

# Clinical aspects of allergic disease

## Diagnosis of soybean-induced asthma

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**Background:** The role of soybean dust as a causal agent of asthma has been clearly established since the Barcelona asthma epidemics in the 1980s. The large number of patients who were first seen with asthma symptoms during those epidemics provided an excellent opportunity to study the possibilities of different diagnostic tests.

**Objective:** This study was designed to evaluate the usefulness of the skin test and amplified ELISA technique for quantifying specific IgE in the diagnosis of soybean asthma.

**Patients and results:** Ninety epidemic asthmatic patients and 95 nonepidemic asthmatic patients were studied 2 years after the last epidemic. Results of prick tests and ELISAs for specific IgE with hull and dust extracts showed a significant difference between the two groups of patients ( $p < 0.001$ ). Sensitivity and specificity of glycerinated prick test with hull extract in epidemic asthmatic patients were 57.7% and 95.3%, respectively, and ELISA values were 56.6% and 93.7%, respectively. Similar results were obtained with dust extracts.

**Conclusion:** Glycerinated skin prick tests and ELISAs with soybean hull and dust extracts have proved effective in the diagnosis of soybean asthma, even 2 years after the epidemics. Taking into account the sensitivity (90.5%) and specificity (93.7%) of ELISA test results for epidemic asthmatic patients found when the epidemic occurred, data from this study suggest that both tests may be very useful for the diagnosis of soybean dust-induced asthma.  
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**Key words:** Asthma, ELISA, soybeans, skin test

Until studies of asthma epidemics related to unloading of soybeans in the port of Barcelona were published in 1989,<sup>1,2</sup> very little was known about the association between soybean dust and asthma. It is now accepted that bronchial hyperreactivity may be the result of inhalation of soybean dust,<sup>3</sup> and the antigens involved have even been identified and characterized.<sup>4-6</sup> Because soybeans are produced extensively worldwide, it would seem logical to assume that further cases of asthma may

### Abbreviations used

|        |                                |
|--------|--------------------------------|
| EAPs:  | Epidemic asthmatic patients    |
| ED:    | Emergency department           |
| NEAPs: | Nonepidemic asthmatic patients |

occur as a result of sensitivity to these antigens during harvesting, transportation, and storage. Indeed, other epidemic cases have already been reported,<sup>7,8</sup> and high concentrations of allergens, similar to those determined in Barcelona during the epidemic days, have been detected in other cities where asthma epidemics have been occurring for quite some time.<sup>9</sup>

In the diagnosis of soybean-induced asthma, the in vitro determination of soybean-specific IgE antibodies has proved to be a good marker of the illness. In fact, when determining specific IgE by an amplified ELISA method in a group of epidemic asthmatic patients (EAPs) in a previous study, we found 90.5% positivity, which is similar to the 84.9% obtained by radioimmunoassay with the

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same extracts.<sup>2</sup> On the other hand, skin tests are also known to be very useful tools in the diagnosis of asthma and other allergic conditions, because a good correlation with the *in vitro* determination of specific IgE has been demonstrated for other antigens.<sup>10</sup> Nevertheless, the diagnostic role of skin tests varies according to the antigenic product studied, the antigenicity, preparative conditions, optimal protein concentration, susceptibility of the extract to breakdown, and the conditions required for conservation. All of these factors should be kept in mind to obtain optimal diagnostic utility, because each may vary from one type of extract to another.

The number of patients who were first seen with asthma symptoms during the epidemic in Barcelona provided an exceptional opportunity to study the diagnostic role of skin tests in soybean-induced asthma by assessing the validity and performance of skin tests, as well as that of specific IgE to the same antigens as determined by an amplified ELISA method.

## METHODS

### Study groups

We studied 90 EAPs who were treated for acute severe asthma in the emergency departments (EDs) of Barcelona city hospitals during an asthma epidemic day.<sup>1</sup> The mean age was 50.12 years, and 54% of patients were male. As a control group, 95 asthmatic patients treated for acute severe asthma in the same ED on a nonepidemic day (NEAPs) were also studied<sup>1</sup>; the mean age was 45.29 years, and 40% of these patients were male.

Both groups of patients were selected from a previous, wider study of 169 EAPs and 149 NEAPs.<sup>11</sup> The cases included represent 53.2% and 63.9%, respectively, of the original sample, and patients are all those for whom the tests reported in this study were simultaneously performed. The fact that in this study only those subjects who had undergone all the tests were selected has probably introduced no bias, because the results obtained in each test were very similar to those found for this same test in the total population. All patients and control subjects were residents of Barcelona and over 14 years of age; no notable increase in hospital admissions of persons under this age was observed during the epidemics.

All patients who attended the ED on an epidemic day were defined as soybean dust-sensitive. With the aim of making this definition more specific, we also studied a group of patients with asthma who had attended the ED on more than 1 day considered an epidemic day (*i.e.*, a "re-incident" group).

Studies were carried out in both groups of patients at least 2 years after the last epidemic when the possible escape of soybean dust into the atmosphere during

unloading had been controlled. A preliminary study was conducted in a group of 10 EAPs to determine the midpoint doses to be used as the ideal extract dilutions for prick tests.<sup>12</sup>

ELISA study of the persistence of specific antibodies to hull and dust extracts in sera of 21 of the 90 EAPs, extracted in 1987 during the last epidemic (*i.e.*, 2 years before the start of this study), has been useful for comparing current positivity with that obtained in 1989 in the same patients. In this study both samples were analyzed in blinded fashion simultaneously.

The protocol of this study was approved by the board for human studies of our institution, and informed written consent was obtained from the subjects.

### Preparation of antigen extract

Extracts of hulls and dust from soybeans stored in Barcelona silos from ships loaded in Toledo, Ohio, were prepared. One hundred grams of each material was defatted with petroleum ether at 37° C in a Soxhlet apparatus (Corning Ltd., Stone, Staffordshire, England) for 1 hour and then air-dried. The extraction was performed (1:20 wt/vol) with 0.2 mol/L of ammonium bicarbonate, pH 9, at 4° C overnight with gentle shaking. The supernatants were isolated by centrifugation at 600 g for 15 minutes and dialyzed extensively against deionized water at 4° C for 2 days with a 3,500 MWCO membrane (Spectra/Por MWCO 3,500; Spectrum, Houston, Texas). Protein concentration determination by bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, Ill.) was performed after centrifugation at 600 g at 4° C for 20 minutes or until transparent solutions were obtained. Final concentrations of proteins obtained for the extracts of hull and dust were 1160 µg/ml and 1550 µg/ml, respectively.

### Preparation of different antigen extract solutions for prick tests

For both antigen extracts, hull and dust, an initial solution with a protein concentration of 1 mg/ml was used, and four serial dilutions at a ratio of 1:10 were performed. The same solutions were prepared with 50% sterile glycerine.

### Prick tests

The skin tests were carried out by the same person with the usual technique<sup>13</sup> throughout the study. A positive (histamine) test and a negative (saline solution) test were performed at the same time as the soybean tests. Reading was blinded at 15 minutes, and the test result was considered positive when the mean of the maximum diameter of the wheal and its perpendicular was equal to or greater than 3 mm. All prick tests with unglycerinated extracts were performed before the fifteenth day of preparation. Tests with glycerinated extract were carried out within 60 days of preparation.

**TABLE I.** Prick and amplified ELISA test positivity (2 years after preventing escape of soybean dust during unloading) with hull extract

|                           | Prick test     |              | ELISA |
|---------------------------|----------------|--------------|-------|
|                           | Unglycerinated | Glycerinated |       |
| EAPs<br>( <i>n</i> = 90)  | 29*            | 52*          | 51*   |
| Sensitivity               | 32.2%          | 57.7%        | 56.6% |
| NEAPs<br>( <i>n</i> = 95) | 3              | 5            | 6     |
| Specificity               | 96.9%          | 94.8%        | 93.7% |

\**p* < 0.001 between EAPs and NEAPs.

### Detection of specific IgE to soybean hull and dust extracts

Specific IgE to soybean hull and dust extracts was evaluated by an amplified ELISA method with a biotin-streptavidin-amplified system (Stravigen BSA; Biogenex Laboratories, San Ramon, Calif.), based on the modified method of Metzger et al.<sup>14</sup> With a minimum of 4 washes with phosphate-buffered saline–0.005% Tween-20 (Sigma Chemical Co, St. Louis, Mo.) intercalated between stages, the method was performed as follows: (1) 100  $\mu$ l/well of 10  $\mu$ g/ml of the aqueous extract of soybean hull and dust in carbonate-bicarbonate 0.1 mol/L, pH 9.6 buffer were coated in flat-bottomed high-affinity microtiter plates (Nunc, Copenhagen, Denmark), overnight at 4° C; (2) the wells were blocked with 350  $\mu$ l/well phosphate-buffered saline–1% bovine serum albumin (Sigma Chemical Co.) for 1 hour at 37° C; (3) 100  $\mu$ l/well of the undiluted samples was incubated in duplicate for 1 hour at 37° C; (4) 100  $\mu$ l/well of mouse monoclonal anti-IgE at a concentration of 2  $\mu$ g/ml was incubated for 2 hours at 37° C; (5) 100  $\mu$ l/well of mouse biotin-anti-Ig was incubated in diluent buffer for 30 minutes at 37° C; (6) 100  $\mu$ l of 1:5 dilution streptavidin-peroxidase was added to each well and incubated for 30 minutes at 37° C; (7) 100  $\mu$ l of tetramethylbenzidine solution in 3% H<sub>2</sub>O<sub>2</sub> was added to each well and incubated for 30 minutes in darkness and at room temperature; (8) the reaction was stopped with 30  $\mu$ l/well of 2 mol/L H<sub>2</sub>SO<sub>4</sub>; (9) the final reaction was read at 450 nm with an ELISA microplate reader (Titertek Multiskan; Flow Laboratories, Helsinki, Finland). Results were expressed as optical density units at 450 nm. All determinations of specific IgE were performed in blinded fashion by the same person.

The optical density in the control group was zero; hence, any number greater than zero was considered a positive result.

### Evaluation of the diagnostic tests

Differences in prevalence of positive prick test and ELISA results for each extract between the two groups

**TABLE II.** Prick and amplified ELISA test positivity (2 years after preventing escape of soybean dust during unloading) with dust extract

|                        | Prick test     |              | ELISA |
|------------------------|----------------|--------------|-------|
|                        | Unglycerinated | Glycerinated |       |
| EAPs ( <i>n</i> = 90)  | 26*            | 48*          | 52*   |
| Sensitivity            | 28.8%          | 53.3%        | 57.7% |
| NEAPs ( <i>n</i> = 95) | 5              | 7            | 7     |
| Specificity            | 94.8%          | 92.6%        | 92.6% |

\**p* < 0.001 between EAPs and NEAPs.

of patients were detected with the chi square test. Comparisons of sensitivity and specificity between prick test and ELISA results for each extract were made by the two-sample test for binomial proportions (normal theory test).<sup>15</sup>

## RESULTS

### Prick test: Midpoint dose

A very preliminary study with 10 EAPs suggested that the midpoint dose was located between dilutions of 1:10 (100  $\mu$ g/ml) and 1:100 (10  $\mu$ g/ml) of the two antigenic extracts because at these dilutions there was a sharp increase in response.<sup>12</sup> Thus we began the study with both dilutions in the first 59 EAPs and 64 NEAPs. Concordance of results between the two dilutions showed 90.6% and 98.1%, respectively. Pearson's correlation coefficients were *r* = 0.6156 and *r* = 0.5732 (*p* < 0.01 for both). Despite the good concordance obtained for both extracts with both dilutions, individualized analysis of data showed the 1:10 dilution to be optimal.

### Positivity of the prick test and the ELISA

Positivities of the prick test at the 1:10 dilution and the ELISA assay with regard to both hull and dust extracts are shown in Tables I and II. It can be seen that all the tests studied discriminated significantly between EAPs and NEAPs (*p* < 0.001). In the EAP group, with regard to the hull extract, we observed the prevalence of positivity of the prick test when the glycerinated extract was used to be higher than that when the unglycerinated extract was used (57.7% vs 32.2%, *p* < 0.001). The same difference was found in positivity for the prick test with unglycerinated extracts and the ELISA (32.2% vs 56.6%, *p* < 0.001). Nonsignificant differences were observed between ELISA results and prick test results when glycerinated extract was used. With respect to the dust extract, it was also

observed that positivity to the prick test with glycerinated extract was higher than that with the unglycerinated extract (53.3% vs 28.8%,  $p < 0.001$ ), and again, that positivity of the ELISA results was higher than that obtained with the unglycerinated prick test.

In the 21 EAPs included in this study (Table III) in whom specific IgE was determined in sera extracted 2 years earlier during the last epidemic (1987), hull extract sensitivity as determined by ELISA was 90.5% (19 of 21) compared with the present 56.6% (14 of 21). This latter result is the same as that found in this study for a total group of 90 EAPs (56, 6%) and shows sensitivity of the ELISA method, when performed at the time of diagnosis, to be higher than that obtained at 2 years. The specificity of this method, calculated in 95 NEAPs, was 93.7% (Table I).

Positivities obtained in the "re-incident" EAP group with hull extracts were: 9 of 23 (39%) for the prick test without glycerine, 13 of 19 (68%) for the prick test with glycerine, and 14 of 23 (61%) for the ELISA; these results do not differ significantly from those obtained in the total EAP group (Table III), which supports the theory that the population defined as EAPs was, in effect, sensitized to soybean antigens.

## DISCUSSION

Because many persons throughout the world are exposed to inhalation of soybean dust, it seems logical to assume that in some of these cases, asthma might be, at least in part, the result of contact with soybeans. Validated diagnostic methods are required to confirm this relationship. During the 26 epidemics of asthma caused by inhalation of soybean dust, which occurred as a result of the unloading of soybeans in the port of Barcelona between 1981 and 1987, 678 asthmatic patients were treated in the EDs of city hospitals, many of them on more than one occasion.<sup>1</sup> These epidemics provided an exceptional framework for assessing the role of soybean dust as a cause of asthma, which in some cases was severe or even fatal.

The large number of patients affected by these epidemics enabled us to evaluate different techniques in the diagnosis of this entity. A case-control study of this population<sup>2</sup> showed the radioimmunoassay technique with commercial soybean extracts to be sensitive and specific enough to discriminate between EAPs and control NEAPs: positivity was 77.4% in EAPs compared with 4.6% in NEAPs. The percentage of positive results was even higher when antigens prepared from soy-

**TABLE III.** ELISA test positivity to hull extract in EAPs: Patients tested in 1989 and in 1987 (during unloading)

| Year        | ELISA Positivity         |                  |                        |
|-------------|--------------------------|------------------|------------------------|
|             | Tested on both occasions | Total population | "Re-incident" patients |
| 1989* (no.) | 14/21                    | 51/90            | 14/23                  |
| Sensitivity | 56.6%                    | 56.6%            | 68%                    |
| 1987* (no.) | 19/21                    |                  |                        |
| Sensitivity | 90.5%                    |                  |                        |

As stated in Table I, the specificity was 93.7%.

beans unloaded in Barcelona were used (i.e., 84.9% vs. 5.8%, respectively).<sup>2</sup>

The high sensitivity (90.5%) obtained with the ELISA method in the evaluation of specific IgE to hull extract in the sera of 21 patients in 1987 has been shown to be somewhat higher than that obtained by radioimmunoassay (84.9%), but specificity was similar (93.7% vs 90.5%). These data permit us to assume that the best technique for in vitro diagnosis of soybean-induced asthma is the amplified ELISA. Thus in this study the ELISA method was used to compare its diagnostic efficacy with that of the skin test. For better interpretation of the performance of the tests, it must be remembered that this study was performed 2 years after measures to avoid the release of soybean dust from ships into the atmosphere had been introduced and the epidemics had ended.

In this study we have been able to show that the skin test with glycerinated extract has a diagnostic performance similar to that of the amplified ELISA (Table I) and significantly differentiates EAPs from NEAPs ( $p < 0.001$ ). According to these results and those obtained in patients studied during the last epidemic in 1987 (Table III), in which determination of specific IgE to soybean hull by ELISA was positive in 90.5% of EAPs compared with 6.3% positivity in NEAPs, it may be assumed that if this study had been conducted at the time of diagnosis, a percentage of positivities similar to those obtained with ELISA would have been obtained with the prick tests carried out with glycerinated extracts.

Moreover, if we consider the probability that a small number of patients attending the ED on epidemic days had symptoms of asthma on that day for other reasons and that a small number of patients with asthma attending the ED on nonepidemic days could have been sensitized to soybean (although exposure to soybean did not provoke

that particular attack), it may be assumed that the real performance of the prick tests and ELISAs could be even better, and that may, in part, explain why sensitivity nearer to 100% was not obtained.

Although the better performance of glycerinated extracts compared with nonglycerinated extracts may be considered to be due to the conserving effect of glycerine, which would impede breakdown of soybean proteins by stabilizing soybean allergens, data from this study and a previous study (Morell et al. Unpublished data) permit preliminary attribution of an adjuvant effect to glycerine, which enables its sensitivity to approach that of in vitro methods. In any event, further studies are required to confirm this assertion.

To date, studies with skin tests and ELISAs in soybean-induced asthma have been sporadic<sup>16-18</sup>; hence, our study appears to be the first carried out prospectively in a large number of patients and control subjects with the aim of assessing these routine diagnostic techniques in this type of asthma. The fact that the results obtained in the group that attended the ED on an epidemic day on more than one occasion ("re-incidents") do not vary significantly from those obtained in the total EAP population (Table III) supports the hypothesis that patients who attended the ED on an epidemic day (EAPs) should really be considered sensitized to soybean proteins.

In view of these results, it seems that the practice of skin prick testing and determination of specific IgE antibodies by ELISA techniques can be strongly recommended for the diagnosis of soybean-induced asthma. The ELISA alone would be indicated when skin tests are unreliable because of dermatographism or drug interference, or when positive and negative prick controls fail, as well as in cases in which the result is doubtful or patients require greater response precision and quantification. This technique would also permit better quantified follow-up of sensitization to soybean in these patients with asthma.

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