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Characterization of Human, Mouse and Rabbit Anti-Gliadin Antibodies by ELISA and Western Blotting

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Dedicated to Prof. MUDr. Ctirad John, DrSc. on the occasion of his 75th birthday

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ABSTRACT. Monoclonal, hyperimmune rabbit and human serum anti-gliadin antibodies were analyzed by ELISA and immunoblotting techniques. In Western blotting the difference in reactivity between monoclonal and human antibodies was quantitative rather than qualitative. Rabbit antisera differed in reactivity according to the protein used for immunization. The rabbits immunized by the peptic-tryptic pancreatic digest of gliadin reacted similarly to the patients. In ELISA, significantly higher reactivity with crude, A-, glyc-gli, α-, β- and ω-gliadins was found in the patients' sera than in controls.

Abbreviations

AGA	anti-gliadin antibodies
BSA	bovine serum albumin
ELISA	enzyme-linked immunosorbent asay
F III	Frazer's fraction III of gliadin
CD	cœliac disease
glyc-gli	glycopeptide of gliadin
Ig	immunoglobulin
mAbs	monoclonal antibodies
PBS	phosphate-buffered saline
PTP	peptic-tryptic-pancreatic digest of gliadin
SDS–PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SwAHuIgAPx	peroxidase-labeled swine anti-human IgA immunoglobulin fraction
SwAHuIgGPx	peroxidase-labeled swine-anti-human IgG immunoglobulin fraction
SwARPx	peroxidase-labeled swine anti rabbit immunoglobulins immunoglobulin fraction

Cœliac disease is a chronic gastrointestinal disease of children and adults. The onset of the symptoms is triggered by ingestion of gluten-containing food but the exact mechanism whereby gluten damages the jejunal mucosa remains unknown. It is clear that the "toxicity" lies in the ethanol-soluble gliadin fraction. It is now generally accepted that immune mechanisms are involved in the pathogenesis of CD. Numerous studies have shown that the patients have elevated serum antibodies to gluten and its fractions (Gonczi *et al.* 1991). All the main gliadin fractions (α-, β-, γ-, and ω-) have been shown to cause damage in cœliac disease patients, but the particular polypeptide or amino acid sequence responsible for triggering the immune response has not been identified so far (Devery *et al.* 1991). In order to find such a peptide, we tested the immunogenicity of gliadin for rabbits, mice and humans by the ELISA method and Western blot analysis.

MATERIALS AND METHODS

Subjects. Sera from 29 patients with cœliac disease diagnozed upon biopsy were examined. All of them were on a gluten-free diet. Sera from 29 healthy subjects were used as controls.

Rabbit antisera. Each rabbit was immunized subcutaneously with either (*1*) gliadin A, or (*2*) Fraser's fraction III (F III) of gliadin, or (*3*) peptic-tryptic-pancreatic (PTP) digest of gliadin emulsified in complete Freund's adjuvant. Samples showed high titres of antigliadin antibodies (AGA) by ELISA.

Monoclonal antibodies. Production of mAbs was described elsewhere (Stoyanov *et al.* 1988). Monoclonal antibodies GL1, GL2 and GL3 were used.

Preparation of gliadin fractions. Gliadin and its fractions used in this study were crude gliadin, A gliadin (*Sigma*, St. Louis, MO, USA), F III fraction, glycopeptide of gliadin (glyc-gli fraction), and α-, β-, γ-, and ω-gliadins. Their preparation was described earlier (Kocna *et al.* 1988, 1990).

ELISA. The use of ELISA for detection of antigliadin antibodies was described earlier (Stoyanov *et al.* 1988; Tlaskalová *et al.* 1983). Wells of polystyrene microtiter plates (*Hardtmuth Koh-i-noor*, Czech Republic) were filled with crude gliadin or A-gliadin diluted in 70 % ethanol. After drying, a 5-min fixation with 10 % formaldehyde solution was performed. Alternatively, gliadin fractions soluble in PBS were bound diluted in PBS (Frazer III fraction, PTP digest of A-gliadin and its fractions separated by HPLC – α-, β-, γ-, ω-gliadins). The plates were incubated overnight at 4 °C in both cases. After repeated washing, nonspecific binding was blocked by 0.02 % gelatin in PBS with 0.1 % sodium azide. The plates were then washed 5 times with Tween PBS. Tested samples (patients' and rabbit sera and ascitic fluids diluted with PBS) were added to the wells in duplicate and incubated overnight at 4 °C. Control wells were filled with PBS. After repeated washings, pig anti-human (SwAHuIgGPx, SwaHuIgAPx), anti-rabbit (SwARPx), anti-mouse (SwAMPx) immunoglobulin peroxidase conjugates, all from the *Institute of Sera and Vaccines*, Prague, were added. The plates were incubated for 2 h at room temperature. After five washings the enzyme reaction was developed with 1,2-phenylenediamine solution, stopped with H_2SO_4 and read on the Microelisa Reader MR 500 Dynatech at 492 nm. The results are expressed in ELISA units as a percentage of reference serum (pool of patients' sera).

SDS–PAGE and Western blotting. Gliadin and its fractions were solubilized into sample buffer according to Laemmli and Favre (1979), with addition of 10 % 2-mercaptoethanol. To ensure reduction, the samples were immersed in boiling water for 20 s. This procedure did not abolish the antigenicity of gliadins and ensured better resolution of peptide bands in SDS–PAGE. The proteins were separated in a vertical SDS–PAGE slab gel with a gradient of 10–12.5 % and 5–20 % (O'Farrell 1975). The amount of gliadin loaded in the slot (30 mm width) was 700 μg. The molar mass markers used were Electrophoresis Low Molecular Weight Calibration Kit (*Pharmacia Fine Chemicals*, Uppsala, Sweden). Gels were either stained with Coomassie brilliant blue in a methanol–acetic acid mixture, or transferred on to a nitrocellulose sheet (0.45 μm *Schleicher Schuell*, Dassel, Germany) (Towbin *et al.* 1975). After the electrophoretic transfer (42 mA, up to 180 V, overnight), the nitrocellulose sheet was saturated with 1 % BSA in PBS for 3 h at room temperature, cut into strips which were then incubated with rabbit antisera (1:50 in PBS 1 %, BSA 0.05 %, Tween 20 0.01 % NaN_3 for 1 h at room temperature) or with mouse monoclonal antibodies GL1, GL2, GL3 from the ascitic fluid (1:50 in PBS for 1 h at room temperature) or with human sera (1:25 in PBS 1 % BSA for 1 h at room temperature). After three washes with PBS 0.05 % Tween 20, the strips were probed with horseradish peroxidase-labelled swine antibodies (*Institute of Sera and Vaccines*, Prague) to rabbit immunoglobulins (1:250 in PBS 1 % BSA), mouse immunoglobulins (1:100 in PBS 1 % BSA), and human IgG (1:250 in PBS 1 % BSA) for 1.5 h at 37 °C. After further three washes with PBS 0.05 % Tween 20 and one wash with PBS, the peroxidase activity was detected by means of a 4-chloro-1-naphthol substrate solution (Hawkes *et al.* 1982). The reaction was stopped in distilled water.

Statistics. Nonparametric statistical tests were used: Nonpaired Wilcoxon's rank sum test was used for comparison of differences between groups. Spearman's rank correlation test was used for estimation of the significance of correlations. A probability value of $p \leq 0.01$ was set to indicate the level of statistically significant difference.

RESULTS

ELISA AGA titres to various gliadin fractions

Reactivity of our three mAb's with crude gliadin, A-gliadin, glyc-gli, F III, α-, β-, γ-, and ω- was tested by the ELISA. The results of the experiments with crude, A, α and FIII were described earlier (Stoyanov *et al.* 1988). Further experiments with glyc-gli, β-, γ- and ω-gliadins confirmed that GL1 and GL3 exhibit positive binding titers to all the gliadins used. This suggests that they recognize an epitope comprised of a common amino acid sequence shared by all gliadin fractions used. GL2 exhibits weak antibody binding to all gliadin fractions tested (Table I).

The patients' serum antibodies exhibited significantly higher binding (when compared to controls) to crude gliadin, A-gliadin, glyc-gli, α-, β-, ω- in the IgG class and to gliadin A and ω- in the IgA

class (Table II). Good correlation of reactivity to all other gliadins in both IgG and IgA classes was found for α- and glyc-gli (Table III).

Table I. ELISA reactivity of monoclonal anti-gliadin antibodies GL1, GL2, GL3 with various gliadin fractions (absorbance at 492 nm)

MAB in dilution 1 : 2500	Crude	A-gli	glyc-gli	Frazer fraction	α-gli	β-gli	γ-gli	ω-gli
GL1	1.30	1.05	1.40	1.35	1.25	1.25	0.42	1.62
GL2	0.24	0.20	0.24	0.15	0.20	0.20	0.13	0.48
GL3	1.18	1.15	1.57	1.45	1.10	1.10	0.23	1.30

Table II. ELISA reactivity of patients' serum IgG and IgA antibodies with various gliadin fractions[a]

Gliadin	α-		β-		γ-		ω-		Crude		A		Frazer		glyc-gli	
Ig class	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA
Patients' sera	**82** ±59*	**114** ±62	**128** ±102*	**110** ±120	**66** ±22	**106** ±52	**90** ±63*	**200** ±140*	**72** ±40*	**60** ±74	**54** ±38*	**84** ±60*	**344** ±152	**118** ±180	**84** ±60*	**74** ±40
Control sera	**64** ±26*	**106** ±40	**50** ±20*	**108** ±40	**68** ±28	**120** ±48	**36** ±24*	**88** ±80*	**20** ±10*	**24** ±16	**24** ±16*	**56** ±26*	**98** ±116	**104** ±60	**42** ±18*	**44** ±20

[a]Expressed in ELISA units as means of the group (29 patients resp. controls) ± SE. Statistically significant difference ($p \leq 0.001$) is *asterisked*.

Table III. Spearman's correlation coefficients (×100) for ELISA reactivity of patients' serum IgG and/or IgA with each of two gliadin preparations[a]

Gliadin	α-		β-		γ-		ω-		Crude		A		Frazer		glyc-gli	
Ig class	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA
α-	34		88*	62*	66*	67*	69*	66*	64*	56*	81*	86*	48*	80*	76*	84*
β-	88*	62*	27		60*	80*	73*	27	70*	18	91*	69*	34	76*	89*	66*
γ-	66*	67*	60*	80*	84*		47*	32	40	30	62*	70*	25	80*	57*	68*
ω-	69*	66*	73*	27	47*	32	60*		79*	80*	75*	55*	25	43	67*	52*
Crude	64*	56*	70*	18	40	30	79*	88*	70*		73*	51*	17	46*	62*	55*
A	81*	86*	91*	69*	62*	70*	75*	55*	73*	51*	39		30	88*	89*	94*
Frazer	48*	80*	34	76*	25	80*	25	43	17	46*	30	88*	13		63*	87*
glyc-gli	76*	84*	89*	66*	57*	68*	67*	52*	62*	55*	89*	94*	63*	87*	60*	

[a]Statistically significant correlation ($p \leq 0.01$) was found between the two samples for which the Spearman's correlation coefficient (×100) was greater than the critical value of 43.2 (*asterisked*).

All the rabbit antisera, prepared as described above, exhibited at least six times higher ELISA titers, compared to control sera from nonimmunized rabbits.

Western blotting

Fig. 1 shows a photograph of a Coomassie-blue-stained gel of separation of crude, A-, α-, β-, γ-, ω-gliadins under reducing conditions, and α-, A, and crude gliadin under nonreducing conditions. Addition of 2-mercaptoethanol to the sample buffer (reducing conditions) did not affect the quality of resolution of the gliadins.

Fig. 2 shows the separation of F III and glyc-gli gliadins (Coomassie-blue-stained gel, reducing conditions). It can be seen that the main gliadin area ranges from 30 to 60 kDa. Crude gliadin is composed of a large number of proteins with molar mass ranging from 30 to 74 kDa. A-gliadin is very sim-

ilar, but has two more bands of 45 and 52 kDa. α-Gliadin is similar to crude gliadin, but the bands of 34 and 45 kDa stain more heavily. In β-gliadin the bands of 42 and 43 kDa appear. γ-Gliadin consists mainly of two strong bands of 42 and 74 kDa and one 52 kDa. ω-Gliadin is more heterogeneous and contains also high-molar-mass proteins of 34, 42, 44, 45, 67, 78 kDa. FIII is comprised of proteins of 12 to 18 kDa and 27 kDa. Glyc-gli is very heterogeneous, the major band is of 16 kDa, the rest ranging from 12 to 67 kDa.

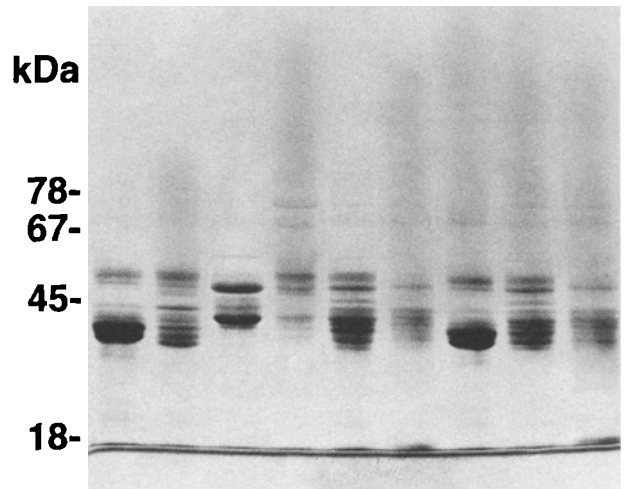

Fig. 1. SDS–PAGE analysis of gliadin: 1 – α, 2 – β, 3 – γ, 4 – ω, 5 – A, 6 – crude in reducing conditions, and 7 – α, 8 – A, 9 – crude in nonreducing conditions.

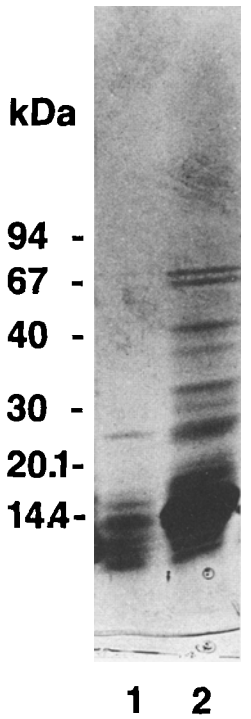

Fig. 2. SDS–PAGE analysis of gliadin: 1 – FIII, and 2 – glyc-gli in reducing conditions.

Immunoblotting with the three monoclonal antibodies exhibited staining of a large number of protein bands from 20 to 80 kDa for α-, β-gliadin, 20–65 kDa for γ-gliadin, 30–94 kDa for ω-gliadin, 30–94 kDa for crude gliadin, 10–70 kDa for A-gliadin, 14.4–67 kDa for glyc-gli, and 15–67 kDa for FIII.

Patient's serum IgG antibodies reacted very similarly to the mAb's. Control sera exhibited only weaker staining, no qualitative differences were found between these two groups.

Each rabbit exhibited reactivity with different protein bands. The PTP-immunized rabbit reacted most similarly to the mAb's and patients (Fig. 3).

DISCUSSION

The aim of this study was to characterize more precisely gliadin as an antigen and antigliadin antibodies present in the patients' sera and produced by immunized animals. First we used the ELISA and, for more detailed study, immunoblotting.

In ELISA, human, rabbit as well as monoclonal antibodies reacted with all gliadin fractions used which suggests the existence of shared immunodominant epitopes on all of them.

This hypothesis was further tested by immunoblotting which showed that no particular protein reacting specifically with the patients' serum IgG antibodies can be detected (in agreement with Vainio *et al.* 1986). The monoclonals reacted with a broad spectrum of proteins. This indicates that the immunodominant epitope is frequently present on all gliadin subfractions tested. The structure of gliadin is known to be different from other proteins. The high content of proline, glutamine and the low

charge of the molecules are of special interest (Hekkens 1978). The molecules are essentially linear and contain repetitive amino acid sequences. Some of these sequences are suggested to be both disease-precipitating (De Ritis *et al.* 1988) and forming the structure of β-turns, as was proposed in computer prediction studies (Kocna *et al.* 1991; Marsh 1992). The β-turn structures are usually located at the surfaces of proteins and therefore may well function as antigenic sites. Similar β-turn-forming repetitive sequences were recently proposed to be present in some human extracellular matrix proteins (Matsushima *et al.* 1990). The cross-reactivity of these peptides and gliadin structures could explain the incidence of autoantibodies in CD patients. Recently it was shown that monoclonal anti-gliadin antibodies cross-react with epitopes on enterocytes (Tučková *et al.* 1995).

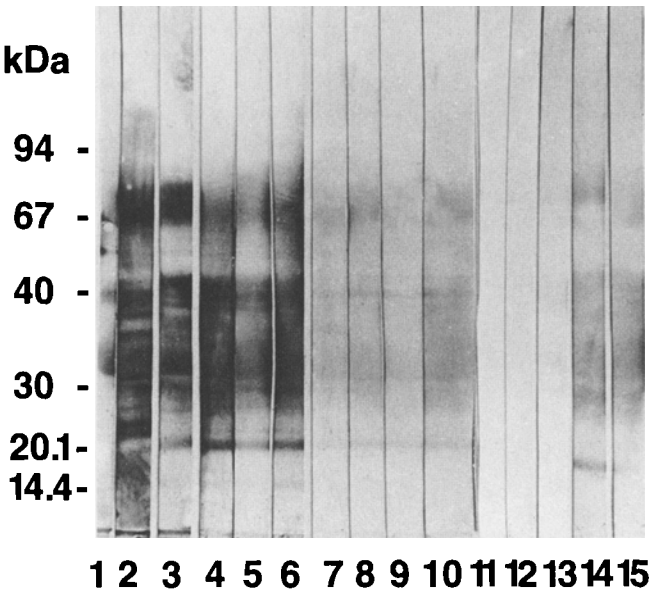

Fig. 3. Western blot analysis of A gliadin with: rabbit antisera 1 – anti-A gliadin, 2 – anti-F III and 3 – anti-PTP digest of gliadin, monoclonal antibodies 4 – GL1, 5 – GL2, 6 – GL3, patients' sera 7–10, control sera 11–15.

To summarize, gliadin contains a number of immunogenic epitopes which are probably involved in eliciting an autoimmune response against the intestinal epithelium and other cross-reacting proteins (Karská *et al.* 1995).

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