Committee Report

A basic policy for allergen standardization in our country and standardization of Japanese cedar (Cryptomeria japonica) pollen extracts*

The Allergen Committee in Japanese Society of Allergology
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INTRODUCTION

Allergen extracts are indispensable for the diagnosis and treatment of allergic diseases. However, the quality of allergen extracts is affected by various factors because the extracts are biological products. Therefore, in order to raise the credibility of diagnosis and the effectiveness of treatment and to ensure safety in clinical use, it is necessary to use standardized allergens.¹-³ In Europe and the United States, the necessity of standardization has been recognized and clinically important allergens have been standardized and are commercially available. However, in our country, standardizations have not been performed. For these reasons, in order to promptly discuss allergen standardization, the Japanese Society of Allergology organized a Committee of Allergen Standardization. The committee, at first, determined essential guidelines for allergen standardization. In addition, the committee picked cedar pollen as the first target for study and performed selection of its standardized extracts and standardization, as cedar pollen is one of the most important allergens in Japan and the quality of its extracts is thought to cause several problems.

METHODS

Candidates for standards of cedar pollen extracts

Eight lots of cedar pollen extract were supplied by the Torii Pharmaceutical Company Ltd, Tokyo, Japan. These extracts were prepared as follows. Fifty per cent glycerin-NaCl solution (glycerin 50%, NaCl 5%) was added to dried pollen powder at a ratio of 20:1 (w/v) and was mixed and stirred with ceramic balls for 24 h at 5°C; the mixture was then centrifuged and the supernatant was sterilized by filtration. An extract of Japanese cedar pollen was obtained from the Hollister-Stier Company Ltd, Spokane, WA, USA, through the Committee of Antigen in the Japanese Allergy Association. This extract contained 50% (w/v) glycerin solution, but the procedure by which the extract was prepared is not known.

Evaluation of the quality of pollen extracts

The quality of candidates for standardized cedar pollen extract was evaluated as follows: (i) observation of characteristics and measurement of pH; (ii) existence of cedar pollen antigen by the double gel diffusion test; (iii) existence of amino residue by the ninhydrin test; (iv) existence of glycerin; (v) measurement of the spectrum that absorbs an ultraviolet beam; (vi) measurement of the fluorescent spectrum; and (vii) isoelectrical electrophoresis.

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**Chief of Committee.
These tests were performed by the Common Rule and Common Examination Rule of the Department of Pharmacology, Japanese Government, and also by its standardized guidelines for biological products.

**Measurement of Cry j 1 and Cry j 2 in pollen extracts**

*Cry j 1* was measured by enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies by the Japanese National Institute for Health, National Sagamihara Hospital and by the Torii Pharmaceutical Company Ltd.4,5 *Cry j 2* was measured by ELISA using monoclonal antibodies by the Japanese National Institute of Health and by the Torii Pharmaceutical Company Ltd.

**Measurement of total allergenic activities by RAST inhibition test or ELISA inhibition test**

The total allergenic activities of cedar pollen extracts were measured by the RAST inhibition test6 or by the ELISA inhibition test.7 Sera were obtained from 30 patients who showed a score over 4 in cedar-pollen RAST and had not received immunotherapy with this allergen. Sera were pooled and were used for the inhibition test. The activity of each extract was determined by the FDA method8 using analysis of counts obtained. The RAST inhibition test was performed by the National Sagamihara Hospital and the ELISA inhibition test was performed by the Torii Pharmaceutical Company Ltd.

**Measurement of activities by skin test**

Measurement of activities by skin test was performed by the method of the FDA (USA)9 with some modifications, most notably the determination of the endpoint. In the FDA method, a degree of extract dilution, which results in a total 50 mm flare of shorter diameter plus longer diameter, is determined from a regression line and patients are selected as the slope of the line is equal to or greater than 10 mm and the correlation efficient is greater than 0.85, when over three concentrations were applied. In contrast, in our method, the endpoint was determined by Ishizaki’s criteria,10 where the skin reaction is regarded as positive if the diameter of the wheal is equal to or greater than 9 mm and/or the diameter of the flare is equal to or greater than 20 mm, by measuring the wheal and flare, although serial three-fold dilutions were similarly applied. The concepts of the two methods are completely different. Another point at which our method differs to that of the FDA method is as follows. In our method 0.02 mL extract was injected intradermally into an upper arm, whereas in the FDA method 0.05 mL extract is injected intradermally into the back. It can be assumed that this difference does not affect the final results. With regard to the determination of activity, if a patient’s endpoint occurs at a $3^{10}$ dilution, the endpoint is assigned as 10 and the mean endpoint is calculated for all patients. In the FDA method, if the endpoint (E) is equal to or greater than 9 and less than 11 ($9 \leq E < 11$), the titer of the extract is assigned 1000 Allergy Units (AU)/mL. If the titer is $11 \leq E < 13$ and $13 \leq E < 15$, the titer is assigned as 10 000 and 100 000 AU/mL, respectively. Therefore, the same titer is assigned to extracts of, at most, $3^2$; that is, an approximate 10-fold difference. Skin tests according to the FDA method were performed in 50 adults (15–50 years old), who showed RAST scores over 2, in six clinics (Japan Clinical Allergy Research Institute; Department of Otorhinolaryngology, School of Medicine, Chiba University; Department of Otorhinolaryngology, Dokkyo Medical School; Department of Otorhinolaryngology, Second Hospital, Japanese Medical University; Department of Allergology, National Sagamihara Hospital). However, patients who were receiving systemic steroid therapy, hyposensitization or antihistamine therapy, or who were pregnant or had atopic skin at the test region were excluded as test subjects. In addition, the skin prick test according to Nordic guidelines11 was performed in 20 children in the National Children’s Hospital.

**RESULTS**

At first, it was determined that the standardization of the allergen extract should be done as follows:

1. Select allergen extracts that can be standards. These standards should be representatives of extracts that are clinically and routinely used.
2. Assign titers of standard extracts on the basis of skin tests that have been performed according to certain procedural rules. A titer common in all allergen extracts should be used.
3. Evaluate in vitro determination of titers (RAST inhibition test, measurement of major allergen etc.) and examine whether these methods can supply correct titers that reflect genuine biologic activities of the extracts.
4. Estimate the titer of commercially obtained extracts by certain in vitro methods. If the titer is consistent with that
of the standard extract, within an acceptable range, the titer of the tested extract can be labeled as equal to the standard. This means that the titer of standard extracts is determined by a skin test and, thereafter, is maintained and managed by in vitro methods in place of skin tests.

Standardization of cedar pollen extract

The standardization of cedar pollen extracts was performed according to the policy of allergen extract standardization mentioned above.

Selection of standard cedar pollen extracts

Eight batches of cedar pollen extracts were supplied by the Torii Pharmaceutical Company Ltd as candidates for standard extracts and their qualities were examined from various viewpoints. There were no problems in the analysis of physicochemical and immunochemical characteristics as described in Methods. Furthermore, similar results were obtained for all extracts. It was revealed by measurement of Cry i 1 and Cry i 2 that Cry i 1 and Cry i 2 were both found in all extracts. The difference in the concentration of these components between extract batches was, at most approximately two-fold, and the ratio of Cry j 1 and Cry j 2 was found to be approximately 1:1. With regard to the relative allergenic activity of each batch, as determined by the RAST inhibition test or by the ELISA inhibition test, the difference in activity between batches was; at most, approximately 2-fold, as was the concentration of Cry j 1 or Cry j 2. Furthermore, the correlation coefficient between the relative activities and the concentrations of Cry j in these lots was 0.771, while that between the relative activities and the total concentration of Cry j 1 plus Cry j 2 was 0.931. Thus, good positive correlations were observed. The stability of the extracts and the effects of freezing and thawing on the extracts was also examined. It was revealed that the extracts were very stable, did not lose activity if frozen at temperatures less than -55°C and that there was no change in activity even after freezing and thawing was repeated 10 times. As mentioned, it was confirmed by in vitro tests that there was no problem with the quality of eight batches of extracts supplied by the Torii Pharmaceutical Company Ltd and this was further confirmed by the skin test, as described below. All extracts had adequate activity. Therefore, one batch, called lot G, which had activity close to the mean activity, was selected as the standard cedar pollen extract in our country.

Determination of titers by the skin test

In the skin test, three types of cedar pollen extracts were used: (i) a standard cedar pollen extract (batch C); (ii) a five-fold diluted extract that was prepared quite similarly to the batch in (i), but which is from a different batch; and (iii) an extract from the Hollister-Stier Company Ltd, USA.

The reason for using three different extracts was not only for the determination of the titer of the standard extract, but also for the examination of the correlation between the titer as determined by in vitro methods and by the skin test. The results from skin tests in 35 of 50 patients were selected. The data from 15 patients were eliminated because they showed a positive reaction to a control solution or demonstrated an irregular relationship between the concentration of extract and the size of the reaction, resulting in a difficulty in the determination of the endpoint. As shown in Table 1, the mean values for the endpoint determined by the intradermal skin test were 12.9, 12.4 and 12.7 for (i), (ii) and (iii), respectively. The relative activity of the three extracts was 3:2.9:3:2.4:3:27 = 1:0.58:0.80. In contrast, the relative activity of (i), (ii) and (iii) extracts as determined by the skin prick test in 16 eligible patients was 1:0.55:0.79 (Table 1).

Determination of titer by in vitro tests

The titers of the three types of extracts, (i), (ii) and (iii), were determined by three types of tests, namely the measurement of Cry j 1 by ELISA, the measurement of Cry j 2 by ELISA and the measurement of total allergenic activities by the RAST inhibition test or by the ELISA inhibition test. The data of total allergenic activity was analyzed according to the methods of the FDA.

It was revealed that the original data and all steps for obtaining relative activities were matched to all the statistical rules. The final results are shown in Table 2. Because the total allergenic activities can not be presented as

<table>
<thead>
<tr>
<th>Table 1. Comparison of allergenic potency of three Japanese cedar pollen extracts by intradermal and skin prick tests</th>
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<tbody>
<tr>
<td>Extract</td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>C</td>
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Values in parentheses are the potency ratios of test extracts relative to extract A.
Table 2. Determination of allergenic potency of three Japanese cedar pollen extracts by in vitro assays

<table>
<thead>
<tr>
<th></th>
<th>Extract A</th>
<th>Potency</th>
<th>Extract B</th>
<th>Potency</th>
<th>Extract C</th>
<th>Potency</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cry j 1 (mg/mL)</strong></td>
<td>12.7</td>
<td>2.9</td>
<td>13.6</td>
<td>5.0</td>
<td>15.6</td>
<td>3.9</td>
</tr>
<tr>
<td><strong>Potency</strong></td>
<td>(1)</td>
<td>(0.23)</td>
<td>(0.32)</td>
<td>(0.25)</td>
<td>(0.26)</td>
<td>(0.45)</td>
</tr>
<tr>
<td><strong>Total allergenic potency</strong></td>
<td>1 (1)</td>
<td>0.26</td>
<td>0.35</td>
<td>0.25</td>
<td></td>
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Values in parentheses are the potency ratios of test extracts relative to extract A.

Determination of a unit of titer in the standard cedar pollen extract and standardization of the in vitro titration method

The mean threshold of standard extract determined by the intradermal skin test was 12.7, which is over 11 and under 13. Therefore, the titer is 10 000 AU/mL if determined according to the FDA method. However, the method of skin test used is not completely the same as the FDA method. Therefore, Japanese Allergy Units (JAU) were introduced as a unique unit in our country and the calculation of the titer was performed according to the FDA method. By using this method the titer of the standard extract was officially determined as 10 000 JAU/mL. Next, after the titer of the standard extract has been determined by the skin test, titers of commercially available cedar pollen extract can be maintained and managed by in vitro tests. For this, the measurement of Cry j 1 was used. The amount of Cry j 1 in the standard extract was 13.1, 11.7 and 13.7 µg/mL when determined in three laboratories. The committee considered that the extract contained 12.5 µg/mL of Cry j 1, which is close to the mean value of the data from the three laboratories. Thereafter, a cedar pollen extract can be labeled as having a titer of 10 000 JAU/mL if it contains a similar amount of Cry j 1 with any difference within the acceptable range. The committee determined that the acceptable range should be 7.3–21 µg/mL; that is, a three-fold difference of approximately 12.5 µg/mL. To summarize, the Allergen Committee of the Japanese Society of Allergology made the following decisions regarding the standardization of Japanese cedar pollen extract:

1. Japanese cedar pollen extract, Lot G (prepared by Torii Pharmaceutical Company Ltd), was determined to be the Japanese standard cedar pollen extract.
2. The titer of the standard extract was determined to be 10 000 JAU/mL.
3. The concentration of Cry j 1 in the standard extract was determined to be 12.5 µg/mL.
4. When the concentration of Cry j 1 is within the range 7.3–21 µg/mL in an extract, the titer of that extract can be labelled as 10 000 JAU/mL.

DISCUSSION

For the standardization of allergen extracts, the most important step is the selection of a standard allergen extract. International standards for allergens important worldwide, such as Dermatophagoides pteronyssinus, timothy pollen and ragweed pollen, have already been selected. However, a standard extract for Japanese cedar pollen should be selected from Japan, because the tree is peculiar to this country. It is desirable that the standard allergen extract is representative of extracts routinely used in clinics. As there is only one company in Japan that can prepare allergen extracts, it was thought appropriate to select a representative batch of Japanese cedar pollen extract produced by this company if the extract could be confirmed as having adequate quality. Therefore, eight batches of cedar pollen extract supplied by this company were examined as candidates for the standard extract. All batches supplied were confirmed to have adequate quality. Therefore, a representative batch was selected as the standard extract. For standardization we believe that the titer of the standard extract should first be determined by the skin prick test and then the titer should be maintained and managed by in vitro methods in place of the skin test. The titration of all allergen extract by the skin test may reflect its own biological activity. Furthermore, the unit of the titer thus determined is the same for any kind of allergen and any kind of extract of the same concentration can be expected to produce the same degree of reaction
in any group of patients who are similarly sensitized. However, the skin test gives a large burden to patients and it is difficult to repeat the test. Therefore, after the titer has been determined by the skin test, it is thought best to use in vitro methods that will reflect the in vivo reaction. The JAU (Japanese allergy unit) was newly created as a common unit for all allergens.

For the calculation of the JAU, a skin test was performed according to the method of the FDA with some modifications. Therefore, when other types of allergens are standardized in Japan, allergen extracts that have the JAU titer and allergen extracts from the USA that have the AU titer can be considered to have almost the same potency. Although the relative activity determined by in vitro methods was consistent between the three types of cedar extract examined, the relative activity determined by the skin test showed some discrepancy (Tables 1, 2). This does not necessarily mean that the in vitro estimation does not reflect in vivo activity but, rather, that in vivo reactions to the extract are not necessarily linear and show individual variations. Batch B is different to batch A, even though batch B is the dilution of a batch that was prepared in a manner similar to batch A. In considering this, the results could suggest that in vitro tests reflect the real activity more so than do the in vivo tests.

An error of one-step concentration may easily occur if an endpoint is determined by measuring the diameters of the wheal and flare. Therefore, it may be necessary to determine endpoints as done in the method of the FDA, where optimal lines are obtained from the relationship between the dilution of the extracts and flare diameters.

In order to confirm equal activity of an extract with the standard extract measurement of Cry j by ELISA is used. There are three reasons why this is an appropriate method to use. First, total allergen activity can be only presented as a relative activity to standard activity when the RAST/ELISA inhibition test is used, but it can be presented as an absolute concentration of allergenic protein (μg/ml) when allergen concentration is measured. Second, the RAST/ELISA inhibition test requires a large amount of patient serum, which could be difficult to obtain. Furthermore, when the serum used is changed, the results may be affected. However, when ELISA using a monoclonal antibody is used, the antibody is perfectly homogeneous and can be produced in large quantities for a long period of time and the method itself is simple and has good sensitivity and reproducibility.

Third, when comparing Cry j 1 with Cry j 2, antibodies to Cry j 2 are difficult to obtain and the number of laboratories that can measure Cry j 2 are limited. In contrast, many monoclonal antibodies to Cry j 1 have been prepared and many laboratories are able to measure Cry j 1 at this time. Furthermore, there is no problem with regards to reproducibility of results, as clearly demonstrated in this study. In the USA, where the method of measurement of major allergens is well established, as in the Amb a of ragweed and the Fel d 1 of cat, in vitro tests are used, and for many allergens the RAST/ELISA inhibition test has been changed to the measurement of major allergens. Of course, the representation of the titer of an allergen extract by the concentration of only one kind of major allergen is the minimum necessary to certify the quality of the extract. For the purposes of commercial production of a standardized cedar pollen extract that is of a high quality and has small variations in quality between batches, the measurement of not only Cry j 1 but also Cry j 2 and the total allergenic activity is desirable, as is the use of many different tests to maintain and manage quality of the extract.

The Allergen Committee is now discussing how best to manage the standardized cedar pollen extract and how to distribute it to individuals who may wish to obtain it.

REFERENCES

9 Turkeltaub PC, Rastogi SC, Baer H. Office of biologics research and review skin test method for evaluation of subject sensitivity to standardized allergenic extracts and for assignment of allergy units to reference preparations using the ID_{50}EAL method (Intradermal Dilution for 50 mm sum of erythema determines the allergy unit). Bethesda: FDA publication, 1986.


