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Antibodies to gluten and reticulin in gastrointestinal diseases

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SUMMARY

Antibodies to reticulin were found in 33% of coeliac patients on a normal diet. These antibodies were found in only 11% of coeliac patients on a gluten-free diet. In patients with dermatitis herpitiformis, 12% had these antibodies, whereas the highest frequency in the other diseases studied (Crohn's disease, ulcerative colitis, cystic fibrosis and 'recurrent diarrhoea') was 7% (compared to 2% in healthy controls).

Antibodies to gluten, demonstrated with the immunofluorescence technique, were found in all coeliac children on a normal diet that were studied, and in half of the adults with the untreated disease. In children and adults on a gluten-free diet these frequencies decreased to 87 and 32%. In Crohn's disease, cystic fibrosis, recurrent diarrhoea, dermatitis herpetiformis and ulcerative colitis, the frequencies were 52, 42, 37, 18 and 18%, respectively (and in 4% of controls).

It was therefore concluded that antibodies to gluten were sensitive markers for gastrointestinal diseases, but were not specific for gluten enteropathy. Antibodies to reticulin, on the other hand, were less sensitive but of far greater specificity for coeliac disease. Gluten antibodies were of the IgA, IgM and IgG classes, whereas antibodies to reticulin were only of the IgA and IgG classes. Both types of antibody were found to be non-complement-fixing. Autoantibodies to smooth muscle were found in 5% of the coeliac patients (and in 0% of controls). No relationship with hepatic complications was found. In the patients with dermatitis herpetiformis, autoantibodies to gastric parietal cells were found in 24% (and in 5% of controls). No relationship was established between the occurrence of HLA-B8 and the presence or absence of any of the antibodies studied.

INTRODUCTION

Antibodies to reticulin (RAb) have been found in significant proportions of patients with coeliac disease, dermatitis herpetiformis and Crohn's disease (Seah *et al.*, 1971a; Alp & Wright, 1971; von Essen, Savilahti & Pelkonen, 1972; Rizzetto & Doniach, 1973; Magalhaes, Peters & Doe, 1974). In coeliac disease and dermatitis herpetiformis, antibodies are more common in patients on a normal diet (ND) than in those on a gluten-free diet (GFD) (Alp & Wright, 1971; Magalhaes *et al.*, 1974; Seah *et al.*, 1973). Moreover, many studies have revealed that antibodies to wheat gluten (GAb), or fractions of this protein, are also frequently present in patients with coeliac disease (Berger, 1958; Taylor *et al.*, 1961; Heiner *et al.*, 1962; Alarcón-Segovia *et al.*, 1964; Rossipal, 1970; Ferguson & Carswell, 1972). These antibodies tend to disappear after the introduction of GFD (Alarcón-Segovia *et al.*, 1964; Carswell & Ferguson, 1973).

The Rab were of the IgG and IgA classes (Magalhaes *et al.*, 1974), but the Ig class of GAb has not yet been studied. Recently, we described an immunofluorescence technique (IFT) by means of which the immunoglobulin class of GAb can be determined (Eterman *et al.*, 1977).

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In the present paper, frequencies and immunoglobulin classes of Rab and GAb in relation to the diet will be given. We studied sera from patients with coeliac disease, dermatitis herpetiformis, Crohn's disease, ulcerative colitis, cystic fibrosis and miscellaneous diseases leading to recurrent diarrhoea. Furthermore, the sera of a number of coeliac patients and of patients with dermatitis herpetiformis were examined for the presence of autoantibodies to smooth muscle, skeletal muscle, thyroid, gastric parietal cells, adrenocortex, salivary duct cells, nuclei and mitochondria.

MATERIALS AND METHODS

Sera from patients and control subjects. Sera were obtained from ninety-five 'adults' over 15 years of age (mean age, 43 years) with coeliac disease (thirty-six on a ND and fifty-nine on a strict GFD for more than 4 months) and fourteen children under 15 years of age (mean age 6 years; six on a ND and eight on a strict GFD for more than 4 months). All subjects showed evidence at biopsy of partial or (sub)total villous atrophy of the small intestinal mucosa (Doniach & Shiner, 1957) and had shown a favourable response to a GFD. Serum samples were taken from thirty-four untreated patients with dermatitis herpetiformis (a disease characterized by a polymorphic vesiculo-bullous eruption on specific predilected sites, the occurrence of 'neutrophilic' micro-abscesses at the tips of the dermal papillae and the deposition of IgA immunoglobulin in the dermal papillary tips of the clinically uninvolved skin; van der Meer, 1972); from fifty patients with Crohn's disease; from forty-four patients with ulcerative colitis (diagnosis based on well-accepted radiological, endoscopical and histological critera; Meuwissen, 1977); from seventeen children with cystic fibrosis (chronic pulmonary disease, pancreatic insufficiency and elevated sweat electrolytes; di Sant'Agnese, 1976); and from forty-five children with miscellaneous disorders leading to recurrent diarrhoea. Healthy blood donors, matched for sex and age with the patient groups, were used as controls. All samples were stored at $-20°C$ until testing.

Preparation of FITC-labelled human anti-reticulin antibodies. Serum from an untreated patient with coeliac disease, containing antibodies to reticulin in a titre of 1:40, was fractionated on a P300 Bio-gel column. The IgG fraction was labelled with FITC, after which the mixture was dialysed against PBS to remove unbound FITC. The final protein concentration was 0·4 mg/ml; the molecular F/P ratio was 3·3 and the final titre was 1:20. The same procedure was followed to label an IgG fraction of normal serum. The final protein concentration was 2·1 mg/ml, and the molecular F/P ratio was 2·1.

Antisera. For the determination of the Ig class of RAb and GAb we used specific rabbit antisera of the FITC-conjugated class. These sera were prepared in the Department of Immune Reagents of our laboratory. They were all tested for specificity by haemagglutination, immunoelectrophoresis, agar precipitation and by IFT on monoclonal bone-marrow preparations (anti-IgG: protein concentration 3·5 mg/ml, molecular F/P ratio 2·8, dilution used 1:80, lot No. KH 16-103-F3; anti-IgM: protein concentration 5·3 mg/ml, molecular F/P ratio 2·3, dilution used 1:40, lot No. KH 15-18-F01; anti-IgA: protein concentration 3·7 mg/ml, molecular F/P ratio 2·5, dilution used 1:40, lot No. KH 14-15-F01).

Determination of antibodies to reticulin and gluten. RAb were determined according to the technique described by Rizzetto & Doniach (1973) with 4 μm thick acetone-fixed frozen sections of rat renal tissue and rat gastric mucosa as substrates. Sera were considered to be positive when the R_1 or R_2 'reticulin' pattern was observed on these two substrates (Rizzetto & Doniach, 1973). For the R_1-staining type, there is a staining of peritublar connective tissue and of fibres around the Bowman's capsule (renal tissue) and a 'honeycomb' appearance on smooth muscle (gastric mucosa); and for the R_2-staining type, there is a staining of long streaks between the gastric glands in rat gastric mucosa. The reticulin specificity was tested by absorption studies, with three distinct preparations: (a) a 'non-collagenous reticulin component' (NCRC), isolated from pig renal cortex according to the technique described by Pras & Glynn (1973); (b) cryostat sections of rat renal tissue; and (c) cryostat sections of rat cartilage.

Five sera known to be reticulin antibody-positive were selected for these absorption studies. The absorption procedure was performed as described previously (Feltkamp & van Rossum, 1968).

GAb were determined with the IFT, performed on sections of wheat grains (Eterman *et al.*, 1977). To that end, cryostat sections were incubated with the serum (1:10 dilution), washed with PBS, incubated with a fluorescent anti-human Ig conjugate from horse, again washed with PBS, and finally mounted in glycerol–PBS (1:1) and read on a fluorescence microscope with incident light and dichroic mirrors.

Ig class typing. For the determination of the Ig class of RAb and GAb, specific rabbit antisera, FITC-conjugated, were used. The fluorescence procedure was performed according to standard conditions.

C fixation. For the demonstration of C-fixing antibodies, a two-step reaction was performed as described previously (Feltkamp & van Rossum, 1968).

Autoantibody determination. Autoantibodies to thyroid, nuclei, skeletal muscle, smooth muscle, adrenal cortex, salivary duct cells, mitochondria and gastric parietal cells were determined according to the method described previously by Feltkamp & van Rossum (1968), slightly modified as described by Lucas *et al.* (1972).

HLA typing. HLA typing was performed with the microcytotoxicity method, antisera being used with which at least thirty-eight HLA-ABC specificities might be detected (Terasaki & McClelland, 1964).

RESULTS

Sera from 109 patients with coeliac disease were studied for RAb and GAb. RAb were found in twenty of these 109 cases (18%) and GAb in fifty cases (45%). Table 1 shows a positive correlation between RAb and GAb ($P<0{\cdot}05$).

TABLE 1. Relation between RAb and GAb in 109 patients with coeliac disease (independent of diet)

	RAb-positive	RAb-negative	Total
GAb-positive	14	36	50
GAb-negative	6	53	59
Total	20	89	109

Table 2 summarizes the results of RAb and GAb determinations in patients and controls and shows that RAb were found in a third of the untreated patients, whereas GAb were found in all untreated children and in half the untreated adults. In treated patients lower numbers were found. In controls the frequencies of the antibodies remained below 5%. All untreated children and nearly 60% of the adults were positive with one or both tests. One can say that 68% of the untreated patients, irrespective of their age, were positive with one or both tests.

TABLE 2. Frequency of antibodies to RAb and GAb in patients with coeliac disease

Patients	Number studied	RAb	GAb	RAb and/or GAb
Children (ND)	6	2 (33)	6 (100)	6 (100)
Children (GFD)	8	0	7 (87)	7 (87)
Controls	14	0	0	0
Adults ND	36	12 (33)	18 (50)	21 (58)
Adults GFD	59	6 (10)	19 (32)	22 (37)
Controls	95	2 (2)	4 (4)	6 (6)

ND, Normal diet; GFD, gluten-free diet; all numbers in parentheses are percentages.

TABLE 3. Frequency of antibodies to RAb and GAb in various diseases

Patients	Mean age	Number studied	RAb	GAb
Untreated coeliacs (adults)	43	36	12 (33)	18 (50)
Dermatitis herpetiformis	51	34	4 (12)	6 (18)
Crohn's disease	39	50	3 (6)	26 (52)
Ulcerative colitis	40	44	3 (7)	8 (18)
Controls (adults)	44	95	2 (2)	4 (4)
Untreated coeliacs (children)	6	6	2 (33)	6 (100)
Cystic fibrosis	6	17	1 (6)	7 (42)
Recurrent diarrhoea	3	45	1 (2)	17 (37)
Controls (children)	6	60	0 (0)	4 (6)

Numbers in parentheses are percentages.

TABLE 4. RAb and GAb in forty-five children with 'recurrent diarrhoea'

Diagnosis	Number studied	RAb-positive	GAb-positive
Coeliac disease	2	1	2
Disaccharidase deficiency	5	0	2
Gastroenteritis	4	0	2
Ulcerative colitis	2	0	2
Gardia lamblia	1	0	1
'Wiscott–Aldrich' syndrome	1	0	1
Immune deficiency	1	0	0
Lactase deficiency	1	0	0
'Irritable colon' syndrome	2	0	1
Intractable diarrhoea	1	0	1
Unknown	25	0	5
Total	45	1	17

The results of RAb and GAb determination in patients with dermatitis herpetiformis, Crohn's disease, ulcerative colitis, cystic fibrosis and recurrent diarrhoea due to miscellaneous disorders are listed in Table 3. The diagnoses of the latter group are specified in Table 4. RAb were only found in low percentages of these diseases. The patients with cystic fibrosis, and another with recurrent diarrhoea, who were RAb-positive later on proved to suffer from coeliac disease as well.

High frequencies of GAb were found in patients with Crohn's disease, cystic fibrosis and recurrent diarrhoea.

Immunoglobulin class of RAb and GAb

The sera from fourteen adult patients (five on a GFD and nine on a ND) with RAb and/or GAb were studied to determine the G, A and M immunoglobulin classes of the antibodies. Tables 5 and 6 show that RAb were only of the IgG and/or IgA class, whereas GAb belonged to all three classes. In only one patient were no GAb found in the IgG class. RAb and GAb of all immunoglobulin classes were found to be non-complement-fixing.

Immunofluorescence on human small intestinal tissue

When the FITC-labelled anti-reticulin antibodies were used, a fluorescence of the fine 'reticulin' structures in the human small intestine was observed. The fluorescence pattern observed was in complete accordance with the 'Gomori' silver staining of the same tissue. In rat renal tissue these antibodies gave a fine fibrillar staining of the tubuli and the Bowman's capsule; and in rat gastric mucosa fluorescence of the sarcolemma of smooth muscle was observed. With a FITC-labelled IgG fraction of normal serum no fluorescence was seen.

TABLE 5. Ig class of antibodies to reticulin (RAb)

Number studied	Ig class
6 (2 GFD)*	G
4 (2 GFD)	G+A
4 (1 GFD)	A
(Total = 14)	

* On a gluten-free diet.

TABLE 6. Ig class of antibodies to gluten (GAb)

Number studied	Ig class
5 (2 GFD)*	G
1 1 (GFD)	G+A
1	G +M
1	G+A+M
1	A+M
(Total = 9)	

* On a gluten-free diet.

Absorption studies revealed that, in five patients, RAb titres were not affected by absorption with 130 mg NCRC per ml, or with cryostat sections of rat cartilage in the same concentration. RAb were completely absorbed with cryostat sections of rat renal tissue in a final concentration of 130 mg/ml.

Autoantibody determination

The sera from seventy-six adult coeliac patients and twenty-five patients with dermatitis herpetiformis were screened for the presence of antibodies to smooth muscle, skeletal muscle, thyroid, gastric parietal cells, adrenal cortex, salivary duct cells, nuclei and mitochondria.

In the coeliac patients only antibodies to smooth muscle were found in a frequency (four out of seventy-four = 5%) significantly different from that found in matched controls (none out of 225 = 0%) ($P<0{\cdot}01$). No relationship was found with liver disease in the positive patients. In the patients with dermatitis herpetiformis, antibodies to gastric parietal cells were found in a significantly higher frequency (six out of twenty-five = 24%) than in matched controls (seven out of 148 = 5%).

HLA typing

Forty-eight coeliacs were screened for HLA determinants. Forty-one of these were HLA-B8-positive (85%). This is in accordance with the findings of Stokes *et al.* (1972), of 88% HLA-B8 positives. When HLA-B8-positive and HLA-B8-negative patients were compared, the frequencies of antibodies to gluten or reticulin did not differ significantly.

DISCUSSION

RAb and GAb were determined in sera from children and adults with coeliac disease, dermatitis herpetiformis, Crohn's disease, ulcerative colitis, cystic fibrosis and recurrent diarrhoea. The prevalence of RAb in the coeliac patients of the present study was lower than that reported in literature (Table 7), especially in the children. This might, however, be due to differences in patient population. The lower

TABLE 7. Incidence of RAb in coeliac disease and controls

	Coeliac adults			Normal adults		Coeliac children			Normal children	
Study	Number studied	Normal diet (%)	Gluten-free diet (%)	Number studied	Percentage positive	Number studied	Normal diet (%)	Gluten-free diet (%)	Number studied	Percentage positive
Alp Wright (1971)	50	57	42	68	4	71	76	44	43	0
Seah *et al.* (1973)	101	60	16	n.d.	n.d.	46	85	29	n.d.	n.d.
Stevens *et al.* (1975)	49	74	0	n.d.	n.d.	20	93	0	n.d.	n.d.
Present study	95	33	10	95	2	14	33	0	14	0

n.d. = Not done.

frequency of RAb in treated patients compared with untreated patients is in accordance with data obtained in other studies (Table 7). In dermatitis herpetiformis and Crohn's disease low numbers of RAb were found compared with those previously reported by Seah *et al.* (1973), who found 22 and 24% respectively. The results in the case of ulcerative colitis are in agreement with the findings of these investigators. The RAb-positive patient with cystic fibrosis and the one with 'recurrent diarrhoea' both turned out to suffer from coeliac disease.

The percentage of control subjects positive for RAb corresponds with the percentage found by Alp & Wright (1971). The finding that RAb were only of the IgG and IgA classes is in accordance with results of other investigators (Magalhaes *et al.*, 1974; Seah *et al.*, 1971a).

With the indirect immunofluorescence test on wheat sections, GAb were found in a large proportion of patients with coeliac disease, which is in accordance with the frequencies found by other smaller studies using haemagglutination, complement fixation and precipitation techniques (Alarcón-Segovia *et al.*, 1964; Ferguson & Carswell, 1972; Taylor, Truelove & Wright, 1964; Berger, Bürgin-Wolff & Freudenberg, 1964; Pokorná, Sourek & Svejcar, 1965). No results of normal control sera were mentioned by some of these authors (Alarcón-Segovia *et al.*, 1964; Ferguson & Carswell, 1972); and in some of these studies only results on children were described (Ferguson & Carswell, 1972; Pokorná *et al.*, 1965). In two studies, in which the passive haemagglutination method was used, high frequencies (69 to 88%) of GAb were found in coeliac patients. However, the results with respect to healthy individuals were not mentioned in one study; and in the other study, frequencies of 30 and 50% were reported in healthy adults and children respectively. GAb tend to disappear in patients tested (Carswell & Ferguson, 1973). However, the study by Alarcón-Segovia (1964) is the only one in which the patients were divided into ND and GFD groups. Both in their study and in the present one the GAb frequency in treated patients was lower than in patients on a ND. In our study this difference was not statistically significant, so that it might be suggested that the relatively high frequency of GAb in treated patients was due to insufficient elimination of gluten from the diet, as the disappearance of these antibodies in adequately treated patients is reported to occur within 2 months after the introduction of a GFD (Carswell & Ferguson, 1973). However, the technique (microgel diffusion) used to determine GAb in these last studies was different from the technique used in our study. The indirect immunofluorescence technique we used might be a more sensitive method to demonstrate GAb in serum, so that minute amounts of gluten present in the diet may be detected by this test. In the patients with Crohn's disease a very high frequency of GAb was found. This is in discordance with the results of Taylor *et al.* (1964), who did not find an increased frequency of antibodies to fraction III in this disease. However, Falchuk & Isselbacher (1976) found a higher number of antibodies to BSA in patients with Crohn's disease, which suggests that an increased absorption of antigenic material and stimulation of antibody production may occur in association with Crohn's disease. Furthermore, a relationship was found between the ingestion of cornflakes and wheat and the incidence of Crohn's disease (James, 1977).

In cystic fibrosis, seven out of seventeen patients (42%) were GAb-positive. Two of these were later found to be coeliacs as well. The coexistence of cystic fibrosis and coeliac disease has occasionally been reported (Goodchild *et al.*, 1973; Taylor & Sokol, 1973), and it has been suggested that an abnormal pancreatic exocrine function favours gluten intolerance (Taylor & Sokol, 1973). The results of GAb determination in patients with recurrent diarrhoea suggest that GAb can be produced secondarily to an aspecific gut lesion, and as such are non-specific. GAb were found in low frequencies in the healthy controls. It may be concluded that antibodies to gluten are sensitive markers for gastrointestinal diseases but not specific for gluten enteropathy. Antibodies to reticulin, on the other hand, are less sensitive but of far greater specificity for coeliac disease.

The finding of a significantly increased frequency of antibodies to smooth muscle in coeliac disease is in discordance with results of Seah *et al.* (1971b), who found normal values for these antibodies but an increased frequency of antibodies to gastric parietal cells. The other autoantibodies tested occurred in a frequency not significantly different from that established in healthy controls. As far as antibodies to nuclei, parietal cells, mitochondria and thyroid are concerned, our results are in accord with those obtained in previous studies (Seah *et al.*, 1971b; Williamson *et al.*, 1976). So far the prevalence of anti-

bodies to skeletal muscle and salivary duct cells has not been studied by others. In patients suffering from dermatitis herpetiformis, a significantly higher number than in controls of autoantibodies to gastric parietal cells was found. This is in agreement with studies by O'Donoghue *et al.* (1976), who found these antibodies in 15% of their patients. We could not confirm the high frequency (34%) of antibodies to nuclei found by Seah *et al.* (1971a). The other autoantibodies were found in normal frequencies in patients with dermatitis herpetiformis. The positive 'reticulin' fluorescence on the human small intestine observed with fluorescent reticulin antibodies gives further support for the autoimmune character of these antibodies, although final evidence for this hypothesis should be provided by testing the patients' tissues.

Many autoimmune diseases are characterized by increased frequencies of the HLA-B8 antigen. In myasthenia gravis it was proved that HLA-B8 occurred especially in the patients without autoantibodies to skeletal muscle (Feltkamp *et al.*, 1974). We therefore studied whether such a discrepancy was also present in relation to antibodies to gluten or reticulin. No significant positive or negative correlation with the prevalence of these antibodies was demonstrated.

An advantage of the IFT on wheat grain sections is that the immunoglobulin classes of GAb can be determined. GAb of all three main immunoglobulin classes were found. This is interesting because it has been reported that the intestinal mucosa of patients with coeliac disease responds to gluten challenge *in vivo* with a striking increase in IgA and IgM synthesis, which is largely due to the synthesis of anti-gliadin antibodies (Falchuk & Strober, 1974). Moreover, a higher number of jejunal mucosal cells bearing immunoglobulins of one of the three major classes occurred in children on gluten-containing diets, whereas the extent of the increase of IgA- and IgM-bearing cells seemed to correlate with the severity of the mucosal lesion (Savilahti, 1972; Lankaster-Smith *et al.*, 1976). This raises the question whether the proliferation of these cells is also due to the production of anti-gliadin antibodies. Studies comparing the number of Ig-bearing cells in the mucosa and the immunoglobulin classes of GAb produced in coeliac disease might give further insights into the pathogenesis of the mucosal lesions in these patients.

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