

Detection of proteins in wheat flour extracts that bind human IgG, IgE, and mouse monoclonal antibodies

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Wheat flour was extracted with phosphate-buffered saline. The biologic significance of the extracted cereal proteins was demonstrated. Basophil leukocytes were obtained from patients suffering from "baker's asthma disease." Histamine release in vitro was induced by addition of the aqueous flour extract. By means of ELISA, as well as immunoblotting technique, IgG and IgE binding to wheat flour proteins was demonstrated. Wheat-specific antibodies could be detected in sera obtained from healthy and atopic individuals. Further analysis of the cereal components by monoclonal antibodies revealed that several wheat molecules exhibit identical epitopes. (J ALLERGY CLIN IMMUNOL 78:470-7, 1986.)

It is well-known that nutritive components can elicit hypersensitivity reactions.^{1,2} Among a wide range of food stuffs, adverse reactions to wheat flour is commonly known. The most prominent examples are the gluten-induced enteropathy and the "baker's asthma disease." The cereal proteins for these disorders have been identified as the causative agents. Although the pathogenic mechanism in gluten-induced enteropathy is still uncertain, the baker's asthma is mediated by IgE antibodies. Thus, it is obvious that not all food allergies are caused by the ingestion of the sensitizing molecules. This is illustrated by the baker's asthma disease, which is provoked by the inhalation of allergens.³ The anaphylactic reaction is then mediated by sensitizing antibodies that can be demonstrated in the sera of these patients.

Recently, the knowledge about food components, which induce pathophysiologic reactions, improved. However, only little progress has been made to identify the responsible allergens. RAST procedures have been developed. Since the cereal extracts were not uniformly standardized, results from different laboratories were not comparable. In order to overcome these difficulties, we produced monoclonal antibodies

Abbreviations used

AS:	Ammonium sulfate
BSA:	Bovine serum albumin
HRP:	Horseradish peroxidase
LMB:	Lymphocytes, monocytes, and basophils
PBS:	Phosphate-buffered saline
SDS:	Sodium dodecyl sulfate
hd:	Healthy donors

directed toward the cereal proteins. Recently, we described two hybridoma-derived antibodies that were immobilized.⁴ The corresponding antigens were purified by immunoabsorption.

It was the purpose of this study to investigate the binding characteristics of human IgE and IgG to cereal antigens. Their reaction pattern is compared with the binding of several monoclonal antibodies.

MATERIAL AND METHODS

Wheat flour extracts

The water-soluble proteins of wheat flour were extracted by a modified procedure, which was originally described by Osborne.⁵ The 10% sodium chloride was replaced by PBS containing NaCl (123 mmol/L), Na₂HPO₄ (8.3 mmol/L), and KH₂PO₄ (3.16 mmol/L). The pH was adjusted to pH 7.4, the conductivity was 14 msec. One gram of dry wheat flour was added to 4 ml of PBS buffer. The slurry was then stirred for 1 hour at room temperature. Insoluble components were removed by centrifugation (1,500 × g for 20 minutes). The protein yield varied between 15 and 20 mg of protein per gram of wheat flour. The insoluble components were resuspended in an equal volume of ethanol

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(70%) and stirred for 30 minutes at room temperature. After clarifying the supernatant by centrifugation ($1.500 \times g$ for 20 minutes), the ethanol was evaporated under a constant flow of nitrogen. The dry gliadins were stored at 4° C.

Immunoblotting

The blotting procedure followed has been reported by Towbin et al.⁶ Briefly, SDS gel electrophoresis was carried out according to the method of Lugtenberg et al.⁷ by use of the LKB (Bromma, Sweden) vertical electrophoresis unit. The separating gel consisted of 12.5% acrylamide with a relative amount of 1.8% methylene-bisacrylamide ($T = 12.5$; $C = 1.8$) and a 3% spacer ($C = 2.7$). Electrophoresis was performed at 40 mA of constant current for 6 hours. The gels were then transferred to a blotting cell (Trans Blot, Bio-Rad Laboratories, Richmond, Calif.) and overlaid with a sheet of pure nitrocellulose (pore size 0.45 μ m, Bio-Rad). The proteins were electroblotted by the application of 27 V, 0.2 A, for 1 hour, and 54 V, 0.4 A, for 2 hours. The blotting buffer contained 286 mmol/L of glycine, 37.1 mmol/L of Tris, and 13.3% (v/v) of methanol.

After blotting, the filter was cut and transferred to a test tube containing 18 ml of incubation buffer (PBS plus 0.05% Tween 20 plus 1% BSA). After an incubation period of 30 minutes at room temperature, the blots were washed five times. Subsequently, 8 ml of PBS/Tween/BSA and 2 ml of serum or culture supernatant was added. The tubes were incubated for an overnight period on a rotating rack and washed five times, again. Then, the HRP-conjugated anti-serum (antimouse immunoglobulin antihuman-IgG, Dako, Denmark) was added (8 ml, diluted 1:200). Six hours later, antibody-binding proteins were detected by conversion of the substrate (3 mg of 4-chloro-1-naphthol/1 ml of methanol plus 5 ml of PBS plus 2 μ l of 30% H_2O_2). The staining of protein bands occurred within 15 to 30 minutes.⁸

Monoclonal antibodies and ELISA techniques

Hybridoma cell lines were obtained as has been described in detail elsewhere.⁹ A mouse β -lymphoma cell line (NS-1) was used for cell fusion. The hybridomas were cloned by the method of "limiting dilution." Cells are grown with this procedure at a statistical density of one cell per well, and thus individual clones are isolated. The ELISA procedures were performed as has been described.¹⁰ In short, flexible microtiter plates (Microtest III, Falcon Labware, Oxnard, Calif.) were coated with the water-soluble antigens in the presence of 1.25% of glutaraldehyde. The addition of glutaraldehyde leads to a conjugation of small molecules, which ensures their attachment to the plastic well. The gliadins were coated by evaporating the solvent. The microtiter plate was placed for this purpose under a nonheating fan for an overnight period. Unbound protein was washed with PBS plus 0.05% Tween 20. Samples (0.2 ml) were incubated for 2 hours at 37° C. The bound immunoglobulin was detected by an enzyme-labeled second antibody. Each experiment was repeated at least five times. The means and standard deviations are calculated from triplicates of one representative experiment. Anti-IgG was purchased from

TABLE I. Detection of IgG antibodies with specificity toward wheat flour proteins
ELISA precoated with gliadin or PBS extract

Patient	Gliadin		PBS extract	
	M	SD	M	SD
Fr	1.67	0.09	1.81	0.15
Pa	1.59	0.04	1.83	0.15
Lu	1.25	0.16	1.34	0.22
Sc	1.01	0.04	0.69	0.03
Ku	1.38	0.02	1.47	0.41
Ri	1.13	0.08	1.51	0.22
hd	0.27	0.01	0.32	0.02
Buffer	0.048	0.02	0.03	0.005

Values represent optical density at 405 nm and incubation period of 30 minutes. M = mean; SD = standard deviation.

Dako, Denmark; anti-IgE was obtained from Miles Laboratories, Slough, Great Britain.

Histamine release

The in vitro stimulation of washed basophil leukocytes was carried out as has been described in detail.¹¹ In short, human basophil leukocytes were obtained from peripheral blood by a Ficoll/metrizoate gradient. The LMB-containing cell fraction (1×10^5 basophils per tube) was incubated with various concentrations of PBS-extracted wheat flour proteins (80 ng to 80 μ g). After an incubation period of 60 minutes at 37° C, the cells were removed by centrifugation. The histamine content was measured fluorophotometrically by a Technicon (Technicon Instruments Corp., Tarrytown, N. Y.) autoanalyzer. The sensitivity of the assay was better than 1 ng/ml.

Chromatography

The interfacial, hydrophobic chromatography was performed according to the method of von der Haar.¹² In short, 20 mg of protein were mixed with 1 ml of unsubstituted Sepharose 4B. Under constant stirring, ammonium sulfate was added to yield a final concentration of 80% salt saturation. Subsequently, the slurry was poured into a column. Elution was performed with decreasing salt saturations by steps of 10%.

Preparation of immunosorbent columns was achieved according to March et al.¹³ The culture supernatant was precipitated at 0% to 33% of ammonium sulfate; 200 mg of the precipitate was added to 10 ml of activated Sepharose 4B. About two thirds of the protein was immobilized by this procedure.

RESULTS

IgG and IgE binding to flour extracts

In order to prove the biologic relevance of the extracted proteins, a specific ELISA was performed. The

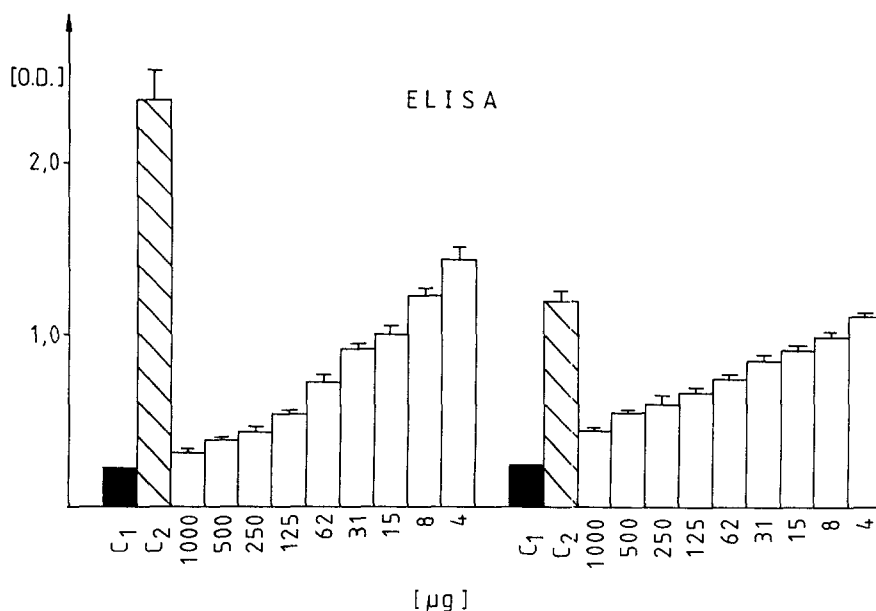


FIG. 1. Dose-dependent inhibition of wheat-specific IgG binding by the addition of cereal protein. To 100 μ l of serum (*left*, baker Ri; *right*, hd) 4 to 1000 μ g of PBS-extracted wheat proteins was added. The *filled bars* (C_1) illustrate background values; *hatched bars* (C_2), the buffer control. ELISA readings were obtained 30 minutes after the substrate was added.

surface of a plastic microtiter plate was coated with the extracted cereal proteins. Subsequently, sera obtained from six individuals suffering from baker's asthma disease (Fr, Pa, Lu, Sc, Ku, and Ri) were added. A serum pool obtained from 20 hd served as control. After a 2-hour incubation period, bound IgG or IgE was detected, respectively. All sera were diluted by 1:81. As observed in Table I, all sera contained wheat flour-specific IgG antibodies. In addition, it became evident that the IgG antibodies were reactive to both fractions, the gliadins as well as the water-soluble proteins. However, the titers of the various sera differed profoundly. Atopic sera were more reactive as compared to the "healthy" control (Table I). This is not due to nonspecific binding, because the total IgG values of all sera were within the physiologic range (10 to 12 mg/ml).

Furthermore, the antibody binding can be competitively inhibited by addition of PBS extract. Fig. 1 illustrates the result with one serum obtained from baker Ri. In this experiment the microtiter plate was coated with cereal protein. Subsequently, 100 μ l of serum (diluted 1:50) and 100 μ l PBS extract containing 4 to 1000 μ g of protein were added. As can be observed, inhibition of IgG binding clearly is dose dependent. A 50% inhibition is achieved with 4 to 8 μ g of cereal protein.

A similar reaction can be observed with "normal"

serum as well. However, because of the lower antibody titer, the pool serum (hd) had to be used at a tenfold higher concentration (dilution 1:5 versus 1:50). As is apparent from Fig. 1, a 50% inhibition is observed with 60 μ g of wheat protein.

The analysis of human IgE binding was carried out similarly. However, for this assay, the sera were used undiluted. Table II illustrates the results obtained with antihuman IgE. As illustrated in Table II, all sera, obtained from allergic bakers, contained IgE antibodies reactive to both cereal protein fractions. In contrast, the serum obtained from hd failed to demonstrate antibodies of this isotype. Table II illustrates that the IgE binding does not correlate with total IgE values.

Characterization of cereal proteins

Further fractionation of the extracted cereal proteins was achieved by interfacial, hydrophobic chromatography. Proteins were eluted with 70% ammonium sulfate saturated PBS buffer. Subsequently, salt saturation of the PBS buffer was decreased by steps of 10%. The resulting eight fractions (I to VIII) were subjected to SDS gel electrophoresis. The results are presented in Fig. 2. Lane No. 1 carries the molecular weight standards ranging from 12,500 to 68,000 daltons. Lane No. 2 exhibits the crude PBS extract, and lanes Nos. 3 to 10 demonstrate proteins as they eluted with increasing ammonium sulfate saturation. Frac-

TABLE II. Detection of IgE antibodies with specificity toward wheat flour proteins
ELISA precoated with gliadin or PBS extract

Patient	Total IgE (ng/ml)	Gliadin		PBS extract	
		M	SD	M	SD
Fr	155	0.20	0.002	0.80	0.13
Pa	128	0.09	0.03	0.23	0.10
Lu	342	0.19	0.08	0.43	0.17
Sc	122	0.44	0.03	0.27	0.02
Ku	592	0.26	0.01	0.49	0.16
Ri	282	0.22	0.004	0.45	0.12
hd	177	0.03	0.02	0.11	0.01
Buffer	—	0.02	0.001	0.13	0.05

Values represent optical density at 405 nm and incubation period of 1 hour. M = mean, SD = standard deviation.

tion VIII reveals some impurities if more protein is applied.

The allergenic relevance of these flour protein preparations was demonstrated by their ability to induce histamine release in vitro. Each of the fractions I to VIII (80 ng to 80 µg) led to a secretory response from washed peripheral leukocytes (1×10^7), which were obtained from wheat flour-sensitive individuals. It was found that the fractions of least solubility (VII and VIII) were the most potent stimuli. Approximately 40% of total histamine content were released (data not presented). Cells obtained from hd were not stimulated by these fractions.

The various preparations were then analyzed by ELISA technique for their ability to bind IgG (Fig. 3). They were diluted to yield a final protein concentration of 20 µg/ml and were coated onto the surface of a microtiter plate. Subsequently, sera of four wheat-sensitive atopic subjects (Pa, Ku, Ro, and Za) were diluted by 1:81 and incubated in the wells. A pool of serum had served as control. After washing with PBS plus 0.05% Tween 20, the bound IgG could be demonstrated. As illustrated in Fig. 3, marked differences can be observed when the sera obtained from bakers and hd were compared. Again, sera obtained from atopic subjects revealed a significantly higher wheat-specific IgG titer than the hd. Furthermore, the antigenicity as measured by IgG binding capacity increases with decreasing solubility of the wheat proteins. This finding contrasts to the results obtained with sera of hd. With these sera an approximately equal amount of IgG antibody binding can be demonstrated for each of the cereal components.

Thus, it became evident that all fractions contain

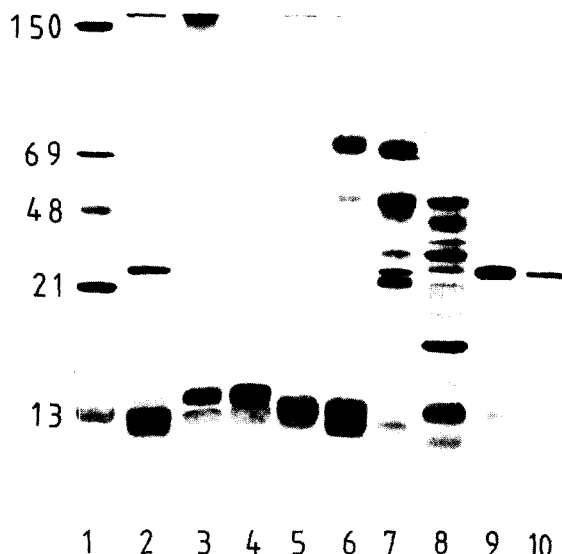


FIG. 2. SDS gel electrophoresis of cereal protein fractions obtained after interfacial hydrophobic chromatography. 1, molecular weight standards; 2, crude wheat flour extract; 3, fraction VIII (eluted with PBS); 4, fraction VII (10% AS); 5, fraction VI (20% AS); 6, fraction V (30% AS); 7, fraction IV (40% AS); 8, fraction III (50% AS); 9, fraction II (60% AS); and 10, fraction I (70% AS).

proteins of immunologic relevance. Therefore, the crude extract was used for further investigations.

Demonstration of cereal antigens and allergens

We then compared the pattern of human antibody binding by the immunoblotting technique. For this purpose the water-soluble proteins of wheat flour were separated by SDS gel electrophoresis. Subsequently, the proteins were electroblotted to a sheet of pure nitrocellulose. The cellulose was cut into strips for analysis of 0.5 by 12 cm in size. The filter strips were incubated with 10 ml of human serum (diluted 1:5). After washing, the bound immunoglobulin was demonstrated by an HRP-labeled second antibody. Fig. 4 demonstrates (from left to right) the IgG-binding profile of two sera obtained from allergic bakers, 24 sera obtained from hd, and six sera obtained from infants (4 to 6 months). It is apparent that virtually every serum exhibits an IgG specificity for several cereal proteins. A group of proteins, which is recognized by most of the sera, migrates with an Rf value of 0.3.

A comparison of the IgG and IgE-binding pattern is presented in Fig. 5. The serum in this assay of one allergic baker (Sc) was incubated with several filter strips. Subsequently, antihuman IgG (I to II) and antihuman IgE (III to V) was added. A great variety of

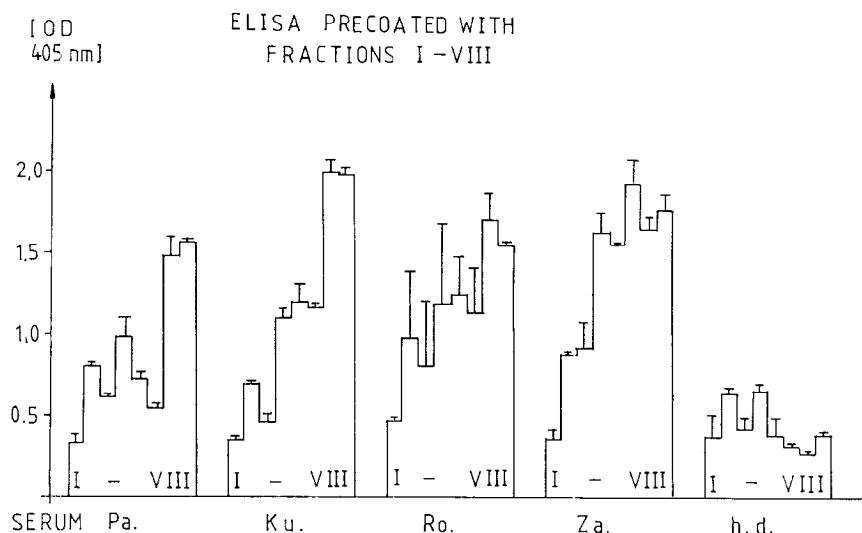


FIG. 3. IgG binding to fractions I to VIII as detected by ELISA. Sera (diluted 1:81) was obtained from four atopic subjects (Pa, Ku, Ro, and Za) and one hd. ELISA readings were obtained 30 minutes after the substrate was added.

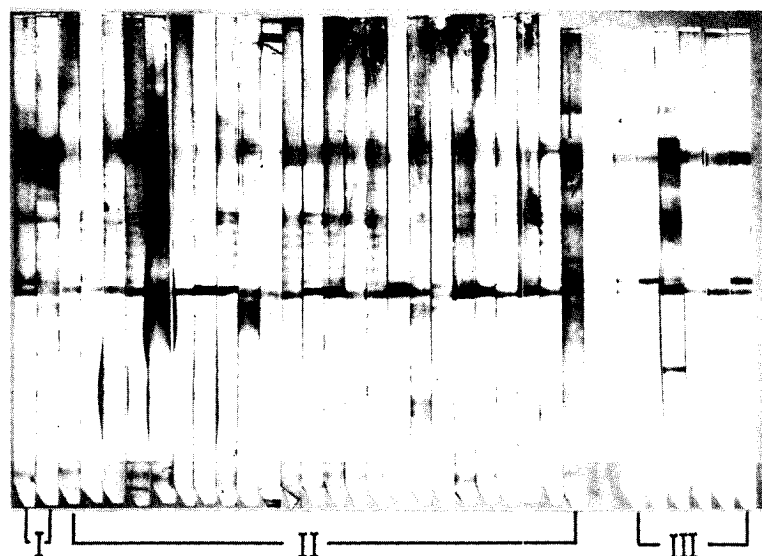


FIG. 4. Demonstration of IgG binding to "Western blotted" cereal proteins. Sera were obtained (left to right) from two atopic subjects (I), 24 hd (II), and six infants (4 to 6 months) (III).

cereal proteins are recognized by IgG (filters I to II). The IgE antibodies are detected in filters III to V. Obviously, most protein bands, which are stained by anti-IgG, are visible in the anti-IgE blot as well.

Binding of monoclonal antibodies to cereal proteins

We attempted to produce monoclonal antibodies against the crude flour extract for further analysis. BALB/c mice were immunized with PBS-extracted wheat flour. In order to achieve better solubility of the

antigens, 10% NaCl was added.⁵ The spleens of the immunized mice were obtained, and fusion was carried out with a mouse B lymphoma cell line (NS-1). A total of 16 antibody-secreting hybridomas were identified. Two of the monoclonal antibodies were partially purified and immobilized on CNBr-activated Sepharose 4B.¹³

As has been previously described, the corresponding antigens were purified from the crude extract by affinity chromatography. However, it was observed that with increasing amounts of adsorbed material

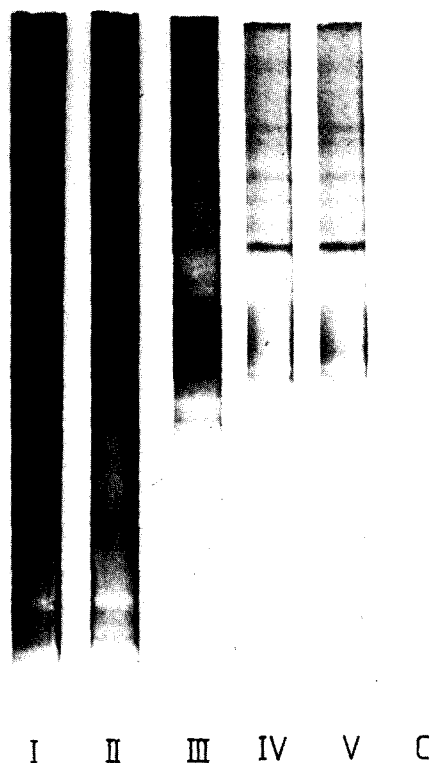


FIG. 5. Comparison of IgG and IgE binding specificity in serum of one atopic baker. Filters I to II demonstrate the IgG binding and filters III to V, the reaction of human IgE with wheat flour proteins.

(about 100 $\mu\text{g}/\text{ml}$), the number of detectable protein bands increased as well. It became apparent that at least two protein bands with approximate molecular weights of 5000 and 10,000 daltons, respectively, were adsorbed by the immunoaffinity columns.⁴ Therefore, an attempt was made to characterize the antibody specificity by the "Western blotting" technique.

Wheat flour was separated in the presence of SDS by a 12.5% acrylamide gel and electroblotted onto a nitrocellulose filter. Subsequently, the filter was incubated with the culture supernatant from wheat flour-specific hybridomas containing 10 to 100 μg of antibody per milliliter. The monoclonal antibody that had bound to the solid support was demonstrated with an HRP-conjugated antimouse immunoglobulin. Fig. 6 illustrates that each monoclonal antibody ($n = 8$) reacted with a multitude of wheat flour proteins. As a control, buffer served instead of culture supernatant (filter strip on the very right). One molecule with an apparent molecular weight of approximately 30,000 daltons (estimated by its R_f value of 0.56) is stained nonspecifically. With the exception of this protein, the staining of all other bands could be inhibited by

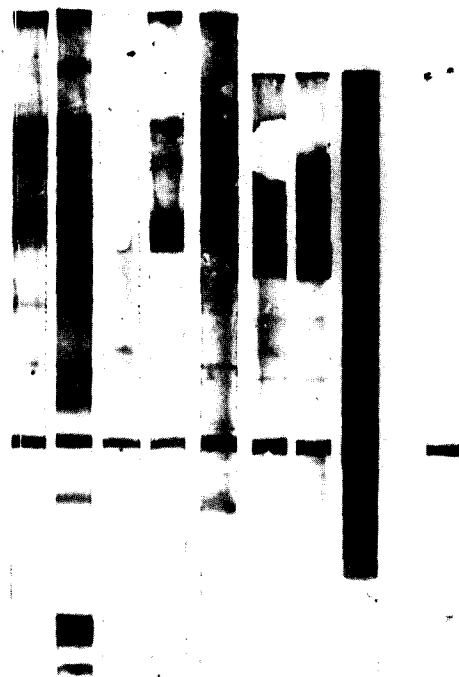


FIG. 6. Binding of monoclonal antibodies to cereal components. The "Western blots" of wheat flour proteins were incubated with hybridoma culture supernatant. The monoclonal antibody is detected with an enzyme-labeled second antibody.

the addition of PBS-extracted wheat flour to the culture supernatant (data not presented). As a further control, we analyzed a culture supernatant containing monoclonal antibodies ($>100 \mu\text{g}/\text{ml}$) with specificity toward human transferrin. No additional nonspecific antibody binding was detectable (data not presented). Furthermore, the nonspecific binding can be reduced by an increased amount of Tween 20, i.e., 0.5%, which does not affect the staining pattern for the other cereal proteins. Filters incubated with buffer instead of enzyme-labeled antibody demonstrated no substrate conversion. Thus, it is obvious that wheat flour does not contain peroxidase activity. Therefore, it is concluded that several wheat flour proteins may exhibit identical epitopes.

DISCUSSION

The extraction of wheat flour proteins was basically performed as described by Osborne⁵ and Baldo and Wrigley.¹⁴ For the preparation of the water-soluble proteins, the addition of sodium chloride (10%) was omitted in order to obtain an extract under conditions similar to the bronchial environment (pH 7.4; conductivity 14 mS). Thus, this extract can be used for

in vitro studies, e.g., the stimulation of histamine release from basophil leukocytes. In addition, severe denaturation, i.e., by acidic conditions (pH 3.0), was avoided. We are well aware of the fact that the described procedure will yield only a relatively small portion of the entire flour protein. However, although we did not extract all of the possible allergens, it is demonstrated by ELISA, as well as "Western blotting" technique (Tables I and II; Fig. 4), that the PBS-extracted proteins are of immunologic significance. Antibodies of both isotypes, IgG as well as IgE, were demonstrated. Furthermore, the IgE-mediated ability to induce in vitro histamine release from washed peripheral leukocytes must be accepted as an indication for the allergenic efficacy of the PBS-extracted proteins. This finding is in agreement with other investigators who demonstrated important allergens in the water-soluble fraction.^{15, 16} For further studies on wheat allergy, the aqueous extract may be used.

Our results demonstrate that the pooled sera of hd contain IgG antibodies with specificity for the extracted wheat flour proteins (Tables I and II; Figs. 1 and 4). The IgG binding appears to be specific, since it cannot be inhibited by mild detergents such as Tween 20. In contrast, adsorption of IgG antibodies can be inhibited by the addition of cereal proteins (Fig. 1). The data are in agreement with the findings of many investigators who described naturally occurring antibodies against wheat flour extracts and marked differences in titers between hd and, i.e., patients with the gluten-induced enteropathy.^{17, 18}

The measurement of IgG antibodies is of importance, since they are able to activate the complement system. This reaction has been described for food allergy.¹⁹ The complement-split products C3a and C5a could then lead to the activation of mucosa mast cells. To our knowledge, no comparable data are available for the respiratory system; however, it may be assumed that complement activation in the bronchial mucosa could play a pathophysiologic role as well.

The sera of atopic individuals by ELISA analysis revealed the highest antibody binding toward the cereal fractions of least solubility. This finding is in accordance with the fact that IgG antibodies against the poorly soluble gliadins were readily demonstrated. However, it can not be concluded that the solubility of a molecule defines its immunogenicity. The sera of hd did not exhibit this binding pattern (Fig. 3).

The immunoblotting experiments revealed a great variety of cereal proteins recognized by human antibodies. In Fig. 5 the comparison of antigen specificity for IgG and IgE antibodies is demonstrated with the serum of one individual (Sc). Apparently, most protein bands that are stained by the anti-IgG antiserum

are detected by IgE as well. Thus, it appears that most antigens are important allergens as well. It is obvious that a clear-cut distinction between antigens and allergens is not obtained.

In order to overcome this problem, monoclonal antibodies against the aqueous wheat flour extract were raised. A detailed analysis as to the antigenic specificity of the monoclonal antibodies was obtained by the "Western blotting" procedure. Each monoclonal antibody (Fig. 6) reacted with a variety of protein bands. The cloning of cells was achieved by the "limiting dilution" technique. We are quite confident in the efficacy of this procedure, which led to reliable results in fusion protocols directed toward other antigens. Furthermore, nonspecific binding of immunoglobulins can be excluded, since hybridomas with other specificities (e.g., antihuman transferrin) did not bind to the cereal protein. Thus, there is no indication for a lectin-like interaction of the monoclonal antibodies with the various fractions. The data are further supported by the fact that all our antibodies belong to the IgM class without exhibiting identical binding profiles.

The proteins were boiled for gel electrophoresis in the presence of 2% SDS in order to achieve a proper solubility. Thus, it was excluded that the proteins are incompletely separated and, furthermore, that antibody binding is due to traces of the same antigen contaminating several protein bands. Since no protease inhibitors were added to our extracts, it can not be excluded, however, that allergenic molecules detected by the immunoblotting technique might represent oligomeric forms or cleavage products derived from the same basic structure. All protein bands detected in fractions I to VIII (Fig. 2) are present in the crude extract. However, because of their low concentration, they can not be presented without overloading the gel.

Irrespective of their origin, it is suggested that a variety of wheat flour proteins exhibit similar or identical determinants. For the gliadins, which are also recognized by some of our hybridomas (data not presented), it has been suggested that the genes are probably derived from a common ancestor through gene duplication.²⁰ Some degree of sequence homology and immunologic cross-reactivity among the proteins is expected. However, it appears that the number of allergenic epitopes might be much smaller than the number of allergenic molecules that has been anticipated by crossed radioimmuno-electrophoretic studies.²¹

The monoclonal antibodies may represent a useful tool in standardizing the cereal extracts with respect to their antigenicity and allergenicity. A more detailed analysis for the antigen-binding specificity of human

sera by immunoblotting technique could be a valuable procedure in the diagnosis of food-related hypersensitivity reactions.

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