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An Investigation on *in vivo* Allergenicity of *Artemisia annua* Leaves and Stems

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SUMMARY **Pollen of *Artemisia annua* is considered to be one of the most important allergens in autumnal hay fever in China, just as ragweed is in North America. In order to clarify the allergenicity of non – pollen containing components of the plant, *Artemisia annua* leaves and stems were collected and extracted before pollination time. The extracts of these pollen – free plant components were studied for allergenic activities using skin prick tests, intradermal tests, intranasal challenge and bronchial provocation tests. In 52 subjects sensitive to *Artemisia* pollen, 92.3% gave positive responses in skin prick tests, 100% gave positive responses in intradermal tests, 66.7% gave positive responses in intranasal challenge and 59.3% gave positive responses in bronchial provocation tests. Negative results to skin prick tests, intradermal tests and bronchial provocation tests were revealed in 30 seasonal asthmatics who gave negative responses to the pollen skin tests. Strict placebo control showed all negative tests in non – atopic adult human volunteers. We concluded that pollen – free plant extracts did have *in vivo* allergenic activities. Analysis of the plant allergenic components *in vitro* will be the subject of further studies.**

Although *Artemisia (Ar.)* pollen is a well-known cause of hay fever in China,[1] patients suffering from pollen allergy often experience symptoms before the pollination season or long after pollen has disappeared from the air. Moreover, about 38% of the patients with allergic rhinitis had episodes of asthma.[2] However, particles of the diameter of *Ar*-pollen (average 20 μm) are too large to penetrate the bronchi and cause allergic reactions in the airways.[3,4] Minute particles are detached from *Ar.* leaves and stems, and become airborne themselves during plant maturation or upon drying by the sun and wind. They may comprise allergenic components in the plant tissues other than pollen. The present investigation was undertaken to study *in vivo* allergenic activities of pollen-free plant extracts.

METERIALS AND METHODS

Plant materials and allergen extract preparation

Leaves and stems of *Artemisia annua* were collected from a field in the west suburb of Beijing on July 15, 1985, shortly before the season of *Ar.* pollination. Thus, they were without pollen contamination. The fresh plant specimens were washed thoroughly in distilled water, cut into small pieces, and then extracted (1:2 W/V) in Coca's solution (0.5% sodium chloride, 0.275% sodium bicarbonate and 0.4% phenol) with continuous stirring at 4°C for 72 hours (pH 7.0). The extract was sterilized by Seitz filtration and stored at 4°C. The filtrate contained 20,600 PNU/ml and platings to assess bacterial and mold contamination were negative.

Subjects

(1). Fifty-two *Ar*-pollen-sensitive patients were chosen by definite positive skin testing and typical histories. There were 41 males and 11 females with an average age of 39 ± 11, ranging from 14 to 74 years old. They were accepted randomly. During the season of *Ar.* pollination, allergic rhinitis was present in 22 patients, asthma alone in 3 and a combination of both in 27 patients. All these patients gave positive responses from + to + + + + in skin intradermal tests to *Ar*-pollen allergen extract, according to Norman's grading system.[5] With

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the pollen-free plant extract, the skin prick and intradermal tests were performed in all of the subjects, intranasal challenge tests were done with 49 patients who had allergic rhinitis and bronchial provocation tests were carried out with 27 seasonal asthmatics. Thirty-three serum samples were analyzed for total IgE.

(2). A second group of thirty patients (17 males, 13 females, average age 42 ± 12, ranging from 12 to 62 years old) who had episodes of asthma in the pollination season but gave negative responses to skin tests with common inhalants were also tested with pollen-free plant extracts by skin prick and intradermal tests, by bronchial provocation and by serum IgE measurement.

(3). As a control, a group of thirty adult volunteers (16 males, 14 females, average age 35 ± 14, ranging from 18 to 71 years old) without known allergen sensitivities were studied using all the tests mentioned above.

Skin prick and intradermal tests

Skin tests were performed with *Ar*-pollen-free plant extracts according to the method described by Norman.[5] The significant skin threshold dilution was determined for each test. The first dilution which gave no positive reaction in the control group was considered as the significant threshold dilution. These were 1:10 for the skin prick test and 1:100 for the intradermal test. After 15 minutes the test sites were inspected. A wheal over 4 mm in mean diameter was considered positive for the prick test and a wheal over 5 mm in mean diameter was for the intradermal test.

Intranasal and bronchial provocation tests

Provocation tests were carried out with *Ar*-pollen-free plant extracts out of the pollination season. The patients were instructed not to take any medication in the 24 hours preceeding the test. Nasal and chest physical examinations were all negative. For intranasal challenge a 10×5 mm^2 paper disc which could absorb about 16 μl of extract was put onto the inferior turbinate. In five minutes all the reactions were recorded by index points. The criteria of the point system are listed in Table 1. To show its reproducibility, the test with 2:1 concentrated extract was repeated for each subject. An average of over 10 points was considered a positive reaction. The bronchial provocation tests were performed according to the standardized procedure.[6] Briefly, Coca's solution (control) as well as the pollen-free plant extract were inhaled in the form of an aerosol by means of a pressurized nebulizer (0.2 kg/cm^2). The forced expiratory 1-second volume ($FEV_{1.0}$) was monitored through an electronic spirometer. Any symptoms or wheezing rales were monitored if they occurred during the test. A drop in $FEV_{1.0}$ of over 20% was considered to be a positive response.

Quantitation of IgE

Serum samples were analyzed for total IgE using a standard ELISA developed in our laboratory and described in a previous paper.[7]

RESULTS

Skin tests

Among 52 *Ar*-pollen-sensitive patients, 48 gave positive prick test reactions (92.3%) to the *Ar*-pollen-free plant extract, while intradermal tests were positive in all (100%). The skin reactions to the *Ar*-pollen-free plant extract closely matched those to the *Ar*-pollen allergen extract (Table 2). All the patients in group II who gave negative skin reactions to *Ar*-pollen also gave negative reactions to the pollen-free plant extracts. In group I, patients with strong skin reactions (++ to ++++) to *Ar*-pollen displayed stronger skin prick and intradermal test reactions to the pollen-free plant extract than those who had only one-plus skin reactions to *Ar*-pollen ($P < 0.01$). However, among those with strong skin reactions to the *Ar*-pollen, there were no statistically significant differences in their skin reactions to the pollen-free plant extract. The negative skin tests of the control group excluded false positive reactions produced by the extracts.

Provocation tests

Table 3 shows the number and percentage of positive reactions in the subjects who received intranasal and bronchial provocation tests. During the provocation tests, all 16

Table 1 The criteria of the point system for responses to intranasal challenge*

	0	1 point	2 points	3 points
Nasal itching	None	Slight	Moderate	Extreme
Sneezing	0	1–5	6–10	Above 10
Mucosa paleness	None	Pink	Slight	Obvious
Nasal blockage	None	Slight	Partial	Total
Watery rhinorrhea	None	Slight	Moderate	Extreme

*When systemic symptoms (itching of eyes, palate, pharynx, tearing, wheezing, etc.) appear, another 3 points were added.

Table 2 Comparison of skin reactions to the pollen–free plant extract among subjects with positive pollen skin reactions

	Group I				Group II	Control
Ar – pollen extract intradermal test	+	+ +	+ + +	+ + + +	Negative	Negative
Number of subjects	4.0	9.0	34.0	5.0	30	30
Pollen – free plant* extract prick test	4.5	8.2	8.3	9.1	<4	<4
Intradermal test*	7.5	10.5	11.0	11.7	<5	<5

*Mean diameter of the wheal in millimeters.

bronchial provocation positive patients developed mild wheezing and expiratory dry rales on chest auscultation. Six of them had nasal itching and running nose simultaneously. In addition, five of them developed late asthmatic episodes. With nasal smear examinations for 32 intranasal challenge positive subjects, eosinophils were present in 62.5 percent. The relative lack of sensitivity to the pollen-free plant extract of group II patients was also reflected in bronchial provocations tests.

Serum total IgE

As shown in Table 4, the control subjects had an average total serum IgE of 585 i.u./ml, while group I patients had 1889 i.u./ml and group II patients had 1412 i.u./ml on average. The difference between patients and the control group was significant ($P < 0.01$). It gave further evidence of the atopic characteristics of the patients, especially for the group II subjects, although they gave negative responses in skin tests with common inhalants.

DISCUSSION

Through this explorative investigation, it is clear that *in vivo* allergenic activities do exist in *Ar*-pollen-free plant extracts. In 1954, Rebhum *et al.*[8] studied the antigenic relationship of the pollen, seeds, and leaves of giant ragweed. This issue has been debated for decades. Busse and his co-workers[9] have further suggested in their study that asthma from ragweed could be caused by airborne allergenic particles smaller than 5 μm in diameter derived from the ragweed stems and leaves instead of the pollen. Shafiee *et al.*[10] were able to isolate AgE from leaves and stems of the short ragweed plant collected before pollination. These workers[11] also isolated two allergenically active fractions from short ragweed leaf cells grown in tissue cultures, but they were unable to detect AgE by immunodiffusion. Baldo and his fellows[12] found that extracts of Plantain leaves and roots reacted in the RAST with sera from some Plantain-pollen-sensitive subjects. However, Lowell[13] was unable to detect allergenic activity in short ragweed pollen-free plant extracts and he suggested that minute particles such as sand, loam, or plant debris lying close to settled pollen grains on the ground could derive allergen activity from the pollen grains by transfer through moisture from light rain or dew. Nor did Larson and Gleich[14] find AgE in ragweed plant prior to the pollination season. Agarwal and his colleagues,[15] nevertheless, have demonstrated AgE activity in short ragweed pollen-free plant parts in their recent study. They[16] also found that ragweed-sensitive individuals showed mild symptoms outside the ragweed pollination season and measurable ragweed allergen activity in filter sheet eluates without pollen counts.

Table 3 Results of intranasal and bronchial provocation tests with *Ar*–pollen – free plant extracts in group I patients

	Number tested	Positive reactions	Percentage
Nasal challenge	48	32	66.7%
Bronchial provocation	27	16	59.3%

Table 4 Total serum IgE

Subjects	Group I (52)	Group II (30)	Control (30)
Serum IgE level (i.u./ml)	1889±30	1412±16	585±81

*Mean ± SEM

They postulated persistent short ragweed plant debris as a source of allergen in the air before and after the pollination season.

We are far from a conclusion. All the studies mentioned above lack further *in vivo* evidence in addition to skin intradermal tests. However, our present study demonstrated allergen activities of *Ar*-pollen-free plant extracts through strictly controlled skin prick tests, intradermal tests, intranasal challenge and brochial provocation tests, and would give further support to the agument for persistent allergen from plant debris.

The components of *Artemisia annua* are rather complex.[17] Apart from the protein allergens that exist in the pollen grains, it is possible that low molecular weight compounds like volatile terpenes may be unique allergen components of the pollen-free plant parts.[18] Other workers[19] have described extraction procedures for terpenes from various plants. As shown in our study, the *Ar*-pollen-free plant extract provoked delayed asthmatic reactions ,after bronchial challenge. Certain low molecular compounds may account for such reactions. Tse, Chan and Chan-Yeung[20] have investigated occupational asthma due to western red cedar. All these points deserve further study.

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