

# Evaluation of an in vitro Mast Cell Degranulation Test in the Context of Food Allergy to Wheat

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## Key Words

Albumin/globulin fraction • ELISA • FcεRI • ω5-Gliadins • Humanized rat cell line • IgE • Mast cells • RBL-2H3 • Wheat allergy

## Abstract

**Background:** Antigenic profiles obtained by ELISA with IgE from patients with wheat food allergy (WFA) established that major allergens are albumins/globulins (AG) for children suffering from atopic eczema/dermatitis syndrome (AEDS), ω5-gliadins for adults suffering from wheat-dependent exercise-induced anaphylaxis (WDEIA), anaphylaxis or urticaria and low-molecular-weight (LMW) glutenin subunits for patients with anaphylaxis. We aimed to characterize a new mast cell transfectant for its ability to degranulate with wheat proteins and patient sera and compare these results to those obtained by ELISA. **Methods:** Thirty sera from patients with WFA were tested: 14 with AEDS (group 1) and 16 with WDEIA, anaphylaxis or urticaria (group 2). An IgE Fc receptor (FcεRI) humanized rat RBL-2H3 line was established by transfection with cDNAs encoding α-, β- and γ-subunits for the human IgE receptor. **Results:** A humanized RBL clone

was selected for its capacity to express mRNA α-, β- and γ-subunits of FcεRI, to bind allergen-specific human IgE and to degranulate. In group 1, sera induced enhanced degranulation with AG extract, but rarely reacted with gliadins and glutenins. In group 2, half of the sera showed degranulation with LMW glutenins whereas the AG fraction and lipid transfer proteins were rarely positive. ω5-Gliadins did not appear as a major allergen in degranulation assays, although functional allergen-specific IgE was measurable in appreciable amounts. **Conclusion:** Our data demonstrate that in wheat food allergen evaluation, correlation exists between mast cell degranulation and IgE measurements, depending on the type of allergen. Therefore, the biological activity of some allergen types may also be affected by other parameters.

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## Introduction

Wheat flour proteins are divided into two classes on the basis of their solubility: water-/salt-soluble albumins and globulins (AG; including lipid transfer proteins: LTP,

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the family of  $\alpha$ -amylase inhibitors) and water-/salt-insoluble gliadins and glutenins, also known as prolamins. Gliadins are classified into  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\omega$ -gliadins, the latter being composed of  $\omega$ 1-,  $\omega$ 2- and  $\omega$ 5-gliadins. Glutenins are multimeric proteins containing high (HMW)- and low-molecular-weight (LMW) subunits [1].

The prevalence of wheat food allergy (WFA) has increased in the last decade in both children and adults [2–5]. However clinical symptoms differ between children and adults. While the atopic eczema/dermatitis syndrome (AEDS) occurs mainly in children, chronic urticaria and wheat-dependent exercise-induced anaphylaxis (WDEIA) are mostly found in adults [5]. Several antigenic profiles were noted in patient IgE depending on age and symptoms [6, 7]. More than 80% of AEDS patients have IgE reacting with the AG fraction, whereas  $\omega$ 5-gliadins represent major allergens for anaphylactic and/or urticaria patients [8–10].  $\alpha$ -/ $\beta$ -Gliadins are frequently recognized by IgE in children with AEDS and LMW glutenin subunits by patients with anaphylaxis. IgE against LTP were detected in about 30% of AEDS sera [10].

The reactivity of patient IgE is commonly evaluated by immunochemical methods like ELISA or Western blotting. These methods indicate an antigen/antibody interaction, but do not necessarily reflect a biological activity such as the capacity to promote mast cell degranulation. Mast cell activation requires two or more epitopes on the allergen to cross-link antigen-specific IgE molecules bound to high-affinity IgE receptors (Fc $\epsilon$ RI) on the mast cell surface. By ELISA, one epitope is sufficient to obtain reactivity, and thus this could lead to false-positive results in the identification of relevant allergens that are biologically active. Additionally, parameters such as the ability of the IgE to bind its receptor or the binding affinity are not taken into account by ELISA [11, 12].

Allergists also use skin prick testing to induce type I allergic reactions, but for ethical reasons, large numbers of antigens cannot be tested by this method. Furthermore, skin prick testing does not always correlate with immunochemical analyses [13, 14] and may also be false positive or negative. Mediator release can also be measured by the basophil assay [15, 16]. However, this test depends on fresh blood samples because of the absence of suitable long-term human basophil cultures. The only cell culture available for research resembling human mast cells are cell lines from patients with mast cell leukemia like HMC-1 [17], KU812 [18] or a human leukocytic basophil-like cell line (LAMA-84) [19]. However, none of these lines release mediators in response to IgE cross-linking. Very recently, LAD-1 and -2, cell lines

from a patient with mast cell leukemia, showing characteristics of human mast cells have been described [20]. These lines released mediators upon Fc $\epsilon$ RI aggregation, but they have the disadvantage of a very long doubling time (2–3 weeks).

The transfection of cDNAs coding for the  $\alpha$ -subunit of the human Fc $\epsilon$ RI into a rat basophilic leukemia (RBL) mast cell line is a means to obtain suitable effectors that can be triggered with human serum IgE and specific allergen [11, 21–25].

In this study, we established a humanized RBL-2H3 line that, in addition to the Fc $\epsilon$ RI  $\alpha$ -chain, was co-transfected with cDNAs coding for human Fc $\epsilon$ RI  $\beta$ - and  $\gamma$ -chains. This transfectant was used to investigate the capacity of wheat protein fractions to trigger a functional degranulation response using sera of patients with WFA. The objective was to characterize this new transfectant for its capacity to bind human allergen-specific IgE and to obtain degranulation profiles for IgE from patients suffering from different symptoms and to compare them with results from ELISAs.

## Materials and Methods

### *Cell Cultures, Plasmids and Transfections*

RBL-2H3 cells were cultured at 37°C in DMEM (Cambrex, Walkersville, Md., USA) containing 2 mM of glutamine, 10% FCS, 100 U/ml penicillin and 100 U/ml streptomycin (Cambrex), as described previously [26].

Expression plasmids (pSR $\alpha$ Neo) containing cDNAs encoding human Fc $\epsilon$ RI $\alpha$  and Fc $\epsilon$ RI $\gamma$  have been described [11, 27]. The human  $\beta$ -chain cDNA cloned into pBJneo [28, 29] was a gift from Dr. H. Küster (Universität Greifswald, Germany).

RBL-2H3 cells ( $4 \times 10^6$  in 400  $\mu$ l) were transfected with a mixture of cDNAs containing 10  $\mu$ g of human Fc $\epsilon$ RI $\alpha$ , human Fc $\epsilon$ RI $\beta$  and human Fc $\epsilon$ RI $\gamma$ , respectively, by electroporation, as described previously [26]. Transfectants were selected in complete medium containing 2 mg/ml G-418 (Gibco BRL, Cergy-Pontoise, France) and 20 mM HEPES (pH 7.3). Resistant clones expressing high levels of human Fc $\epsilon$ RI were considered as clones expressing the complete receptor and were maintained in G-418 at 1 mg/ml. A selected clone (6) was routinely used for all the experiments described hereafter.

### *Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction*

mRNA expression of human Fc $\epsilon$ RI subunits in non-transfected and transfected (clone 6) RBL-2H3 cell lines was studied by real-time quantitative RT-PCR. Cells were directly lysed in a culture dish and total RNA was isolated with TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. RNA samples were treated for 45 min at 37°C with 2 IU RQ1 DNase (Promega, Madison, Wisc., USA). RNA samples were then heated for 10 min at 75°C in order to denature RQ1 DNase. The yield of

each RNA sample was determined by ultraviolet spectrophotometry (VICTORIII, Perkin-Elmer, Waltham, Mass., USA) at a wavelength of 260 nm. One microgram of RNA was used for reverse transcription, performed with 100 IU Superscript III reverse transcriptase (Invitrogen), according to the manufacturer's instructions. The 5× diluted retrotranscription reaction mixture was amplified by PCR using Quantitect SybrGreen buffer (Qiagen, Courtabœuf, France) and 2.5 μM of each forward and reverse primers specific for human FcεRI subunits (α, β and γ) and rat β-actin. We used a set of primers for each chain designed in our laboratory (Q set) with Beacon designer 2.0 software (Biosoft International, Palo Alto, Calif., USA) and then synthesized by MWG-Biotech. Quantitative RT-PCR was realized with the following primers: human α (NCBI sequence, accession No. NM-002001 [30]) Q set forward 5'-CCGCGTGAGAAAGTACTGGC-TAC-3' and reverse 5'-TGTGACCTGCTGCTGAGTTGAG-3'; human β (NCBI sequence, accession No. NM-000139 [31]) Q set forward 5'-GGAAGCCTGGGAGCAAACAC-3' and reverse 5'-TTCTGGCAACTGTGGATGTGG-3'; human γ (NCBI sequence, accession No. NM-004106 [32]) Q set forward 5'-GTCCTCA-CCCTCCTCTACTGTG-3' and reverse 5'-TCGTAAGTCTCCT-GGTTCCCTGG-3'. The samples were subjected to 45 PCR cycles (iQCyler; BioRad, Hercules, Calif., USA). Each PCR cycle consisted of 30 s at 95°C and 10 s at 60°C. Gene expression was analyzed by the 2<sup>-ΔCt</sup> method and the β-actin gene was used as an internal control. Each analysis of relative mRNA expression was repeated three times and the results presented correspond to the mean expression obtained.

#### Flow-Cytometric Analysis

Cells were cultured for 1 or 48 h in medium with or without human IgE (0.4 μg/ml). Cells were harvested by flushing the wells and then stained in PBS with 1% FCS with rabbit anti-human IgE (1:200) followed by anti-rabbit IgG-phycoerythrin (1:200; Jackson-ImmunoResearch, Newmarket, Suffolk, UK). Cells were analyzed by cytofluorometry using Cellquest software (Becton-Dickinson, Le Pont-de-Claix, France).

#### Antibodies and Sera

Human IgE from U266BL myeloma of unknown specificity and rabbit anti-human IgE have been described previously [22].

Sera were obtained from 30 patients with WFA (table 1). They included 13 children: 12 with AEDS and 1 child with anaphylaxis. The 17 adult patients had recurrent urticaria (2 patients), anaphylaxis (1 patient), WDEIA (12 patients) or AEDS (2 patients). Sera were sampled from patients during prick-in-prick (PIP) tests to natural wheat flour, total ω-gliadins, α-gliadins, LMW glutenins and LTP (table 1). PIP was considered positive when the response obtained was ≥75% of the codeine control. Patients were described as food allergic to wheat 1 month later by a positive, double-blind, placebo-controlled food challenge (DBPCFC) [33, 34] after a wheat-free diet of 3 weeks or by an evident positive effect of avoidance diet if DBPCFC could not be performed for ethical reasons. DBPCFC corresponded to the consumption of increasing doses of wheat flour in apple sauce and of placebo that corresponded to corn starch or fecula of potatoes in apple sauce every 20 min. The cumulative reactive dose or the cumulative highest test dose of wheat proteins obtained at the time of DBPCFC is indicated in table 1. PIP and DBPCFC were performed with informed consent of the patients or their parents. We re-

ceived ethical committee approval. Sera from 21 non-wheat allergic subjects (16 non-atopic and 5 allergic to grass pollen) were used as controls.

#### Wheat Protein Fractions

α-, ω5-gliadins, LMW glutenin subunits [6] and LTP [10] were obtained using chromatography steps and an AG fraction by extraction in a phosphate buffer as described. Purity and composition of these fractions were analyzed by SDS-PAGE and reverse-phase HPLC [10]. Wheat protein fractions were also characterized using polyclonal or monoclonal anti-peptide antibodies specific for different gliadin and glutenin fractions [35–37].

#### β-Hexosaminidase Release Assay

Because of cytotoxicity, diluted sera (1:10) were routinely adsorbed twice on untransfected RBL-2H3 cells (5 × 10<sup>4</sup> in 100 μl) at 37°C for 2 h. Transfected RBL-2H3 cells (clone 6) were plated at 10<sup>4</sup> cells/well in a 96-well plate. After adherence (4 h at 37°C), the supernatant was discarded and replaced by the medium containing control myeloma IgE (0.4 μg/ml) or adsorbed sera and cells were cultured for 48 h. After two washes, IgE-sensitized cells were stimulated in 100 μl Tyrode buffer containing 50% deuterium oxide (D<sub>2</sub>O; Sigma, St. Louis, Mo., USA) with anti-human IgE (1 μg/ml) or indicated concentrations of wheat proteins for 45 min at 37°C, and β-hexosaminidase activity was measured as described [11, 22]. All results are expressed as a percentage of total β-hexosaminidase (determined after lysis in 0.3% Triton X-100) after correction for spontaneous release in supernatants from unstimulated cultures (net release). Measurements were considered positive if the percentage of β-hexosaminidase release was ≥4%. This limit was established by analyzing 13 non-wheat-allergic control sera (8 non-atopic and 5 allergic to grass pollen) and corresponded to the mean + 2 SD.

#### Fluorimetric ELISA for IgE Binding Profiles

The reactivity of sera to wheat protein fractions was analyzed as described previously [10]. Briefly, gliadins, LMW glutenins and AG were coated (5 μg/ml) in carbonate buffer, except for LTP, which was coated at 1 μg/ml in PBS (100 μl/well). After 3 washes with PBS/0.05% Tween 20 (PBS/T), saturation with 200 μl of 0.5% gelatin (Sigma, G-2500) in PBS/T (PBS/T/G) and 3 washes with PBS/T, the plates (Falcon 3944, BD Biosciences, San Jose, Calif., USA) were incubated for 2 h at 37°C with patient and control sera diluted 1:10 in PBS/T/G (100 μl/well). Goat anti-human IgE alkaline phosphatase conjugate (ε-chain specific, Sigma A-3525) was added for 2 h at 37°C (100 μl/well, 1:1,000) before addition of fluorescent substrate, 4-MUP (4-methylumbelliferyl phosphate, Sigma M-3168) diluted 1/5 in 1 M Tris/HCl (pH 9.8) buffer (150 μl/well) and incubation for 90 min at room temperature in the dark. Fluorescence was measured at 440 nm (excitation 360 nm). Values reported are the ratio of the mean fluorescence for a given antigen to the fluorescence obtained for control wells containing no antigen. They were considered positive if the ratio was ≥3, and this limit of positivity was established with 8 non-atopic control sera from the mean + 2 SD.

#### Fluorimetric ELISA for Total and Specific IgE Quantification

The ELISA used for the quantification of human IgE comprised two parts.

**Table 1.** Characteristics of the WFA patients

| Patient No. | Symptoms | Age years | PIP         |                  |            |               |     | DBPCFC reactive or highest dose mg protein equivalent |
|-------------|----------|-----------|-------------|------------------|------------|---------------|-----|-------------------------------------------------------|
|             |          |           | wheat flour | total ω-gliadins | α-gliadins | LMW glutenins | LTP |                                                       |
| Group 1     |          |           |             |                  |            |               |     |                                                       |
| 1           | AEDS     | 1         | +           | NT               | NT         | NT            | NT  | 7.8                                                   |
| 21          | AEDS     | 2         | +           | NT               | NT         | NT            | NT  | 4,000                                                 |
| 25          | AEDS     | 4         | +           | NT               | +          | NT            | NT  | 1,000                                                 |
| 27          | AEDS     | 4         | +           | NT               | NT         | NT            | NT  | 294 <sup>1</sup>                                      |
| 33          | AEDS     | 4         | +           | NT               | +          | NT            | +   | 480                                                   |
| 50          | AEDS     | 2         | +           | +                | +          | +             | NT  | 1,000                                                 |
| 56          | AEDS     | 2         | +           | NT               | NT         | NT            | NT  | 1,560                                                 |
| 65          | AEDS     | 3         | +           | –                | +          | –             | NT  | 97.5 <sup>1</sup>                                     |
| 67          | AEDS     | 34        | +           | –                | –          | –             | +   | NT <sup>1</sup>                                       |
| 79          | AEDS     | 2         | +           | +                | +          | +             | +   | 97.5                                                  |
| 8           | AEDS     | 9         | –           | NT               | NT         | NT            | –   | 850                                                   |
| 53          | AEDS     | 3         | +           | NT               | NT         | NT            | NT  | NT (LT+) <sup>3</sup>                                 |
| 15          | AEDS     | 29        | +           | NT               | NT         | NT            | NT  | 3,200                                                 |
| 70          | AEDS     | 3         | +           | +                | +          | –             | +   | 97.5 <sup>2</sup>                                     |
| Group 2     |          |           |             |                  |            |               |     |                                                       |
| 3           | U        | 65        | +           | NT               | NT         | NT            | NT  | 9,000 <sup>1</sup>                                    |
| 45          | U        | 50        | +           | +                | –          | –             | +   | 4,200 <sup>1</sup>                                    |
| 41          | A        | 3         | +           | +                | +          | +             | NT  | NT <sup>2</sup>                                       |
| 48          | A        | 32        | –           | +                | +          | –             | NT  | 11,200                                                |
| 52          | WDEIA    | 42        | –           | +                | +          | +             | NT  | NT <sup>2</sup>                                       |
| 60          | WDEIA    | 24        | +           | +                | –          | –             | NT  | 8,150 <sup>1</sup>                                    |
| 31          | WDEIA    | 37        | +           | +                | –          | –             | NT  | NT <sup>2</sup>                                       |
| 58          | WDEIA    | 44        | +           | +                | +          | –             | NT  | 1,500                                                 |
| 59          | WDEIA    | 27        | –           | +                | –          | +             | NT  | 6,600                                                 |
| 61          | WDEIA    | 41        | +           | +                | –          | +             | NT  | 11,240                                                |
| 71          | WDEIA    | 54        | +           | +                | +          | –             | NT  | 15,300                                                |
| 80          | WDEIA    | 49        | +           | +                | –          | –             | NT  | NT <sup>2</sup>                                       |
| 69          | WDEIA    | 43        | +           | NT               | +          | +             | NT  | 12,350                                                |
| 63          | WDEIA    | 31        | –           | NT               | +          | +             | NT  | 11,240 <sup>1</sup>                                   |
| 66          | WDEIA    | 38        | +           | –                | –          | –             | NT  | 12,350                                                |
| 77          | WDEIA    | 29        | +           | –                | –          | –             | NT  | NT <sup>2</sup>                                       |

A = Anaphylaxis; LT = labial test; NT = not tested; U = urticaria. PIP tests to natural wheat flour and purified wheat proteins are positive when the responses obtained are  $\geq 75\%$  of the codeine control. DBPCFC was positive when the cumulative dose of proteins induced a clinical reaction. Positive values are italic and negative results corresponded to various situations.

<sup>1</sup> Positive effect of avoidance diet.

<sup>2</sup> DBPCFC not performed or discontinued for ethical reason because of severe symptoms.

<sup>3</sup> DBPCFC not performed because of a severe reaction to the labial test.

(1) A sandwich ELISA was applied for the standard curve and the dosage of total IgE: a primary rabbit anti-human IgE antibody ( $\epsilon$ -chain-specific A0094, DAKO) diluted in 100 mM carbonate buffer, pH 9.6, was coated onto plates (436007; Nunc, Roskilde, Denmark) at ambient temperature with shaking for 2 h; then washes and saturation were performed as above except that Tween (0.1%) was used; known concentrations of standard human IgE for the standard curve (human serum IgE; 2nd WHO Interna-

tional Reference Preparation, established in 1981; NIBSC Code No. 75/502), or dilutions of the sera tested for total IgE quantification were incubated for 15 h at 37°C; PBS/T/G was used for dilutions, and 8 control wells with PBS/T/G (no IgE) were included in the test.

(2) An antigen-coated plate ELISA was used for the quantification of specific IgE: on the same plate, the antigens were coated at 5  $\mu$ g/ml in 100 mM carbonate buffer, pH 9.6, and after washes and

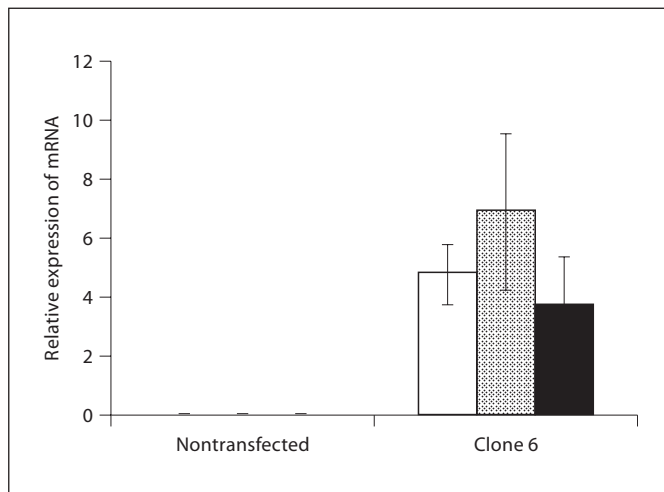


**Fig. 1.** Verification of the expression of mRNA coding for the  $\alpha$ - ( $\square$ ),  $\beta$ - ( $\square$ ) and  $\gamma$ -subunits ( $\blacksquare$ ) of human Fc $\epsilon$ RI of transfected clone 6 and the untransfected cell line RBL by quantitative RT-PCR. A set of primers was used for each chain ( $\alpha$ -,  $\beta$ - and  $\gamma$ -chains) called the Q set (established in our laboratory).

saturation, sera were diluted (1:10) and incubated for 15 h at 37°C; two control wells with 10 mM ethanolamine in carbonate buffer instead of antigen were included for each serum.

IgE fixed on the primary antibody or the antigens were revealed as above.

To relate fluorescence intensity to IgE concentration, a sigmoid 4-parameter model [ $y = d + (a - d)/(1 + (x/c)^b$ )] was adjusted to the standard curve data by non-linear regression using the Solver option from Microsoft® Excel 2000 by minimizing the sum of the squared differences. Limits of detection (means + 3 SD) and quantification (means + 10 SD) were calculated from fluorescence data of the 8 wells with no IgE. Fluorescence data measured for each antigen and serum in the antigen-coated plate ELISA were corrected by subtracting the fluorescence of the control with no antigen and corresponding specific IgE concentrations were calculated from the adjusted standard curve provided that the fluorescence exceeded the quantification limit.

## Results

### Characterization of Fc $\epsilon$ RI Humanized RBL Mast Cell Transfectants

#### Generation of an RBL Clone Transfected with Human Fc $\epsilon$ RI $\alpha$ -, $\beta$ - and $\gamma$ -Chains

RBL-2H3 cells were transfected with cDNAs encoding human Fc $\epsilon$ RI  $\alpha$ -,  $\beta$ - and  $\gamma$ -chains. One clone (clone 6) was selected and the relative expression of human  $\alpha$ -,  $\beta$ - and  $\gamma$ -chains was tested by quantitative RT-PCR with the Q set of primers for each chain (fig. 1). Clone 6 was

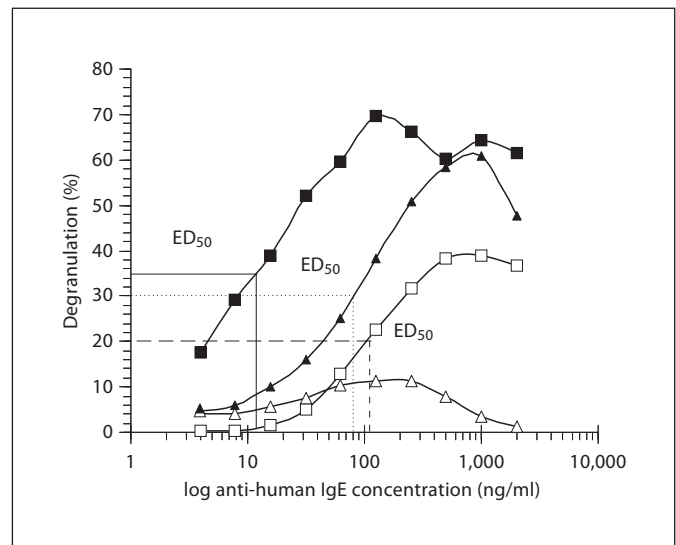


**Fig. 2.** Comparison of degranulation efficiency between the  $\alpha$ -,  $\beta$ - and  $\gamma$ -chain transfectant (clone 6) and the previous  $\alpha$ -chain transfectant (clone 12.8). Degranulation of huFc $\epsilon$ RI RBL-2H3 clone 6 cells ( $\blacksquare$ ,  $\square$ ) and huFc $\epsilon$ RI RBL-2H3 clone 12.8 ( $\blacktriangle$ ,  $\triangle$ ) after sensitization with human IgE in response to an increase in anti-human IgE concentration in the presence ( $\blacksquare$ ,  $\blacktriangle$ ) or absence ( $\square$ ,  $\triangle$ ) of D<sub>2</sub>O. ED<sub>50</sub> is shown for clones 6 and 12.8 in the presence of D<sub>2</sub>O and in clone 6 in the absence of D<sub>2</sub>O.

positive for all chains. The mRNA expression level was approximately the same for  $\alpha$ - and  $\gamma$ -chains and was higher for the  $\beta$ -chain. As expected, the control parent cell line RBL-2H3 was negative for the three human chains.

### RBL Transfectant (Clone 6) Is Highly Efficient in Degranulation through Human IgE

To confirm the advantage of the clone transfected with all Fc $\epsilon$ RI chains (clone 6), we compared its degranulation potential with that of a previously established clone (clone 12.8) [11] transfected with the  $\alpha$ -chain only (fig. 2). IgE-sensitized cells were stimulated with anti-human IgE in the absence or presence of D<sub>2</sub>O [38].

In the presence of D<sub>2</sub>O (fig. 2), the clones displayed very different degranulation profiles. ED<sub>50</sub> values for clone 6 and clone 12.8 were estimated to be 12 and 80 ng/ml, respectively, i.e. clone 6 was more effective and sensitive than clone 12.8. In contrast to clone 12.8, clone 6 also strongly degranulated in the absence of D<sub>2</sub>O with an ED<sub>50</sub> of approximately 120 ng/ml (fig. 2). The response was, however, stronger in the presence of D<sub>2</sub>O. To maximize sensitivity, all further experiments were thus carried out in the presence of D<sub>2</sub>O.

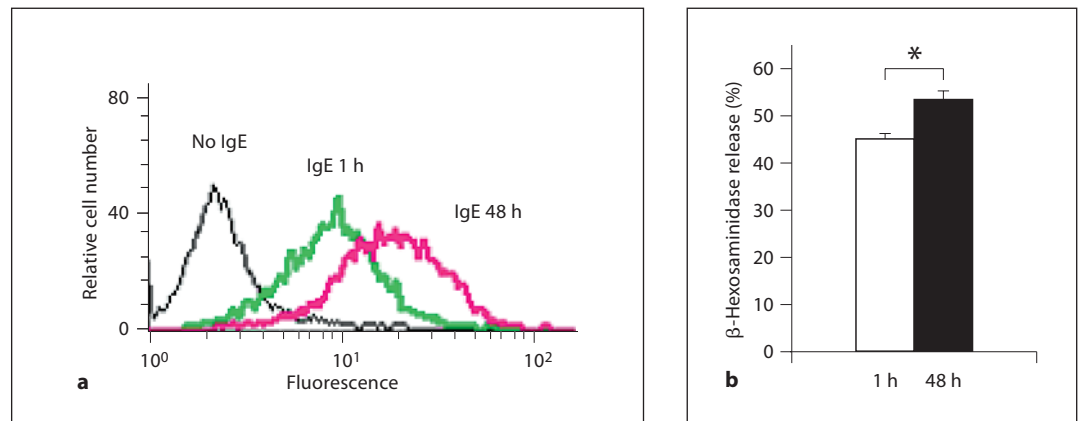


**Fig. 3.** Establishment of RBL mast cell  $\alpha$ -,  $\beta$ - and  $\gamma$ -chain transfectant. **a** Flow-cytometric analysis of human IgE binding in human Fc $\epsilon$ RI RBL-2H3 clone 6 cells. Cells were either unincubated or incubated with human IgE for the indicated times. Expression of human IgE binding was measured by cytofluorometry using

rabbit anti-human IgE and anti-rabbit phycoerythrin. **b** Degranulation response of human Fc $\epsilon$ RI RBL-2H3 clone 6 cells after sensitization with human IgE for 1 or 48 h in the presence of D<sub>2</sub>O. Data are means  $\pm$  SEM (n = 5), \* p < 0.05.

Clone 6 also showed high human IgE binding capacity after incubation for 1 h and this binding capacity was increased after prolonged incubation (48 h; fig. 3a). A strong degranulation response of clone 6 was obtained after 1 h of sensitization with IgE in the presence of D<sub>2</sub>O. This response significantly increased after 48 h (fig. 3b). All further experiments were carried out after sensitization for 48 h.

#### *Characteristics of Sera from Patients with WFA*

Thirty sera (table 1) were selected based on their reactivity against the major IgE-binding antigens of wheat flour determined previously by ELISA [10].

Table 1 lists the two groups of patients separated on the basis of their symptoms and results of PIP tests to natural wheat flour and to some purified wheat proteins: group 1 included 12 children and 2 adult patients suffering from AEDS and group 2 comprised patients suffering from WDEIA (12 adults), chronic urticaria (2 adults) and anaphylaxis (1 adult and 1 child). All patients from group 1, except 1 (patient 8), showed reactivity against wheat flour in PIP. They frequently responded to  $\alpha$ -gliadins (6 of 7 were positive). Despite the fact that few of them were tested against the other wheat proteins, we noted that 4 were positive against LTP.

The second group of patients frequently reacted to wheat flour, except for 4 patients (52, 48, 59 and 63) that were at least positive against one purified wheat protein. Group 2 showed increased reactivity against total  $\omega$ -gliadins (11 of 13 positive) and about half of the patients re-

sponded to  $\alpha$ -gliadins (7 of 15 positive) and LMW glutenins (6 of 15 positive). Only 1 patient in this group was tested against LTP and was positive.

Table 2 summarizes the reactivity of sera in the degranulation assay and in ELISA against the five indicated wheat fractions. Sera from group 1 were preferentially analyzed with the AG,  $\alpha$ -gliadin and LTP fractions, since these fractions were the most frequent IgE-binding antigens in ELISA for patients with AEDS, but some were also tested against  $\omega$ 5-gliadins and LMW glutenins depending on serum availability. Sera from group 2 were preferentially analyzed with  $\omega$ 5-gliadins and LMW glutenin fractions, since these fractions were the antigens that bound more frequently IgE in patients with WDEIA, urticaria and anaphylaxis. Regarding group 1, some were also tested with AG,  $\alpha$ -gliadin and LTP fractions. Table 3 shows the total IgE concentrations (ng/ml); in all sera tested, the total IgE concentration was  $\geq 244$  ng/ml except for serum 63.

#### *Serum Reactivity in Mast Cell Degranulation Assays for Group 1: Patients with AEDS (Table 2)*

Figure 4a shows an example of dose response for mast cell degranulation obtained with one serum (patient 65) using different wheat proteins. Appreciable degranulation was observed in the presence of LTP and AG fractions. Out of the 13 sera analyzed, the AG extract induced degranulation in 11 of the 12 sera that were positive for these proteins by ELISA. Five of the 8 sera with IgE binding to LTP in ELISA gave a positive signal in the presence

**Table 2.** Reactivity of the two groups of sera against wheat proteins obtained by ELISA or mast cell degranulation

| Patient No. | Symptoms | $\alpha$ -Gliadins |      | $\omega$ 5-Gliadins |       | LMW glutenins |       | AG fraction |      | LTP   |       |
|-------------|----------|--------------------|------|---------------------|-------|---------------|-------|-------------|------|-------|-------|
|             |          | ELISA              | mast | ELISA               | mast  | ELISA         | mast  | ELISA       | mast | ELISA | mast  |
| Group 1     |          |                    |      |                     |       |               |       |             |      |       |       |
| 1           | AEDS     | 2.9                | 0    |                     |       |               |       | 3.2         | 2.4  |       |       |
| 21          | AEDS     | 2.3                | 0    |                     |       |               |       | 2.2         | 0    |       |       |
| 25          | AEDS     | 0                  | 1.2  | 0                   | 0     | 0             | 1.9   | 4           | 5.8  | 12    | 0.28  |
| 27          | AEDS     | 2.8                | 1.4  | 0                   | 1.79  | 2.1           | 0     | 7.1         | 9.8  | 2.6   | 1.5   |
| 33          | AEDS     | 6                  | 4.3  | 5                   | 2     | 3.3           | 7.7   | 13.6        | 17.5 | 13.7  | 18.7  |
| 50          | AEDS     | 15                 | 1.1  |                     |       |               |       | 16.5        | 6.4  | 3.5   | 1.9   |
| 56          | AEDS     | 4.7                | 1.4  |                     |       |               |       | 8.1         | 12.4 |       |       |
| 65          | AEDS     | 3.7                | 3.2  | 0                   | 1.9   | 0             | 0.7   | 8.2         | 20.5 | 4.6   | 12.3  |
| 67          | AEDS     |                    |      |                     |       |               |       |             |      | 7.97  | 23.06 |
| 79          | AEDS     | 28.98              | 16.5 | 21.66               | 13.58 | 21.66         | 34.29 | 26.75       | 57.8 |       |       |
| 8           | AEDS     | 6.6                | 1    |                     |       | 10.3          | 0     | 4.3         | 6.9  |       |       |
| 53          | AEDS     |                    |      |                     |       |               |       | 16.5        | 45.5 | 10.4  | 9.9   |
| 15          | AEDS     |                    |      |                     |       |               |       | 13.2        | 25.9 | 6.9   | 14.3  |
| 70          | AEDS     | 8.95               | 4.8  |                     |       |               |       | 22.05       | 29.3 | 4.94  | 1.6   |
| Group 2     |          |                    |      |                     |       |               |       |             |      |       |       |
| 3           | U        | 0                  | 2.56 | 21.3                | 7.4   | 0             | 19.1  |             |      | 0     | 0.2   |
| 45          | U        | 0                  | 0    | 16.8                | 3.1   |               |       | 2           | 0    | 2.2   | 0     |
| 41          | A        | 21.9               | 19.6 | 17.1                | 37.2  | 9.7           | 49.2  | 21.7        | 9.6  | 13.7  | 16.7  |
| 48          | A        |                    |      | 3.9                 | 0     |               |       | 2           | 0.2  |       |       |
| 52          | WDEIA    |                    |      | 3.1                 | 6.97  | 0             | 1.8   |             |      |       |       |
| 60          | WDEIA    |                    |      | 22.8                | 0     | 10.2          | 4.77  |             |      |       |       |
| 31          | WDEIA    |                    |      | 15.9                | 0.67  |               |       |             |      |       |       |
| 58          | WDEIA    | 0                  | 0    | 34.4                | 4.7   | 3.3           | 0     | 0           | 0.69 | 0     | 0.1   |
| 59          | WDEIA    | 0                  | 0    | 3                   | 0.3   | 2.5           | 1.25  | 2.3         | 0    | 0     | 2.8   |
| 61          | WDEIA    | 8.8                | 0    | 76.5                | 5.7   | 7.9           | 1.7   | 2.5         | 0.4  |       |       |
| 71          | WDEIA    |                    |      | 2.8                 | 1.52  | 1.5           | 2.9   | 0           | 0    |       |       |
| 80          | WDEIA    |                    |      | 5.2                 | 0     | 10.39         | 4.3   |             |      |       |       |
| 69          | WDEIA    |                    |      | 6.99                | 0     | 2.4           | 4.35  |             |      |       |       |
| 63          | WDEIA    |                    |      | 2                   | 0     | 3.2           | 6.21  | 2.8         | 0.6  | 3.3   | 4.34  |
| 66          | WDEIA    |                    |      | 2.4                 | 1.51  |               |       |             |      |       |       |
| 77          | WDEIA    |                    |      | 55.97               | 3.9   |               |       |             |      |       |       |

For ELISA, results are expressed as the signal/background ratio. The test is positive when the ratio is  $\geq 3$ . For mast cell tests, percent degranulation is given. Values correspond to the maximal response obtained with three different antigen concentrations. The test is positive when the percentage is  $\geq 4$ . The limits of positivity for both tests were established with control sera. Positive values are shown in italics and negative values are shown in normal print.

of LTP in the mast cell assay. One gave a negative response in both tests (patient 27). Eleven sera were tested with  $\alpha$ -gliadins. From the 7 sera positive in ELISA, only 3 sera gave a positive signal in the presence of  $\alpha$ -gliadins in the mast cell test. LMW glutenins were recognized by 2 and 3 of the 6 sera tested in the mast cell test and ELISA, re-

spectively.  $\omega$ 5-Gliadins were rarely recognized by these patient sera in both assays (only patient 79).

Figure 4b shows the correlation of signal intensity between ELISA and mast cell degranulation tests with the two classes of wheat proteins (soluble: LTP and A/G fraction and insoluble fractions:  $\alpha$ - and  $\omega$ 5-gliadins, and

LMW glutenins). The signal intensities recorded in the tests were correlated in the two classes of wheat proteins ( $R^2 = 0.63$  for soluble proteins and  $0.55$  for insoluble proteins), but with a higher slope for soluble proteins.

In group 1, degranulation was observed in 12 of the 14 sera with at least one of the wheat proteins, 5 gave only a positive response with the AG extract and 1 only with LTP, whereas 6 reacted with two or more fractions. One serum (patient 21) was negative in both tests, although it had shown a positive reaction to wheat flour in the PIP test. Interestingly, the patient (patient 8) that had shown a negative PIP test against wheat flour and LTP showed a positive reaction against the AG fraction by ELISA and degranulation assays.

#### *Serum Reactivity in the Mast Cell Degranulation Assays for Group 2: Patients with Urticaria, Anaphylaxis and WDEIA (Table 2)*

Figure 5a shows an example of dose response for mast cell degranulation obtained with 1 serum (patient 3) from an urticaria patient using different wheat proteins. Degranulation was observed with  $\omega 5$ -gliadins and LMW glutenins. Sixteen sera were tested with  $\omega 5$ -gliadins, 3 of which were negative in both tests. Only 5 patients were positive for  $\omega 5$ -gliadins in the mast cell degranulation test (31%), whereas 13 had IgE binding to this protein by ELISA. From 11 sera tested against LMW glutenin subunits, 4 gave positive responses in both tests, 2 were positive only by ELISA and another 2 were positive only in the mast cell test. However, the latter (patients 3 and 69) also reacted with a glutenin extract by Western blotting (not shown). Eight sera of this group were tested against the AG fraction and only 1 (patient 41) responded in both the mast cell degranulation test and ELISA. Two of the 6 sera (patients 41 and 63) analyzed had IgE binding to LTP in the mast cell test and ELISA. Only 1 serum (patient 41) was positive to  $\alpha$ -gliadins in both tests. Another gave a positive result by ELISA. Serum 41, the only child of group 2, presented a particular reactivity. Indeed, this serum strongly recognized all protein fractions. Its reactivity profile was similar to that of serum 79 (in group 1), a child suffering from AEDS.

Figure 5b shows the correlation of signal intensity between ELISA and mast cell degranulation tests with the two classes of wheat proteins. The signal intensities were correlated for soluble proteins ( $R^2 = 0.66$ ) but not for insoluble proteins ( $R^2 = 0.01$ ). The slope obtained with insoluble proteins was weak because of the absence of  $\omega 5$ -gliadin recognition in the mast cell test.

**Table 3.** Quantification of total IgE in the sera of both patient groups

| Patient No.    | Symptoms | Total IgE kU/l |
|----------------|----------|----------------|
| <i>Group 1</i> |          |                |
| 1              | AEDS     | 123            |
| 21             | AEDS     | 832            |
| 25             | AEDS     | >13,115        |
| 27             | AEDS     | NT             |
| 33             | AEDS     | 240            |
| 50             | AEDS     | NT             |
| 56             | AEDS     | NT             |
| 65             | AEDS     | 237            |
| 67             | AEDS     | 536            |
| 79             | AEDS     | 2,648          |
| 8              | AEDS     | 6,486          |
| 53             | AEDS     | NT             |
| 15             | AEDS     | NT             |
| 70             | AEDS     | 380            |
| <i>Group 2</i> |          |                |
| 3              | U        | 2,278          |
| 45             | U        | 282            |
| 41             | A        | 1,484          |
| 48             | A        | 267            |
| 52             | WDEIA    | 428            |
| 60             | WDEIA    | 420            |
| 31             | WDEIA    | 316            |
| 58             | WDEIA    | 1,582          |
| 59             | WDEIA    | 105            |
| 61             | WDEIA    | 5,377          |
| 71             | WDEIA    | 142            |
| 80             | WDEIA    | 170            |
| 69             | WDEIA    | 213            |
| 63             | WDEIA    | 36             |
| 66             | WDEIA    | 252            |
| 77             | WDEIA    | NT             |

A = Anaphylaxis; NT = sera were not tested; U = urticaria.

In this group of patients with urticaria, anaphylaxis and WDEIA, degranulation was observed more frequently with LMW glutenins (55%) followed by  $\omega 5$ -gliadins (31%).

#### *Summary of Wheat Protein Reactivity in the Mast Cell Degranulation Assay*

Combining all wheat proteins, 37% of ELISA-positive sera were unable to induce mast cell activation. Yet, the results differed depending on the proteins. Disregarding the clinical status of WFA patients, 56 and 60% of sera positive to  $\alpha$ - and  $\omega 5$ -gliadins, respectively, by ELISA did

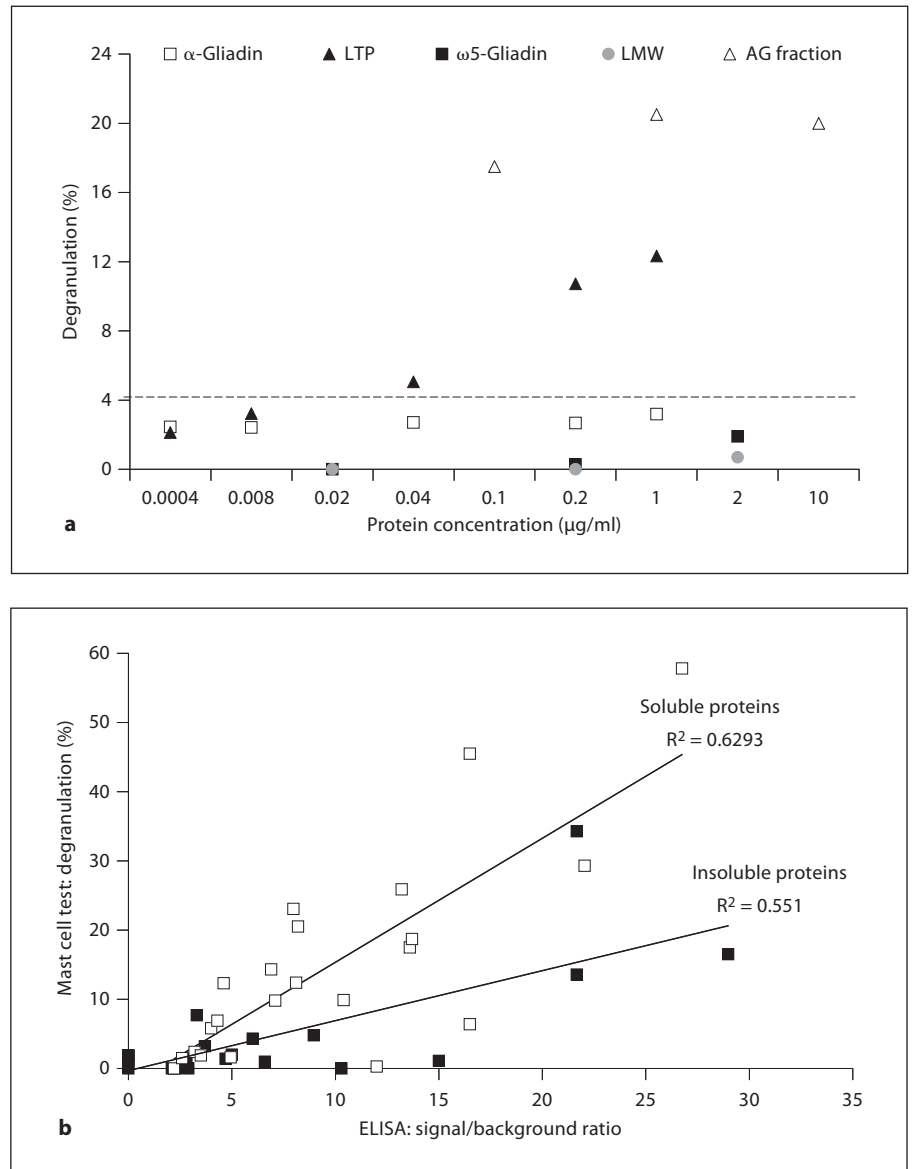


**Fig. 4.** AEDS patients. **a** Degranulation obtained with wheat proteins ( $\alpha$ -gliadin,  $\omega$ 5-gliadin, LMW glutenin, AG fraction and LTP) at different concentrations for patient 65. The positive threshold is indicated by the stippled line. **b** Correlation of signal intensity between ELISA and mast cell degranulation tests with the two classes of wheat proteins [soluble ( $\square$ ) and insoluble fractions ( $\blacksquare$ )]. The correlation was expressed by  $R^2$ .

not induce mast cell degranulation. With LTP and LMW glutenins, approximately 30% of ELISA-positive sera were unable to induce mast cell degranulation. It is important to indicate that 2 sera negative to LMW glutenins by ELISA but positive by Western blot were able to induce degranulation in the presence of these proteins (patients 3 and 69). Only 8% of sera positive to AG extract by ELISA did not induce degranulation in the presence of this fraction.

Thus, in WFA patients, the capacity of IgE to bind to soluble proteins and LMW glutenins was a possible prognosticator of the biological activity of mast cell degranulation, but not in the case of gliadins.

#### *Correlation of Mast Cell Degranulation with the Concentration of Specific IgE against Wheat Proteins*

An ELISA was developed to quantify specific IgE against wheat proteins. Due to the scarcity of available sera, we could not determine specific IgE concentrations for all patients and proteins. In figure 6, the percentage of mast cell degranulation obtained in response to wheat proteins ( $\alpha$ - and  $\omega$ 5-gliadins, LMW glutenins, AG extract and LTP) in different WFA sera (diluted 1:10) was compared to specific IgE concentrations. Sera with specific IgE concentration lower than the limit of quantification were not included. Mast cell degranulation was not-

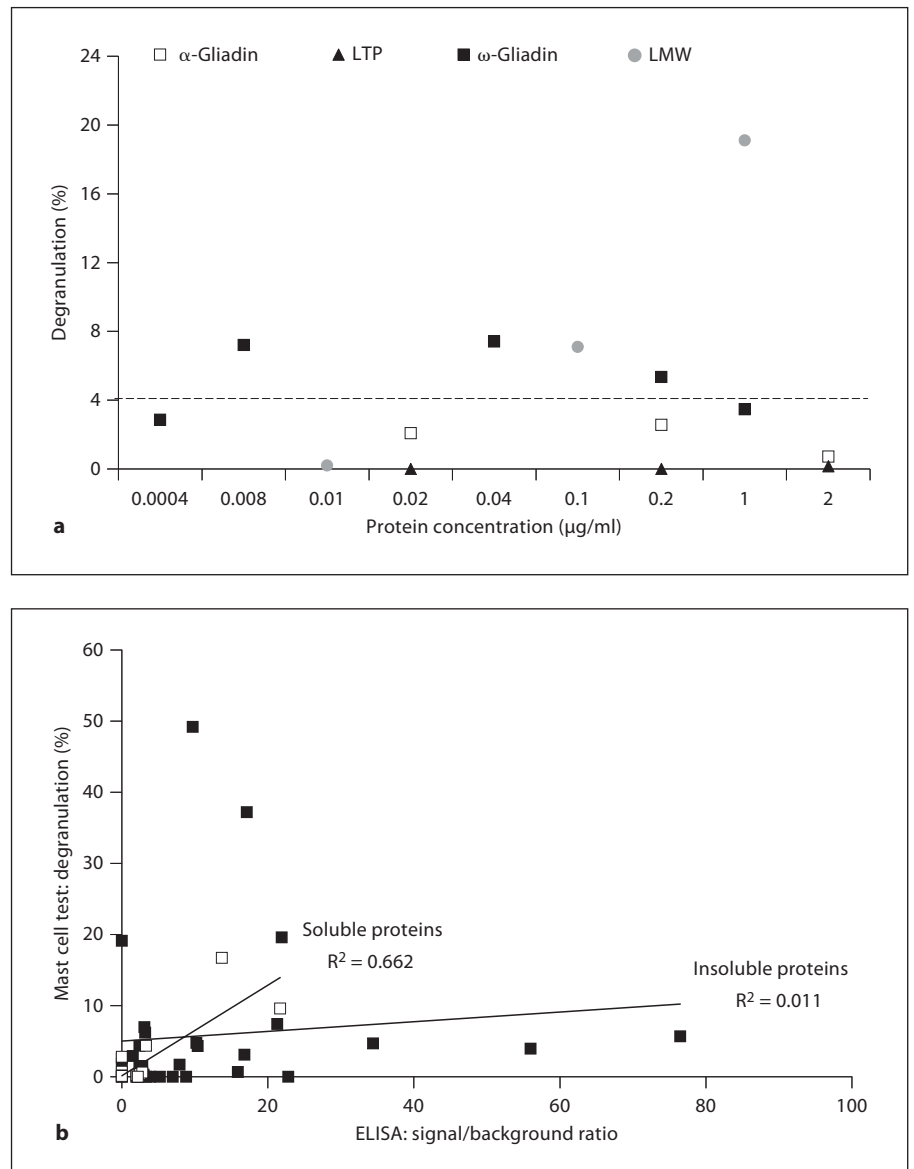


**Fig. 5.** Patients with urticaria, anaphylaxis and WDEIA. **a** Degranulation obtained with wheat proteins ( $\alpha$ -gliadin,  $\omega$ 5-gliadin, LMW glutenin, AG fraction and LTP) at different concentrations for patient 3. The positive threshold is indicated by the stippled line. **b** Correlation of signal intensity between ELISA and mast cell degranulation tests with the two classes of wheat proteins [soluble ( $\square$ ) and insoluble fractions ( $\blacksquare$ )]. The correlation was expressed by  $R^2$ .

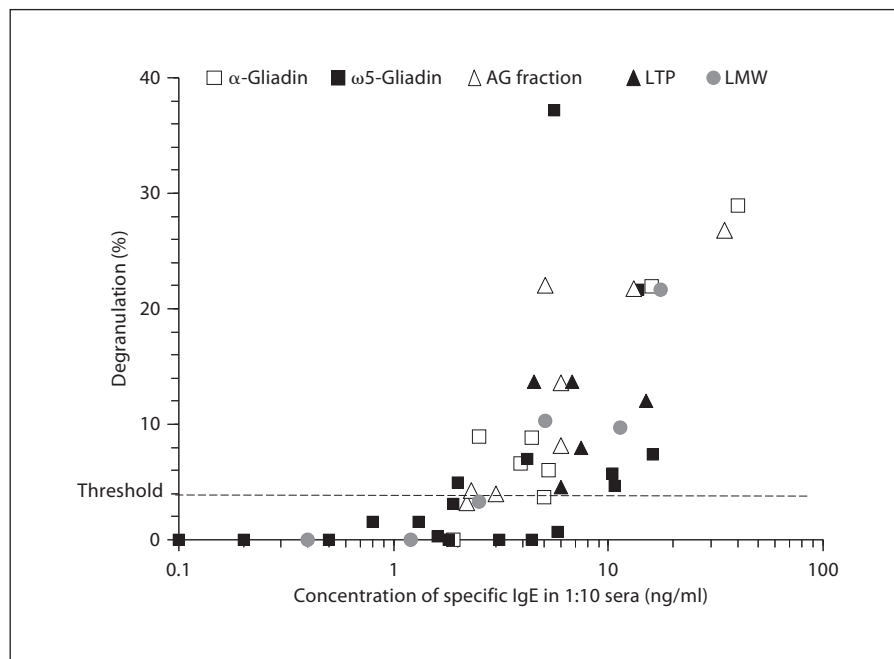
ed when specific IgE to wheat protein exceeded 2 ng/ml in diluted serum except for  $\omega$ 5-gliadins. Indeed, 3 sera of group 2 did not induce degranulation in response to  $\omega$ 5-gliadins despite specific IgE concentrations between 2.5 and 5 ng/ml. Thus, we concluded that mast cell degranulation is related to the concentration of specific IgE to  $\alpha$ -gliadins, LMW glutenins, AG extract and LTP, but not to  $\omega$ 5-gliadins. The weak response of sera from group 2 against  $\omega$ 5-gliadins was not linked to a weak concentration of specific IgE.

## Discussion

In this study, we used a new RBL mast cell line transfected with cDNAs encoding the three subunits of human Fc $\epsilon$ RI (clone 6) to study degranulation activity in wheat-allergic patient sera. The relative expression of the three human Fc $\epsilon$ RI  $\alpha$ -,  $\beta$ - and  $\gamma$ -chains was confirmed by quantitative RT-PCR.

Compared to the previously obtained clone 12.8, which was only transfected with the  $\alpha$ -chain [11], the  $\alpha$ -,  $\beta$ - and  $\gamma$ -expressing clone 6 showed higher levels of human IgE receptor expression after 1 and 48 h of incuba-

**Fig. 6.** Mast cell degranulation and its correlation with the concentration of specific IgE against wheat proteins. Degranulation of a mast cell line with wheat proteins ( $\alpha$ -,  $\omega$ 5-gliadins, LMW glutenins, AG fraction and LTP) is shown as a function of the concentration of specific IgE in sera from both groups.



tected by the majority of sera in both the degranulation assay and ELISA. By PIP, the same fraction induced 40% of the positive responses in this group. Strikingly, the mast cell degranulation assay failed to detect  $\omega$ 5-gliadins as major allergens, although these proteins have been described as major allergens for patients with WDEIA [9, 40, 41]. In vivo, 11 of the 13 patients of group 2 were positive to total  $\omega$ -gliadins using PIP. This result was confirmed later by Moneret-Vautrin [42], who showed that 11 of 15 WDEIA patients were positive to  $\omega$ 5-gliadins in PIP. It is unlikely that the absence of reactivity in the mast cell assay is due to total IgE levels since the total IgE concentration in these patients did not significantly differ from AEDS patients. This was also not due to a weak concentration of specific IgE against  $\omega$ 5-gliadins. Indeed, specific IgE concentrations against  $\omega$ 5-gliadins exceeding the limit of specific IgE concentrations (2 ng/ml) able to induce degranulation with all other proteins was not sufficient to induce mast cell activation in response to this gliadin, suggesting that other parameters may interfere in degranulation assays that prevent proper cross-linking of allergen-specific IgE bound to the surface of mast cells. In vivo transformations may exist that increase the affinity of IgE to allergens. In this group of patients with urticaria, anaphylaxis and WDEIA, signal intensities between mast cell tests and ELISAs were not correlated for insoluble proteins. By contrast, despite the weak number of sera tested, the two tests seemed to correlate in the case of soluble fractions.

WDEIA is a particular symptom that is induced by the combination of both exercise and wheat ingestion. Studies in humans [43] and mice [44] have shown that exercise facilitates allergen absorption from the gastrointestinal tract. Palosuo et al. [45] hypothesized that transglutaminase (intestinal enzyme) could play a role in increasing  $\omega$ 5-gliadin allergenicity during exercise. The cross-linking of  $\omega$ 5-gliadin-derived peptides by transglutaminase could then increase cross-linking activity in vivo. As in celiac disease, gluten deamidation by transglutaminase could also be involved. In our study, only native  $\omega$ 5-gliadins were tested. It would therefore be interesting to study if transglutaminase-modified  $\omega$ 5-gliadins could trigger mast cell degranulation with negative sera. Other factors could play a role like the diversity of the epitope repertoire [24]. In addition, other allergens may be important for this second group of patients, e.g. LMW glutenins. Indeed, a recent study [39] did not confirm the importance of  $\omega$ 5-gliadins, but found IgE against a component of the soluble fraction in patients with WDEIA.

For all wheat proteins except for  $\omega$ 5-gliadins, the mast cell degranulation test appeared to be related to allergen-specific IgE levels. This result disagrees with the study of Purohit et al. [12] who showed that the concentration of allergen-specific IgE antibodies capable of binding to Fc $\epsilon$ RI and biological sensitivities were not necessarily associated. However, it is in agreement with the study of McDermott et al. [46] who stated that the ability of Ara h 2 to effectively cross-link IgE from a specific serum was related to the proportion of anti-Ara h 2 antibodies in that serum. Kaul et al. [47] also reported that the degranulation assay with humanized RBL cells was comparable with the CAP-IgE inhibition assay.

Characterization of IgE responses to wheat flour allergens showed a broad spectrum [48]. It raised questions about the ability of all these antigens to activate mast cells in view of their clinical relevance. The degranulation test of Fc $\epsilon$ RI humanized rat mast cells is a good tool to study this question excluding the variability linked to the use of patient basophils. Indeed, basophils of polysensitized patients often degranulate spontaneously because of continuous stimulation by numerous allergens. This preliminary study shows that large IgE responses against wheat proteins obtained by ELISA were concordant with a positive response in the in vitro mast cell degranulation test except for gliadins. However, degranulation was observed in vivo by PIP with such proteins suggesting that other parameters may also be involved in the degranulation of these proteins in vivo or in vitro.

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