

Original article

Monoclonal antibody-based method to quantify Gly m 1. Its application to assess environmental exposure to soybean dust

Background: The demonstration that some asthma epidemics have been caused by allergens of soybean-hull dust prompted us to develop a two-site ELISA, suitable for the quantification of the major allergen (Gly m 1), to be used for the prevention of new episodes.

Methods: BALB/c mice were injected with Gly m 1 purified from soybean hulls. After fusion and screening, 10 monoclonal antibodies (mAbs) were obtained that were shown to be specific for Gly m 1. Two of them (6G1 as the capture antibody; 1G10 as the tracer) were selected to develop a quantitative two-site ELISA for the indoor and outdoor determination of Gly m 1.

Results: The two-site ELISA developed is very sensitive, with a detection limit of less than 0.2 ng/ml and a practical working range of 0.4–10 ng/ml. The assay is also highly reproducible with an intra-assay coefficient of variation of 3.5% and an interassay coefficient of variation of 12.5%. The method was applied to measure the concentration of Gly m 1 in air-sampler filters and in house-dust samples. Our results illustrate that there is a good correlation between the content of Gly m 1 in a number of samples and the allergenic activity as measured by ELISA inhibition.

Conclusions: A specific and sensitive method is presented that can be used for the quantification of Gly m 1. The application of this method may allow the establishment of risk limits for soybean dust, and thus may contribute to the control of environmental contamination and to the prevention of new asthma epidemics.

**R. González, O. Duffort,
B. Calabozo, D. Barber,
J. Carreira, F. Polo**

Research and Development Department, ALK-Abelló
SA, Madrid, Spain

Key words: allergen; asthma epidemics; epitopes;
Glycine max; Gly m 1; monoclonal antibody;
soybean asthma.

F. Polo, PhD
Product Development Department
ALK-Abelló SA
Miguel Fleta 19
28037 Madrid
Spain

Accepted for publication 27 August 1999

Allergic reactions to soybean proteins have been known to occur since the introduction of soybean preparations to feed infants with intolerance to cow's milk (1). Several proteins have been identified and characterized, to different extents, as potentially responsible for food allergy (2, 3).

Occupational allergy has also been described (4–7), showing that asthma, rhinitis, and other allergic manifestations can be caused by inhalation of soybean derivatives in predisposed individuals with or without food allergy.

On the other hand, several asthma outbreaks in Barcelona and Cartagena (Spain) in the 1980s were shown to be linked to unloading of soybean at the harbor, on days with concurrent favorable weather conditions for spreading soybean dust over the cities (8–10). Moreover, the recent analysis of the asthma epidemics that occurred in New Orleans (USA) between 1957 and 1968 disclosed a relation between the outbreaks and the presence of soybean at the harbor (11).

The major allergen responsible for the asthma outbreaks, Gly m 1, has been purified and characterized

(12, 13). This allergen, localized in soybean hulls, was shown to be different from proteins causing food allergy to soybean (14). Gly m 1 consists of two isoforms, Gly m 1A (MW 7500) and Gly m 1B (MW 7000), differing only in the absence of a tripeptide at the N-terminus in Gly m 1B. The purified isoforms completely cross-reacted for the binding of specific human IgE, and they accounted for 75% of the total IgE-binding capacity of a whole soybean-hull extract (12).

The severe asthma attacks and deaths caused by proteins of airborne hull dust from soybean have created deep concern. Industrial activities are being controlled to avoid release of hull dust into the open air, and both health authorities and industrial manufacturers have been urged to develop methods that can measure quantitatively the concentration of hull-dust contaminating proteins in the air.

In the present paper, we describe a mAb-based ELISA that can be used to monitor, in a very sensitive and specific way, amounts (ng) of airborne Gly m 1. The application of this technology will allow us to establish

the levels of hull-dust contamination, both inside factories and in the open air, and, thereby, to secure adequate means to avoid asthma outbreaks in the future.

Material and methods

Source materials and preparation of extracts

Soybean samples were obtained from materials unloaded at the seaport of Cartagena during the asthma epidemic in October 1987. These legumes had been imported from the USA. The extract was prepared from soybean hulls as previously described (14). Briefly, the soybean hulls were ground with a coffee grinder and extracted with 0.9% NaCl at a 5% (w/v) ratio, by stirring for 1 h at room temperature. The extract was separated from the solid material by centrifugation at 22 000 g for 20 min, and dialyzed against distilled water for 24 h. Afterward, the extract was again centrifuged as before, filtered through a 0.22- μ m pore size membrane, aliquoted, and freeze-dried. The lyophilized extract was stored at 4°C until use.

Filters from air samplers at different places in the city of Barcelona were extracted as follows. Small pieces (3 cm²) of the filters were incubated with 3 ml of PBS, containing 1% BSA and 0.1% Tween 20 (PBS-BSA-T), in an orbital shaker overnight at 4°C. Afterward, the solution was recovered, filtered through a 0.22- μ m filter (Sartorius, Göttingen, Germany), and stored frozen in aliquots at -20°C. Air samples were collected on nonepidemic days between May 1997 and January 1999.

House-dust samples from Barcelona and Madrid dwellings, randomly chosen from our collection, were extracted at a 10% (w/v) ratio in PBS-BSA-T overnight at 4°C. The solution was recovered by centrifugation, passed through a 0.22- μ m filter, and stored frozen in aliquots at -20°C.

Other allergenic extracts were obtained from ALK-Abelló, SA (Madrid, Spain).

Purification of Gly m 1 and ¹²⁵I-labeling

The isoforms of Gly m 1, the major soybean allergen, were purified from soybean-hull extract by the procedure previously described (12) that consists of sequential size-exclusion and reverse-phase HPLC.

Ten micrograms of purified Gly m 1 (1:1 mixture of the two isoforms) and 20 μ g of soybean extract were ¹²⁵I-labeled with 0.5 mCi of ¹²⁵INa (Medgenix Diagnostics, Fleurus, Belgium) by the chloramine-T method (15).

Production of mAbs and screening assays

For mAb production, four female BALB/c mice were immunized with Gly m 1 (1:1 mix of the two isoforms).

The OECD principles of good laboratory practice (as revised in 1997) and European Council Directive No. 609 (1986) were followed for use and care of experimental animals throughout this work. Mice were injected intraperitoneally with 50 μ g of Gly m 1 in Freund's complete adjuvant. At 15 and 30 days later, mice were boosted with an identical amount of antigen in incomplete adjuvant. Ten days after the last injection, mouse sera were screened for anti-Gly m 1 antibodies by direct ELISA with Gly m 1 in solid phase. The mouse with the highest antibody titer was injected intravenously with the same dose of antigen in PBS. Three days later, this mouse was killed, and its spleen cells were fused with X63-Ag8.653 myeloma cells, as previously described (16). Ten days after the fusion, hybridoma supernatants were screened, and the positive hybrids were cloned and subcloned by limiting dilution.

Hybridomas were screened for Gly m 1-specific antibody production with the direct ELISA mentioned above and radioimmunoassay with polyclonal rabbit antibodies against mouse Ig (DAKO, Glostrup, Denmark) in solid phase and ¹²⁵I-labeled Gly m 1 as the tracer. In both screening assays, the P3-X63-Ag8 mouse myeloma culture supernatant was used as a negative control.

mAbs positive for Gly m 1 were then used in experiments of cross-competition for ¹²⁵I-labeled Gly m 1 binding to identify mAbs that recognize nonoverlapping epitopes.

mAb isotype, purification, and labeling

The isotype of the mAbs was determined by ELISA with anti-mouse subclass antisera (Nordic, Tilburg, The Netherlands). mAbs were purified from the hybridoma culture supernatants by protein G Sepharose-CL4B affinity chromatography (Pharmacia, Uppsala, Sweden), according to the manufacturer's instructions.

Purified mAbs were labeled with biotin with the reagent EZ-LinkTM NHS-LC-Biotin (Pierce, Rockford, IL, USA), by the procedure recommended by the manufacturer. Labeled antibodies were separated from excess reagents by size-exclusion chromatography on PD-10 columns (Pharmacia), with PBS as the eluent, and finally stored at -20°C in PBS containing 0.5% BSA and 50% (v/v) glycerol.

SDS-PAGE and immunoprecipitation

SDS-PAGE was performed essentially as previously described (17), with an 8–25% acrylamide gradient in a high molarity Tris-buffer system, but excluding urea from the sample buffer and without prior reduction of the samples. SeeBlueTM Pre-Stained Standards (Novex, San Diego, CA, USA) were used as markers.

Immunoprecipitation was carried out as described for the radioimmunoassay screening, except that, after incubation with hybridoma culture supernatants and

washing, the wells were incubated with ^{125}I -labeled soybean-hull extract (5×10^5 cpm/well). After four washes with PBS containing 0.1% Tween 20 (PBS-T) and a final wash with water, the radioactivity bound to the solid phase was detached by incubating for 5 min at 90°C with 50 μl of SDS-PAGE sample buffer, and then the supernatant was subjected to electrophoresis. Finally, the gels were dried and autoradiographed.

Soybean-specific IgE-binding activity of extracts

The IgE-binding capacity of extracts prepared from environmental filters was determined by inhibition ELISA. For this purpose, soybean-hull extract was adsorbed at 100 $\mu\text{g}/\text{ml}$ in PBS to microtiter 96-well plates (EIA/RIA plates, Costar, Cambridge, MA, USA) overnight at 4°C . After blocking with PBS-BSA-T, the wells were incubated overnight at room temperature with a mixture of 25 μl of a 1:5 dilution of a serum pool from soybean-allergic patients who were admitted to the emergency room during asthma outbreaks in Barcelona, and 25 μl of serial dilutions of the samples. The wells were then washed and incubated for 1 h at room temperature with HRP-conjugated rabbit anti-human IgE (DAKO), diluted 1:1000 in PBS-BSA-T. Bound IgE was detected with *o*-phenyldiamine (OPD, DAKO), according to the manufacturer's instructions. For evaluation of the activity of samples, an arbitrary value of 1 was given to the amount of protein (in μg), in the soybean-hull extract used as a standard, needed to inhibit by up to 50% the binding of specific IgE.

Gly m 1 two-site ELISA

Microtiter 96-well plates (EIA/RIA plates, Costar) were coated overnight at 4°C with 100 μl of anti-Gly m 1 mAb 6G1 at 5 $\mu\text{g}/\text{ml}$ in PBS. The coated wells were washed with PBS-T and blocked with PBS-BSA-T. The wells then were washed and sequentially incubated with samples and references, biotin-labeled anti-Gly m 1 mAb 1G10, and streptavidin/peroxidase conjugate (Amersham, UK). Samples, controls, and reagents were conveniently diluted in PBS-BSA-T, and all the incubations were carried out for 1 h at room temperature with intermediate washes between successive steps. Finally, the wells were incubated for 30 min at room temperature in the dark with a solution of OPD (DAKO), and the color reaction was stopped by adding 2 M HCl. The optical density was then read at 490 nm with a 650-nm reference filter. Samples were assayed in duplicate at several twofold dilutions. The Gly m 1 content of samples, in mass units, was obtained by interpolating in a standard curve from serial twofold dilutions of purified Gly m 1 (1:1 mixture of the two isoforms), the protein concentration of which had been determined by amino-acid composition analysis.

Results

Analysis and specificity of mAbs

From the fusion of spleen cells of a mouse immunized with Gly m 1 and X63-Ag8.653 myeloma cells, 10 mAbs of IgG class were obtained. Cross-competition experiments for the binding of radiolabeled Gly m 1 allowed us to select two mAbs (6G1 and 1G10, both G1 isotype) that recognized nonoverlapping epitopes to develop a sandwich ELISA for Gly m 1 quantification.

The specificity of the two mAbs for Gly m 1 was demonstrated by immunoprecipitation of ^{125}I -labeled soybean-hull extract. Both mAbs clearly produced a broad band in the autoradiogram, corresponding to an apparent MW of about 7000–7500, which is the MW range described for the two Gly m 1 isoforms (Fig. 1). Besides, each antibody gave a similar response in a direct ELISA to the isoforms Gly m 1A and 1B (data not shown).

ELISA for quantification of Gly m 1

We tested the two possible combinations of the selected mAbs in a two-site ELISA for quantifying Gly m 1. The mAbs were biotinylated, and their concentration required to give optimal results, both for coating the plates and detection, was determined empirically. Curves constructed with serial dilutions of Gly m 1 (1:1 mixture of the two isoforms) were assayed, and the

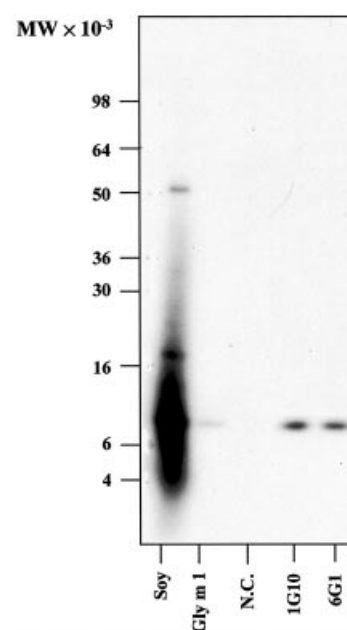


Figure 1. Autoradiogram of SDS-PAGE analysis of immunoprecipitates obtained with ^{125}I -labeled soybean-hull extract and anti-Gly m 1 mAbs. Lanes: Soy, radiolabeled soybean-hull extract; Gly m 1, radiolabeled pure Gly m 1 as control; N.C., negative control with anti-Par j 1 mAb 6.4.4.; 1G10, 6G1, immunoprecipitates with anti-Gly m 1 mAbs indicated.

best results were obtained with mAb 6G1 as the capture antibody and the biotin-labeled mAb 1G10 as the tracer. This particular combination was used thereafter.

The specificity of the two-site ELISA is presented in Table 1. Gly m 1A, Gly m 1B, and soybean hull-extract yielded a very strong signal, whereas there was no significant response in wells incubated with different extracts, including Leguminosae seeds, a number of indoor and outdoor allergens, and crops that may be linked to harbor activities and can cause dust exposure, such as sorghum, corn, or wheat.

The dose-response curves obtained with both Gly m 1 isoforms in the two-site ELISA were completely coincident (not shown); thereafter, a 1:1 mixture of the isoforms was used for the reference curve. This curve is illustrated in Fig. 2. The assay has a detection limit of <0.2 ng/ml and a practical working range of 0.4–10 ng/ml. The same figure shows the dose-response curve for a soybean-hull extract. This curve is parallel to that obtained with pure Gly m 1, indicating that the allergen had not been altered during the purification process, and that the assay was measuring the same molecule in the different preparations. Moreover, the assay is highly reproducible. The intra-assay variability was determined by analyzing five replicates of the same sample in eight independent assays. The mean of the intra-assay coefficient of variation in these assays was 3.5%, and the interassay coefficient of variation was 12.5%.

Gly m 1 content and correlation with allergenic activity

The feasibility of using this two-site ELISA for the environmental monitoring of Gly m 1 was studied by analyzing air samples collected on 24 filters of air samplers located at different places in downtown Barcelona. The concentration of Gly m 1 in the samples analyzed ranged from 4 to 797 ng of Gly m 1 per m³,

and the curves obtained showed good parallelism with the standard curve.

To assess the relationship between Gly m 1 content and the total allergenic activity of soybean hull, we determined the IgE-binding capacity in the same samples by means of inhibition ELISA, as described in Material and methods, using a soybean-hull extract on the solid phase and as the reference curve. Fig. 3 shows the comparison of the results obtained by both techniques. A very good correlation was observed, demonstrating that the content of Gly m 1 is representative of the overall allergenic activity of soybean dust.

As soybean dust dispersed in the atmosphere may persist for a long time, we investigated whether it might penetrate into homes and accumulate in house dust. With this aim, we undertook a preliminary study in which 11 house-dust samples from Barcelona and 11 samples from Madrid, randomly chosen from our collection, were analyzed for Gly m 1 content. Table 2 shows the results of this experiment. The statistical comparison of the averages revealed a significant difference between the two groups of samples.

Discussion

A great number of papers published over the last few years have demonstrated that there is a cause-and-effect relationship between the concentration of both indoor and outdoor allergenic substances and the development or exacerbation of various allergic diseases. Typical outdoor allergenic substances, such as pollen, have been considered the cause of hay fever for more than a century (18), and the number of pollen grains in the air is regarded as a predictive guide for the worsening or improvement of the allergic condition of patients (19). Regarding indoor allergens, the identification and characterization of mite allergens, together with allergens from other sources such as cat and dog, has afforded the opportunity to quantify the level of important allergen markers in the environment, and, interestingly, has led to the possibility of suggesting exposure levels of markers as risk thresholds for sensitization (20–22).

In the 1980s, several outbreaks of asthma occurred in the cities of Cartagena and Barcelona, clearly associated with the inhalation of soybean dust (8–10). Responsible allergen molecules were identified in the soybean hulls and were fully characterized (12, 13). Unlike most environmental allergens, soybean inhalant allergens contaminate limited geographic areas situated around places of soybean industry. Thus, total or at least partial avoidance of pertinent allergens is possible whenever a risk threshold of Gly m 1 for sensitization could be proposed. This paper describes a method to

Table 1. Specificity of two-site mAb ELISA for Gly m 1 quantitation

Sample	Protein concentration (μg/ml)	OD ₄₉₀
Soybean hull	0.125	3.603
Gly m 1A	0.020	3.823
Gly m 1B	0.020	3.726
Pea	1000	0.002
Peanut	1000	0.002
<i>Lolium perenne</i>	1000	0.025
<i>Parietaria judaica</i>	1000	0.004
<i>Olea europaea</i>	1000	0.002
<i>Dermatophagoides pteronyssinus</i>	1000	0.008
Cat hair	1000	0.004
<i>Alternaria alternata</i>	1000	0.001
Sorghum	300	0.001
Corn	300	0.005
Wheat	300	0.000

Samples were tested in two-site mAb ELISA at total protein concentration indicated in second column. Signal of blank with PBS-BSA-T (0.017) was subtracted from optical density (OD) of samples at 490 nm.

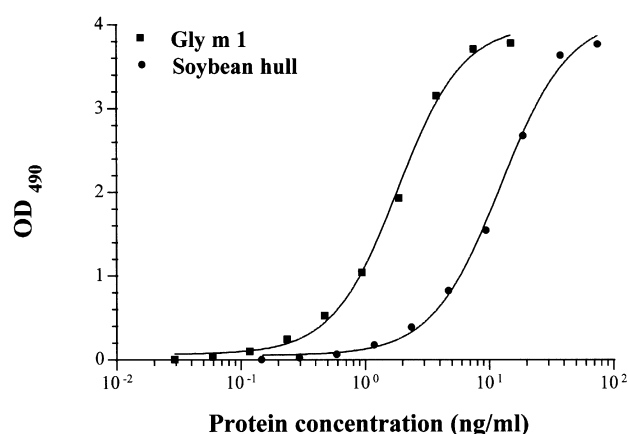


Figure 2. Dose-response curves in two-site mAb ELISA for soybean-hull extract and purified Gly m 1 allergen.

quantify in a very specific and sensitive way the environmental content of Gly m 1.

The suitable characteristics of 6G1 and 1G10 antibodies allowed us to develop a two-site ELISA for the quantification of Gly m 1. The method has proved to be reproducible, with very low coefficients of variation, and is at least 10 times more sensitive than RAST inhibition when both methods are compared to quantify the same samples. Apart from that, 6G1 and 1G10 antibodies are the only reagents relevant to Gly m 1 quantification, and both can be produced in an unlimited amount, for as long as necessary and with the same specificity and affinity.

We had already shown that 90% of patients who suffered an asthma crisis during the asthma outbreaks in Cartagena had specific IgE to Gly m 1, and that Gly m 1 was responsible for over 70% of the allergenic activity of soybean hulls (12). Thus, we assumed that it would be a good marker to evaluate the allergenic pressure of soybean dust in areas of potentially contaminated air. Our results clearly support the assumption: the method not only assesses the concentration of a dust protein marker but also measures it in allergenic active form, since a good correlation was found when Gly m 1 concentrations were plotted against allergenic activities in samples extracted from air-sampler filters, indicating that Gly m 1 can be taken to be representative of the total allergenic activity of contaminant soybean dust. On the other hand, the ELISA described here is very sensitive, with a detection limit as low as 0.2 ng/ml and a quantification limit of 0.4 ng/ml. These values are far below the minimum concentrations of Gly m 1 obtained in the samples analyzed, which were 4 ng/m³ of air. Besides this, one has to bear in mind that each milliliter of sample solution may contain the contaminant hull dust from several cubic meters of air. In conclusion, Gly m 1 is a good marker that can be used to quantify the risk level needed to elicit an asthma crisis in patients sensitized to

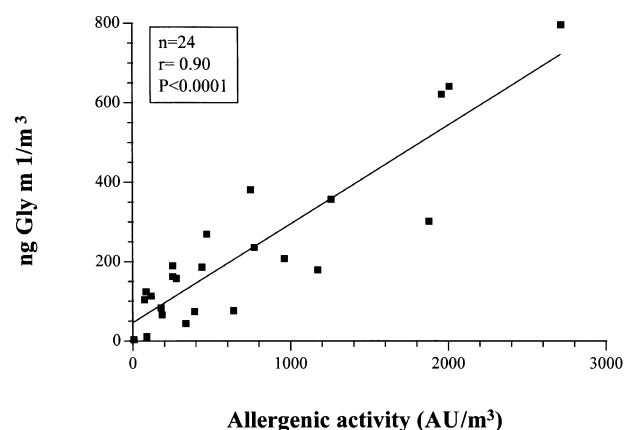


Figure 3. Correlation between allergenic activity, determined by inhibition ELISA and expressed in arbitrary units (AU), and Gly m 1 content, measured by two-site mAb ELISA, in filters from air samplers. Results refer to volume of air filtered, and are expressed in corresponding units per m³.

soybean-hull proteins, and the method to quantify it is described in this paper.

Since the asthma outbreaks of the 1980s, soybean unloading and transport activities in Barcelona have been subject to strict conditions. Filters and other devices have been installed, wherever necessary, in order to avoid as much as possible the spreading of soybean-hull dust into the air of the city. Consequently, the concentration of airborne soybean allergens decreased by about 10 times (23), although occasional releases of high amounts of soybean dust were reported on 29 October 1994 (24) and 12 June 1996 (25). In agreement with these observations, the values of the samples analyzed in this paper (up to 797 ng/m³) indicate that the concentration of Gly m 1 in the air seems to be, at times, still too high. Therefore, the homes of Barcelona where soybean dust is a risk factor contain small but significant amounts of Gly m 1 when

Table 2. Determination of Gly m 1 content in house-dust samples

n	$\mu\text{g Gly m 1/g}$	
	BCN	MAD
1	0.96	0.04
2	0.05	0.01
3	0.07	0.02
4	0.01	0.01
5	0.75	0.04
6	0.14	0.02
7	0.08	0.02
8	1.21	0.06
9	1.61	0.09
10	0.65	0.03
11	0.25	0.03
Geometric mean	0.23	0.03

House-dust samples from Barcelona (BCN) and Madrid (MAD) were analyzed for Gly m 1 content by two-site mAb ELISA. Results were expressed as μg of Gly m 1 per gram of dust. Values were transformed into logarithm for statistical analysis. Separate Student's *t*-test value was 4.03 ($P=0.0022$).

compared with homes of Madrid, where soybean allergens are not a risk factor.

Admittedly, these results are preliminary, but, considering that they were performed only to demonstrate the usefulness of the ELISA presented here, they are encouraging. The use of this method will allow rapid quantification of hundreds of samples, thus enabling the mapping of areas contaminated as a result of the soybean industry. Further studies will be necessary to establish a correlation between levels of Gly m 1 and patient sensitization in order to propose a risk threshold, so that the measures adopted to control the spreading of soybean dust can be effective.

Allergic symptoms caused by unknown allergen substances have been frequent in the past, including the first asthma outbreaks to soybean allergens. From

now on, this situation may change, since the presence of soybean inhalant allergens can be measured both indoors and outdoors. Apart from the help that this two-site ELISA may bring to clinicians and patients, local health authorities will find it to be a reliable method to determine the levels of environmental contamination, and, consequently, they will have adequate information to lay down rules to control it.

Acknowledgments

We thank Dr M.J. Rodrigo (Hospital General Vall d'Hebron, Barcelona) for her generous gift of the epidemic patient serum pool, Drs D. Carrascal and J. Vila (Port Autònom de Barcelona, Serveis de Medi Ambient) for supplying us with the air-sampler filters, Dr S. Martín for his expert advice on statistical analyses, L. Puebla and J. Muñoz for language revision, and I. Courinha for secretarial assistance.

References

- HILL LW, STUART HC. Soybean food preparation for feeding infants with mild idiosyncrasy. *JAMA* 1929;**93**:985–989.
- OGAWA T, BANDO N, TSUJI H, OKAJIMA H, NISHIKAWA K, SASAKA K. Investigation of the IgE-binding proteins in soybeans by immunoblotting with the sera of soybean sensitive patients with atopic dermatitis. *J Nutr Sci Vitaminol* 1991;**37**:555–565.
- HELM RM, COCKRELL G, HERMAN E, BURKS AW, SAMPSON HA, BANNON GA. Cellular and molecular characterization of a major soybean allergen. *Int Arch Allergy Immunol* 1998;**117**:29–37.
- BUSH RK, COHEN M. Immediate and late onset asthma from occupational exposure to soybean dust. *Clin Allergy* 1977;**7**:309–312.
- BOURGEOIS M. Asthme sévère par inhalation de poussière de soja. *Rev Fr Allergol* 1984;**24**:210–211.
- ZUSKIN E, KANCELJAK B, SCHACHTER EN, et al. Immunological respiratory changes in soybean workers. *Int Arch Occup Environ Health* 1991;**63**:15–20.
- BUSH RK, SCHROECKENSTEIN D, MEYER-DAVIES S, BALMES J, REMPEL D. Soybean flour asthma: detection of allergens by immunoblotting. *J Allergy Clin Immunol* 1988;**82**:251–255.
- SUNYER J, RODRIGO MJ, ANTÓ JM, MORELL F. Case-control study of serum immunoglobulin E antibodies reactive with soybean in epidemic asthma. *Lancet* 1989;**1**:178–182.
- ANTÓ JM, SUNYER J, RODRÍGUEZ-ROISIN R, SUÁREZ-CERVERA M, VÁZQUEZ L. Community outbreaks of asthma associated with inhalation of soybean dust. *N Engl J Med* 1989;**320**:1097–1102.
- NAVARRO C, MÁRQUEZ M, HERNANDO M, GALVÁN F, ZAPATERO L, CARAVACA F. Epidemic asthma in Cartagena (Spain) and its association with soybean sensitivity. *Epidemiology* 1993;**4**:76–79.
- WHITE MC, ETZEL RA, OLSON DR, GOLDSTEIN IF. Reexamination of epidemic asthma in New Orleans (Louisiana) in relation to the presence of soy at the harbor. *Am J Epidemiol* 1996;**145**:432–438.
- GONZÁLEZ R, POLO F, ZAPATERO L, CARAVACA F, CARREIRA J. Purification and characterization of major inhalant allergens from soybean hulls. *Clin Exp Allergy* 1992;**22**:748–755.
- GONZÁLEZ R, VARELA J, CARREIRA J, POLO F. Soybean hydrophobic protein and soybean hull allergy. *Lancet* 1995;**346**:48–49.
- GONZÁLEZ R, ZAPATERO L, CARAVACA F, CARREIRA J. Identification of soybean proteins responsible for respiratory allergies. *Int Arch Allergy Appl Immunol* 1991;**95**:53–57.
- HUNTER WM, GREENWOOD FC. Preparation of iodine-131 labeled human growth hormone of high specific activity. *Nature* 1962;**194**:495–498.
- GALFRÉ G, MILSTEIN C. Preparation of monoclonal antibodies: strategies and procedures. In: LANGONE JJ, VAN VUNAKIS H, editors. *Methods in enzymology*. Vol 73, part B. New York: Academic Press, 1981:1–46.
- FLING SP, GREGERSON DS. Peptide and protein molecular weight determination by electrophoresis using a high-molarity Tris buffer system without urea. *Anal Biochem* 1986;**155**:83–88.
- WYMAN M. *Autumnal catarrh*. Cambridge: Hurd and Houghton, 1872.
- D'AMATO G, SPIEKMA FTHM, LICCARDI G, et al. Pollen-related allergy in Europe. Position paper. *Allergy* 1998;**53**:567–578.
- PLATTS-MILLS TAE, CHAPMAN MD. Dust mites: immunology, allergic disease and environmental control. *J Allergy Clin Immunol* 1987;**80**:755–775.
- PLATTS-MILLS TAE. Allergens and asthma. *Allergy Proc* 1990;**11**:269–271.
- INGRAM JM, SPORIK R, ROSE G, HONSINGER R, CHAPMAN MD, PLATTS-MILLS TAE. Quantitative assessment of exposure to dog (Can f 1) and cat (Fel d 1) allergens: relation to sensitization and asthma among children living in Los Alamos, New Mexico. *J Allergy Clin Immunol* 1995;**96**:449–456.
- ANTÓ JM, SUNYER J, REED CE, et al. Preventing asthma epidemics due to soybeans by dust-control measures. *N Engl J Med* 1993;**329**:1760–1763.
- SORIANO JB, ANTÓ JM, PLASENCIA A. Repeaters count: a sentinel method for asthma outbreaks. *Thorax* 1994;**50**:1101–1103.
- SORIANO JB, ERCILLA G, SUNYER J, et al. HLA class II genes in soybean epidemic asthma patients. *Am J Respir Crit Care Med* 1997;**156**:1394–1398.