

Identification and partial characterization of the soybean-dust allergens involved in the Barcelona asthma epidemic

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Asthma epidemics in Barcelona, Spain, have been attributed to dust generated by the unloading of soybeans in the harbor. Sera of four different groups of 10 subjects in each group were studied: (1) patients attending an emergency room in Barcelona for an asthma attack on epidemic days, group A, (2) patients attending an emergency room for an attack on nonepidemic days, group B, (3) patients with asthma from other cities, group C, and (4) patients without asthma from Barcelona matched by age and sex with group A, group D. All subjects in group A had IgE to allergens in extracts of various soybean samples. In contrast, only one of the 10 subjects in each of groups B and C and none of those subjects in group D had IgE to uncleaned bean and hull extracts. Radioimmunoassay demonstrated that in sera from patients with asthma during an asthma outbreak reacted primarily to soybean hull and dust extracts. Sodium dodecyl sulfate-polyacrylamide and gel electrophoresis thin-layer isoelectrofocusing demonstrated protein bands of 97.4 to <14.4 kd and isoelectric point between 6 and 3.5. By Western blot and thin layer isoelectrofocusing/blotted radioimmunoisoelectrofocusing, IgE of patients with asthma during an asthma outbreak reacted weakly to two protein bands of molecular weight ranging from 42 to 21 kd, strongly to glycoprotein bands with molecular weight <14.4 kd, and isoelectric point <6, which appeared to be the major allergens. (J ALLERGY CLIN IMMUNOL 1990;85:778-84.)

Since 1981, 26 asthma outbreaks in Barcelona, Spain, have involved 688 subjects who have had 958 hospital admissions. More than 20 deaths were registered during these outbreaks (unpublished data). Space and hourly time-clustering analysis suggested a relationship with the unloading of soybeans from ships in the harbor.¹ This conclusion was supported by preliminary immunologic studies² and later by a

Abbreviations used

BRIEF:	Blotted radioimmunoisoelectric focusing
BSA:	Bovine serum albumin
PBS:	Phosphate-buffered saline
RIA:	Radioimmunoassay
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TLIEF:	Thin-layer isoelectrofocusing
MW:	Molecular weight
pI:	Isoelectric point
IEF:	Isoelectric focusing

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case-control study that confirmed the presence of high levels of specific IgE to soybean in 84.9% of sera from patients with asthma during an asthma outbreak and in only 5.8% of control subjects.³ Preliminary studies have been performed on the identification and characterization of soybean allergens that may cause asthma,⁴⁻⁶ and the allergens associated with acute allergic reactions from eating soybeans have been investigated recently.⁷

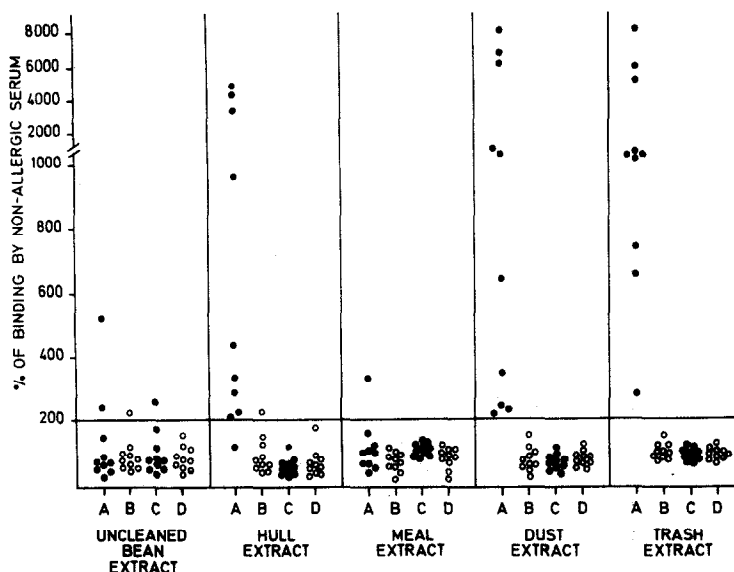


FIG. 1. RIA results for the four study groups. *A*, Patients with asthma during the asthma epidemic in Barcelona; *B*, Patients with asthma during nonepidemic days in Barcelona; *C*, Patients with asthma from another Spanish city; *D*, normal Barcelona residents without asthma. Ordinate represents the percent binding of the IgE antibody with the test sera compared to negative nonexposed serum pool. The abscissa represents the extracts from different soybean sources.

We report our studies on the evaluation of specific IgE by RIA in the sera of patients with asthma during an asthma epidemic to extracts of crude soybeans, cleaned soybeans, soybean flakes and meal, soybean hulls, dust from soybeans, and material left over from cleaning the beans, called trash. Cross-reactivity of allergens in these extracts was studied by RAST-inhibition analyses. Allergens were characterized by TLIEF and SDS-PAGE, followed by capillary blotting and electroblotting, respectively, onto nitrocellulose membrane, followed by incubation with IgE containing sera and a radioiodinated anti-IgE. The major allergens appear to be glycoproteins with an MW <14 kd found primarily in the hulls and dust.

MATERIAL AND METHODS

Patients

The sera of four different groups of 10 subjects each, all older than the age of 14 years, were studied. Group A consisted of patients who had been treated for acute asthma in the emergency rooms at one of the five major city hospitals on 2 or more epidemic days. Group B was similar patients with asthma who attended the emergency rooms on a nonepidemic day. Group C was patients with asthma from Spain outside the city of Barcelona. Group D was normal residents of Barcelona without asthma. Groups B, C, and D served as control subjects. Groups B and D were matched with Group A for age, sex, and geographic residence within the city. Group C was matched with Group A for age and sex only. In addition, 250 ml of plasma from 10 patients with asthma was used to compare and characterize the an-

tigen as described below. Some, but not all, of these plasma donors were in Group A. Also, a pool of sera from patients from Minnesota with systemic allergic reactions after eating soybeans was included for a positive soybean IgE antibody control.

Antigen extracts

We prepared extracts of bulk uncleaned soybeans, cleaned soybeans, hulls, processed soybean meal and flakes, dust and trash (the residue left after the beans were cleaned) from soybeans unloaded from the ship *Argus Traveler* during the epidemic days, Sept. 4 and 7, 1987. These beans had been loaded in Ohio. We also prepared extracts of uncleaned soybean, hulls, and processed meal from another ship, *Maritime Baron*, that unloaded during a nonepidemic day. These beans had been loaded in Brazil.

Batches of each material were defatted with petroleum ether in a Soxhlet (Corning Ltd., Stone, Staffordshire, England) apparatus for 4 hours and dried. Then, extraction was performed with 0.2 mol/L of ammonium bicarbonate, pH 9, at 4° C for 18 hours with gentle shaking. The supernatants were isolated by centrifugation (600 g for 15 minutes) and dialyzed exhaustively against deionized water at 4° C for 2 days. Total protein content was determined by bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, Ill.) after centrifugation at 20,000 rpm at 4° C for 10 minutes or until transparent solutions were obtained.

Identification of IgE antibodies against soybean extracts

Allergens were absorbed to wells of Immulon II microtiter Removawell strips (Dynatech, Alexandria, Va.). Each

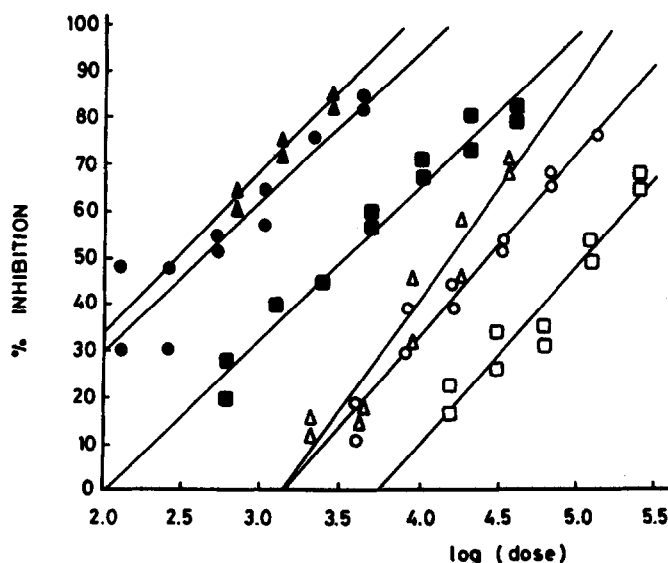


FIG. 2. Inhibition of IgE antibody binding to soybean-dust extract as the solid-phase antigen by other extracts. Fluid-phase inhibition was by the following extracts: dust ($\blacktriangle$), hull ($\bullet$), trash ($\blacksquare$), meal ($\triangle$), clean bean ($\circ$), and flakes ($\square$). The 50% inhibition dose for each of the different extracts is 0.25, 0.32, 3.2, 15.8, and 126.0, respectively.

well received 1 μ g of protein in 100 μ l of 200 mmol/L of sodium bicarbonate buffer titrated with 200 mmol/L of sodium carbonate to pH 9.2. The plates were incubated overnight at room temperature in a humid box and washed five times with 0.1 mol/L of phosphate buffer, pH 7.5, and 1% Tween 20. Fifty microliters of undiluted serum to detect IgE was added to each well, along with 50 μ l of buffer. Samples were incubated overnight and washed. After addition of 100 μ l of rabbit ^{125} I-labeled antihuman IgE, the plates were incubated overnight, again washed, and counted in a gamma counter. Binding of radioiodinated anti-IgE antibodies that was 200% above the normal control serum pool was interpreted as positive but borderline, and >400% was interpreted as specific proportionately elevated titers of antibody. The donors of this normal serum pool were not the same individuals as in Group D.

Cross-inhibition studies

The different extracts were compared by solid-phase RIA-inhibition analysis.^{8,9} The procedure was performed by adding 50 μ l of an undiluted pool of plasma from the allergic patients with asthma during the epidemic, along with 50 μ l of various extracts in different concentrations to wells of microtiter plates coated with one or another of the extracts. The reactants were incubated overnight and washed. One hundred microliters of ^{125} I-labeled antihuman IgE was added, the plates incubated again overnight, rewashed, and counted by a gamma counter. The relative potencies of allergenic extracts were compared by the quantity of each extract needed to produce 50% inhibition. In addition, the inhibition lines have a measurable slope, and comparison

of the slopes of the extracts is a measure of allergenic relatedness. The results were evaluated with a covariance analysis program with a Hewlett-Packard (Palo Alto, Calif.) computer.

Electrophoresis of extracts

SDS-PAGE was carried out in 13 cm long, 12.5% acrylamide gels with the discontinuous buffer system of Laemmli.¹⁰ Antigen extracts containing 39 to 42 μ g of protein were treated with 5% mercaptoethanol and applied in a 3% stacking gel. Electrophoresis was carried out at 20 mA per gel until the tracing dye reached the bottom of the gel. After electrophoresis, gels were either stained with Coomassie brilliant blue (Bio-Rad Laboratories, Richmond, Calif.) or periodic acid Schiff stain, or the protein bands were transferred to nitrocellulose membranes according to the method of Towbin et al.¹¹

Transfer was effected in a water-cooled (10°C) Bio-Rad Trans-blot apparatus (Bio-Rad Laboratories) at a constant voltage of 30 V for 18 hours or at room temperature at 60 V for 3 hours. Blotted gels were stained with Coomassie brilliant blue to evaluate effectiveness of the transfer. After transfer, nitrocellulose membranes were washed in PBS containing 0.5% BSA for 1 hour to saturate additional protein-binding sites.

Sera were diluted 1:3 in PBS with 5% BSA buffer (10 ml volume) and incubated with blotted membranes overnight at room temperature with constant agitation. Serum was then removed, and each membrane was washed three times with PBS containing 0.5% BSA, and PBS respectively. Each membrane was then incubated with ^{125} I-labeled

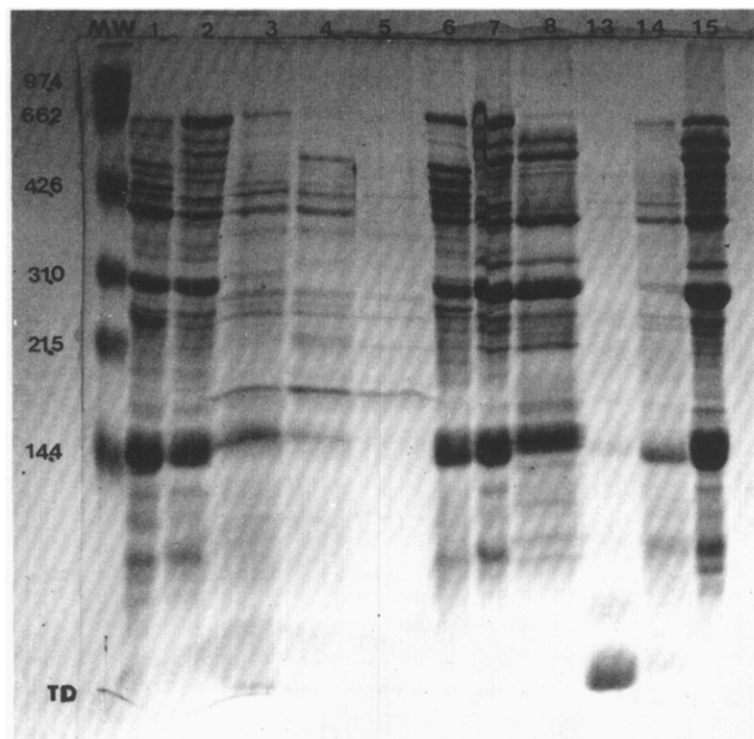


FIG. 3. Coomassie brilliant blue stain of 12.5% SDS-PAGE gel of soybean extracts. *Lanes 1 and 2, unclean beans (39 and 39 μ g); lanes 3, 4, and 5, hulls (41, 40, and 40 μ g); lanes 6 and 7, cleaned beans (39 and 42 μ g); lane 8, meal (40 μ g); lane 13, dust (40 μ g); lane 14, trash (41 μ g); and lane 15, flakes (39 μ g); TD, tracking dye.*

antihuman IgE (20 ng/ml) in a final volume of 10 ml for 16 hours with constant agitation. Membranes were then washed as above, blotted dry, and wrapped in polyethylene film. Autoradiographs were prepared by incubating membranes with x-ray film (Kodak X-Omat, Eastman Kodak Co., Rochester, N.Y.) and exposing in a cassette equipped with a Cronex intensifying screen (Dupont Co., Wilmington, Del.) for 6 to 18 hours at -70°C . Nonspecific binding of anti-IgE was determined with a serum pool prepared from four subjects with asthma who lived in a nonepidemic area. These donors were not the same individuals as group C.

TLIEF/BRIEF

TLIEF (PAG plate; LKB Instruments, Bromma, Sweden, pH 3.5 to 9.5) was performed with the Multiphor system (LKB Instruments). Antigen extracts were applied at the cathode margin and run under constant current for 45 minutes at 10°C ; wicks containing the antigen were removed, and electrofocusing was continued at 900 V for an additional 60 minutes. After electrofocusing, the gel was removed and either stained with Coomassie brilliant blue or the proteins were transferred by capillary action to nitrocellulose membranes.¹² Transfer was carried out for 1 to 3 hours. After the blot, the membrane was carefully removed from the gel,

and IgE-binding zones were determined with the same procedure as outlined for SDS-PAGE/immunoblotting.

RESULTS

Measurement of specific IgE antibodies

Solid-phase RIA demonstrated IgE antibodies to allergens in hull extracts from both lots of soybeans; nine of 10 (90%) sera from patients during the asthma epidemic had levels $>200\%$ of the serum pool from normal control subjects. Five of these subjects had a level $>400\%$, and three of these five subjects demonstrated 10 times the values of normal control subjects. One Barcelona patient with asthma (group B) demonstrated low-level binding to uncleaned bean and hull extract, and one non-Barcelona patient with asthma (group C) demonstrated low-level binding to uncleaned bean extract (Fig. 1).

Similar results were obtained with dust and trash, of which 100% of the sera during the epidemic but none of the sera from control subjects demonstrated levels $>200\%$ of the results obtained from the serum pool of normal subjects.

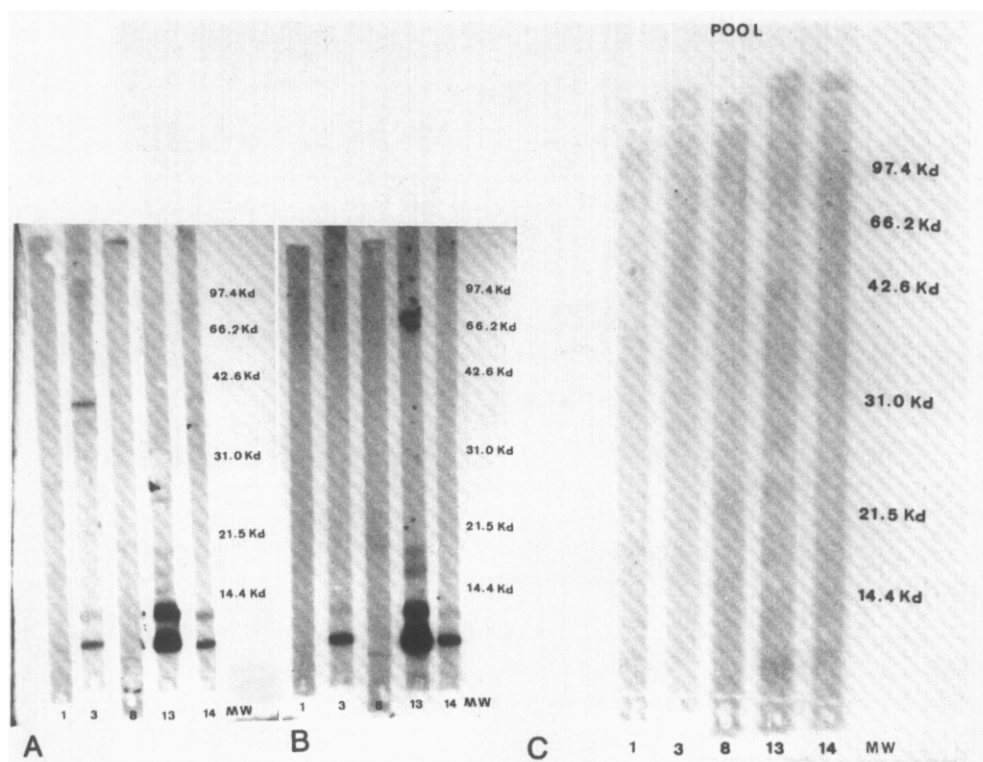


FIG. 4. A-B, Autoradiograph of an SDS-PAGE/immunoblot of soybean-extract preparations with plasma from two patients with asthma from an epidemic area. **C,** A plasma pool from four subjects with asthma from a nonepidemic area and radiolabeled anti-IgE. 1, Unclean beans (39 μ g); 3, hulls (41 μ g); 8, meal (41 μ g); 13, dust (40 μ g); 14, trash (41 μ g). MW, standard markers.

The sera of only two patients proof positive to uncleaned soybean extract on epidemic days demonstrated positive results to uncleaned soybean extract. Moreover, only one patient during the asthma epidemic was found to be positive to meal and flake extracts.

Cross-inhibition studies

With dust extract absorbed to the wells as a solid phase, we compared the inhibition of the same extract with extracts of hull, meal, dust, unclean soybean, trash, clean beans, flakes, and meal as fluid-phase inhibitors.

The slopes of the inhibition curves were similar. The dust and hull contained about the same concentration of the significant allergens, but the trash and cleaned beans, meal, and flakes contained considerably less (Fig. 2). We detected no differences between extracts prepared from materials obtained from the *Argus Traveler* or from the *Maritime Baron* (data not presented).

SDS-PAGE/immunoblot

Approximately 40 μ g of protein of the various extracts was applied to a 12.5% SDS-PAGE gel (Fig.

3). Although considerable differences in the intensity of Coomassie brilliant blue–stained bands were demonstrated, multiple stained bands were evident in all extract preparations. The dust extract demonstrated predominant bands <14.4 kd MW marker but remained above the tracking dye front. Major staining bands appeared at approximate MWs of 42.6, 31, and 14.4 kd in all extracts and only faintly in the hull and dust extracts (lanes, 3, 4, 5, and 13, respectively).

For immunoblot analysis, we selected representative extract preparations of unclean beans, hulls, meal, dust, and trash extracts. Autoradiography of immunoblot analysis of these extracts with serum from 10 different subjects from the epidemic area and radiolabeled anti-IgE (Fig. 4) demonstrated two patterns: (1) faint bands of MWs of 21.5 to 42 kd, but especially two distinct radiostained bands in the dust extract (lane 13) corresponding to the <14.4 kd Coomassie brilliant blue– and periodic acid-Schiff–stained bands in SDS-PAGE. Similar radiostained bands at the lowest MW position for the dust extract were identified in both the hull and trash extracts and (2) only the protein bands of MWs <14.4 kd to dust, hulls, and trash extracts. Specific binding of IgE for the same group of extracts was supported by the absence of radio-

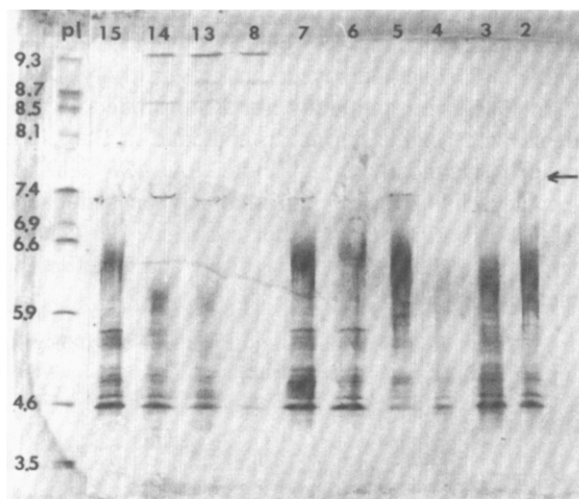


FIG. 5. Coomassie brilliant blue stain of TLIEF gel (pH, 3.5 to 9.5). Legend as in Fig. 4. Arrow indicates application site.

stained IgE-binding proteins with a plasma pool from four individuals with asthma and not during the asthma epidemic from Group B. When we tested serum from individuals with soy anaphylaxis after eating, radiostained bands appeared in high (>21.5 kd) zones of the transfer; however, no radiostained bands were evident in the 14.4 kd or less zone of the transfer (data not presented).

TLIEF/BRIEF

Ten plasmas were studied by immunoblot analysis after an IEF/capillary blot protocol of the five extracts described for SDS-PAGE/immunoblot method. Multiple Coomassie brilliant blue-stained bands were observed principally for hull, dust, and trash with pI <6 . Only a few cathodic bands were detected at pIs of 8.4 to 9.3 in meal dust and trash extracts. The extracts that demonstrated faintest protein bands were unclean beans and meal (Fig. 5). Autoradiography of the blotted radioisoelectrofocusing pattern demonstrated several radiostained bands in all but the unclean bean extracts (Fig. 6) to the acidic components, especially pI 3 to 5. Predominant radiostained bands were demonstrated at pIs of 6.55, 5.85, 5.20, and 4.55 for both hull and dust extracts.

DISCUSSION

The asthma epidemics detected in Barcelona were attributed both epidemiologically² and by RAST in a case-control study³ to the unloading of soybean in the port. Our present studies in patients and in three different control groups have demonstrated the positivity of RAST to be associated with an allergen predominantly located in hulls, dust, and trash. The RIA for

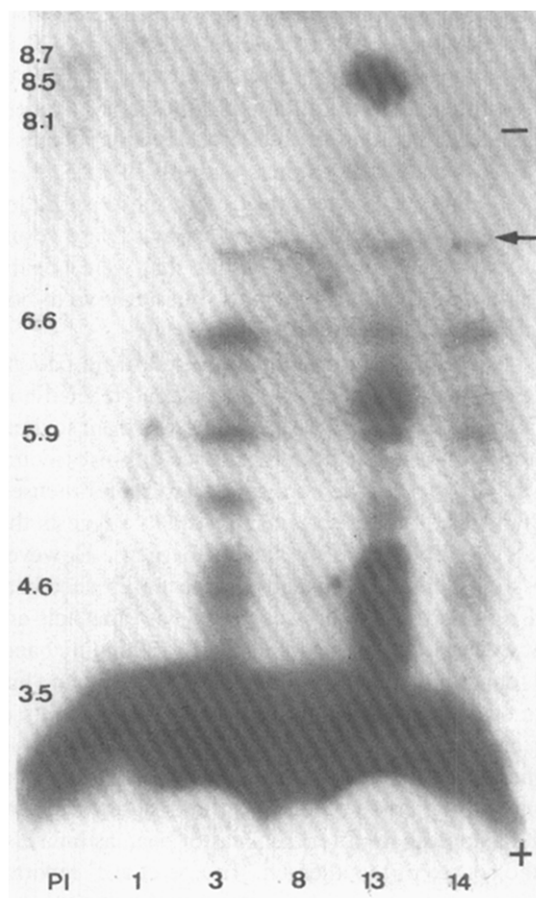


FIG. 6. Blotted BRIEF pattern of various soybean-extract preparations with plasma from a patient with asthma during an asthma epidemic and radiolabeled antihuman IgE. Legend as in Fig. 4. Arrow indicates application site.

IgE antibody to the allergens associated with soybean is sensitive and specific for the epidemic cases. The consistency of these results with results obtained in the other studies of Barcelona asthma outbreaks^{2, 3} elicits additional evidence on a causal relationship between asthma epidemics and soybean unloadings in the harbor of the city.

SDS-PAGE analysis of different soybean extracts revealed approximately 30 different Coomassie brilliant blue-stained bands located between 97.5 and 14.4 kd. The patterns of different soybean extracts found by SDS-PAGE and IEF were similar, indicating that most proteins and glycoproteins in hulls and dust are also found in meal. However, it is technically difficult to separate hulls from the beans during the processing, and some contamination of the meal with bits of hull may occur.

By Western blot analysis, the 10 sera from patients with asthma during the asthma epidemic reacted with different apparent MW bands from the different extracts; these results were similar to results of Busch

et al.⁴ with soy flour extract. However, in addition to these bands, we also detected IgE-binding bands <14.4 kd in hull, dust, and trash extracts. IEF followed by capillary blotting onto nitrocellulose membrane confirmed that the major IgE binding occurs in hull, dust, and trash extracts with pIs of 6.55, 5.85, 5.20, and 4.55. Characterization of these acidic low MW materials is currently underway. These results are coincident with RAST results that were positive to hull, dust, and trash extracts, but negative to both uncleaned bean and meal extracts.

Thus, it may be concluded that sera from patients with asthma during the asthma epidemic reacted both by RIA and immunoblotting to allergens from soybean material unloaded in the port of Barcelona on epidemic days. The fact that most sera reacted more intensely to hull, dust, and trash antigen extracts suggests that these sources contain the major allergens. However, cross-inhibition studies demonstrate that cleaned bean and meal extracts share reactivity with the hull and dust extracts. In contrast, the major IgE binding bands are found in the low MW components of dust and hull extracts, rather than the higher MW components of the cleaned beans or meal. The minor reactions to higher MW seed proteins are consistent with the immunoblot patterns reported by Burks et al.⁷ Soybean asthma appears to differ from castor bean asthma also in the source of the allergen. Thorpe et al.¹³ reported the major allergen in the castor bean dust to be a 2 sedimentation coefficient storage albumin of the seed itself, but our data indicate the major allergen in soybean dust to be a low MW polypeptide associated with the hull rather than the bean. The sera from patients with soybean-dust asthma did contain a small amount of IgE antibody that reacted to high MW proteins of the cleaned soybeans. These proteins appear to be the major allergens to which IgE from food-sensitive patients reacted.

At this time it is not possible to be absolutely certain that the major low MW soybean-dust allergens are either natural or degradation products of the soybean plant itself or are derived from contamination during storage and shipping. Several observations argue against this last possibility. First, direct examination of these materials did not disclose insects or mites, and cultures for fungi, such as *Aspergillus*, were negative. Second, cross-inhibition studies with extracts of pyroglyphid and glycophus mites, insects, and several molds were either completely negative or only partially inhibitory at high concentrations. Third, in the case-control study, multivariate analysis demon-

strated that IgE antibody to mites, molds, or pollens did not confound the association between asthma during an epidemic and soybean hypersensitivity.

In conclusion, our study has demonstrated that patients with asthma during an epidemic in Barcelona reacted specifically to an acidic, low MW allergen located in hulls, dust, and trash of soybeans unloaded in the harbor. Further studies are needed to characterize these allergens more precisely and to determine their origin.

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REFERENCES

1. Antò JM, Sunyer J. A point source asthma outbreak. *Lancet* 1986;1:900-3.
2. Antò JM, Sunyer J, Rodriguez Roisin R, Suarez M, Vazquez L. Asthma epidemics associated with soybean dust released during harbor unloading activities. *N Engl J Med* 1989; 320:1997-1102.
3. Sunyer J, Antò JM, Rodrigo MJ, Morell F. Epidemic asthma and soybean IgE antibodies. *Lancet* 1989;1:179-82.
4. Busch RK, Schroeckenstein D, Meier-Davis S, Balmes J, Kempel D. Soybean flour asthma: detection of allergens by immunoblotting. *J ALLERGY CLIN IMMUNOL* 1988;82:251-5.
5. Taylor SL, Herian AM, Bush RK, Chu FS. Allergen content of commercial skin test extracts for soybeans [Abstract]. *J ALLERGY CLIN IMMUNOL* 1988;81:264.
6. Herian AM, Taylor SL, Bush RK, Chu FS. Identification of soybean allergens by immunoblotting [Abstract]. *J ALLERGY CLIN IMMUNOL* 1988;81:264.
7. Burks AW Jr, Brooks JR, Sampson HA. Allergenicity of major component proteins of soybean determined by enzyme-linked immunosorbent assay (ELISA) and immunoblotting in children with atopic dermatitis and positive soy challenges. *J ALLERGY CLIN IMMUNOL* 1988;81:1135-42.
8. Adolphson CR, Yunginger JW, Gleich G. Standardization of allergens. In: Rose NR, Friedman H, eds. *Manual of clinical immunology*. 2nd ed. Washington, DC: American Society for Microbiology, 1980:778-88.
9. Swanson MC, Rosanoff E, Gurwith M, Deitch, Schnurrenberger P, Reed CE. IgE and IgG antibodies to β -propiolactone and human serum albumin associated with urticarial reactions to rabies vaccine. *J Infect Dis* 1987;155:909-13.
10. Laemmli UK. Cleavage of extructural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227: 680-5.
11. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979;9:4350-4.
12. Reinhardt MP, Malamud D. Protein transfer from isoelectrofocusing gels: the native blot. *Anal Biochem* 1982;123:229-35.
13. Thorpe SC, Kemeny DM, Pazani RC, McGurl B, Lord M. Allergy to castor bean. II. Identification fo the major allergens in castor bean seeds. *J ALLERGY CLIN IMMUNOL* 1988;82:67-72.