

Specific Immunoglobulins to Soybean Hull Allergens in Soybean Asthma*

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Soybean asthma, which occurred as an epidemic among patients in Barcelona, Spain, is associated with specific IgE to soybean hull allergens. The purpose of this study was to investigate the possible role of specific IgG, IgG subclasses, IgA, and IgM in the pathogenesis of soybean asthma. We studied 3 groups of subjects from Barcelona: group 1, 12 asthmatic epidemic patients; group 2, 23 asthmatic nonepidemic patients; and group 3, 32 nonallergic subjects. Specific IgE was determined by radioimmunoassay and specific IgG, IgG subclasses (1, 2, 3, and 4), IgA, and IgM by amplified enzyme-linked immunosorbent assay. Cross-inhibition studies were performed for specific IgE and IgG4. We partially characterized the soybean hull allergens that bind specific IgE, IgG, and IgG4 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis/Western blot. Percentage of positive results for the assays of the 8 Igs are as follows: for group 1, 100% (IgE), 75% (IgG), 16.6% (IgG1), 8.3% (IgG2), 0% (IgG3), 66.6% (IgG4), 25% (IgA), and 25% (IgM); for group 2, 4.3% were positive for specific IgE only; and for group 3, 0% (IgE), 0% (IgG), 6.2% (IgG1), 9.4% (IgG2), 9.4% (IgG3), 9.4% (IgG4), 6.2% (IgA), and 6.2% (IgM). The correlation between the specific IgE and the other specific Igs was significant between IgE and IgG4 in group 1 only ($r=0.752$, $p<0.01$). Cross-inhibition studies demonstrated a higher inhibitory capacity for IgG4 than for IgE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis/Western blot demonstrates three low molecular weight protein bands that bind specific IgE, IgG, and IgG4. This study suggests that specific IgG4 to soybean hull allergens plays a role in the pathogenesis of soybean asthma and corroborates the role of specific IgE in the same disease.

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Key words: Asthma outbreaks; IgG4; soybean asthma; specific immunoglobulins

Abbreviations: AEP=asthmatic epidemic patients; ANEP=asthmatic nonepidemic patients; BSA=bovine serum albumin; MW=molecular weight; NC=nitrocellulose; OD=optical density; SDS-PAGE=sodium dodecyl sulfate-polyacrylamide gel electrophoresis; %TCB=percentage of total counts bound; TTBS=polysorbate (Tween)-Tris buffer saline solution

From 1981 to 1987, there were 26 asthma outbreaks in Barcelona, Spain, affecting 687 people and causing 20 deaths.¹⁻³ Subsequent studies linked the asthma epidemics to soybean dust released during the unloading of soybeans into harbor silos of Barcelona.^{4,5} Similar outbreaks of asthma from soybean dust were subsequently reported in Cartagena, Spain.^{6,7}

Soybean hull allergens have been partially characterized⁸⁻¹⁰ and 2 main allergens *Gly m 1* and *Gly m*

2 have been described.¹⁰⁻¹² The allergen *Gly m 1*, with two isoallergens, *Gly m 1 A* and *Gly m 1 B*, have been associated with the asthma outbreaks that took place in Cartagena, Spain¹⁰ and their N-terminal aminoacid sequence has been reported.¹¹ In addition, the allergen *Gly m 2*, associated with the asthma outbreaks in Barcelona, Spain, has been recently purified and its N-terminal aminoacid sequence has been registered at the Protein Data Submission, National Biomedical Research Foundation, Georgetown University Medical Center, Washington, DC, with the code A57106.¹²

There is a good correlation between the soybean-induced asthma and the presence of specific IgE to soybean hull allergens.¹³ However, Barcelona asthma outbreaks showed some intriguing characteristics: a high severity, occurrence most often in men, and a rapid recovery after treatment. Some of these characteristics have been partially explained,¹³⁻¹⁶ but

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there is no adequate explanation for some clinical features, *eg*, there seems to be a lack of a late-phase allergic response.¹⁷

The mechanism of production of IgG4 is similar to that for IgE, *eg*, interleukin-4 stimulates switching by human B cells to both IgE and IgG4, and it is postulated that the switch from IgM to IgE involves an initial switch to an IgG isotype.¹⁸ Some studies of type I hypersensitivity to dust mites or to soybean demonstrated the presence of specific IgG or specific IgG subclasses in addition to the specific IgE.¹⁹⁻²¹ However, the role of IgG4 in type I hypersensitivity is still not well understood.²²

The purpose of this study was to ascertain if, in addition to IgE, other specific immunoglobulins that reacted with soybean hull allergens play a role in the pathogenesis of soybean asthma.

MATERIALS AND METHODS

Patient Population

Serum samples from 3 groups of subjects from Barcelona were kept at -20°C until analysis: group 1 consisted of 12 asthmatic epidemic patients (AEPs) attended in an emergency department during one of the asthma outbreaks; group 2 consisted of 23 asthmatic nonepidemic patients (ANEPs) attended in an emergency department during a nonepidemic day; and group 3 consisted of 32 nonallergic subjects.

Allergen Extract

Allergen extract was prepared from soybean hull unloaded from a ship that caused one of the asthma outbreaks in September 1987 in Barcelona. Extraction was performed at 1:20 (w/v) with 0.2 mol/L ammonium bicarbonate buffer, pH 9, overnight at 4°C . The supernatant was isolated by filtration under vacuum and sterilized through a $0.45\text{-}\mu\text{m}$ cellulose nitrate filter (Nalgene Company; Rochester, NY). The total protein content was measured by the bicinchoninic acid protein assay (Pierce Chemical Co; Rockford, Ill).

Specific IgE Determination

Specific IgE determination was performed by radioimmunoassay as follows: soybean hull proteins were adsorbed to wells of removawell strips (Immulon 4, removawell strips; Dynatech Laboratories Inc; Chantilly, Va). Each well received $1\text{ }\mu\text{g}$ of protein in $100\text{ }\mu\text{L}$ of 0.1 mol/L of sodium carbonate/bicarbonate buffer, at a pH of 9.6. The wells were incubated overnight at room temperature in a humid box and washed four times with 0.1 mol/L phosphate buffer, pH 7.5 containing 1% polysorbate 20 (Tween 20) (washing buffer). After adding $100\text{ }\mu\text{L}$ of undiluted serum to each well, the wells were again incubated overnight and then washed 4 times with washing buffer. One hundred microliters of rabbit ^{125}I -anti-human IgE (Sanofi Diagnostics Pasteur Inc; Chaska, Minn) diluted to 1:2 in 0.1 mol/L phosphate buffer, pH 7.5 containing 1% polysorbate 20 (Tween 20) and 1% bovine serum albumin (BSA) (diluent buffer) was then added to all wells, which were incubated overnight, again washed, and the counts per minute were counted in a gamma counter. The results were

expressed as the percentage of total counts bound (%TCB) and were considered positive if exceeding the reference value.

Specific IgG, IgG Subclasses, IgA, and IgM Determinations

Specific IgG, IgG1, IgG2, IgG3, IgG4, IgA, and IgM determinations were performed by an amplified enzyme-linked immunosorbent assay method based on the biotin-streptavidin complex as follows: soybean hull proteins were adsorbed to wells of high-binding microtiter plates (Costar Corporation; Cambridge, Mass) by incubating $1\text{ }\mu\text{g}$ of protein in $100\text{ }\mu\text{L}$ of 0.1 mol/L of sodium carbonate/bicarbonate buffer, pH 9.6, overnight at room temperature in a humid box. Then the wells were washed 4 times with washing buffer and blocked with $150\text{ }\mu\text{L}$ per well of 0.1 mol/L phosphate buffer, at a pH of 7.5 containing 1% polysorbate 20 (Tween 20) and 3% BSA (blocking buffer) for 1 h at room temperature. Washing the plates 4 times between steps, the 7 immunoglobulin assays were performed in duplicate by incubating for 1 h at 37°C $100\text{ }\mu\text{L}$ of each serum, diluted in diluent buffer to 1:100, for specific IgG and diluted to 1:10, for the other 6 specific immunoglobulins. Monoclonal biotin conjugated anti-human IgG, IgG1, IgG2, IgG3, and IgG4 (Sigma Chemical Co; St. Louis) were diluted in diluent buffer to 1:1,000, 1:500, 1:20,000, 1:4,000, and 1:15,000, respectively. Since monoclonal antihuman IgA and IgM were not available, polyclonal biotin-conjugated antihuman IgA and IgM (Sigma Chemical Co) were diluted at 1:20,000 and incubated for 1 h at 37°C . A solution of streptavidin-peroxidase (Sigma Chemical Co) diluted at 1:10,000 in diluent buffer was added and the plates were incubated for 30 min each at 37°C ($100\text{ }\mu\text{L}$ per well), after which, a substrate working solution of 0.1 mmol/L 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (Sigma Chemical Co) in 0.1 mol/L citrate buffer, pH 4.2, was incubated for a minimum of 5 min ($100\text{ }\mu\text{L}$ per well) until the color was developed. The reaction was stopped with $100\text{ }\mu\text{L}$ of 2 mmol/L sodium azide. Optical density (OD) at 414 nm was measured with a microplate reader (Dynatech Laboratories Inc; Chantilly, Va). Results were expressed as OD units at 414 nm and were considered positive if exceeding the reference value.

Cross-Inhibition Study

A cross-inhibition study for specific IgE and IgG4, using the hull extract as solid and fluid phases, was performed using a pool of 4 serum samples from group 1. The assays were performed essentially as those for specific IgE and IgG4 determinations, except that the coated solid phases with hull extract were made to react with a mixture of $50\text{ }\mu\text{L}$ of the pooled serum samples diluted to 1:3 for IgE and to 1:20 for IgG4 and $50\text{ }\mu\text{L}$ of serial dilutions to 1:2 of the hull extract.

Statistical Analysis

The statistical analysis was performed with a software program (Statview; Macintosh Apple Computer Inc; Cupertino, Calif). To ascertain if the values were normally distributed, the Kolmogorov-Smirnov test was applied to the values found for each technique in group 3.

For each technique the means between groups 1 and 3 and groups 2 and 3 were compared with the Wilcoxon signed rank test. The Spearman rank correlation coefficient was calculated for each group between techniques.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis of the Extract

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in an 8-cm-long 5 to 20% poly-

acrylamide gradient gel (Ready Gels; BIO-RAD Laboratories; Richmond, Calif) according to the discontinuous buffer system of Laemmli.²³ Twenty-five microliters of the soybean hull extract containing 10 µg of protein was treated with 10% mercaptoethanol, boiled for 5 min, and applied in a 4% polyacrylamide stacking gel. A set of molecular weight (MW) markers (prestained SDS-PAGE Standards; low range; BIO-RAD Laboratories) was loaded with the hull extract. Electrophoresis was carried out (in a Mini Protein II Dual Slab Cell; BIO-RAD Laboratories) at 200 V until the tracing dye reached the bottom of the gel. After the electrophoresis, the proteins were immunoblotted.

Western Blot

Transfer of the proteins to a 0.2 µm nitrocellulose (NC) membrane (BIO-RAD Laboratories) was carried out at 150 mA constant power for 3 h (in a Mini-Trans-Blot Cell; BIO-RAD Laboratories), according to the method of Towbin et al.²⁴ After the transfer, to saturate the unbound sites, the NC was blocked overnight with phosphate buffer, at a pH of 7.5, containing 1% polysorbate 20 (Tween 20) and 3% BSA.

The detection of specific IgE was performed with five serum samples of AEP and with two control pools, one made from four ANEP serum samples and one from four serum samples of nonallergic subjects. The serum samples and pools were diluted to 1:2 in 0.1 mol/L phosphate buffer, at a pH of 7.5, containing 1% polysorbate 20 (Tween 20) and 1% BSA and were incubated overnight with the blotted membranes, at room temperature and with continuous shaking. The membranes were washed 4 times with 0.1 mol/L phosphate buffer, pH 7.5, containing 1% polysorbate 20 (Tween 20) and then incubated with rabbit ¹²⁵I-antihuman IgE (Sanofi Diagnostics Pasteur Inc) diluted to 1:10 in diluent buffer, overnight at room temperature and with continuous shaking. After washing, the blots were exposed to an X-ray film (Kodak X-Omat; Sigma Chemical Co) for 3, 7, or 25 days, at -80°C.

The detection of specific IgG and IgG4 was performed using a pool made from four AEP serum samples and a pool made from four ANEP serum samples. The two pools were diluted 1:10 in Tris buffer saline solution containing 0.1% polysorbate 20 (Tween 20) (polysorbate [Tween]-Tris buffer saline solution [TTBS]) and were incubated overnight with the blotted NC membranes, at room temperature and with continuous shaking. The membranes were washed four times with TTBS and then incubated with monoclonal antihuman-IgG and IgG4 (Sigma Chemical Co), diluted in TTBS at 1:500 and 1:700, respectively, for 30 min with shaking. After four washes with TTBS, the membranes were incubated with a biotin-streptavidin-peroxidase complex (Vector; Burlingame, Calif) for 30 min with shaking. After four more washes with TTBS, the immunochemical reaction was developed with diaminobenzidine-containing nickel chloride (Vector) by incubating the NC membranes for 10 min. The reaction was stopped by washing the NC membranes with distilled water for 5 min. The determination of MWs was performed according to the method of Weber and Osborn.²⁵

RESULTS

As the values for specific IgE, IgG, IgG1, IgG2, IgG3, IgG4, IgA, and IgM in group 3 do not follow a normal distribution, the reference values were established as the percentile 97.5. The reference value for specific IgE is less than 0.04 %TCB and for specific IgG, IgG1, IgG2, IgG3, IgG4, IgA, and IgM,

less than 0.300, less than 0.076, less than 0.051, less than 0.094, less than 0.046, less than 0.096, and less than 0.069 OD units, respectively.

As can be seen in Table 1, the mean values found for specific IgG in the three groups of subjects are higher than those found for the other specific immunoglobulins; however, the mean OD value in group 1 is higher than the values for groups 2 and 3. In group 1 only, the OD value for specific IgG4 is also higher. The comparison of the means between groups 1 and 3 is significant for IgE, IgG, and IgG4 ($p<0.001$, $p<0.02$, and $p<0.01$, respectively) and between groups 2 and 3 is significant for IgE, IgG3, IgA, and IgM ($p<0.05$).

The percentages of positive results for specific IgE in groups 1, 2, and 3 are 100%, 4.3%, and 0%, respectively. In group 1 the percentage of positive results for specific IgG and IgG4 are 75% and 66.6%, respectively. For specific IgA and IgM, the percentages are 25%, but all the positive values are borderline (Table 1, Fig 1).

There is a significant correlation between specific IgE and IgG4 in group 1 only (Table 2); however, the correlation between IgE and IgG is not significant.

Cross-inhibition study shows that the amount of protein to produce the 50% inhibition is approximately 0.61 µg for specific IgE and 0.0013 µg for specific IgG4 (Table 2, Fig 2).

The 5 serum samples of AEP recognized 3 protein bands, with MWs of 8, 7.5, and 7 kd that bind specific IgE (Fig 3). The pooled AEP serum samples recognized 3 protein bands, with the same MWs that bind specific IgG and IgG4 (Fig 4). The specificity of

Table 1—Mean and SD for Specific IgE, IgG, IgG Subclasses, IgA, and IgM in the Three Groups of Patients*

	Group No.		
	1 (AEP)	2 (ANEP)	3 (Nonallergies)
Immunoglobulin			
IgE	13.6±12.8 [†]	0.188±0.091 [‡]	0.072±0.04
IgG	0.442±0.245 [§]	0.133±0.117	0.174±0.02
IgG1	0.092±0.182	0.010±0.00	0.04±0.092
IgG2	0.026±0.028	0.010±0.00	0.02±0.024
IgG3	0.014±0.008	0.010±0.00	0.044±0.033
IgG4	0.300±0.334	0.010±0.00	0.021±0.016
IgA	0.085±0.111	0.012±0.008 [‡]	0.042±0.035
IgM	0.05±0.035 [†]	0.010±0.00 [†]	0.024±0.027

*Note: Specific IgE is expressed as %TCB and the other specific immunoglobulins as OD units. p values are comparison of groups 1 and 2 with group 3.

[†] $p<0.001$.

[‡] $p<0.05$.

[§] $p<0.02$.

^{||} $p<0.01$.

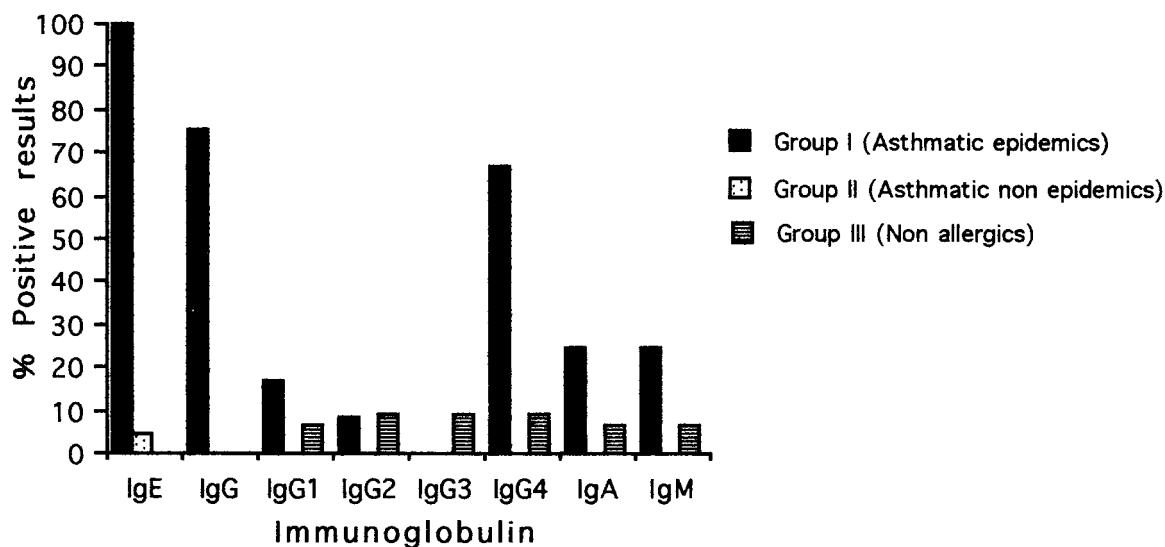


FIGURE 1. Percentages of positive results for the eight specific immunoglobulins in the three groups of subjects.

the immunoreactions is confirmed by the nonrecognition of bands by the pooled control serum samples.

DISCUSSION

In this study of 12 patients with soybean asthma, we found a higher mean serum concentration of specific IgE ($p < 0.001$) and IgG4 ($p < 0.01$) than the mean of a group of nonallergic subjects. The results of this study support the hypothesis that specific IgG4, in combination with IgE, plays a role in the pathogenesis of soybean asthma.

Asthma outbreaks that took place in Barcelona and Cartagena, Spain, were due to the inhalation of soybean dust and there is no doubt about the role of

the specific IgE to soybean hull allergens in the pathogenesis of soybean asthma.⁵⁻⁹

The finding of high values for specific IgG and IgG4 in group 1 led us to characterize the soybean hull allergens involved in the IgG4 immunoreponse by SDS-PAGE/Western blot, in addition to the allergens that bind specific IgE. Our results demonstrate the presence of 3 bands with MWs of 7, 7.5, and 8 KD that bind specific IgE, IgG, and IgG4. These allergens probably are *Gly m 1 A*, *Gly m 1 B*, and *Gly m 2*, the three main allergens from soybean hull that bind specific IgE.¹⁰⁻¹² The significant correlations in group 2 between specific IgE and IgG1, IgG2, IgG3, and IgM and in group 3 between specific IgE and IgM are explained by the low concentration for these immunoglobulins found in both groups.

Table 2—Spearman Rank Correlation Coefficient Between Specific IgE and the Other Seven Specific Immunoglobulins in the Three Groups of Subjects

Immunoglobulin	Group No.		
	1 (AEP)	2 (ANEP)	3 (Nonallergics)
IgG	-0.280	0.169	-0.246
IgG1	0.400	0.480*	0.253
IgG2	0.036	0.510*	-0.308
IgG3	0.422	0.510*	0.162
IgG4	0.752 [†]	0.200	0.309
IgA	0.348	0.100	0.092
IgM	0.021	0.510*	0.381*

* $p < 0.05$.

[†] $p < 0.01$.

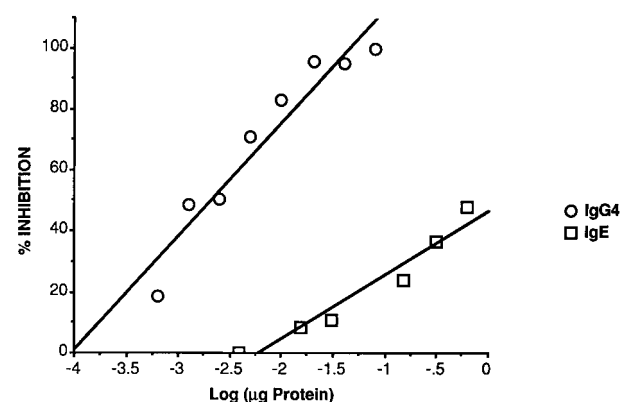


FIGURE 2. Cross-inhibition studies for specific IgE and IgG4 using soybean hull extract as solid and fluid phases.

MW
(KD)

142.9—
97.2—
50—
35.1—
29.7—
21.9—
16.9—
14.4—
10.7—
8.1—
6.2—
2.5—

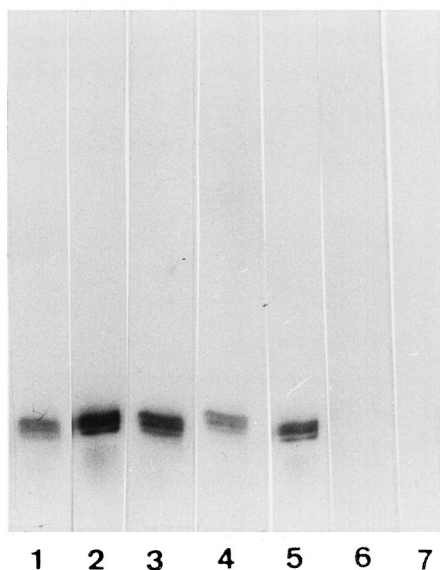


FIGURE 3. SDS-PAGE/Western blot of soybean hull corresponding to the detection of specific IgE. Lanes 1, 2, 3, 4, and 5 using 5 serum samples from AEPs; lane 6 using a pool made from 4 ANEP serum samples, and lane 7 using a pool made from 4 serum samples of nonallergic subjects.

Cross-inhibition assay shows that the amount of protein to inhibit the specific IgG4 binding is almost 500 times lower than for specific IgE. This fact, together with the finding of a more pronounced slope for IgG4, indicates that IgG4 has a higher avidity than IgE for soybean hull allergens. Moreover, the IgG4 concentration is higher than IgE, which is demonstrated by the lower dilution of the serum samples necessary for its detection. These observations suggest that IgG4 plays a role in the mechanisms of soybean asthma.

Burks et al,²¹ employing an analogous technology to that used in our study, demonstrated high levels of both specific IgE and IgG to different fractions obtained from an extract made from soybean flakes in patients responding positively to double-blind placebo-controlled challenges with soybean. They demonstrated several protein bands that bind specific IgE and IgG in the SDS-PAGE/Western blot.

High serum concentrations of IgG4 had been found in other conditions, such as atopic dermatitis,²¹ allergic asthma due to *Dermatophagoides pteronyssinus*,²⁶ *Lepidoglyphus destructor*,²⁰ occupational asthma,²⁷ allergic rhinitis,²⁸ and aspirin-induced asthma,²⁹ among others. The high percentage of positive results found for specific IgE and IgG4 in group 1, together with the high mean values for these immunoglobulins and the significant cor-

MW
(KD)

112—
84—
53.2—
34.9—
28.7—
20.5—

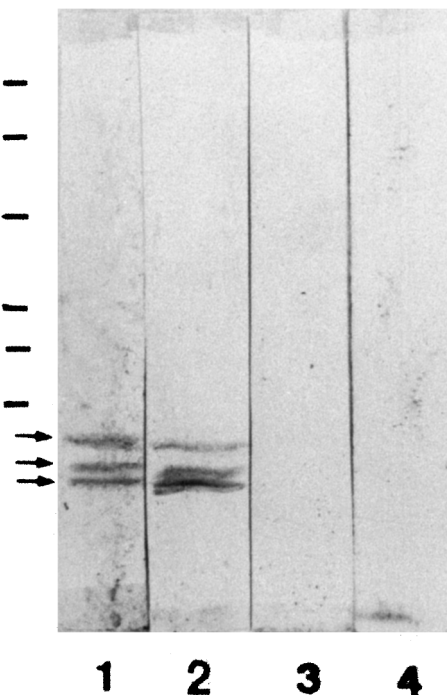


FIGURE 4. SDS-PAGE/Western blot of soybean hull. Lanes 1 and 2 correspond to the detection of specific IgG and IgG4, respectively, using a pool made from 4 AEP serum samples; lanes 3 and 4 correspond to the same immunoglobulins using a pool made from 4 ANEP serum samples.

relation between them, suggests that the synthesis of IgG4 is increased probably by the same factors that regulate the IgE production, such as interleukin-4.

In conclusion, this study demonstrates higher levels of specific IgE and IgG4 to soybean hull in AEPs from Barcelona compared with ANEPs and with nonallergic subjects, suggesting that both immunoglobulins might be involved in the pathogenesis of soybean asthma. The possible involvement of IgG4 is intriguing and will merit further investigation.

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