

Optimization of Allergen Standardization

Kyoung Yong Jeong,¹ Chein-Soo Hong,¹ Joo-Shil Lee,² and Jung-Won Park¹

¹Department of Internal Medicine and Institute of Allergy, Yonsei University College of Medicine, Seoul, Korea.

²Center for Immunology and Pathology, Korea National Institute of Health, Osong, Korea.

Received: March 9, 2011

Corresponding author: Dr. Jung-Won Park,
Department of Internal Medicine and
Institute of Allergy,
Yonsei University College of Medicine,
250 Seongsan-ro, Seodaemun-gu,
Seoul 120-752, Korea.
Tel: 82-2-2228-1961, Fax: 82-2-362-8647
E-mail: parkjw@yuhs.ac

The authors have no financial conflicts of interest.

Preparation of high quality allergen extracts is essential for the diagnosis and immunotherapy of allergic disorders. Standardization of allergen extracts concerns determination of the allergen unit, development of reference material and measurement of the overall IgE binding capacity of an allergen extract. Recently, quantification of individual allergens has been the main focus of allergen standardization because the allergenicity of most allergen extracts is known to be mainly dependent on the content of a small number of allergen molecules. Therefore, characterization of major allergens will facilitate the standardization of allergens. In this article, we review the current state of allergen standardization. In addition, we briefly summarize the components of allergen extracts that should be under control for the optimization of allergen standardization, since its adjuvant-like activities could play an important role in allergic reactions even though the molecule itself does not bind to the IgE antibodies from subjects.

Key Words: Allergen extracts, natural adjuvant, polymorphism, standardization

INTRODUCTION

Extracts from various allergenic sources have been used for the diagnosis and allergen-specific immunotherapy of type I hypersensitivity since 1911.^{1,2} However, the quality of commercial allergen extract has been repeatedly reported to be variable.³⁻⁶

Biological standardization, which mainly determines overall IgE-binding potency, is generally carried out to assess the quality of allergen extracts. The Center for Biologics Evaluation and Research (CBER) in the US Food and Drug Administration (FDA) maintains 19 US reference standards for potency; six Hymenoptera venoms, two house dust mites, two cat dander extracts, one short ragweed pollen, and eight grass pollens.⁷ More specifically, grasses and mite allergen extracts are standardized on the basis of overall allergens with pooled serum by ELISA. Allergen potency of cat and ragweed allergen extracts is evaluated by determination of specific allergen with a sheep antibody by immunodiffusion. Allergenicity of Hymenoptera venoms are mainly assessed by total protein content and enzymatic activities. All manufacturers in the US are required to demonstrate batch-to-batch consistency and compliance with the standard. However, in-house reference (IHR) preparations from the companies are utilized for standardization in European

© Copyright:

Yonsei University College of Medicine 2011

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

countries.⁸ Therefore, each manufacturer uses a different unit for labelling the allergenic activity, making it difficult to interchange products between different companies.

SELECTION OF ALLERGEN SOURCE MATERIALS

Selection of raw materials is the first step for the preparation of an allergen extract. The quality of an allergen extract directly reflects the identity and purity of the allergenic source materials. Samples should be collected by qualified personnel. The content of all relevant allergens should be verified by appropriate methods. It may be necessary to culture or cultivate plants and animals of the allergen source in order to control the possible contamination of microbes, which can produce natural adjuvant-like substances in the allergen extract. Interestingly, it has been reported that allergen extracts prepared even from the same species of house dust mite, *Dermatophagoides pteronyssinus*, obtained from manufacturers in geographically distinct regions such as Sweden, Chile, and Australia, were found to exhibit remarkably different IgE reactivity.⁹ In contrast, the populations of mite-allergic patients in different geographic regions also showed different IgE reactivity to purified natural and

recombinant allergens.¹⁰

The extraction procedure should reflect the physiological conditions of the airway. Generally, raw source materials are freeze-dried, and proteins are extracted using buffers resembling the physiological conditions of human airway. Extracted proteins are dialyzed to eliminate chemicals and hapten molecules. Effort should be made to suppress proteolytic degradation and contamination and the growth of microorganisms.

ALLERGEN UNIT

Commonly used allergen units are summarized in Table 1. The Noon unit and the protein nitrogen unit (PNU) were widely used for allergen products. A Noon unit denotes the quantity of water-soluble protein extractable from 1 µg of pollen.¹ An allergen extract prepared from one gram of pollen in 10 mL of extraction buffer contains 100,000 Noon units per mL. Pollen derived from different sources is assumed to have the same allergenic activity. The weight per volume system is still widely used. PNU was introduced because most allergens were found to be proteins. PNU corresponds to 0.01 µg phosphotungstic acid-precipitable nitrogen, which is approximately 0.06 µg of protein.¹¹ Recently,

Table 1. Allergen Units Currently Used by Individual Allergen Manufacturers

Manufacturer	Allergen Unit	Standardization
Alk-Albero	Biologic unit (BU)	Skin prick test
	Histamine equivalent unit (HEP)	
	Standard treatment unit (STU)	
	Standardized quality units tablet (SQ-U)	
Allerbio	Index of reactivity (IR)	Skin prick test
	Biologische Einheiten (BE)	
	Standard biological unit (SBE)	
Allergopharma	Therapeutic unit (TU)	Skin prick test
	Sublinguale Einheiten (SE)	
Allergy therapeutics	Therapeutic unit (TU)	Skin prick test
	Standard oral unit (SOU)	
	Diagnostic unit (DU)	
ARTU Biologicals	Biologische Einheiten (BE)	Skin prick test
HAL Allergy	Bioequivalent allergen unit (BAU)	Intradermal skin test
HolisterStier	Bioequivalent allergen unit (BAU)	Intradermal skin test
Immunotek	Therapeutic unit (TU)	Skin prick test
IPI-ASAC	Unidades biológicas equivalentes (UBE: equivalent biological unit)	Skin prick test
Laboratorios LETI	Histamine equivalent prick leti (HEPL)	Skin prick test
Stallergenes	Index of reactivity (IR)	Skin prick test
	Index of concentration (IC)	

histamine equivalent in prick testing (HEP), biologic unit (BU), and bioequivalent allergen unit (BAU) have come to be widely used on standardized products. Ten HEP is equivalent to the allergen concentration, which elicits the same wheal size on a skin prick test as 10 mg/mL of histamine dihydrochloride.¹² BU represents 1/1,000 of HEP since 10,000 BU/mL is equivalent to 10 HEP. However, the detailed method determining the unit differs between manufacturers even though most of the companies utilize the skin prick test.⁴ BAU is based on intradermal skin tests.¹³ The 3-fold dilution (0.05 mL) is calculated to induce a sum of erythema of 50 mm (D50). The allergen extract with a D50 of 14th dilution is arbitrarily assigned 100,000 BAU/mL [BAU/mL = 100,000 × 3^(D50-14)]. However, these approaches can't be applied to the chemically modified allergens (allergoids) or hypoallergenic allergens (which are currently not commercially available).¹⁴

For *in vitro* standardization, competitive IgE-binding inhibition test (e.g., ELISA or RAST) has proven to be reliable and is most commonly applied for measurement of total allergenic activity.¹⁵ The relative potency may be calculated by comparing activity with the potency of reference allergen extracts. Various biochemical and immunological methods such as SDS-PAGE, immunoelectrophoresis, circular dichroism spectrum analysis, immunoblotting, mass-spectrometric analysis, dot blot analysis, and proteomic approaches, etc. could be performed to check and control the consistent composition and activity of allergen product. However, IgE-reactivity does not reflect ability to cause allergic symptoms. The basophil activation test utilizes the same mechanism as a skin test and is expected to more closely reflect the biological activity of allergens, although it has not been widely utilized until now.^{16,17}

ALLERGEN STANDARD

Production of allergen standard is essential for the standardization of allergen extracts. In fact, international reference standards were produced under the auspices of WHO according to the guidelines established by the Allergen Standardization Subcommittee under the International Union of Immunological Societies (IUIS) for short ragweed (*Ambrosia artemisiifolia*), timothy grass (*Phleum pratense*), birch (*Betula verrucosa*) pollen, house dust mite (*Dermaphagoides pteronyssinus*), and dog (*Canis familiaris*) between 1984 and 1989. These allergen extracts are obtain-

able from the National Institute of Biological Science and Control (NIBSC), London. However, these standard materials have not been widely utilized for the standardization of commercial products because they were determined based on IgE potency tests, which use various serum pools and cannot distinguish the ratios of major allergens that are not identical between companies.

MAJOR ALLERGEN CONTENT

An alternative approach is necessary to overcome these problems caused by using allergen extracts containing a mixture of antigens and toxic substances such as endotoxin, enterotoxin, and/or proteases, which may play a role as an allergic adjuvant. The use of combinations of optimized concentrations of purified allergens, recombinant or native proteins, seems to be advantageous.^{18,19} Measurement of the concentration of individual major allergens is reported to correlate with the biological potency and IgE reactivity of allergen extracts.^{20,21} Furthermore, clinical trials using cocktails of recombinant allergens have demonstrated clinical efficacy, supporting the importance of major allergen content for immunotherapy.^{22,23} It also facilitates direct comparison between allergen products.²⁴

The CREATE project (Development of Certified Reference Materials for Allergenic Products and Validation of Methods for their Quantification) was initiated by the European Commission from nine European Union member states in order to evaluate the potential of purified natural or recombinant allergens as certified reference materials, and to validate available two-site ELISAs for the measurement of major allergens.²⁵ So far, two reference materials (rBet v 1 and rPhl p 5a) and relevant ELISA assays are now being validated for future establishment as international references under the guidance of the European Directorate for the Quality of Medicine (EDQM) after extensive biological and biochemical investigation. Currently, these two reference materials (rBet v 1 and rPhl p 5a) are under further validation by BSP090 project. The CREATE project leads us to conclude that standardization with major allergens is possible, even though recombinant allergens and monoclonal antibody-based immunoassays are not always ideal. Still, standardization based on major allergen content seems to be reasonable for determining the optimal dose of allergen vaccine, inducing a clinically relevant effect in the majority of subjects without unacceptable side effects. It is

generally thought that 5-20 µg of major allergen (50-250 µg/yr) for house dust mite, cat, ragweed, and Hymenoptera venom are optimal for subcutaneous allergen specific immunotherapy. Much higher concentrations are needed for sublingual immunotherapy.

SEQUENCE POLYMORPHISMS OF ALLERGENS

A mixture of similar molecules with some variation in the amino acid sequences, isoallergens or variants often constitutes allergenic proteins.²⁶ This phenomenon, often called polymorphism, may be caused by the presence of several gene alleles or families. Sequence diversity of major allergens is one of the important factors affecting complete allergen characterization and standardization.^{21,27} RNA editing as well as multiple genes were found to produce sequence variations, which may influence its allergenicity in case of cockroach allergen, Bla g 4.²⁸ Importantly, mite group 2 allergens and birch pollen allergen Bet v 1 showed sequence diversity, which could influence IgE-binding reactivity, T cell response, and cytokine production from various immune cells.²⁹⁻³² Extraction procedure and storage can also influence allergen contents. Different isoforms could be included in different batches of allergen extract. IgE or IgG recognition could be affected even by a single amino acid substitution on the surface of the allergens.³³

Sequence polymorphism also influences reactivity to monoclonal antibodies. Commonly used immunoassay kits to quantify major allergens in Korea could underestimate the quantity of dominant isoforms in Korea.³⁴ In case of Der f 2, amino acid sequence variations were described from Korean isolates of house dust mite.³⁵ Differences in the capture antibody (monoclonal or polyclonal), storage buffer, or microplates showed up to 2-fold differences in two-site ELISA. Furthermore, distinct combinations of materials or incubations from different steps also could produce up to 5-fold differences in Can f 1 and egg white analyses.³⁶ Epidemiological studies using immunoassay kits are also believed to underestimate the allergen content in dust samples. It is necessary to investigate the sequence polymorphism of major allergens that are thought to play important roles in the sensitization of Korean allergic patients. Studies of the sequence polymorphism and identification of major isoforms in each country/geographical region are thought to be necessary for more accurate determination of allergen content from a given

environment or allergen extract by using immunoassay kits.³⁷

NATURAL ADJUVANT IN ALLERGENS EXTRACT

Some component of allergen extract could activate innate and/or adaptive immunity. In particular, house dust mites have been known to contain many substances that could induce innate immunity, subsequently affecting the development of allergic responses.³⁸ Some proteins in allergen extract, whether they are allergens themselves or not, can activate the various human cell and induce inflammatory responses.³⁹

Some common contaminants from the indoor environment can play a role in the development of allergic disorders through their adjuvant-like activity. Several lines of evidence have been described on the relationship between pathogen-associated molecular patterns (PAMPs).⁴⁰ Endotoxin has been implicated as an important modulator of allergic responses.^{41,42} Natural adjuvant derived from various microbes may affect allergy diagnosis and immunotherapy as well as pathogenesis of allergic disorders. Interestingly, the major house dust mite allergen Der p 2, homologous to MD-2, was shown to reconstitute lipopolysaccharide-induced TLR4 signalling in MD-2-deficient mice.⁴³

Endotoxin, one of the well-described cell wall components of gram-negative bacteria, is known to induce inflammatory responses in human cells, subsequently affecting allergic diseases.⁴⁴ Several lines of evidence have shown that sequence variation of TLR-2 and -4 genes are associated with the development of allergic disorders.⁴⁵ In a normal indoor environment, 300-18,000 ng/g dust of endotoxin was detected.⁴⁴ This concentration implies that a significant amount of endotoxin is breathed in and can have influence on immune reactions. Exposure to the high level of endotoxin is thought to protect against the allergy. However, low level of endotoxin is believed to worsen allergic symptoms.

β-glucan, a component of fungal cell walls, is another common component of the indoor environment. It is also known to modulate allergic responses, influencing both the Th1 and Th2 responses.^{46,47} Notably, 0.4-41.8 ng/mL of β-glucan was detected in standardized allergen extracts.⁴⁸

Various enzyme activities have been detected from the allergen extracts and house dust.^{49,50} Mite group 1, 3, 6, and 9 allergens are cysteine protease, trypsin-like serine protease, chymotrypsin-like serine protease, and collagenolytic

serine protease, respectively.⁵¹ Proteins with cysteine or serine protease activity are well known to induce Th2 immune response. Protease allergens can cleave CD23 (low affinity IgE receptor) of activated B cells⁵² and CD25 (IL-2 receptor) of T cells.⁵³ Tight junctions in the lung epithelium could be degraded and cause various cells to release inflammatory cytokines.^{54,55} Degraded epithelium is vulnerable even to bystander allergens.^{56,57} Protease allergens also can cleave several component of the complement system, generating active anaphylatoxins, C3a, C4a, and C5a.⁵⁸⁻⁶⁰ Furthermore, protease-activated receptor 2 is activated and induces proinflammatory responses.^{61,62} Human basophils are known to be directly activated by cysteine protease (Der p 1 and Der f 1) and to release cytokines, which can induce allergic inflammation.⁶³ Some serine protease inhibitors and defects of the protease gene are known to be associated with allergic conditions.⁵⁴ Interestingly, cystatin A, a cysteine protease inhibitor in secreted sweat, has been shown to eliminate the activity of mite group 1 allergens.⁶⁴ It is also conceivable that some proteases originating from symbionts or parasites of allergen sources (house dust mite or cockroach) can influence allergic inflammation.⁶⁵⁻⁶⁸ In fact, various microorganisms, which may secrete or excrete proteases, have been isolated from the cockroach hindgut.⁶⁹

Flagellin, a principal constituent of bacterial flagellum, is one element that may elicit an inflammatory response.^{70,71} However, much remains to be investigated.

Chitin, a major component of the exoskeletons of arthropods, fungal walls and the microfilarial sheath of parasites, is the second most abundant polysaccharide in nature. Inhalation or infection of chitin-containing pathogen could acti-

vate chitinases and chitinase-like protein in humans and play a role in the pathogenesis of Th2 inflammation.^{72,73} Interestingly, mite allergen Der f 15 and 18 show significant homology to chitinases. It would be interesting to explore the role of chitinases in the pathogenesis of allergic diseases and to examine the chitin content and chitinase activity in allergen extracts.

Superantigen, endotoxin B from *Staphylococcus* in skin, is also known to be associated with the development of atopic dermatitis by activating T cells.⁷⁴

DNA fragments derived from microorganisms may have various effects on the pathogenesis of allergic disorders. *Herpes simplex* is known to induce production of TNF- α by activation of TLR-9.⁷⁵ DNA fragments may contain the CpG motif, which could be protective against allergy.⁷⁶ Bacterial DNA are detectable even from house dust mite cultures for allergen extract preparation,⁷⁷ indicating that DNA fragments are included in the extracts.

It is noteworthy that many adjuvant-like activities are derived from a microbial component. It is known that commensals in intestine and skin can influence allergic disorders.⁷⁸ Recently, it has been reported that there are a large number of microbiota that can affect allergic diseases.⁷⁹ Viral infection can also exacerbate the symptoms via TLR-3, -7, -8, -9 activation.⁸⁰ Concomitant infectious diseases and hygiene are important not only for the development of allergic disorders and exacerbation of the symptoms, but also for diagnosis and allergy immunotherapy. Currently, it is thought to be almost impossible to remove all those elements that may play adjuvant-like roles. Therefore, it should be emphasized that every effort must be made to minimize possi-



Fig. 1. (A) A Japanese hop, an annual climbing vine with 5-lobed leaves and covered with hooked hairs. The pollen of the Japanese hop is an important cause of seasonal rhinitis in Asia in August and September. (B) An adult male *Chironomus kiiensis*, a dominant species of non-biting midge in Korea. The emergence of a large number of non-biting midges often causes a nuisance as well as allergies.

ble contamination and suppress microbial growth. It is desirable to check the activity and content of substances that may play adjuvant-like roles.

ALLERGEN STANDARDIZATION IN KOREA

The allergen extracts produced at the Research Center for the Standardization of Allergic Diseases, Department of Internal Medicine and Institute of Allergy, Yonsei University College of Medicine, Seoul, Korea, exhibit good allergen potency, in terms of *in vitro* (ELISA inhibition) and *in vivo* (intradermal skin test) tests, when compared with commercially available extracts, even though immunosassays detected low levels of major allergens. Comparisons were performed between endotoxin levels of house dust mite extracts both commercially available and produced in our laboratory. The endotoxin level was determined to be comparable with commercial products even without the addition of phenol (unpublished data).

It is urgently necessary to develop standard references and immunoassays for the detection of major allergens, such as Japanese hop (*Humulus japonicus*) (Fig. 1A),⁸¹ spider mites (*Tetranychus urticae* and *Panonychus citri*),⁸² non-biting midge (*Chironomus* spp) (Fig. 1B),⁸³ Pharaoh ant (*Monomorium pharaonis*),⁸⁴ and Asian needle ant (*Pachydonyla chinensis*),⁸⁵ which are frequent causes of allergic illness in Korea.

CONCLUDING REMARKS

The recent research trend for allergen standardization has changed from IgE potency test to characterization of major allergens and assays for the quantification of major allergen concentrations. Still, measurement of biological potency of extracts remains the basis of allergen standardization. Further study is required to characterize the major allergens that are thought to be important in Korea. Studies on the correlation between major allergen concentrations and the potency of allergen extracts will facilitate the development of advanced allergen-specific immunotherapeutics.

ACKNOWLEDGEMENTS

This study was supported by a grant of the Korea Health-

care Technology R&D Project, Ministry for Health, Welfare & Family Affairs, Republic of Korea (A092076).

REFERENCES

- Noon L. Prophylactic inoculation against hay fever. *Lancet* 1911; 1:1572-3.
- Freeman J, Noon L. Further observation on the treatment of hay-fever by hypodermic inoculation of pollen vaccine. *Lancet* 1911; 2:814-7.
- Patterson ML, Slater JE. Characterization and comparison of commercially available German and American cockroach allergen extracts. *Clin Exp Allergy* 2002;32:721-7.
- Larenas-Linnemann D, Cox LS; Immunotherapy and Allergy Diagnostics Committee of the American Academy of Allergy, Asthma and Immunology. European allergen extract units and potency: review of available information. *Ann Allergy Asthma Immunol* 2008;100:137-45.
- Brunetto B, Tinghino R, Braschi MC, Antonicelli L, Pini C, Iacovacci P. Characterization and comparison of commercially available mite extracts for *in vivo* diagnosis. *Allergy* 2010;65:184-90.
- Rossi R, Monasterolo G, Passalacqua G. The biological potency of different extracts for sublingual immunotherapy assessed by skin prick tests. *Eur Ann Allergy Clin Immunol* 2010;42:112-4.
- Morrow KS, Slater JE. Regulatory aspects of allergen vaccines in the US. *Clin Rev Allergy Immunol* 2001;21:141-52.
- Larsen JN, Dreborg S. Standardization of allergen extracts. *Methods Mol Med* 2008;138:133-45.
- Burazer L, Milovanovic K, Milovanovic M, Vuckovic O, Velekovic TC, Gavrovic-Jankulovic M. Impact of *Dermatophagoides pteronyssinus* mite body raw material on house dust mite allergy diagnosis in a Serbian population. *Med Vet Entomol* 2011; 25:77-83.
- Weghofer M, Thomas WR, Kronqvist M, Mari A, Purohit A, Pauli G, et al. Variability of IgE reactivity profiles among European mite allergic patients. *Eur J Clin Invest* 2008;38:959-65.
- May JC, Sih JT, Miller JR, Seligmann EB Jr. Optimization of parameters in protein nitrogen unit precipitation procedure for allergenic extracts. *J Allergy Clin Immunol* 1979;63:87-97.
- Dreborg S. Precision of biological standardization of allergenic preparations. *Allergy* 1992;47:291-4.
- Turkeltaub PC, Rastogi SC, Baer H, Anderson MC, Norman PS. A standardized quantitative skin-test assay of allergen potency and stability: studies on the allergen dose-response curve and effect of wheal, erythema, and patient selection on assay results. *J Allergy Clin Immunol* 1982;70:343-52.
- Ibarrola I, Sanz ML, Gamboa PM, Mir A, Benahmed D, Ferrer A, et al. Biological characterization of glutaraldehyde-modified *Parietaria judaica* pollen extracts. *Clin Exp Allergy* 2004;34:303-9.
- Grier TJ. Laboratory methods for allergen extract analysis and quality control. *Clin Rev Allergy Immunol* 2001;21:111-40.
- Kleine Budde I, de Heer PG, van der Zee JS, Aalberse RC. The stripped basophil histamine release bioassay as a tool for the detection of allergen-specific IgE in serum. *Int Arch Allergy Immunol* 2001;126:277-85.
- Kaul S, Lüttkopf D, Kastner B, Vogel L, Hölzt G, Vieths S, et al. Mediator release assays based on human or murine immunoglobulin E in allergen standardization. *Clin Exp Allergy* 2007;37:141-50.

18. Ferreira F, Briza P, Inführ D, Schmidt G, Wallner M, Wopfner N, et al. Modified recombinant allergens for safer immunotherapy. *Inflamm Allergy Drug Targets* 2006;5:5-14.
19. Jeong KY, Hong CS, Yong TS. Recombinant allergens for diagnosis and immunotherapy of allergic disorders, with emphasis on cockroach allergy. *Curr Protein Pept Sci* 2006;7:57-71.
20. Dreborg S, Einarsson R. The major allergen content of allergenic preparations reflect their biological activity. *Allergy* 1992;47:418-23.
21. van Ree R. Indoor allergens: relevance of major allergen measurements and standardization. *J Allergy Clin Immunol* 2007;119:270-7.
22. Jutel M, Jaeger L, Suck R, Meyer H, Fiebig H, Cromwell O. Allergen-specific immunotherapy with recombinant grass pollen allergens. *J Allergy Clin Immunol* 2005;116:608-13.
23. Creticos PS, Schroeder JT, Hamilton RG, Balcer-Whaley SL, Khattignavong AP, Lindblad R, et al. Immunotherapy with a ragweed-toll-like receptor 9 agonist vaccine for allergic rhinitis. *N Engl J Med* 2006;355:1445-55.
24. van Ree R, Dorpema JW, Vieths S. Allergy vaccines: a need for standardisation in mass units of major allergen. *Pharmeuropa Bio* 2005;2005:27-30.
25. van Ree R; CREATE Partnership. The CREATE project: EU support for the improvement of allergen standardization in Europe. *Allergy* 2004;59:571-4.
26. King TP, Hoffman D, Lowenstein H, Marsh DG, Platts-Mills TA, Thomas W. Allergen nomenclature. WHO/IUIS Allergen Nomenclature Subcommittee. *Int Arch Allergy Immunol* 1994;105:224-33.
27. van Ree R. Analytic aspects of the standardization of allergenic extracts. *Allergy* 1997;52:795-805.
28. Jeong KY, Yi MH, Jeong KJ, Lee H, Hong CS, Yong TS. Sequence diversity of the Bla g 4 cockroach allergen, homologous to lipocalins, from *Blattella germanica*. *Int Arch Allergy Immunol* 2009;148:339-45.
29. Smith WA, Hales BJ, Jarnicki AG, Thomas WR. Allergens of wild house dust mites: environmental Der p 1 and Der p 2 sequence polymorphisms. *J Allergy Clin Immunol* 2001;107:985-92.
30. Piboonpocanun S, Malainual N, Jirapongsananuruk O, Vichyanond P, Thomas WR. Genetic polymorphisms of major house dust mite allergens. *Clin Exp Allergy* 2006;36:510-6.
31. Ferreira F, Hirtenlehner K, Jilek A, Godnik-Cvar J, Breiteneder H, Grimm R, et al. Dissection of immunoglobulin E and T lymphocyte reactivity of isoforms of the major birch pollen allergen Bet v 1: potential use of hypoallergenic isoforms for immunotherapy. *J Exp Med* 1996;183:599-609.
32. Arquint O, Helbling A, Cramer R, Ferreira F, Breitenbach M, Pichler WJ. Reduced in vivo allergenicity of Bet v 1d isoform, a natural component of birch pollen. *J Allergy Clin Immunol* 1999;104:1239-43.
33. Reyes-López CA, Pedraza-Escalona M, Mendoza G, Hernández-Santoyo A, Rodríguez-Romero A. A single amino acid substitution on the surface of a natural hevein isoform (Hev b 6.0202), confers different IgE recognition. *FEBS Lett* 2006;580:2483-7.
34. Park JW, Kim KS, Jin HS, Kim CW, Kang DB, Choi SY, et al. Der p 2 isoallergens have different allergenicity, and quantification with 2-site ELISA using monoclonal antibodies is influenced by the isoallergens. *Clin Exp Allergy* 2002;32:1042-7.
35. Jeong KY, Jin HS, Oh SH, Hong CS, Lee IY, Ree HI, et al. Monoclonal antibodies to recombinant Der f 2 and development of a two-site ELISA sensitive to major Der f 2 isoallergen in Korea. *Allergy* 2002;57:29-34.
36. Grier TJ, Hazelhurst DM, Duncan EA, West TK, Esch RE. Major allergen measurements: sources of variability, validation, quality assurance, and utility for laboratories, manufacturers, and clinics. *Allergy Asthma Proc* 2002;23:125-31.
37. Löwenstein H, Sparholt SH, Klysner SS, Ipsen H, Larsen JN. The significance of isoallergenic variations in present and future specific immunotherapy. *Int Arch Allergy Immunol* 1995;107:285-9.
38. Jacquet A. The Role of the House Dust Mite-Induced Innate Immunity in Development of Allergic Response. *Int Arch Allergy Immunol* 2010;155:95-105.
39. Osterlund C, Grönlund H, Gafvelin G, Bucht A. Non-Proteolytic Aeroallergens from Mites, Cat and Dog Exert Adjuvant-Like Activation of Bronchial Epithelial Cells. *Int Arch Allergy Immunol* 2010;155:111-8.
40. Willart MA, Lambrecht BN. The danger within: endogenous danger signals, atopy and asthma. *Clin Exp Allergy* 2009;39:12-9.
41. Liu AH. Endotoxin exposure in allergy and asthma: reconciling a paradox. *J Allergy Clin Immunol* 2002;109:379-92.
42. Michel O. Role of lipopolysaccharide (LPS) in asthma and other pulmonary conditions. *J Endotoxin Res* 2003;9:293-300.
43. Trompette A, Divanovic S, Visintin A, Blanchard C, Hegde RS, Madan R, et al. Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. *Nature* 2009;457:585-8.
44. Doreswamy V, Peden DB. Modulation of asthma by endotoxin. *Clin Exp Allergy* 2011;41:9-19.
45. Tulic MK, Fiset PO, Manoukian JJ, Frenkiel S, Lavigne F, Eidelman DH, et al. Role of toll-like receptor 4 in protection by bacterial lipopolysaccharide in the nasal mucosa of atopic children but not adults. *Lancet* 2004;363:1689-97.
46. Rylander R, Holt PG. (1→3)-beta-D-glucan and endotoxin modulate immune response to inhaled allergen. *Mediators Inflamm* 1998;7:105-10.
47. Thorn J, Rylander R. Airways inflammation and glucan in a row-house area. *Am J Respir Crit Care Med* 1998;157:1798-803.
48. Finkelman MA, Lempitski SJ, Slater JE. β-Glucans in standardized allergen extracts. *J Endotoxin Res* 2006;12:241-5.
49. Simonen-Jokinen T, Maisi P, Tervahartiala T, McGorum B, Pirie S, Sorsa T. Direct activation of gelatinase B (MMP-9) by hay dust suspension and different components of organic dust. *Vet Immunol Immunopathol* 2006;109:289-95.
50. Jeong KY, Kim C, Yong TS. Enzymatic activities of allergen extracts from three species of dust mites and cockroaches commonly found in Korean home. *Korean J Parasitol* 2010;48:151-5.
51. Thomas WR, Heinrich TK, Smith WA, Hales BJ. Pyroglyphid house dust mite allergens. *Protein Pept Lett* 2007;14:943-53.
52. Hewitt CR, Brown AP, Hart BJ, Pritchard DI. A major house dust mite allergen disrupts the immunoglobulin E network by selectively cleaving CD23: innate protection by antiproteases. *J Exp Med* 1995;182:1537-44.
53. Ghaemmaghani AM, Robins A, Gough L, Sewell HF, Shakib F. Human T cell subset commitment determined by the intrinsic property of antigen: the proteolytic activity of the major mite allergen Der p 1 conditions T cells to produce more IL-4 and less IFN-gamma. *Eur J Immunol* 2001;31:1211-6.
54. Smith PK, Harper JI. Serine proteases, their inhibitors and allergy. *Allergy* 2006;61:1441-7.
55. Chapman MD, Wünschmann S, Pomés A. Proteases as Th2 adjuvants. *Curr Allergy Asthma Rep* 2007;7:363-7.
56. Gough L, Schulz O, Sewell HF, Shakib F. The cysteine protease

- activity of the major dust mite allergen Der p 1 selectively enhances the immunoglobulin E antibody response. *J Exp Med* 1999;190:1897-902.
57. Gough L, Sewell HF, Shakib F. The proteolytic activity of the major dust mite allergen Der p 1 enhances the IgE antibody response to a bystander antigen. *Clin Exp Allergy* 2001;31:1594-8.
 58. Nagata S, Glovsky MM. Activation of human serum complement with allergens. I. Generation of C3a, C4a, and C5a and induction of human neutrophil aggregation. *J Allergy Clin Immunol* 1987;80:24-32.
 59. Maruo K, Akaike T, Ono T, Okamoto T, Maeda H. Generation of anaphylatoxins through proteolytic processing of C3 and C5 by house dust mite protease. *J Allergy Clin Immunol* 1997;100:253-60.
 60. Karp CL, Grupe A, Schadt E, Ewart SL, Keane-Moore M, Cuomo PJ, et al. Identification of complement factor 5 as a susceptibility locus for experimental allergic asthma. *Nat Immunol* 2000;1:221-6.
 61. Asokanathan N, Graham PT, Fink J, Knight DA, Bakker AJ, McWilliam AS, et al. Activation of protease-activated receptor (PAR)-1, PAR-2, and PAR-4 stimulates IL-6, IL-8, and prostaglandin E2 release from human respiratory epithelial cells. *J Immunol* 2002;168:3577-85.
 62. Miike S, Kita H. Human eosinophils are activated by cysteine proteases and release inflammatory mediators. *J Allergy Clin Immunol* 2003;111:704-13.
 63. Sokol CL, Barton GM, Farr AG, Medzhitov R. A mechanism for the initiation of allergen-induced T helper type 2 responses. *Nat Immunol* 2008;9:310-8.
 64. Kato T, Takai T, Mitsuishi K, Okumura K, Ogawa H. Cystatin A inhibits IL-8 production by keratinocytes stimulated with Der p 1 and Der f 1: biochemical skin barrier against mite cysteine proteases. *J Allergy Clin Immunol* 2005;116:169-76.
 65. Ribas A, Martínez-Girón R. Protozoal forms in house-dust mites and respiratory allergy. *Allergy Asthma Proc* 2006;27:347-9.
 66. Martínez-Girón R, Ribas A, Astudillo-González A. Flagellated protozoa in cockroaches and sputum: the unhygienic connection? *Allergy Asthma Proc* 2007;28:608-9.
 67. Yao G. Bronchopulmonary infection with *Lophomonas blattarum*: two cases report and literature review. *J Med Coll PLA* 2008; 23: 176-182.
 68. Martínez-Girón R, van Woerden HC. Is the dust mite allergen Der p1 protozoal in origin? *Scand J Immunol* 2009;70:75-6.
 69. Peregrine PC. Host dietary changes and the hindgut fauna of cockroaches. *Int J Parasitol* 1974;4:645-56.
 70. Im J, Jeon JH, Cho MK, Woo SS, Kang SS, Yun CH, et al. Induction of IL-8 expression by bacterial flagellin is mediated through lipid raft formation and intracellular TLR5 activation in A549 cells. *Mol Immunol* 2009;47:614-22.
 71. Le TA, Takai T, Vu AT, Kinoshita H, Chen X, Ikeda S, et al. Flagellin Induces the Expression of Thymic Stromal Lymphopoietin in Human Keratinocytes via Toll-Like Receptor 5. *Int Arch Allergy Immunol* 2010;155:31-7.
 72. Zhu Z, Zheng T, Homer RJ, Kim YK, Chen NY, Cohn L, et al. Acidic mammalian chitinase in asthmatic Th2 inflammation and IL-13 pathway activation. *Science* 2004;304:1678-82.
 73. Elias JA, Homer RJ, Hamid Q, Lee CG. Chitinases and chitinase-like proteins in T(H)2 inflammation and asthma. *J Allergy Clin Immunol* 2005;116:497-500.
 74. Schubert MS. A superantigen hypothesis for the pathogenesis of chronic hypertrophic rhinosinuitis, allergic fungal sinusitis, and related disorders. *Ann Allergy Asthma Immunol* 2001;87:181-8.
 75. Hochrein H, Schlatter B, O'Keeffe M, Wagner C, Schmitz F, Schiemann M, et al. *Herpes simplex* virus type-1 induces IFN- α production via Toll-like receptor 9-dependent and -independent pathways. *Proc Natl Acad Sci U S A* 2004;101:11416-21.
 76. Lazarczyk M, Grzela K, Grzela T. Immunostimulatory oligonucleotides in therapy of allergic diseases. *Expert Opin Biol Ther* 2005; 5:525-36.
 77. Valerio CR, Murray P, Arlian LG, Slater JE. Bacterial 16S ribosomal DNA in house dust mite cultures. *J Allergy Clin Immunol* 2005;116:1296-300.
 78. Huffnagle GB. The microbiota and allergies/asthma. *PLoS Pathog* 2010;6:e1000549.
 79. Huang YJ, Nelson CE, Brodie EL, Desantis TZ, Baek MS, Liu J, et al. Airway microbiota and bronchial hyperresponsiveness in patients with suboptimally controlled asthma. *J Allergy Clin Immunol* 2011;127:372-81.
 80. Jackson DJ, Johnston SL. The role of viruses in acute exacerbations of asthma. *J Allergy Clin Immunol* 2010;125:1178-87.
 81. Park JW, Ko SH, Kim CW, Jeoung BJ, Hong CS. Identification and characterization of the major allergen of the *Humulus japonicus* pollen. *Clin Exp Allergy* 1999;29:1080-6.
 82. Kim YK, Kim YY. Spider-mite allergy and asthma in fruit growers. *Curr Opin Allergy Clin Immunol* 2002;2:103-7.
 83. Jeong KY, Yum HY, Lee IY, Ree HI, Hong CS, Kim DS, et al. Molecular cloning and characterization of tropomyosin, a major allergen of *Chironomus kiiensis*, a dominant species of nonbiting midges in Korea. *Clin Diagn Lab Immunol* 2004;11:320-4.
 84. Kim CW, Choi SY, Park JW, Hong CS. Respiratory allergy to the indoor ant (*Monomorium pharaonis*) not related to sting allergy. *Ann Allergy Asthma Immunol* 2005;94:301-6.
 85. Yun YY, Ko SH, Park JW, Hong CS. Anaphylaxis to venom of the *Pachycondyla* species ant. *J Allergy Clin Immunol* 1999;104:879-82.