Current Status of Standardization of Inhalant Allergen Extracts in Korea

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Allergy diagnosis and immunotherapy in Korea rely mostly on imported allergen extracts. However, some allergens that are not important in Western countries are not commercially available, and even the same species of allergen source often displays differences in allergenicity due to amino acid sequence polymorphisms. Therefore, it is essential to prepare allergen extracts that reflect regional characteristics. Allergen standardization has been performed since 2009 with the support of the Korea Center for Disease Control and Prevention. Here, we summarize the current status of allergen standardization, focusing on the house dust mite and cockroach. Pollen allergens that are under investigation are also briefly described.

Key Words: Allergen; allergen extract; standardization

INTRODUCTION

It is essential to standardize allergen extracts for commercial allergy diagnostic and immunotherapeutic reagents.1 A project for the standardization of allergen extracts has been supported by the Korea Center for Disease Control and Prevention since 2009. Here, we summarize the results from 4 years of this project. A standardization procedure needs to be established in order to ensure the consistency and reproducible preparation of allergen extracts. Allergen standardization can be performed through preparation of standard reference material and determination of the unit that denotes IgE binding potency. The overall potency of the extracts was determined by using an in vitro method, measurement of the bioequivalent unit (BAU) by an intradermal skin test. This potency has been used as a reference for the estimation of the potency of extracts from other batches by an in vitro method, competitive IgE-binding assay. The concentration of major allergens has also been assessed because it has been known to be proportional to the potency of the extracts.2 Currently, 7 allergen extracts, including house dust mites (Dermatophagoides pteronyssinus and D. farinae), German cockroach (Blattella germanica), pollens of Humulus japonicus, Artemisia vulgaris, Ambrosia artemisiifolia, and Quercus acutissima, have been prepared or are under investigation. Various recombinant proteins are now being generated and characterized for better allergen standardization.

Standardization of indoor allergens

House dust mite is the most important source of indoor allergens,3,4 therefore we first focused on the preparation and characterization of house dust mite extract, followed by German cockroach extract. Commercial dust mite extracts are well characterized and are provided at a concentration of 5,000-30,000 allergy units (AU)/mL. The AU indicates that the potency of the extract was measured by in vitro methods with the reference standard. To achieve successful immunotherapy for house dust mite allergy, a maintenance dose of 500-2,000 AU/mL is suggested.5 However, commercial cockroach allergen extracts are not standardized and are provided at a dilution of 1:10 w/v. The suggested dose during the maintenance phase for effective cockroach immunotherapy is the highest tolerated dose.

House dust mite

House dust mite extracts were prepared from Korean isolates.6 The allergen potency of D. pteronyssinus extract was compared to reference material obtained from the Center for Biologics

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Received: November 4, 2013; Accepted: December 2, 2013

• There are no financial or other issues that might lead to conflict of interest.
Evaluation and Research (CBER, Food and Drug Administration, USA) (Fig. 1). Interestingly, compared to the CBER reference, the Korean reference material showed only 11.6% allergenic potency in terms of 50% inhibitory concentration, and the commercial extract (HollisterStier, Spokane, WA, USA) showed 14.6% potency. In addition to allergen potency, allergen composition is also important in terms of the consistency of allergen preparations. However, the concentration of major allergens of the house dust mite is known to be variable, even in commercial skin test reagents. For example, the ratio of Der p 1 (12-30 μg/mL)/Der p 2 (3-15 μg/mL) ranges from 1.1:1 to 6:1.2 Extracts prepared from raw materials rich in fecal pellets contain higher ratios of mite group 1/2 allergens than those from pure mite bodies. In the extracts from Korean house dust mite isolates, we measured concentrations of 5.0 μg/mg Der f 1, 12.0 μg/mg Der f 2, 11.6 μg/mg Der p 1, and 12.4 μg/mg Der p 2 by 2-site ELISA (Indoor Biotechnologies Inc., Charlottesville, VA, USA).6

Quantification of mite allergens, particularly group 2 allergens, is known to be influenced by amino acid sequence polymorphism.10 Sequence polymorphism analysis of Korean mite isolates revealed the predominant and novel variants of mite group 1 and 2 allergens.11 Specifically, for group 1, Der f 1.0101 (64.0%) appeared as a predominant variant. Der p 1.0102 (44.0%) was a predominant variant, followed by Der p 1.0105 (14.0%), which contains Ala124 and Gly182. A new variant (GenBank Accession No. JN222803) (8.0%) with Ala124 and Ser182 was also identified. For group 2 allergens, Der f 2.0102 (40.7%) and a new variant (GenBank Accession No. JQ698663) with Gly42 (27.8%) were predominant. The SLD-motif at positions 57-59, along with Ile63 and Phe75, suggest large differences from the European and Thai populations. Two variants of Der p 2—Leu40, Thr49, and Asn114 (26.6%) and Val40, Thr49, and Asn114 (20.0%)—were predominant. Interestingly, all Der p 2 sequences had Thr instead of Lys at position 49. Five new variants of Der p 2 were deposited in GenBank under accession No. JN222805-9. Moreover, most of the variants contained Ser47 which is known to have a higher IgE-binding affinity, and Asn114 which is responsible for strong binding to the monoclonal antibodies 1D8 and 4G7 in the commercial ELISA kit. In a study from Thailand, rabbit polyclonal antibodies were used for the quantification of Der f 1.12 The problems caused by polymorphisms may be overcome by the production of a regional standard using a mixture of predominant variants and/or development of monoclonal antibodies recognizing non-polymorphic regions. Stability of mite extracts have also been investigated.13 Storage temperature is the most important factor in the preservation of allergenicity of the extract, and 92.0%-97.0% of IgE reactivity is retained at 1 year regardless of buffer composition when stored at 4°C. The mite extract reconstituted in 50% glycerol retained more than 89.9% IgE reactivity at 1 year even at room temperature.

Cockroach

Cockroach is another important source of indoor allergens associated with asthma exacerbation.14 However, cockroach extract has not been standardized, and manufacturers commonly use the unit of weight (raw material) to volume (extraction buffer) (v/w). Strong protease activity in the cockroach extract makes it difficult to standardize the extract.15 We prepared an extract of German cockroach and analyzed its allergenic properties.16 The cockroach population used in this study originated from a single pregnant female captured in Seoul.17 The cockroach extract showed allergenic potency (94.2%) similar to the commercial extract (Hollister-Stier) in a competitive IgE-binding analysis. However, there was a substantial difference in Bla g 1 and Bla g 2 concentrations between the extracts: the Korean extract contained 404 U/mg Bla g 1 and 273 ng/mg Bla g 2, whereas the Hollister-Stier extract contained 187 U/mg Bla g 1 and 56 ng/mg Bla g 2. A difference in endotoxin concentration between the extracts was also observed (3,440 EU/mL for Korean extract and 6,580 EU/mL for HollisterStier extract); however, endotoxin is not considered essential18 even though alveolar macrophages play a key role in a cockroach-induced airway inflammation19 in a mouse model. Interestingly, proteins of approximately 60 and 70 kDa showed strong IgE reactivity in both extracts. Proteomic analysis of the extract showed that 60 and 70 kDa allergens are α-amylase and Bla g 3, respectively (Fig. 2, Table 1). Amylase was also identified as an important allergen in German cockroach fecal extract.20 An interesting finding of fecal extract analysis is the IgE reactivity of various digestive enzymes, such as α-amylase, trypsin, chymotrypsin, metalloprotease, and midgut carboxypeptidase A. Furthermore, we detected glycinin-like protein, which may have been derived from the cockroach diet and may lead to false diagnosis. Further studies on α-amylase and Bla g 3 allergens are needed because they appear to have various isoforms.
ever, Siberian silver birch (B. platyphylla var. japonica) is often planted as a landscape tree.\textsuperscript{25} In the case of oak species, Mongolian oak (Q. mongolica) is known to be a predominant species in Korea,\textsuperscript{30} whereas sawtooth oak (Q. acutissima) is often found only at human dwellings. Pathogenesis-related 10 proteins (PR-10, Bet v 1 homologues) from the order Fagales are known to be important allergens, but cross-reactivity could be limited.\textsuperscript{27} Therefore, there is an urgent need to determine cross-reactivity between pollen extracts from trees native to Korea and the commercially-available extracts from foreign trees. A preliminary study suggested that sawtooth oak is a major cause of oak allergy in Korea. Therefore, we are characterizing the PR-10 protein, a putative Que ac 1, from sawtooth oak and the potency of the sawtooth oak pollen extract is being evaluated by an in vivo method.

**Weed pollen**

Pollens from Japanese hop, sagebrush, and ragweed constitute about 90\% of weed pollens in the air in Korea during autumn.\textsuperscript{28} In particular, 2 invasive plants, Japanese hop (Humulus japonicus) and ragweed (Ambrosia artemisiifolia var. elatior), have become able to displace native species as a result of global warming.\textsuperscript{29} Most importantly, Japanese hop pollen extract is not standardized despite its allergenic importance in East Asia. A study from China reported that profilin (a putative Hum j 2) is a major allergen from the Japanese hop.\textsuperscript{31} However, 2 isoforms of recombinant profilin were found to play a minor role in Korea.\textsuperscript{32} An allergen with a molecular mass of approximately 11 kDa is believed to be a major allergen.\textsuperscript{32,33} We are currently attempting to characterize this allergen of interest. The potency BAU of the allergen extract is also being determined by an intradermal skin test. Detailed information on in vivo standardization and characterization of 11 kDa allergen of Japanese hop will be published elsewhere.

At CBER, the potency of the ragweed allergen extract is evaluated through determination of a specific allergen, Amb a 1 by immunodiffusion with sheep polyclonal antibodies. However, a commercial 2-site ELISA kit (Indoor Technologies Inc.) uses monoclonal antibodies for the quantification of Amb a 1. Therefore, it is necessary to compare these 2 methods.

**Artemisia vulgaris** is commonly used for the diagnosis of mugwort allergy in Korea. However, this species is not found in Korea, and A. princeps is reported to be a dominant species.\textsuperscript{34} Comparison of 6 different species of Artemisia (A. vulgaris, A. sco-

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**Table 1. Identification of IgE reactive spots from German cockroach extract**

| Spot | Identification | Species | Molecular weight | Isoelectric point | Mascot score | Coverage (%) |
|------|----------------|---------|------------------|------------------|--------------|--------------|
| B1   | Bla g 3 isoform | Blattella germanica | 78867 | 6.44 | 193 | 10 |
| B2   | Bla g 3 isoform | Blattella germanica | 78730 | 6.35 | 219 | 8 |
| B3   | α-glucosidase  | Culex quinquefasciatus | 67755 | 4.90 | 78 | 3 |
| B4   | α-amylase      | Blattella germanica | 56759 | 5.74 | 163 | 7 |

**Fig. 2. Proteomic analysis of German cockroach extract.** Proteins were separated by 2D-electrophoresis and visualized by Coomassie Blue staining (A), and IgE-reactive components were probed with a pooled serum from cockroach-sensitized subjects (B). Selected proteins were identified by MS/MS analysis (B1-4). The data are summarized in Table.
paria, A. princeps, A. tridentate, A. annu, and A. campestris) revealed essentially the same pattern of IgE reactivity both to A. vulgaris and A. princeps. Therefore, we are conducting in vivo standardization of mugwort extract using A. vulgaris.

Recombinant allergens

The concentration of major allergens could be affected by environmental stresses. The mite culturing diet and the temperature at which the mites are cultured are known factors that determine allergen concentrations in extracts. The expression of cockroach allergen Bla g 1 is known to be influenced by food intake, whereas excretion of Bla g 2 is increased after exposure to a sublethal dose of boric acid, a common pesticide. Glutathione S-transferases, mite group 8 and cockroach group 5 allergens, are thought to be influenced by environmental chemicals because of their detoxification activity. A similar situation exists for pollen allergens. Pollen production can be increased by exposure to environmental pollutants, high CO₂ concentrations, and high temperatures. High-quality recombinant allergens should ensure the absence of non-allergenic antigens and adjuvant-like molecules, such as endotoxin, β-glucan, superantigen, and chitin. Even if high-quality allergen extracts are available, production of recombinant allergens is desirable for the quantification of major allergens. Quantification of major allergens is often hampered by polymorphisms. Therefore, it is necessary to identify dominant isoforms and produce regional standard molecules. Currently, various recombinant allergens from house dust mite, cockroach, Asian needle ant, buckwheat, and pollens are being characterized for standardization.

Concluding remarks

Allergen standardization is performed not only by the measurement of IgE-binding potency but also by the quantification of major allergens. However, many of the major allergens from Korean trees and weeds have not been characterized. Furthermore, the clinical value of component-resolved diagnosis has been proven. Continued effort is needed to produce validated recombinant allergens and to develop a quantification system for better standardization. Finally, allergen standardization could facilitate the development of advanced diagnostic and immunotherapeutic reagents.

ACKNOWLEDGMENTS

This study was supported by a grant of the Korea Healthcare Technology R&D Project, Ministry for Health, Welfare & Family Affairs, Republic of Korea (A092076).

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