 minutes after allergen challenge by instillation of 5 ml normal saline (preheated to 37° C) into each nostril. After 10 seconds, the fluid was expelled into a plastic beaker and immediately placed on ice. Diluent control and four prewashes were done before the provocation.²²

Assay methods

BAL and NAL fluids were collected in aliquots (500 mm³), after centrifugation (10 minutes, 500 g) and removal of cellular elements, in cooled plastic tubes containing 0.5 μ g/tube bacitracin dissolved in distilled water and frozen immediately. Bacitracin was used as protease inhibitor and antibacterial preservative. SP-LIR was directly measured in 100 mm³ lavage fluid in triple sets and calculated to picograms per milliliter lavage fluid. A highly specific radioimmunoassay (RIA) was used (detection limit 1.5 fmol = 2 pg per incubate; minimal detectable concentration 15

fmol/L = 20 pg per ml lavage fluid). The RIA was performed in 0.05 mol/L sodium barbital buffer, pH 8.6, containing 0.02% (wt/vol) NaN₃ and 2.5% (vol/vol) outdated human serum, and all dilutions were made in this buffer. A 100 mm³ volume of standard substance P or unknown was incubated with 100 mm³ of antiserum and 300 mm³ buffer for 2 hours at 4° C. Then, 100 mm³ tracer (5000 cpm) was added and kept incubated further for 20 hours at 4° C. The antigen-antibody complex was separated from free antigen by charcoal adsorption as follows. A 200 mm³ volume of 1% (wt/vol) charcoal slurry (Norit supra med) was added to the RIA buffer (without human serum) and incubated for 20 minutes at 4° C. After centrifugation the supernatant was decanted and the radioactivity of the bound fraction was measured.

¹²⁵I-(p-HP)-Argl-SP was used as a tracer, with a specific activity of 60 to 65 TBq/mol/L with 50% to 60% binding to the antibody in a dilution of 1:240,000.²³ The antibody (M 27 from guinea pig) was directed against the C-terminal as well as the N-terminal sequence of SP.

βE-LIR was directly measured in 900 mm³ lavage fluid concentrated by lyophilization and desolved in 300 mm³ RIA buffer containing 0.01 mol/L sodium phosphate buffer, pH 7.4 with 0.12 mol/L NaCl, 0.1% (wt/vol) NaN₃ and 0.25 % (wt/vol) human serum albumin. The βE-LIR content was calculated to picograms per milliliter lavage fluid. A highly specific RIA was used for detection (detection limit 1.4 fmol/L = 5 pg per incubate; minimal detectable concentration 5 fmol/L = 17 pg per ml lavage fluid).

¹²⁵I-β-E was used as a tracer with a specific activity of 70 TBq/mmol/L, with 20% to 30% binding to the antibody in a final concentration of 1:15,000.

The antibody M 50 from rabbit was used (prepared and generously supplied by A.D. Dimitriev, Institute of Psychiatry, Academy of Medical Sciences, Moscow). The RIA was performed as follows: 100 mm³ volume of standard or reconstituted unknown was incubated with 100 mm³ tracer (5000 cpm), 300 mm³ RIA buffer and 100 mm³ antiserum for 20 hours at 4° C. The separation of free from bound antigen was made as described above.

SP-LIR and βE-LIR were determined with nearly quantitative recoveries because of the direct measurements in the lavage fluids, avoiding any extraction or prepurification steps in the two used RIAs.

Statistical analysis

All results represent the mean ± SEM of six subjects. Independent samples (allergics/volunteers) were compared with the *t* test. A *p* value less than 0.05 was considered significant. Statistical analysis of the multiple time points was performed by analysis of variance (ANOVA) by use of the program SPSS/PC, version 4, procedure anova. An alpha value less than 0.05 was considered significant.

RESULTS

Baseline concentration of SP-LIR and βE-LIR in lavage fluids from allergics and volunteers

Before performing experiments on allergics and nonallergic controls, it was necessary to demonstrate

that the RIAs could provide quantitative measurements and that the changes in immunoreactivity reflect changes in the target substance. This was done in preliminary experiments. These experiments showed that serial dilutions of nasal and bronchial samples produce an RIA displacement curve that paralleled the standard curve. Furthermore, it was shown that recovery of standard SP-LIR or βE-LIR that was added to nasal or bronchial fluids at collection was 95% + 6% (*n* = 6) for SP-LIR and 98% + 5% (*n* = 6) for βE-LIR. Recovery of added neuropeptides showed no significant variation at any stage in the experimental protocol. In addition, the cross-reactivity with fragments or related peptides was investigated. It was shown that the antibody M 27 was directed against the C-terminal as well as the N-terminal sequence of SP and showed cross-reactivity to a remarkable degree only with the following partial sequences: decapeptide SP (2-11) 60%; nonapeptide SP (3-11) 30%; octapeptide SP (4-11) 26% eledoisin, neurokinin A, neurokinin B, angiotensin II, cholecystokinin-octapeptide, (met5)-enkephalin, (leu5)-enkephalin, gonadotropin-releasing hormone, thyrotropin-releasing hormone, or bradykinin did not show any cross-reactivity (less than 0.5%). The antibody M 50 showed cross-reactivity to β-lipotropin of 10% and to α-endorphin of 2%. Gamma-endorphin, (leu5)-enkephalin, and (met5)-enkephalin did not cross-react with the antibody (less than 0.2%).

Since peptides are known to be easily destroyed in vivo, early experiments (*n* = 3) were performed by collecting fluids in the presence and absence of selected concentrations of bacitracin (0, 0.25, 0.5 μg/sample) and synthetic substance P (8.4 fmol/L/sample). These results, SP-LIR concentration 6.80 + 0.30 fmol/L/sample without bacitracin and 8.57 + 0.25 fmol/L/sample with 0.5 μg/sample bacitracin, indicate protease activity in the fluids. The selected concentration of bacitracin did not influence the radioimmunologic determination of the peptides. Therefore the determination of peptides was performed in the presence of bacitracin.

The data obtained from allergics and volunteers indicate a significant difference (*p* < 0.01) in the baseline concentration of SP-LIR and βE-LIR in BAL fluids. The baseline concentration of SP-LIR was 6.8 times higher in allergics compared with the volunteers, whereas the elevation of βE-LIR in the allergics was 14.3 times compared with the volunteers (Table I).

In NAL fluids there was also an elevation of SP-LIR in allergics compared with the baseline concentration of volunteers (1.8 times, *p* < 0.05). But in NAL fluids the elevation was not so drastic as in BAL fluids (Table I).

The baseline concentration of βE-LIR in NAL fluids was under the detection limit.

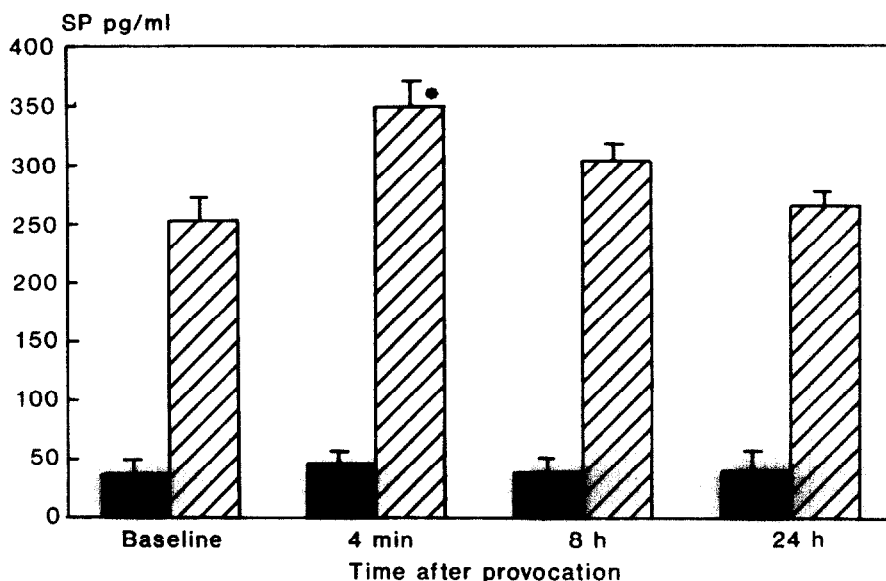


FIG. 1. SP-LIR concentration in BAL fluids before and after intrasegmental allergen provocation in nonallergic subjects (volunteers, $n = 6$) and patients allergic to grass pollen (allergics, $n = 6$). * $\alpha < 0.05$ after versus before provocation.

SP-LIR in BAL and NAL fluids after allergen provocation

After allergen provocation there was a significant rise (37.9% $p < 0.05$) of SP-LIR in BAL fluids of allergics 4 minutes after provocation. The allergen-induced rise of the SP-LIR concentration gradually returned to the baseline within 24 hours (Fig. 1).

In BAL fluids of volunteers no significant difference in the SP-LIR concentration before and after allergen provocation was found (Fig. 1).

In NAL fluids of allergics the saline challenge resulted in a slight but not significant increase of SP-LIR concentration. But allergen challenge increased the SP-LIR concentration significantly by 53.7% ($p < 0.05$) within 10 minutes. SP-LIR concentration was not significantly affected by a second allergen challenge 10 minutes later. The elevated SP-LIR concentration returned to the baseline 20 minutes after provocation (Fig. 2). Allergen provocation was without significant effect on SP-LIR concentration in all BAL and NAL fluids of volunteers (Fig. 2).

β E-LIR in BAL fluids after allergen provocation

β E-LIR in BAL samples of allergics was affected by allergen provocation in the same way as SP-LIR. Four minutes after allergen provocation a significant rise of the β E-LIR concentration was detectable. The average rise was 110%. Twenty-four hours after allergen provocation the β E-LIR concentration returned to the baseline (Fig. 3).

TABLE I. Baseline concentration of SP and β -E in BAL and NAL fluids of healthy nonatopic subjects (volunteers) and patients allergic to grass (allergics).

	Volunteers	Allergics
Substance P pg/ml		
BAL	37.0 $\pm$ 9.0	253 $\pm$ 24*
NAL	22.1 $\pm$ 4.5	41 $\pm$ 18**
B-endorphin pg/ml		
BAL	3.5 $\pm$ 0.6	50 $\pm$ 16.3*
NAL	Not detectable	Not detectable

* $p < 0.01$.

** $p < 0.05$.

Mean $\pm$ SEM of six subjects in each group.

The allergen provocation had no detectable effect on the β E-LIR concentration in BAL fluids of volunteers (Fig. 3).

DISCUSSION

The airway narrowing associated with asthma has previously been regarded as predominantly caused by contraction of smooth muscle. Now it is recognized that asthma is an inflammatory disease.²⁴ The mechanism of inflammation in asthma is not completely understood but is associated with many inflammatory cells and several mediators. Since mediator release in the airway lumen or from the airway mucosa is likely

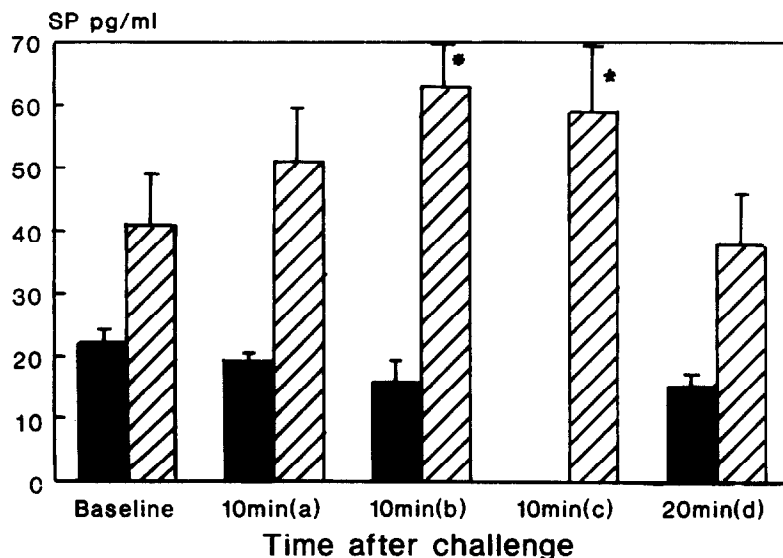


FIG. 2. SP-LIR concentration in NAL fluids before and after nasal allergen provocation in non-allergic subjects (volunteers, $n = 6$) and patients allergic to grass pollen (allergics, $n = 6$). NAL was performed 10 minutes after saline challenge (a), 10 minutes after first (b) or second (c) allergen challenge, and repeated 20 minutes after last allergen challenge (d). * $\alpha < 0.05$ after versus before provocation.

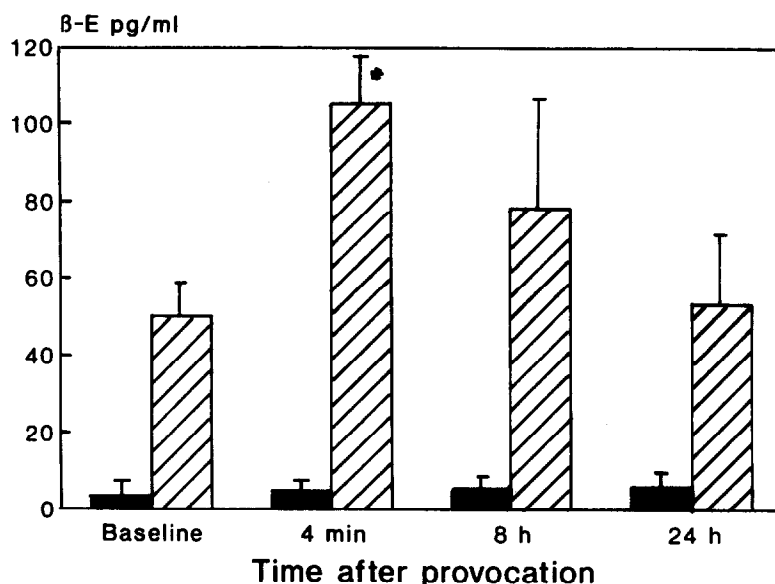


FIG. 3. βE-LIR concentration in BAL fluids before and after intrasegmental allergen provocation in nonallergic subjects (volunteers, $n = 6$) and patients allergic to grass pollen (allergics, $n = 6$). * $\alpha < 0.05$ after versus before provocation.

to be important in the pathogenesis of airway disease,²⁵ lavage techniques have been performed in patients with bronchial asthma or rhinitis to study the mechanisms underlying airway diseases.²⁶⁻²⁸ In attempts to understand the pathophysiology, considerable interest has focused on the airway neuropeptides, especially SP-LIR, since sensory neuropeptides and neural

mechanisms may have an important contribution to the inflammatory reaction in the airway in addition to other mediators, for example, histamine, prostaglandins, leukotrienes, platelet activating factor, and the like. On the other hand, opioids, for example, endorphins, enkephalins, and dynorphin, play an important role in regulatory disorders, especially after

activation of the sympathetic adrenomedullary axis,²³ but the participation of opioids in airway diseases is not yet clear.

To clarify some questions concerning the role of SP-LIR and β E-LIR in the pathogenesis of allergic asthma it was necessary to develop a specific analytic assay for determination. The described RIAs for SP-LIR and β E-LIR specifically permit the determination of the undecapeptide of SP-LIR and β E-LIR (β -lipotropin 61-91). With use of specific antibodies, no cross-reactivities were found with fragments or related peptides. Therefore these assays are suitable to determine SP-LIR and β E-LIR in human BAL and NAL fluids.

The present results give clear evidence that allergic asthma is accompanied by an elevated baseline concentration of SP-LIR and β E-LIR in lavage fluids. Moreover, the findings indicate that allergen provocation induces an immediate rise of the two neuropeptides but only in allergic patients, which is probably due to increased activity in sensory nerves and epithelial alterations. Up to now the reason for an increased neuropeptide concentration is speculative. As to SP-LIR, the following explanation is possible. Following the hypothesis of Barnes⁷ and our previous results³⁰ in allergics an abnormally elevated activity exists of nonadrenergic noncholinergic nerves with liberation of SP-LIR. Normally SP-LIR is cleaved by the enzyme-neutral endopeptidase (NEP). As described for virus-infected airway hyperactivity,³¹ damage of airway epithelium leads to a loss of NEP and subsequently increased concentration and effects of SP-LIR. Both the increased nonadrenergic noncholinergic activity and the decreased NEP activity may contribute to an elevated baseline concentration of SP-LIR.

The physiologic role of β E-LIR in the airways is unknown. Therefore an interpretation of the findings is difficult. With respect to our results it seems of interest that the elevation of β E-LIR differs from that of SP-LIR. In volunteers the baseline concentration of β E-LIR (3.5 pg/ml) is lower compared with SP-LIR (37.0 pg/ml). These data support the findings of Bartho et al.²⁰ that no opioid control is present in airways. On the other hand, the baseline concentration of SP-LIR suggests a participation in the control of airway function. The elevation of SP-LIR and β E-LIR concentrations in allergics in relation to volunteers is approximately in the same range, but the elevation of β E-LIR (110%) after allergen provocation is more pronounced compared with SP-LIR (38%). It seems possible that under certain conditions, endogenous released SP-LIR and β E-LIR may have different functions in the pathogenetic pathways of allergic asthma.

This assumption is supported by in vitro experiments on guinea pig isolated main bronchi indicating that capsaicin-induced bronchial contractions were—in contrast to electrical stimulation—not influenced by a stable enkephalin peptide, which suggests a different site of action.²⁰

In conclusion, our results give clinical evidence about the participation of SP-LIR and β E-LIR in the pathogenesis of allergic asthma.

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