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Molecular Mimicry as a Possible Cause of Autoimmune Reactions in Celiac Disease? Antibodies to Gliadin Cross-React with Epitopes on Enterocytes

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Structural similarities between external antigen and self components are believed to be one of the possible causes of autoimmunity. This study describes the presence of similar structures shared by gliadin and enterocyte surface molecules recognized by antigliadin mAbs. The reactivity of mAbs to gliadin was followed by ELISA using fixed enterocytes, their brush-border membranes, or purified enterocyte antigen. The specificity of reaction was confirmed by ELISA inhibition studies and by immunohistochemical staining of rat tissue sections using biotin–avidin–peroxidase technique. Immunoprecipitation analysis of ^{125}I-labeled intestinal epithelial cells using antigliadin mAb revealed the presence of two main cross-reactive molecules of 28 and 62 kDa. The 62-kDa and an associated 66-kDa protein were isolated by affinity chromatography. Immunoblotting analysis showed that a 28-kDa protein detected by immunoprecipitation also reacted with IgA of celiac disease patient sera.

INTRODUCTION

The pathogenetic mechanism causing mucosal lesions in the jejunum of patients with celiac disease remains unknown, despite the fact that the inducing agent (gluten) is defined. In the past few years, hypotheses about immunologically mediated reactions involved in the etiology of this chronic disease have prevailed (1–4).

In sera of celiac patients, higher amounts of antigliadin antibodies as well as the presence of various autoantibodies have been described. Among these autoantibodies, antireticulin, antiendomysial, and antijejunal antibodies are often used as diagnostic tools for screening the disease (4–7). The presence of autoantibodies in celiac sera, strong association with HLA class II gene products, and local inflammatory patterns suggest that celiac disease can be classified as an autoimmune disease (8–10). However, definition of the target structures (autoantigens) and knowledge of the origin and real effects of autoantibodies along with possible participation of T cells in impairment of intestinal mucosa present in celiac disease are still lacking.

In our preliminary study, we have found that sera of celiac patients and media from cultured jejunal biopsy specimens contain autoantibodies reacting with isolated enterocytes (11). Here we present a study showing reactivity of antigliadin antibodies (hybridoma and patient-derived) with enterocyte antigens which may demonstrate a similarity in structure between external antigen and autoantigen such as is believed to be one of the possible causes of autoimmune reactions.

MATERIALS AND METHODS

Gliadin Preparations

Commercially available gliadin crude (Sigma Chemical Co., St. Louis, MO), α-gliadin (part of gliadins characterized by its electrophoretic mobility containing proteins of M_r 30,000–40,000), Frazer III fraction (peptic–tryptic digest of crude gliadin, peptides of M_r 10,000–20,000), and peptic–tryptic pancreatic digest of α-gliadin prepared as described earlier (12) were used.

Monoclonal and Patient Antigliadin Antibodies

Preparation and characterization of mouse monoclonal antigliadin antibodies used in this study were described previously (12). Sera from three patients suffering from active celiac disease (confirmed bioptically) and containing high levels of antigliadin IgA antibody were used.

Isolation of Villus and Crypt Epithelial Cells and Brush-Border Membrane Preparation

Female rats of Wistar strain, 2–3 months old, bred at the Institute of Physiology CAS, Prague, and 14-day-old germ-free rats of the same origin, reared in plastic isolators and fed by sterilized granulated diet composed of dried cow milk, soya, and corn flour (gluten-

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free diet) (13, 14), were used for preparation of isolated enterocytes.

Intestinal cells were isolated in sequential populations essentially according to Raul *et al.* (15) and Kolínská *et al.* (16) at 37°C with gentle shaking of everted rat jejunum in a phosphate-buffered saline with addition of 1.5 m*M* Na_2EDTA and 0.5 m*M* DL-dithiothreitol. Ca^{2+} and Mg^{2+} were omitted.

Brush-border membranes were prepared from jejunal scraping of adult rat by the method of Schmitz *et al.* (17).

Radioactive Labeling and Cell Lysis

Cells were ^{125}I-labeled using the lactoperoxidase technique (18). Briefly, cells 1–2 × 10^7/ml in 0.5 ml PBS and 50 μl $Na^{125}I$ (1 mCi, Amersham, UK) were mixed with 40 μl of lactoperoxidase (1 mg/ml, Sigma) and 20 μl 0.03% H_2O_2 in PBS. After 5 min incubation at room temperature another 20 μl 0.03% H_2O_2 was added, and again after 5 min incubation the cells were washed three times in PBS.

Labeled cells, as well as nonlabeled cells, were lysed with 1 ml of Laemmli sample buffer (19) (0.75 *M* Tris–HCl buffer, pH 8.8) containing 0.5% Nonidet P-40 (0.2% SDS), 1 m*M* PMSF, 1 m*M* EDTA, and 10 μg/ml aprotinin. After 10–30 min incubation on ice, cellular debris was removed and the nuclei were pelleted by centrifugation at 90,000*g* for 30 min using a Beckman 95 ultracentrifuge rotor. Supernatants were subjected to SDS–PAGE and immunoblotting, or they were immunoprecipitated and analyzed by SDS–PAGE.

Immunoprecipitation and Immunoblotting

Lysate (100 μl) from radiolabeled cells was immunoprecipitated by a two-step procedure (20) using mAb to gliadin, normal Balb/c or human serum, and corresponding second antibody (sheep anti-mouse Ig or swine anti-human Ig). After precipitation and centrifugation, precipitates were washed with cold PBS, resuspended in sample buffer, boiled 10 min at 90°C, loaded onto 10% SDS gel, and subjected to electrophoresis (21). The gels were then dried and autoradiographed using Kodak X-OMAT films (Eastman Kodak, Rochester, NY) for 1–5 days at −70°C.

Blotting was performed at room temperature for 4 hr at 400 mA or 16 hr at 80 mA in 12.5 m*M* Tris, 96 m*M* glycine, and 20% methanol (21) using the Transblot system (Bio-Rad, Richmond, CA). The nitrocellulose (0.2 μm, Schleicher & Schuell, Feldbach, Switzerland) was then cut into strips and blocked for 1 hr at room temperature in 5% BSA in PBS. The strips were stepwise incubated with mAb or human sera and with peroxidase-labeled second antibody (swine anti-mouse, swine anti-human IgG or IgA). The binding proteins were either (a) stained with 1.6 m*M* *o*-diaminobenzidine (DAB) and 0.01% H_2O_2 containing 0.1 *M* Tris–HCl buffer, pH 7.6, or (b) detected after incubation of the strips with ECL (enhanced chemiluminiscence) Western blotting detection reagents (Amersham, UK) on autoradiography film (Hyperfilm-ECL, Amersham, UK).

Affinity Chromatography

Immunoadsorbents were prepared by coupling of gliadin crude, α-gliadin, Frazer fraction III of gliadin, pepsin–trypsin pancreatic digest of α-gliadin, mAb to gliadin, or enterocyte cell lysate to CNBr-activated Sepharose 4B (22). Immunorsorbent gels were used in ELISA inhibition studies and for isolation of cross-reactive molecules from enterocyte lysates. Cell lysate (4 ml) was applied on an immunosorbent column, and adsorbed molecules were subsequently eluted with 0.3 *M* borate buffer, pH 8.0, and 0.018 *M* glycine–HCl buffer, pH 2.4 (eluate II and III).

ELISA

ELISA for antigliadin antibodies was performed as described earlier (12). Briefly, wells of polystyrene microtiter plates (Hardtmuth Koh-i-noor, České Budějovice) were filled with 50 μl of gliadin samples diluted in 70% ethanol (concentration 0.1 mg/ml). Alternatively, rat enterocytes isolated as described above at a concentration of 2 × 10^6 cells/ml or their lysates were applied in 100-μl volumes. The microplates were incubated overnight at 4°C, and adsorbed molecules or sedimented cells were fixed for 10 min with 0.1% glutaraldehyde solution. After blocking (1% BSA) and repeated washings, 50 μl of serum or antibody samples diluted in 1% BSA was added to the wells in duplicate and incubated overnight at 4°C. Wells filled with 1% BSA and PBS were used as blank (negative control). After another wash, 50 μl of pig anti-mouse immunoglobulin conjugated to peroxidase (SWAM-Px, Institute of Sera and Vaccines, Prague) diluted 1:1000 in PBS containing 10% pig serum was added. The plates were then incubated for 2 hr at 37°C. After another wash the enzymatic reaction was developed by 1,2-*o*-phenylenediamine solution, stopped by 2 *M* H_2SO_4, and read on the Microelisa MR 500 Minireader (Dynatech) at 470 nm.

Monoclonal Antibody Inhibition Assay

For inhibition experiments, mAbs at a concentration giving 50% of the maximal optical density in a titration curve were mixed with increasing amounts of immunoadsorbent gels with bound gliadin, enterocyte lysate, or albumin. After overnight incubation at 4°C, the mixture was shaken for 1 hr and centrifuged. The supernatants were then transferred into microtitration plates precoated with α-gliadin or enterocyte lysate, and the mAbs bound to solid phase were measured by

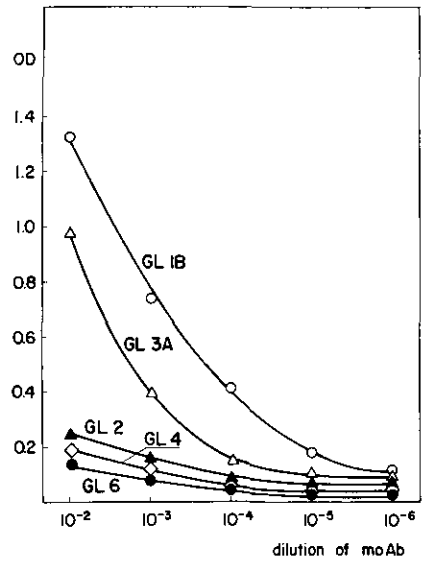

FIG. 1. Reactivity of antigliadin mAbs (Gl1B, Gl2, Gl3A, Gl4, and Gl6) with enterocytes fixed to microplate wells was measured in ELISA.

ELISA. The percentage inhibition was calculated compared to the binding in the absence of inhibitor.

Tissue Section Staining

The immunohistochemistry of acetone-extracted cryostat sections (6 μm) of unfixed frozen rat tissue blocks of jejunum, colon, lung, myocardium, thymus, spleen, kidney, and adrenal gland was determined by the biotin–avidin–peroxidase method. The 24-hr incubation with the primary antigliadin mAb (1:25–1:250, at 4°C) was followed by the biotinylated horse anti-mouse Ig antibody (1 hr), ABC reagent (Vector, Burlingame, CA; 1 hr), and DAB–H_2O_2 with light hematoxylin counterstaining. In the control tests the primary mAb and/or secondary mAb and ABC complex were omitted to visualize the cross-reaction with rat Ig, endogenous biotin, and endogenous peroxidase, respectively.

RESULTS

Reaction of Antigliadin Antibodies with Enterocytes

In order to establish the reactivity of mAbs to gliadin with enterocytes, we performed experiments studying the binding of antigliadin mAbs by ELISA using enterocytes fixed to microplate wells. Results demonstrated in Fig. 1 reveal that two of the mAbs tested exhibited high reactivity against enterocytes. We have tested the binding capacity of these mAbs with relevant structures such as isolated brush-border membranes as well as with affinity-purified enterocyte antigen (Fig. 2).

Specificity of the reaction was tested in an inhibition study. The results showed that the antienterocyte activity of antigliadin antibodies was inhibited by insolubilized gliadin (Fig. 3A), and furthermore, antigliadin activity was inhibited in a concentration-dependent manner by insolubilized isolated enterocyte antigen (Fig. 3B). Moreover, the specificity of antigliadin mAb binding was followed by immunohistochemical staining of rat tissue sections.

The antigliadin mAb 1B produced a fluctuating but distinct surface positivity on the epithelial cells of jejunal mucosa with a weak or negative reaction in the crypts. The lamina propria, submucosa, and muscular layer gave no reaction. The colonic mucosal glands were negative but a local positivity appeared on the surface epithelial cells. The hepatic, splenic, thymic,

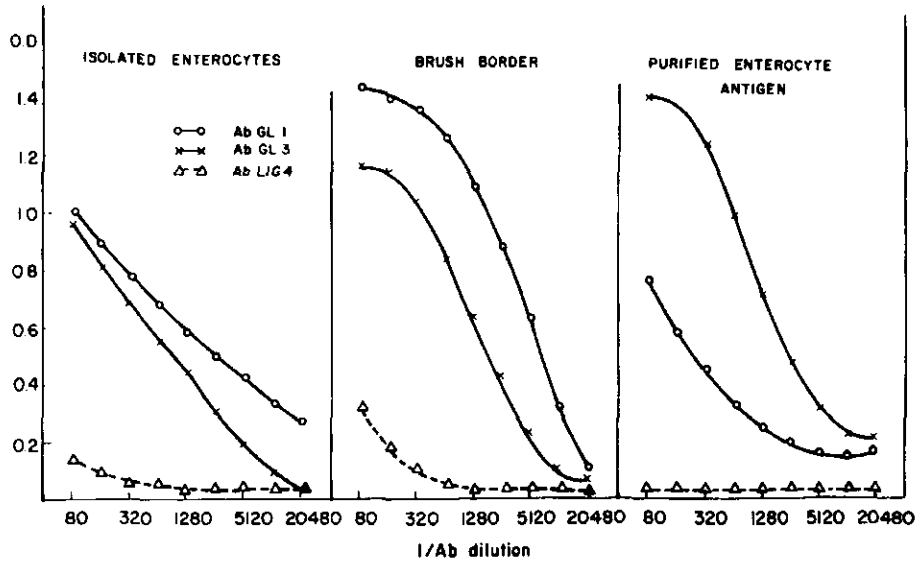

FIG. 2. Interaction of selected monoclonal antigliadin Abs Gl1B and Gl3A with enterocytes and their fractions. In controls the unrelated mAb LIG4 (to pig IgM) was used.

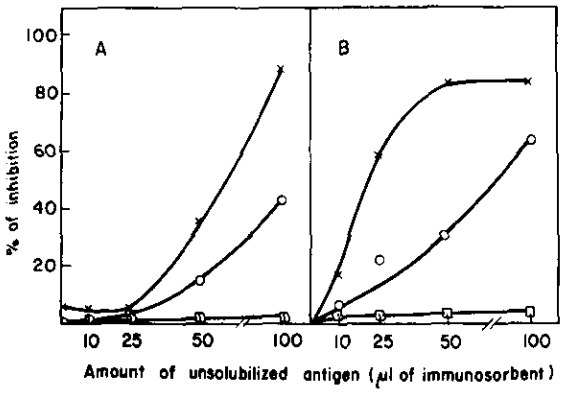

FIG. 3. Inhibition of reactivity of monoclonal antigliadin Abs with enterocytes by immobilized α-gliadin (A) and reactivity against gliadin by immobilized enterocyte lysate (B) were analyzed by ELISA using Gl1B, (○), Gl3A (×), and LIG4 (□) mAbs.

pulmonary, myocardial, renal, and adrenal tissues displayed no specific product. In the control tests the intestinal epithelial cells remained negative. After the omission of primary antigliadin mAb, the secondary anti-mouse Ig gave a weak surface cross-reaction with lymphocytes in the B zones of the spleen; scattered labeled cells also appeared in the intestinal lamina propria, and sporadic elements were seen in some hepatic sinuses and pulmonary alveolar septa and in the interstitium of heart and kidney. Similar results were obtained in experiments in which mAb to gliadin was replaced by mAb of different specificity but of the same isotype (LIG4). The incubation with ABC reagent (endogenous biotin) showed a weak granular positivity of renal proximal tubules. The DAB–H_2O_2 alone (endogenous peroxidase) revealed scattered granular mono-

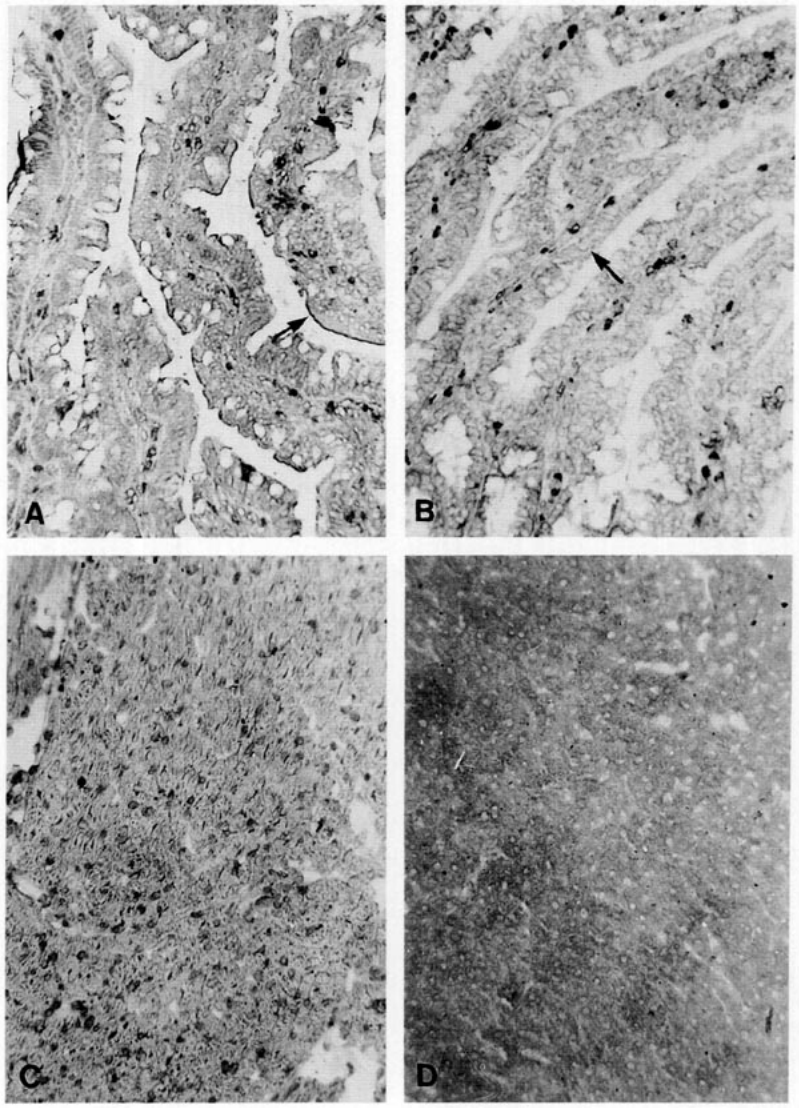

FIG. 4. (A) The reactivity with antigliadin mAb is seen in the brush-border of jejunal villi. Scattered cells in tunica propria express cytoplasmic granular or surface membrane positivity. Mouse antigliadin mAb, biotynylated horse anti-mouse Ig, and ABC reagent were used. (B) Lack of reactivity in the brush-border after omission of antigliadin mAb. Reaction of cells in lamina propria is unchanged. Absence of reactivity with antigliadin mAb in the myocardium (C) and hepatic parenchyma (D) after the same processing as in (A) (160× magnification).

nuclear cells in the red pulp of spleen and intestinal lamina propria and single sporadic cells in the lung alveoli and thymic capsules and septa (Figs. 4A–4D).

Characterization of Cross-Reactive Molecules in Enterocyte Lysate

Enterocytes isolated from jejunum of normal rats were ^{125}I-labeled and immediately lysed using a solubilizing buffer, pH 8.8, containing 0.2% SDS. Immunoprecipitation with mouse antigliadin mAb and SDS–PAGE analysis revealed two major protein bands of 62 and 28 kDa and two bands of lesser intensity corresponding to 34- and 52-kDa molecules (Fig. 5). In control experiments, antigliadin mAb was omitted or replaced by normal Balb/c sera. In controls only one or two weak bands representing proteins nonspecifically adsorbed on the precipitate were detected.

To confirm the specificity of binding, ^{125}I-labeled coelomocytes of an annelid (*Lumbricus terrestris*) were lysed, instead of enterocytes, and immunoprecipitated using the same antigliadin mAb. As the other controls, no reactivity with antigliadin mAb was observed (Fig. 5).

Material with higher molecular mass of about 60 kDa was also detected in experiments in which the enterocytes were solubilized in buffer, pH 8.8, in the absence of SDS.

In order to exclude the possibility that gliadin and/or its fragments originating from food participate in the reaction with antigliadin mAb, enterocytes from 17-day-old rats kept on the gliadin-free diet were isolated and solubilized. Similar results were obtained: immunoprecipitation analysis of cell lysates confirmed the presence of two intense bands corresponding to 28- and 62-kDa molecules (as in Fig. 5).

To compare the reactivity of mAb to gliadin with that of lysates of different cell origin, nonlabeled rat enterocytes, thymocytes, and splenocytes were isolated and lysed, and supernatants were subjected to ECL–Western blot analyses. The two main binding proteins of 62 and 28 kDa were detected only in enterocyte lysates when the gels were run under both reducing and nonreducing conditions. The reactivity with antigliadin mAb in lysates of cell suspensions isolated from other tissues was weak and the spectrum of proteins detected was different (Fig. 6). No binding was observed in controls where mAb was ommitted (results not shown).

To isolate the binding proteins from enterocyte lysates, affinity chromatography on Sepharose 4B with coupled antigliadin mAb (purified Ig fraction) was employed, as described above. Eluted fractions II and III were concentrated using ultrafiltration and analyzed on SDS–PAGE using silver staining. From the whole spectra of solubilized proteins of enterocytes, only two of molecular mass 62 and 66 kDa were isolated on antigliadin immunoadsorbent (Fig. 7).

To summarize, the results indicate that on enterocytes, epitopes of two main proteins of 28 and 62 kDa (and three additional proteins 34, 52, and 66 kDa), solubilized under various conditions, cross-react with mouse mAb against gliadin.

Reactivity of Celiac Disease Serum IgA with Enterocyte Antigens

In these experiments, nonlabeled rat enterocytes were solubilized (buffer pH 8.8, 0.2% SDS) and proteins in lysate characterized by immunoblotting using antigliadin mAb, normal human sera, and sera from patients with celiac disease. Antigliadin mAb, as well as

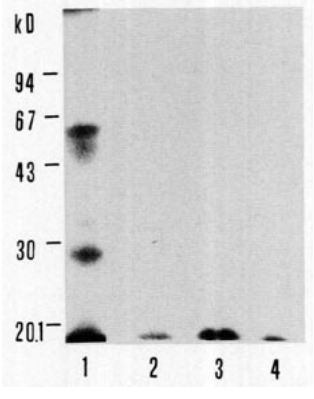

FIG. 5. SDS–PAGE analysis of immunoprecipitates formed after interaction of extracted ^{125}I-labeled rat enterocytes with mAb to gliadin and Px-labeled sheep anti-mouse Ig antibodies (lane 1). The precipitates were solubilized and subjected to electrophoresis on 10% acrylamide gel under nonreducing conditions, and the mAb binding molecules were detected by autoradiography. Low-molecular-weight standards (Pharmacia, Uppsala, Sweden) were stained with Coomassie blue R-250. In controls where mAb was omitted (lane 2) or replaced by normal Balb/c serum (lane 3) or coelomocytes of annelids (*Lumbricus terrestris*) were used instead of enterocytes (lane 4), no specific binding was observed.

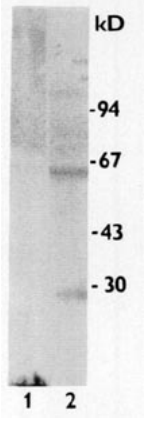

FIG. 6. Comparison of reactivity of rat thymocyte (lane 1) and enterocyte (lane 2) lysates with mAb to gliadin. Cell lysates were separated on SDS–PAGE under nonreducing conditions and transferred to nitrocellulose. The mAb binding was detected after incubation with secondary antibody (Px-labeled ovine anti-mouse Ig) and ECL–Western blotting detection reagents on autoradiography film.

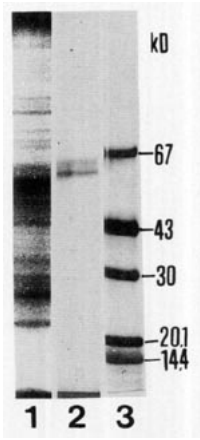

FIG. 7. SDS–PAGE of affinity chromatography-purified enterocyte molecules. Silver staining of enterocyte extract (lane 1) and isolated proteins (lane 2) and molecular weight markers (Pharmacia, Uppsala; lane 3) is shown.

patient sera (selected by ELISA), reacted in enterocyte lysate (using SWAHu IgA) with a protein of molecular mass 28 kDa, which represents one of the main proteins labeled on the surface and detected by immunoprecipitation technique (Fig. 8). The absence of 62-kDa (and/or 52, 34 kDa) binding proteins in this assay could be explained by the lower sensitivity of immunoblotting compared to that of immunoprecipitation using ^{125}I-labeled proteins (as documented in Figs. 5 and 8). Furthermore, the weaker binding capacity of patient sera is probably caused by broad heterogeneity of patients' antibodies to gliadin that recognize different epitopes on gliadin molecule.

In a preliminary experiment, human intestinal cells of jejunal mucosa, prepared by scraping from the gut of a 15-week-old human fetus, were subjected to lysis and immunoblotting analysis using antigliadin mAb. Also in this case, a 28-kDa molecule cross-reactive with antigliadin antibody was detected.

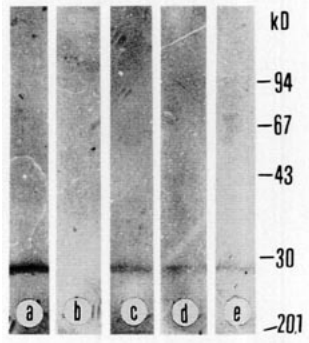

FIG. 8. Western blot analysis of rat enterocyte lysate with antigliadin mAb (a), normal human sera (b), and sera from patient 3 with celiac disease (c–e). The reaction was visualized after interaction with Px-labeled secondary antibodies: (a) swine anti-mouse Ig; (b–e) swine anti-human IgA. Lack of reactivity was observed with normal sera used as a control.

DISCUSSION

Reactivity of antigliadin mAbs with epitopes present on enterocyte membranes could be a consequence of structural features (similarities) of gliadin and other molecules. Structural similarities between self components and external antigens that could contribute to development of autoimmune disease were reviewed (23, 24).

Wheat gliadins (prolamins) responsible for celiac disease activity are markedly homologous in sequence to corresponding prolamins of rye (secalins) and barley (hordeins). These molecules are essentially linear, containing repetitive sequences and are highly immunogenic. The major disease-associated repetitive motifs are supposed to be PSQQ and other variations including PSDQ, PSEQ, QQQP, and QPFP. The structure prediction suggests beta turn occurrence involving sequences PSQQ and PQQP (3). The same features, primary repetitive motif and secondary beta turn formation, as well as high P, Q, and S content characteristic of oligopeptides of gliadin, are also present in rhodopsin, synaptophysin, synexin, and RNA polymerase II; these are proteins important in Ca^{2+} binding, intermembrane contacts, and mRNA synthesis, respectively (25).

Immunologically mediated impairment of jejunal mucosa is now generally considered to be a main pathogenic mechanism operating in celiac disease (26). Among a variety of autoantibodies, those directed to extracellular matrix components prevail (3, 6, 7, 27–29). The IgA reactivity against enterocytes present in the sera of patients and supernatants from cultured intestinal biopsy showed that at least some of the patients possess B and probably also T cells originating in mucosa and specific for enterocyte membrane components (30). Perhaps IgA, despite its inability to bind complement, does participate in pathogenic mechanisms through its interactions with FcαR present on effector cells. This suggestion is also supported by the finding of increased levels of FcαR receptors in the lamina propria cells of patients with celiac disease (26).

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