

Humoral and cellular responses to gliadin in wheat-dependent, exercise-induced anaphylaxis

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Summary

Background Wheat-dependent, exercise-induced anaphylaxis (WDEIA) is a severe allergy where wheat ingestion together with physical exercise induces anaphylaxis. We have previously shown that patients with WDEIA have IgE antibodies against gliadin proteins and identified ω -5 gliadin (Tri a 19) as a major allergen.

Objective The aim of this study was to examine gliadin-specific IgG subclass, IgA and IgE antibodies, basophil histamine release and cell-mediated responses in WDEIA.

Methods Sera and peripheral blood mononuclear cells (PBMC) were obtained from patients with WDEIA and from controls without wheat allergy. Serum antibodies to crude gliadin extract (CGE) and purified ω -5 gliadin were measured by ELISA and basophil reactivity by histamine-release test. Gliadin-induced cell-mediated responses were assessed by lymphocyte proliferation assay, and cytokine mRNA expression with real-time quantitative PCR.

Results All patients with WDEIA, but none of the controls, had IgE antibodies to CGE and ω -5 gliadin. Both allergens released high levels of histamine from the basophils of patients with WDEIA. Levels of IgA antibodies to CGE and ω -5 gliadin were significantly elevated in the patients, but the distribution of IgG subclass antibodies showed no statistically significant differences between the two groups. Proliferative responses of PBMC to CGE were increased in patients with WDEIA, and stimulation of PBMC with CGE caused, both in patients and in controls, a clear induction of IL-10 mRNA. Compared with the controls, induction of IL-10 mRNA expression in patients with WDEIA was significantly ($P < 0.01$) suppressed.

Conclusion These results suggest that, in addition to IgE antibodies against ω -5 gliadin, specific IgA antibodies may be involved in the pathogenesis of WDEIA. Decreased expression of IL-10 mRNA in PBMC during gliadin stimulation may facilitate the development of gliadin-specific T cell responses.

Keywords anaphylaxis, cytokine, ELISA, gliadin, histamine release, IgA, IgE, IL-10, proliferation

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Introduction

Food-dependent, exercise-induced anaphylaxis is a life-threatening allergy where ingestion of a specific food in connection with physical exercise causes severe allergic reactions [1]. The symptoms vary from local or generalized urticaria and angioedema to dyspnoea, hypotension, collapse and shock [2, 3]. Patients typically have IgE antibodies to the incriminated food, but no symptoms appear unless food ingestion is followed by exercise [1]. Although various foods (e.g. shellfish [4], nuts [5], celery [1], tomato and apple [3]) can trigger exercise-induced anaphylaxis, one of the most frequent causes is wheat [2, 3, 5–9]. We have previously shown that patients with wheat-dependent,

exercise-induced anaphylaxis (WDEIA) mount IgE antibodies to gliadins [8] and identified ω -5 gliadin (Tri a 19) as a major allergen [2, 9]. Cross-reactive allergens are present in rye (γ -70 and γ -35 secalins) and barley (γ -3 hordein) and patients with WDEIA are treated with a gluten-free diet [9]. In addition, we have shown that ω -5 gliadin is a significant wheat allergen in young children with atopic dermatitis; the presence of IgE antibodies to ω -5 gliadin associated strongly with immediate symptoms on oral wheat challenge [10].

Sensitization to wheat and other cereals can occur via inhalation leading to baker's asthma, a well-known occupational respiratory disorder. In contrast to ingested wheat allergy, the IgE response in baker's asthma is directed mainly against the water/salt-soluble wheat proteins, rather than the gliadins [11, 12]. The ethanol-soluble gliadins together with the acid/alkali-soluble glutenins form a complex mixture of proteins known as gluten [13]. Ingested gluten may, in addition to IgE-mediated allergy, cause coeliac disease. This is a chronic

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inflammatory disorder of the small intestine resulting from an aberrant T cell response to ingested gluten and related proteins in rye and barley [14]. Patients with active coeliac disease have high levels of serum IgA antibodies to various antigens, including gliadin [15].

Cellular immune responses to gliadin, and humoral responses apart from IgE antibodies, have not been studied in WDEIA. The aim of this study was to examine gliadin-specific IgG subclass and IgA antibodies, basophil histamine release, proliferative responses and cytokine production in patients with WDEIA.

Materials and methods

Patients

Venous blood samples were obtained from 27 adult patients with WDEIA (11 females, 16 males, mean age 42, range 24 to 68 years). All patients had positive skin prick test reactions to wheat (1 : 10 w/v 0.9% NaCl) and gliadin (1 mg/mL in 20% ethanol; crude, Sigma Chemical Co., St. Louis, MO, USA), and they had experienced anaphylactic reactions when exercising after ingestion of wheat products. Twenty-one adults (14 females, 7 males, mean age 38, range 18 to 56 years), with no symptoms of wheat allergy or exercise-induced anaphylaxis, and negative skin prick tests to wheat and gliadin, served as controls. This study was approved by the ethics committee of the Helsinki University Hospital for Skin and Allergic Diseases.

Gliadin antigens

Wheat (*Triticum aestivum*, cultivar Tjalve) ω -5 gliadin was purified by reversed-phase chromatography as previously described in detail [2]. Wheat grains were ground, and proteins were extracted with 50% acetonitrile containing 0.1% trifluoroacetic acid. ω -5 gliadin was purified on a Resource RPC 3 mL column (Pharmacia, Uppsala, Sweden) using a linear gradient of acetonitrile (10 to 60% in 60 min) in 0.1% trifluoroacetic acid. Crude gliadin extract (CGE) was prepared by dissolving 10 mg of gliadin (crude, from Sigma) in 1 mL 0.1 M acetic acid and boiling for 30 min. The antigen solutions were irradiated with 12 000 rads to destroy possible microbial contamination, and stored at -20°C .

Both ω -5 gliadin and CGE were used in ELISA and histamine release tests. The proliferation and lymphocyte stimulation tests were performed with CGE only, because the amount of antigen required for these tests was large, and the results of ELISA, skin prick and histamine release tests were very similar with both antigens.

ELISA

ELISA was performed as described earlier [2, 16] with sera from 27 patients with WDEIA and 21 controls. Briefly, the concentration of ω -5 gliadin was adjusted to 2 $\mu\text{g/mL}$ and that of CGE to 20 $\mu\text{g/mL}$ for coating microtitre plates (100 $\mu\text{L/well}$). The wells were then post-coated with 1% human serum albumin in 50 mM sodium carbonate buffer, and 100 μL of patient or control serum (diluted 1 : 150 for IgG1, 1 : 80 for IgG2, 1 : 40 for IgG3, 1 : 20 for IgG4, 1 : 60 for IgA and 1 : 10 for IgE) was added. Biotinylated mouse anti-human IgG1, IgG2, IgG3, IgG4, IgA (Zymed, San Francisco, CA, USA, diluted 1 : 1000)

or biotinylated goat anti-human IgE (Vector, Burlingame, CA, USA, diluted 1 : 1000) was added, followed by streptavidin-conjugated alkaline phosphatase (Zymed, diluted 1 : 1000) and colour substrate (Sigma). The optical density was measured at 405 nm with an automated ELISA reader (Titertek Multiscan, Eflab, Turku, Finland).

Histamine release

The basophil histamine-release test was performed as previously described [17] in nine patients with WDEIA and in five controls. In brief, washed whole blood cell suspensions containing basophils were incubated in calcium PIPES (piperazine-N, N¹-bis (2-ethanesulphonic acid) buffer with serial dilutions of CGE (ranging from 100 $\mu\text{g/mL}$ to 0.1 $\mu\text{g/mL}$), ω -5 gliadin (ranging from 10 $\mu\text{g/mL}$ to 0.1 $\mu\text{g/mL}$), goat anti-human IgE (Sigma, diluted 1 : 2000), or buffer only at 37°C for 45 min. The samples were heated at 100°C for 10 min for the measurement of total blood histamine. After centrifugation (10 min at 700 g or 2900 g for total histamine) the histamine content of the supernatants was measured by a commercial kit (Immunotech, A Beckman Coulter Company, Marseille, France). The results were expressed as the percentage of histamine released after antigen stimulation from the amount released by anti-human IgE. The spontaneous histamine release was less than 10% of total histamine release and most frequently below 2%.

Proliferation assays with PBMC and lymphocyte stimulation test

Proliferation assays with peripheral blood mononuclear cells (PBMC) and lymphocyte stimulation tests were performed in complete RPMI 1640 (with Glutamax-1) medium (Gibco BRL, Paisley, UK) containing 5% inactivated human AB serum (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland), 1 mM sodium pyruvate MEM, MEM non-essential amino acids, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid) buffer (Gibco BRL), 200 IU/mL sodium benzylpenicillin (Orion, Espoo, Finland), 152 U/mL streptomycin sulphate, and 20 μM 2-mercaptoethanol (Sigma).

PBMC from 12 patients with WDEIA and 10 controls were isolated from heparinized venous blood by using Ficoll (Nycomed Pharma As, Oslo, Norway) density gradient centrifugation and washed twice with phosphate buffered saline. Recovered cells were incubated in triplicate (10^5 cells/200 $\mu\text{L/well}$) at 37°C under 5% CO_2 in 96-well microplates (Costar, Corning Incorporated, Corning, NY, USA) in the presence or absence of different concentrations (500 $\mu\text{g/mL}$ to 1 $\mu\text{g/mL}$) of CGE. Purified tetanus toxoid (10 $\mu\text{g/mL}$, National Public Health Institute, Helsinki, Finland) and phytohaemagglutinin (0.5%, Murex Biotech Ltd, Dartford, England) were used as positive controls. After 6 days of culturing, tritiated methylthymidine (1 $\mu\text{Ci/well}$; Amersham Life Science, Buckinghamshire, UK) was added, and the cells were harvested into glassfibre filters after 18 h (Tomtec, Hamden, CT, USA). Thymidine incorporation was measured with a betaplate counter Trilux (Wallac, Turku, Finland). The results were expressed as stimulation indexes (uptake of isotope in stimulated culture/uptake of isotope in non-stimulated control culture). The c.p.m. values of control cultures were always less than 2000.

Gliadin-specific cytokine mRNA expression was screened in five patients with WDEIA and in five controls. Gliadin-induced

IL-10 mRNA expression was examined in 10 patients and 10 controls. Ovalbumin (grade V, Sigma), used as a control antigen, was tested in five patients and five controls. Isolated and washed PBMC were stimulated on 24-well plates (Costar, Corning Incorporated) for 6 or 24 h in the presence or absence of CGE or ovalbumin (75 µg/mL). Each 1.5 mL well contained 2×10^6 cells, the total number of cells per stimulation being 8 to 12×10^6 . After incubation in a humidified 5% CO₂ atmosphere at 37 °C, cell pellets were collected and suspended in Trizol Reagent (Gibco BRL) and stored at -70 °C before RNA isolation.

RNA isolation

PBMC were homogenized in Trizol (Gibco BRL), and RNA extraction was performed according to the Trizol instructions; all chemicals were from Sigma. The concentration and purity of the RNA was determined by measuring the absorbance at 260 and 280 nm in a spectrophotometer (Pharmacia Biotech (Biochrom) Ltd, Cambridge, England). Ribosomal RNAs (28S rRNA, 5 kb and 18S rRNA, 1.9 kb) were visualized on a 1.0% agarose gel following electrophoresis to demonstrate the integrity of the RNAs. Isolated total cellular RNA was stored at -70 °C in nuclease-free water.

cDNA synthesis and quantitative real-time PCR amplification of reverse-transcribed cDNA

cDNA was synthesized from 2 µg of total RNA in a 100-µL reaction mix using MultiScribe™ Reverse Transcriptase and random hexamers from the TaqMan® Reverse Transcription Reagents (PE Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Real-time quantitative PCR was performed with a commercial kit, TaqMan® Cytokine Gene Expression Plate I, on an ABI PRISM™ 7700 Sequence Detector System (PE Applied Biosystems). The following cytokines were measured: IL-1β, IL-4, IL-5, IL-10, IFN-γ and TNF-α. Each well on the cytokine plate contained dried primers and probes for the detection of one cytokine cDNA target and the endogenous 18S rRNA control. Two dye layers, a FAM layer that detects the cytokine target sequences and a VIC layer that detects the endogenous control, were used to detect amplification of both species. One hundred nanograms of total RNA converted to cDNA was applied into each well. Initial steps in thermal cycler conditions were 2 min at 50 °C (for optimal AmpErase UNG enzyme activity) and 10 min at 95 °C (to activate AmpliTaq Gold DNA Polymerase and to inactivate AmpErase UNG enzyme). Thereafter, real-time PCR reactions were made up to 40 cycles: 15 s at 95 °C (melting) and 1 min at 60 °C (annealing/extension). FAM C_T (threshold cycle) values greater than or equal to 36, and VIC C_T values greater than 13, were not used for calculation. The results were expressed as relative units (RU), i.e. the ratio of PCR amplification efficiency of sample cDNA to that of the calibrator. The calibrator C_T values were obtained by incubation of unstimulated PBMC for 6 h or 24 h, and used for calculation of mRNA expression after 6 h or 24 h stimulation, respectively.

Relative units were calculated by the comparative C_T method. First, the C_T for the target amplicon and the C_T for the endogenous control (18S rRNA) was determined for each sample. Differences in the C_T for the target and endogenous

control, called ΔC_T , were calculated to normalize the differences in the amounts of total nucleic acid added to each reaction mixture. The ΔC_T of the calibrator was subtracted from the ΔC_T of each experimental sample and termed as $\Delta\Delta C_T$. The amount of target, normalized to an endogenous control and relative to the calibrator, was then calculated by the equation $2^{-\Delta\Delta C_T}$.

Statistical analysis

Non-parametric Mann-Whitney or unpaired *t*-test was used to compare the groups. A *P*-value smaller than 0.05 was considered statistically significant.

Results

Patients with WDEIA have elevated levels of gliadin-specific IgE and IgA antibodies

All 27 patients with WDEIA had IgE antibodies to purified ω-5 gliadin (mean ELISA value 1.0) and CGE (mean ELISA value 0.77), whereas the 21 subjects without wheat allergy remained negative in ELISA (Fig. 1). Patients with WDEIA showed significantly higher levels of IgA antibodies to ω-5 gliadin (mean ELISA value 0.68, *P* < 0.001) and CGE (mean ELISA value 1.0, *P* < 0.01) than the control subjects (mean ELISA values 0.38 and 0.74) (Fig. 2). IgG3 levels to ω-5 gliadin were slightly elevated in the patients (mean ELISA value 0.22), but compared with the controls (mean ELISA value 0.11), the difference was statistically insignificant. Levels of IgG1, IgG2 and IgG4 antibodies to ω-5 gliadin were low, both in patients with WDEIA and in controls (mean ELISA values 0.05, 0.03 and 0.15 for WDEIA, and 0.04, 0.15 and 0.03 for controls, respectively). The range of IgG1-4 antibody levels to CGE (mean ELISA values 0.17, 0.32, 0.33 and 0.52 for WDEIA, and 0.23, 0.56, 0.30 and 0.54 for controls, respectively) was large and overlapped extensively between the two groups, showing no significant differences.

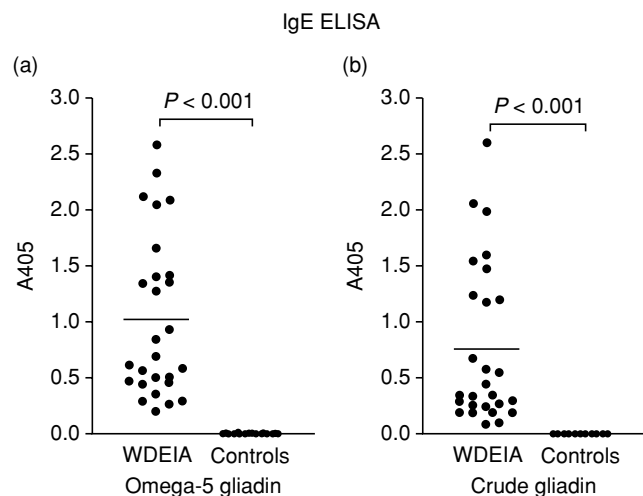


Fig. 1. IgE antibody levels to (a) purified ω-5 gliadin (2 µg/mL) and (b) crude gliadin extract (20 µg/mL) in ELISA (absorbance units) in 27 patients with WDEIA and 21 controls. *P* < 0.001 (Mann-Whitney test).

Gliadin-induced basophil histamine release and PBMC proliferative responses are increased in WDEIA

Purified ω -5 gliadin and CGE released significantly more histamine from the basophils of patients with WDEIA ($n = 9$) than from controls without wheat allergy ($n = 5$) ($P < 0.001$) (Fig. 3). In the patients, the mean histamine release induced by ω -5 gliadin was 47.6%, compared with 4.5% in the controls. The corresponding values for CGE were 35% and 3.4%.

Proliferative responses of PBMC to CGE (50 $\mu\text{g}/\text{mL}$) were significantly higher in patients with WDEIA ($n = 12$) than in controls ($n = 10$) ($P < 0.01$), although the ranges showed some overlapping (Fig. 4). The mean stimulation indexes were 3.80 for patients and 1.44 for controls. Proliferative responses to tetanus toxoid and phytohaemagglutinin showed no differences between the two groups (data not shown).

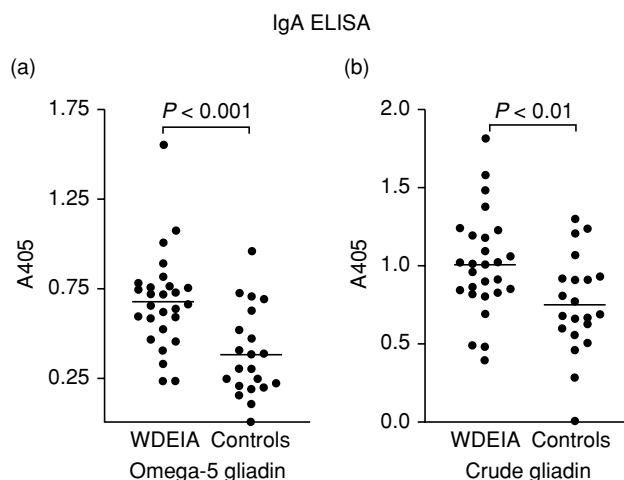


Fig. 2. IgA antibody levels to (a) purified ω -5 gliadin (2 $\mu\text{g}/\text{mL}$) and (b) crude gliadin extract (20 $\mu\text{g}/\text{mL}$) in ELISA (absorbance units) in 27 patients with WDEIA and 21 controls. $P < 0.001$, $P < 0.01$ (unpaired t -test).

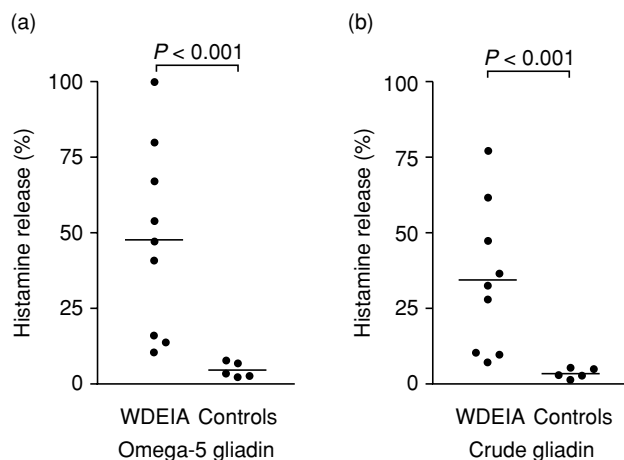


Fig. 3. Histamine release from whole blood induced by (a) ω -5 gliadin and (b) crude gliadin extract in nine patients with WDEIA and five controls. The values are percentages of anti-IgE-induced histamine release. $P < 0.001$ (Mann-Whitney test).

Gliadin-induced expression of IL-10 mRNA is decreased in patients with WDEIA

Stimulation of PBMC for 6 h with CGE induced, in patients with WDEIA ($n = 10$) as well as in controls ($n = 10$), strong expression of IL-10 mRNA (Fig. 5). However, compared with the controls induction of IL-10 mRNA expression was significantly suppressed in the patients ($P < 0.01$). The mean gliadin-specific IL-10 mRNA levels in relative units were 81.7 in the patients and 156.8 in the controls. Ovalbumin-specific IL-10 mRNA expression showed no differences between the two groups (data not shown). Gliadin stimulation for 6 h induced no significant expression of major Th2-type (IL-4, IL-5), Th1-type (IFN- γ) or proinflammatory (IL-1 β , TNF- α) cytokines in patients or controls, and no significant differences in any

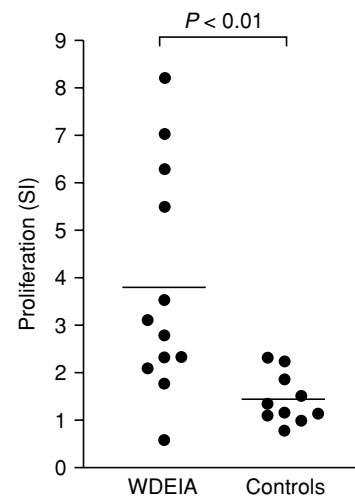


Fig. 4. Proliferative responses of PBMC after 6 days stimulation with crude gliadin extract (50 $\mu\text{g}/\text{mL}$) in 12 patients with WDEIA and in 10 controls. The values are shown as stimulation indices (SI). $P < 0.01$ (Mann-Whitney test).

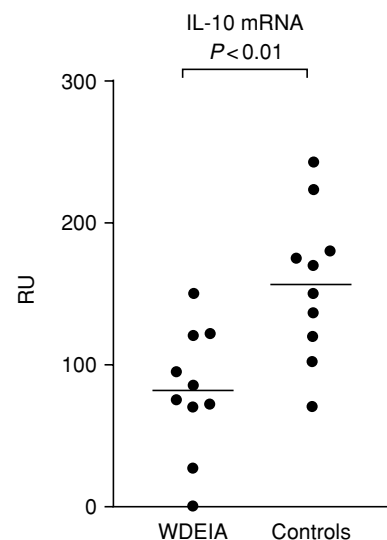


Fig. 5. IL-10 mRNA expression after 6 h stimulation of PBMC with crude gliadin extract (75 $\mu\text{g}/\text{mL}$) in 10 patients with WDEIA and in 10 controls. The values (RU) are expressed as fold differences relative to the calibrator. $P < 0.01$ (unpaired t -test).

cytokine levels were observed after 24 h stimulation (data not shown).

Discussion

Consistent with our previous findings, all patients with WDEIA had elevated levels of IgE antibodies to CGE [8] and purified ω -5 gliadin [2, 9]. Basophil histamine release induced by ω -5 gliadin was elevated as well, confirming the role of IgE in WDEIA and the importance of ω -5 gliadin as a major allergen. In addition, our results showed that ω -5 gliadin triggers increased production of serum IgA antibodies. The sensitive ELISA method detected IgA antibodies to CGE and ω -5 gliadin both in patients with WDEIA and in healthy controls, but IgA levels, especially to ω -5 gliadin, were significantly elevated in the patients. IgA antibodies to gliadin are most frequently linked with dietary exposure to ingested gluten in coeliac disease [15], but these antibodies are found also in healthy individuals, and their prevalence seems to increase with age [18–20].

IgA is the most predominant immunoglobulin at mucosal surfaces, where it is involved in protection against microorganisms. It constitutes 15 to 20% of serum immunoglobulins, yet the biological role of serum IgA remains incompletely understood [21]. It has been proposed that serum IgA might provide a second line of defence in mucosal immunity by IgA-mediated phagocytosis of immune complexes via the IgA Fc α RI receptor on liver Kupfer cells [22]. An intriguing thought is that ω -5 gliadin-specific IgA antibodies could have a role in the removal of antigen from the circulation in WDEIA.

The distribution of IgG subclass antibodies to ω -5 gliadin was similar in patients with WDEIA and controls. The mean antibody levels were low, with the exception of slightly increased IgG3 levels in patient sera. Several studies in food-allergic patients have demonstrated specific IgG4 antibodies in parallel with specific IgE [23, 24], but only four patients with WDEIA had notable levels of IgG4 to ω -5 gliadin, although IgE antibodies were found in all 27 patient sera. Mean antibody levels to CGE were higher than those to ω -5 gliadin in all four IgG subclasses, with similar patterns in patients and controls, most likely reflecting a normal immune response to dietary antigens.

Proliferative responses of PBMC to gliadin were significantly greater in patients with WDEIA than in controls, although they overlapped to some extent between the groups. Previously, gliadin-induced proliferative responses have been demonstrated in patients with coeliac disease [25] as well as in healthy individuals [26], but no data exist on cell-mediated immunity to gliadin in wheat-allergic patients. Several investigators have demonstrated significantly increased PBMC proliferative responses to cow's milk casein in milk-allergic children [27–29] and adults with milk-responsive atopic dermatitis [29, 30], while others have failed to show differences between allergic and non-allergic individuals [31].

Stimulation of PBMC with gliadin induced marked expression of IL-10 mRNA both in patients with WDEIA and in controls. Levels of IL-10 mRNA were, however, significantly lower in the patients. Similarly, Jenmalm et al. [32] observed lower levels of β -lactoglobulin-induced IL-10 in milk-allergic children, when compared with non-allergic controls. Evidence exists that IL-10 can inhibit several activation and effector

functions of T cells, monocytes and macrophages [33]. Via its down-regulatory effects on antigen presenting cells, IL-10 can strongly suppress proliferation and cytokine production of CD4⁺ T cells and T cell clones [34, 35]. Moreover, recent studies show that activation of T cells in the presence of IL-10 can induce anergy [36–38], and that IL-10 is required for induction of protective oral tolerance [39]. It may, thus, be hypothesized that T cell non-responsiveness to gliadin in healthy individuals could be induced by frequent wheat ingestion with simultaneous gliadin-induced IL-10 expression. On the other hand, impaired IL-10 production in response to gliadin stimulation could explain the enhanced cell-mediated responses seen in patients with WDEIA.

Gliadin stimulation caused no significant expression of major Th1-(IFN- γ) or Th2-type (IL-4, IL-5) cytokines in patients with WDEIA or in controls. It should, however, be noted that only a very small proportion of PBMC are gliadin-specific T cells, and the sensitivity of the mRNA assay in detecting trace amounts of T cell-derived cytokines expressed after gliadin stimulation, may not be high enough. Nevertheless, IL-10 mRNA was expressed at high levels, possibly because it was derived both from antigen-presenting cells and T cells. Baseline mRNA levels of proinflammatory cytokines (IL-1 β , TNF- α) were high (data not shown), but gliadin stimulation induced no further mRNA expression. This suggests that proinflammatory cytokines do not play a critical role in the regulation of cell-mediated responses in WDEIA.

In summary, the results of this study show that patients with WDEIA exhibit, in addition to IgE antibodies, increased levels of gliadin-specific IgA. Gliadin-induced basophil histamine release was high, supporting the role of IgE in the pathogenesis of WDEIA. Enhanced proliferative responses of peripheral blood cells to gliadin and significantly decreased gliadin-induced IL-10 production suggest an imbalance in the control of cell-mediated immune responses to gliadin in WDEIA.

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