Autoantibodies to gliadin-binding 90 kDa glycoprotein in coeliac disease

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SUMMARY Patients with untreated coeliac disease were found to have high concentrations of circulating antibodies to 90 kDa glycoprotein, a mannose rich protein found in skin and intestinal mucosa. In contrast, patients with active Crohn's disease or ulcerative colitis had antibody concentrations within the normal range. In coeliac disease the antibody concentrations fell significantly after gluten withdrawal. 90 kDa glycoprotein bound gliadin in a carbohydrate and calcium dependent manner. The results show that circulating antibodies directed against a gliadin-binding antigen are present in coeliac disease. 90 kDa glycoprotein may be a receptor for gliadin; in susceptible subjects ligand receptor interaction may result in cytotoxicity and antibody formation.

The small intestinal mucosal damage in coeliac disease is induced by gluten. The mechanism of the gluten induced injury is, however, unclear. Local antigen antibody reaction as well as cell mediated immune mechanisms have been implicated in the damage.¹ The possibility that dietary gluten may act as a lectin that is toxic to the jejunal mucosa in patients with coeliac disease has also been suggested.^{2,3}

90 kDa Glycoprotein is a newly described tissue protein which accumulates extracellularly in the skin and to some extent in the gut in a rare hyaline disease, massive cutaneous hyalinosis.⁴ Our recent studies have shown that tissue 90 kDa glycoprotein is an antigen in circulating IgG immune complexes in dermatitis herpetiformis and coeliac disease.⁵ In this study we show high concentrations of circulating autoantibodies to 90 kDa glycoprotein in coeliac disease and present immunohistochemical evidence that 90 kDa glycoprotein is a normal constituent of the skin and small intestinal mucosa. These findings, together with the observation that 90 kDa glycoprotein binds gliadin in a carbohydrate and calcium dependent manner, suggest that 90 kDa glycoprotein may be intimately involved in the pathogenesis of coeliac disease.

Methods

PATIENTS

Coeliac disease

Thirty three patients, mean age 37 years (range 16 to 73 years), with coeliac disease were studied. The diagnosis was based on a characteristic jejunal lesion and subsequent satisfactory clinical and morphological response to gluten free diet. Pretreatment sera were available from all patients; in 13 patients sera were also available at the time of repeat biopsy (mean time of gluten free diet nine months, range three to 36 months). Villous height was determined as described elsewhere.⁶

Inflammatory bowel disease

Thirty patients, mean age 31 years (range 20 to 54 years), with biopsy verified inflammatory bowel disease were studied. Fifteen of the patients had ulcerative colitis, and 15 Crohn's disease. In each patient serum samples were studied in an active-phase of the disease and in remission.

Rheumatic disease

Forty four patients, mean age 45 years (range 23 to 77 years), with rheumatic disorders were studied. Eighteen of the patients had rheumatoid arthritis, 15 systemic lupus erythematosus, six systemic sclerosis and five mixed connective tissue disease. All patients were clinically considered to have an active disease. One serum sample was studied in each case.

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NORMAL CONTROLS

Fifty blood donors, mean age 38 years (range 21 to 72 years) served as normal control subjects.

PREPARATION OF ANTIGEN

90 kDa Glycoprotein was prepared from the skin nodules of patients with massive cutaneous hyalinosis.⁴ The purified 90 kDa glycoprotein was homogeneous on sodium dodecylsulfate polyacrylamide gel electrophoresis. The protein has a molecular mass of 90 000 daltons, a pI of 5.0, and a carbohydrate content of approximately 20%. It is sialylated, highly mannosylated and rich in glutamine and serine, but lacks the amino acids typical for collagen (hydroxylysine and hydroxyproline) and keratin (cystine, methionine). It is immunochemically distinct from the normal serum proteins, including the immunoglobulins, α_1 -acid glycoprotein, amyloid A and P proteins, fibronectin, as well as from keratin, elastin, collagen, procollagen and laminin.

ANTISERUM TO 90 kDa GLYCOPROTEIN

Rabbits were given four consecutive subcutaneous injections of 90 kDa glycoprotein (each 1 mg) in complete Freund's adjuvant at two week intervals. The first bleeding took place one week after the last injection and the final bleeding one week later. The antiserum precipitated the purified antigen (1 mg/ml) at a dilution of 1:32. By enzyme immunoassay the antiserum was shown to react with 90 kDa glycoprotein only, but not with human serum proteins, including the immunoglobulins and immunoglobulin light chains. The antiserum did not react with collagen, keratin, elastin, gluten or gliadin.

IMMUNOCHEMICAL TECHNIQUES

Indirect immunofluorescence

Frozen sections of biopsy or surgical specimens of human tissues (liver, kidney, spleen, skin, small bowel, pancreas, prostate, salivary gland, respiratory tract and uterine cervix) were fixed in methanol and cooled to -20°C for 10 minutes. The rabbit antiserum to 90 kDa glycoprotein⁴ was diluted 1:50 and exposed to the sections for 30 minutes. The specimens were then incubated with fluorescein-isothiocyanate coupled (FITC) goat anti-rabbit antibodies (Cappel Laboratories, Cochranville, PA, USA) for 30 minutes. After washing the specimens were embedded in veronal-glycerol and examined in a Zeiss-Universal microscope equipped with filters for FITC-fluorescence.

Immunoperoxidase staining

Four μm sections were cut from formalin-fixed, paraffin embedded blocks of human tissues obtained

at surgery or as biopsy specimens. The tissue sections were rehydrated in a graded alcohol series and then washed in PBS. The intrinsic peroxidase activity was blocked with 0.3% H_2O_2 in methanol for 30 minutes. Rabbit antiserum to 90 kDa glycoprotein, immunoglobulins, or normal rabbit serum (50-fold diluted) were applied for 30 minutes. As a second layer peroxidase conjugated swine antirabbit serum (diluted 1:100) (Dakopatts, Copenhagen, Denmark) was used for 30 minutes. The sections were thereafter incubated for 10 minutes with 3',3'-diaminobenzidine tetrahydrochloride and H_2O_2 (DAB 0.5 mg/ml PBS, 0.003% H_2O_2) (Sigma, St Louis Mo.). The specimens were counterstained with haematoxylin and eosin; as well as with methyl green. Controls in the immunohistochemistry studies included omission of the primary antiserum and its replacement with normal rabbit serum. Sections of massive cutaneous hyalinosis skin was used as positive control.

ENZYME-LINKED IMMUNOSORBENT ASSAY OF ANTIBODIES TO 90 kDa GLYCOPROTEIN

Polystyrene tubes were coated with 90 kDa glycoprotein 1 ml of antigen solution (0.5 $\mu\text{g}/\text{ml}$) in 0.05 mol phosphate buffer, pH 7.3, with 0.05% NaN_2 . One ml of serum diluted 1:200 with phosphate-buffered saline containing 0.05% Tween 20, was added to each tube. The antibodies bound to 90 kDa glycoprotein were detected with alkaline phosphatase labelled antiserum to human immunoglobulins (Orion Diagnostica, Espoo, Finland). The amount of alkaline phosphatase fixed to the tubes was determined in diethanolamine buffer (pH 10.0) with p-nitrophenylphosphate as substrate. Details of the assay are described elsewhere.⁷

BINDING EXPERIMENTS

Polystyrene tubes were coated with 1 μg of 90 kDa glycoprotein (2 $\mu\text{g}/\text{ml}$ in 50 mmol phosphate buffer, pH 7.3) by incubation at $+4^{\circ}\text{C}$ overnight. After careful washing of the tubes 1 ml ^{125}I -labelled proteins (wheat gliadin, transferrin, ovalbumin, α_2 -macroglobulin, α_1 -acid glycoprotein and bovine and rabbit albumin (Sigma Chemical Co., St Louis, USA) and purified human immunoglobulins G, M and A (a gift from Orion Diagnostica, Finland) was added to each tube (0.1 mol TRIS-HCl, pH 8.0, containing 0.05% Tween 20, 10 mmol CaCl_2 and 10 mmol MgCl_2) and the tubes were incubated at room temperature for two hours. The amount of protein attached to the 90 kDa glycoprotein was determined by measuring the residual radioactivity in the tubes. Residual radioactivity in the uncoated tubes was used as a measure of unspecific binding.

OTHER ASSAYS

Antireticulin antibodies were studied by indirect immunofluorescence technique. Total and IgA class antigliadin antibodies were measured by enzyme immunoassays.

STATISTICS

Student's *t* test for paired or unpaired data and Wilcoxon's signed rank test and rank sum test were used to test statistical significances. Linear regression analysis (*r*) and Spearman's rank correlation (*r_s*) were used to study the relationship between two variables. Two-tailed tests, where appropriate, were used in the analyses.

Results

LOCALISATION OF ANTIGEN

Immunohistochemical staining of normal tissue sections with monospecific antiserum to 90 kDa glycoprotein showed positivity in the skin and intestinal mucosa, whereas sections of liver, kidney, spleen, pancreas, prostate and salivary gland did not stain. The epithelium of uterine cervix and trachea stained weakly in some sections. In the skin, bright staining was found in the basal cells of the hair follicle; specifically staining material was to some extent also located extracellularly (Fig. 1). In the gut, a few lamina propria cells stained brightly (Fig. 2); counter staining for plasma cells and macrophages showed that the 90 kDa glycoprotein positive cells were distinct from these cells. In coeliac mucosa, specific staining was found mostly at extracellular sites.

CIRCULATING ANTI-90 kDa GLYCOPROTEIN ANTIBODIES

High concentrations of antibodies to 90 kDa glycoprotein were found in patients with untreated adult coeliac disease, whereas the antibody concentrations in active ulcerative colitis and Crohn's disease, as well as in various rheumatic diseases, did not significantly differ from those in normal controls (Table). In the patients with inflammatory bowel

Fig. 1 Indirect immunofluorescence staining of normal human skin with antibodies to 90 kDa glycoprotein. Note, the bright positivity of the epithelial cells of the hair follicle. Positive staining is also seen extracellularly. E-epidermis. $\times 200$ (original magnification).

disease there were no differences in antibody concentrations in sera obtained in the active phase of the disease or in remission.

After gluten withdrawal in coeliac patients antibody levels fell significantly ($p < 0.005$, Fig. 3). During therapy the decrease in anti-90 kDa glycoprotein antibody concentrations correlated with the decrease in IgA-antigliadin antibody levels ($r = 0.68$, $p < 0.05$). The levels of 90 kDa glycoprotein antibody did not significantly correlate with antireticulin antibody titres ($r_s = 0.41$, NS).

Table Circulating autoantibodies to gliadin binding 90 kDa glycoprotein in patients with coeliac disease, inflammatory bowel disease, rheumatic disorders, and in controls

Group	Subjects (no)	Antibodies to 90 kDa glycoprotein, Δ A/30 min		
		Mean $\pm$ SE	Median	Range
Coeliac disease	33	0.465 $\pm$ 0.050*	0.400*	0.085–1.420
Inflammatory bowel disease	30	0.189 $\pm$ 0.021	0.192	0.020–0.495
Rheumatic disorders	44	0.179 $\pm$ 0.036	0.180	0.055–0.450
Control subjects	50	0.168 $\pm$ 0.025	0.123	0.010–0.570

* $p < 0.001$ versus the other groups.

Fig. 2 Indirect immunofluorescence of small bowel mucosa with antibodies to 90 kDa glycoprotein. Note the positive cells (arrow) close to the mucosal glands (G). $\times 400$ (original magnification).

Fig. 3 Effect of gluten-free diet on the levels of anti-90 kDa glycoprotein antibodies and villous height in 13 patients with coeliac disease. The difference in antibody levels before and after gluten withdrawal is significant (paired Student's *t*-test, $p < 0.005$). Exclusion of the three highest pairs of values from the statistical analysis, does not change the significance. The difference is significant also using the Wilcoxon's signed rank test ($p < 0.01$; after exclusion of the three highest pairs of values, $p = 0.02$).

BINDING OF GLIADIN TO 90 kDa GLYCOPROTEIN

Purified 90 kDa glycoprotein bound gliadin (Fig. 4). The binding of gliadin to 90 kDa glycoprotein was calcium dependent and could be inhibited by the addition of 50 mmol α -D-mannose. On a weight basis α_2 -macroglobulin was bound to 90 kDa glycoprotein to a similar degree as gliadin, whereas only trace amounts of α_1 -acid glycoprotein, α_1 -antitrypsin and ovalbumin were bound. No binding of transferrin, IgG, IgA, IgM or albumin was observed.

Discussion

This study shows that patients with untreated coeliac disease have high concentrations of circulating autoantibodies directed against a gliadin binding protein, 90 kDa glycoprotein. In contrast, patients with active Crohn's disease or ulcerative colitis have normal antibody concentrations suggesting that the raised antibody levels do not reflect mucosal injury *per se*. Beside our initial report of the assay method⁷ anti-90 kDa glycoprotein antibodies have not previously demonstrated in coeliac disease. It is of interest, however, that two case reports of malabsorption and anti-epithelial antibodies have been

Fig. 4 Binding of gliadin to 90 kDa glycoprotein. Various concentrations of 125 I-labelled gliadin were incubated in uncoated control tubes (C) and in polystyrene tubes containing 1 μ g of 90 kDa glycoprotein (A). The amount of labelled gliadin bound was measured. Values are means $\pm$ SD of five determinations.

published.^{8,9} These patients did not have gluten sensitive enteropathy and it seems unlikely that the antiepithelial antibodies were related to the 90 kDa glycoprotein antibodies.

Antibodies to different fractions of wheat proteins are frequently found in patients with coeliac disease. Antiglutin and antigliadin antibodies are sensitive markers of gastrointestinal diseases, but are not specific for coeliac disease.^{10,11} The IgA class antibodies to gliadin correlated with the anti-90 kDa glycoprotein antibody levels during the response to gluten withdrawal in the patients with coeliac disease. As our antiserum to 90 kDa glycoprotein is monospecific and does not react with gliadin or gluten, the possibility of cross reaction as an explanation for the findings is excluded. The decrease in the anti-90 kDa glycoprotein antibody levels was associated with a marked improvement of the jejunal mucosal changes. IgA antigliadin antibody levels reflect the mucosal injury, too, but in contrast with these antibodies, which may also be raised in Crohn's disease,¹¹ the anti-90 kDa glycoprotein antibody concentrations were normal in active Crohn's disease as well as ulcerative colitis.

Although there exist some structural similarities between the 90 kDa glycoprotein and the non-collagenous component of reticulin,^{4,12} no clear relationship was found between the anti-90 kDa glycoprotein concentrations and antireticulin antibody titres. Firm conclusions about the relationship are difficult to draw, however, as reticulin antibodies were measured by a semiquantitative immunofluorescence technique.

Based on immunohistochemical evidence 90 kDa glycoprotein has a very limited distribution in normal human tissues. Skin and small intestinal mucosa as well as, possibly, the epithelium of uterine cervix and trachea, were the only of the tested tissues that specifically stained for 90 kDa glycoprotein. The strong staining of the basal cells of the hair follicle and of some of the intestinal lamina propria cells (distinct from plasma cells and macrophages) may indicate the cellular sites of synthesis of 90 kDa glycoprotein.

With regard to the pathogenesis of coeliac disease, and the inter-relationship between the gut and the skin,¹³ the tissue distribution of 90 kDa antigen is of interest. Interaction of dietary gluten with the intestinal mannose-rich 90 kDa glycoprotein, could result in antibody formation and in cytotoxicity, as shown for some other lectins.¹⁴ The presence of circulating antibodies to 90 kDa glycoprotein and to gliadin, and the relationship between their levels could thus be explained. As the circulating anti-90 kDa glycoprotein antibodies have targets in the skin, deposition of these antibodies and of 90 kDa

glycoprotein containing immune complexes⁵ could explain some of the dermal manifestations in some malabsorption patients. It is noteworthy that 3/10 patients with untreated dermatitis herpetiformis had antibodies to 90 kDa glycoprotein (unpublished). Mild gluten sensitive enteropathy is an almost invariable finding in dermatitis herpetiformis,¹⁵ and gluten withdrawal also appears to improve the skin manifestations.¹⁶ Thus, the anti-90 kDa glycoprotein antibodies with antigen targets in the skin and gut may be the pathogenetic link between these disease manifestations.

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