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Short Communication

Determination of anti-secalin antibodies in sera from coeliac patients by ELISA-based assay[1]

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1. Introduction

Coeliac disease (CD) is a permanent intolerance to dietary prolamins (alcohol soluble protein fraction of cereal gluten) from selected cereals (wheat, rye, barley and oats) resulting in small intestinal villus atrophy with consequent malabsorption and malnutrition and the presence of high level of sera anti-prolamin antibodies [1–3]. Prolamins from maize and rice are considered non toxic for coeliac patients [4]. Gliadins (wheat prolamins) and secalins (rye prolamins) are major storage proteins of cereal kernel endosperm.

Diagnostic procedure is long lasting and highly invasive procedure including at least three duodenal biopsies in a three year period [5]. A gluten-free diet (i.e. a diet without coeliac toxic prolamins) is a lifelong treatment for coeliac patients.

Wheat is the most common consumed cereal and that is the reason why anti-gliadin antibodies are more frequently investigated [3,6] than antibodies to other cereal prolamins. There is not a lot of evidence regarding the relation between the content of anti-gliadin and anti-secalin antibodies in sera from coeliac patients and the applicability of anti-secalin antibodies in a diagnostic procedure.

The main goal of our work was the development of specific ELISA-based assay for detection and quantitation of sera anti-prolamin antibodies that can be used in a diagnostic procedure and to investigate the applicability of this method for the determination of anti-secalin antibodies in sera from coeliac patients.

2. Experimental

2.1. Material and reagents

Sera was collected from 18 diagnosed coeliac patients: six in active status after positive result of the first biopsy, six on a long term gluten free diet and six on relapse. Sera was also collected from six healthy human subjects (control group).

Commercial integral cereal flour from wheat, rye and maize was from Macrobiotics center Trim (Yugoslavia). Gliadin, fast *o*-phenylenediamine (OPD) and rabbit anti-gliadin antibody labeled

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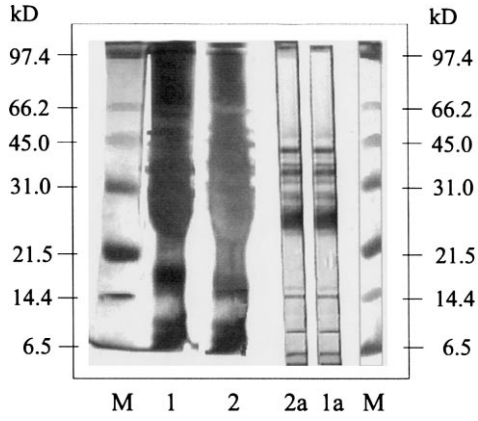

Fig. 1. Electrophoretic and immunochemical identity of commercial gliadin from Sigma and gliadin extracted by our procedure. M, Molecular weight markers (Bio Rad): phosphorylase b 97.4 kDa, serum albumin 66.2 kDa, ovalbumin 45.0 kDa, carbonic anhydrase 31.0, trypsin inhibitor 21.5 kDa, lysozyme 14.4 kDa and aprotinin 6.5 kDa. 1 and 2, SDS-PAGE of gliadin (Sigma) and our gliadin; 1a and 2a, Western blot of SDS-PAGE gels 1 and 2 developed with rabbit anti-gliadin HRP conjugated antibody.

with horseradish peroxidase (HRP) were obtained from Sigma (St. Louis, MO), bicinhonic acid (BCA) protein assay kit from Pierce (USA), rabbit anti-human immunoglobulins of M, A and G classes (IgM, IgA and IgG), were from Behring (Germany) and all other reagents: sodium dodecyl sulfate (SDS), gelatin, Tween 20, Tris and goat anti-rabbit IgG HRP labeled antibody were from Bio-Rad (USA).

Immulon II ELISA plates with 12 × 8 wells were purchased from Nunc (Denmark). For measurement of apsorbance at 492 nm automatic micro plate reader Titertek Multiscan Flow Laboratories (USA) was used.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot were performed in Mini Protean II Apparatus with Transblot system from Bio Rad with all reagents from the same manufacturer.

2.2. Methods

Extraction of prolamins from dry defatted wheat, rye and maize flour was performed with 70% ethanol in water after the albumin and globulin fraction have been removed by 0.34 mol l^{-1} sodium chloride extraction. Total proteins were determined by BCA protein assay with commercial gliadin from Sigma as a standard. For investigation of purity and immunochemical properties of extracted prolamins SDS-PAGE according to Laemlli [7] and Western blot according to Towbin [8] with the commercial gliadin from Sigma and the gliadin extracted by ourselves were performed. Gliadins were stained with Silver stain procedure [9].

Optimal concentrations of antigen and secondary antibodies for ELISA were estimated performing the assay with their serial dilution, representing the

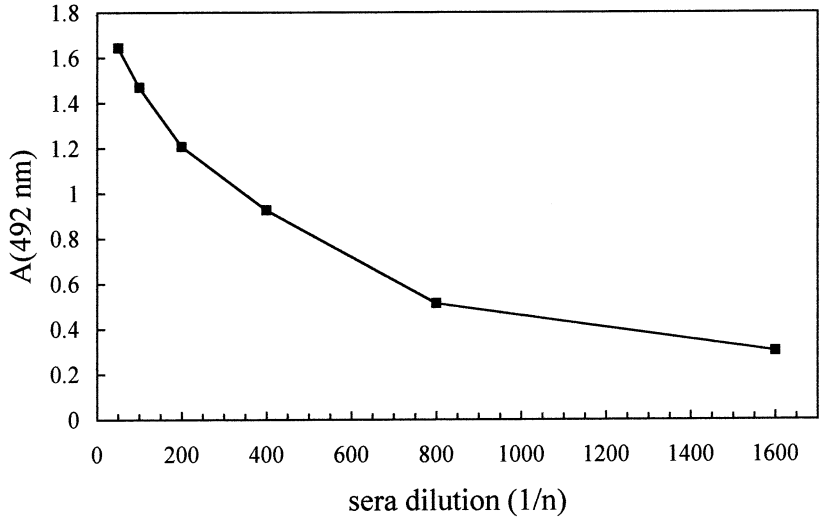

Fig. 2. The influence of dilution of CD sera for detection of IgA anti-gliadin antibodies. In our further work, dilution of 1/500 in 1% GTTBS was used.

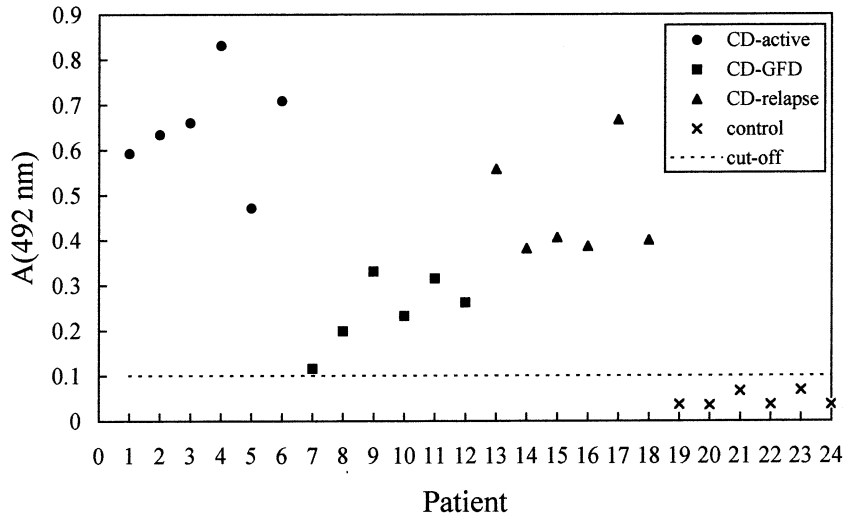

Fig. 3. Detection of IgM anti-gliadin antibodies in sera (1/500 in 1% GTTBS). CD, sera from patients with coeliac disease: in active status, on gluten-free diet (GFD) and on relapse. Control, sera from healthy human subjects. Sensitivity = 100%, specificity = 100%.

lowest concentrations that allow complete binding of primary antibodies in all contents within measurement range. Regions of linearity ($r^2 > 0.99$) between the dilution of sera (primary antibody) and A_{492} was investigated by serial dilution of three samples of CD sera.

Prolamins are water insoluble proteins and can not be dissolved in a standard carbonate/bicarbonate or similar buffer for sensitization of ELISA plates. Therefore a specific strategy was developed for attachment of prolamins to plastic. Antigens (wheat, rye and maize prolamins) 50 μl ml^{-1} in 70% ethanol were dispensed onto ELISA plates (100 μl per well) and left open over night on room temperature. After the evaporation of ethanol and the attachment of antigens to plastic, wells were blocked in 0.05% Tween 20/Tris-HCl, 0.02 M/NaCl, 0.5 M, pH 7.4 solution (TTBS) with 3% gelatin (3% GTTBS) within a 1-h period. Wells were incubated first with 50 μl of investigated sera 1/500 for IgM and IgA and 1/6000 for IgG detection in 1% GTTBS, then with rabbit anti-human

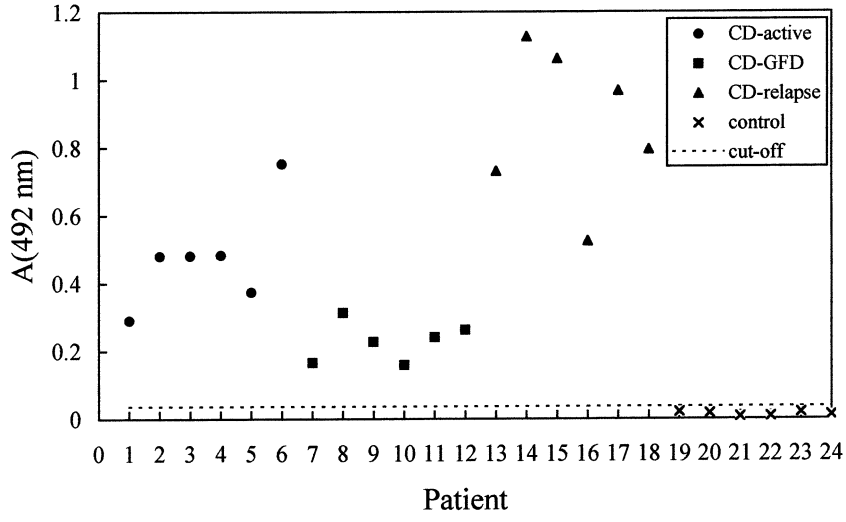

Fig. 4. Detection of IgA anti-gliadin antibodies in sera (1/500 in 1% GTTBS). CD, sera from patients with coeliac disease: in active status, on gluten-free diet (GFD) and on relapse; Control, sera from healthy human subjects. Sensitivity = 100%, specificity = 100%.

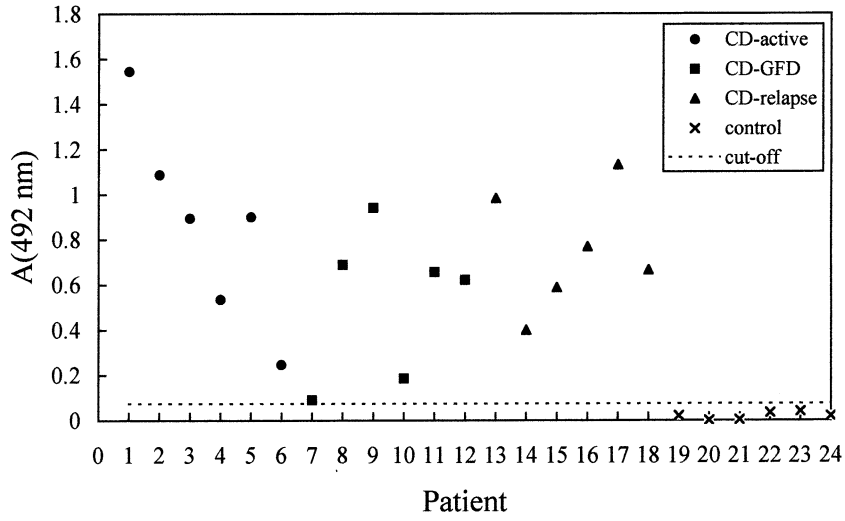

Fig. 5. Detection of IgG anti-gliadin antibodies in sera (1/6000 in 1% GTTBS). CD, sera from patients with coeliac disease: in active status, on gluten-free diet (GFD) and on relapse; Control, sera from healthy human subjects. Sensitivity = 100%, specificity = 100%.

anti-IgM, anti-IgA and anti-IgG antisera 1/1600 in 1% GTTBS and finally with goat anti-rabbit antisera (H+L) labeled with horseradish peroxidase (HRP) 1/1000 in 1% GTTBS. The period of each incubation was 1 h and between incubations wells were washed extensively with TTBS. Peroxidase specific substrate fast OPD (0.4 mg ml^{-1}) with 1 μl ml^{-1} 10% hydrogen peroxide was added and after 20 min in the dark at room temperature, color developing was stopped with 2 M sulfuric acid. Developed color was measured at 492 nm in an automatic micro plate reader. All samples were analyzed in duplicate. Commercial gliadin from Sigma was used as a positive control and maize prolamins (extracted by ourselves) as a negative control. The solvent (70% ethanol) served as a blind.

After the calculation of cut-off values (mean of control group + 3SD), sensitivity and specificity of anti-gliadin and anti-secalin antibodies of all isotypes (IgM, IgA and IgG) for diagnosis of coeliac

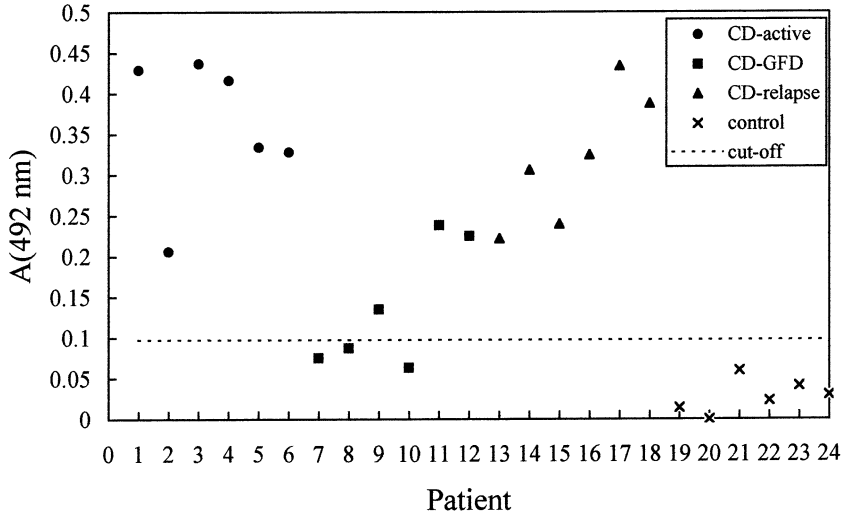

Fig. 6. Detection of IgM anti-secalin antibodies in sera (1/500 in 1% GTTBS). CD, sera from patients with coeliac disease: in active status, on gluten-free diet (GFD) and on relapse; Control, sera from healthy human subjects. Sensitivity = 83.33% (three patients on GFD have concentrations of investigated antibodies under the cut-off value), specificity = 100%.

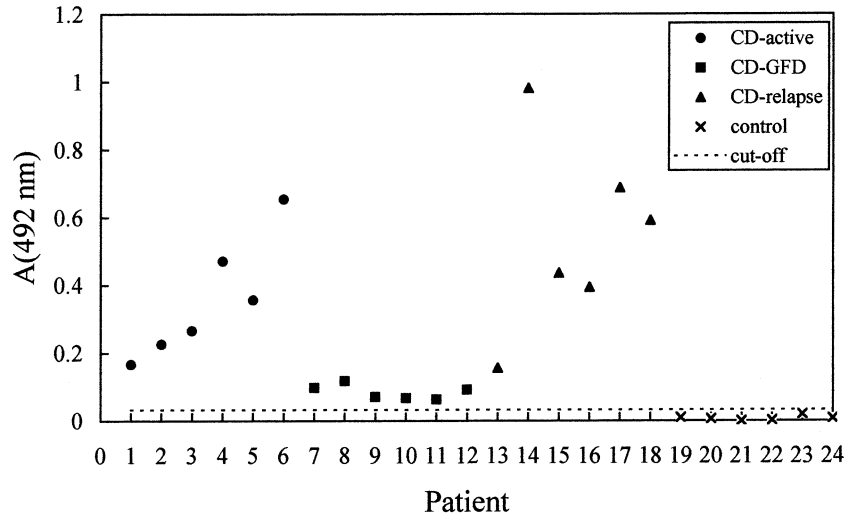

Fig. 7. Detection of IgA anti-secalin antibodies in sera (1/500 in 1% GTTBS). CD, sera from patients with coeliac disease: in active status, on gluten-free diet (GFD) and on relapse; Control, sera from healthy human subjects. Sensitivity = 100%, specificity = 100%.

disease was determined. Sensitivity is expressed by the percentage of CD patients that have concentration of investigated antibodies over, and the specificity by the percent of healthy individuals (control group) with concentration of same antibodies under the cut-off value. For statistic analysis, a t-test was used.

3. Results

Fig. 1 shows that in SDS-PAGE and Western blot developed with rabbit anti-gliadin peroxidase labeled antibody, gliadin extracted by our procedure and commercial gliadin from Sigma are electrophoretic and immunochemically identical.

The region of linearity was examined by ELISA with serial dilution of sera from three CD patients and the dependence of A_{492} of dilution of one CD sera for the determination of antigliadin antibodies of IgA class is shown on Fig. 2. In further work, concentrations of 1/500 in 1% GTTBS were used for the determination of IgM and IgA class of antibodies and 1/6000 for the determination of IgG isotype. Optimal concentration of antigen was 50 $\mu g\ ml^{-1}$ in 70% ethanol and of secondary antibody 1/1600 in 1% GTTBS.

The results of determination of anti-gliadin and anti-secalin antibodies of IgM, IgA and IgG isotype in sera are shown in Figs. 3–8. The reaction with coeliacly non toxic prolamins from maize was negative in all investigated concentrations. Experiments were carried out in duplicates and the correlation between results was very good ($r^2 = 0.997$)

Sensitivity of assay for diagnosis of CD was 100% for all classes of anti-gliadin antibodies and for IgA anti-secalin antibody, while it was 83.33% for IgM and 88.89% for IgG anti-secalin antibodies. Specificity was 100% for all classes of both antibodies.

The difference in contents of anti-gliadin and anti-secalin antibodies between the group of CD patients and the control group (healthy human subjects) was statistically significant for all isotypes ($P < 0.01$). The content of anti-gliadin antibodies was slightly higher than anti-secalin antibodies but differences were not significant.

The correlation of antibody contents with clinical status was very good. Patients in active status (patients 1–6) and on relapse (patients 13–18) showed significantly higher content ($P < 0.05$) of all investigated antibodies than patients on gluten-free diet (patients 7–12).

4. Discussion and conclusions

The incidence of CD is 1/1500 [10], but silent type

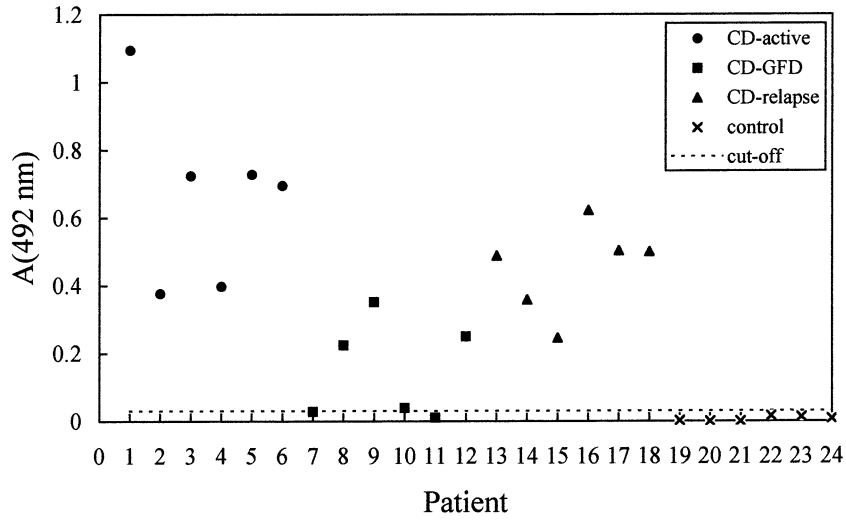

Fig. 8. Detection of IgG anti-secalin antibodies in sera (1/6000 in 1% GTTBS). CD, sera from patients with coeliac disease: in active status, on gluten-free diet (GFD) and on relapse; Control, sera from healthy human subjects. Sensitivity = 88.89% (two patients on GFD have concentrations of investigated antibodies under the cut-off value), specificity = 100%.

or latent disease is much more common affecting3.7/1000 blood donors in Sweden and even 15/250 persons in Iceland [11]. The first description of CD is more then 100 years old, but in last 50 years the toxicity of gliadin (and other prolamins), therapeutical effect of gluten free diet and diagnostically importance of duodenal biopsy were discovered [12,13]. The European Society for Pediatric Gastroenterology and Nutrition (ESPGAN) [5] estimated diagnostic a procedure for CD that requires dietary manipulation and jejunal biopsies. This is invasive procedure especially difficult in young patients. The same organization suggested [14] that serological markers (anti-gliadin, anti-reticulin and anti-endomysium antibodies) might be used in diagnostic instead of biopsy, but there are no standardized methodology for their detection and quantification. The ELISA-based assay described here can be used for diagnostic purpose (sensitivity 100% for all anti-gliadin and IgA anti-secalin antibodies, 83.33% for IgM and 88.89% for IgG anti-secalin antibodies with specificity of 100% for all istypes of both antibodies) as a quick non invasive procedure with the very good correlation with clinical status. Problem of sensitization of ELISA plates with water insoluble prolamins was successfully solved in this procedure. Slight modifications (different dilution and using of rabbit anti-gliadin antisera as a primary antibody) can be easily introduced to allow determination of coeliacly toxic prolamins in meals and for control of gluten free dietary products for gluten sensitive persons.

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