Raised number of jejunal IgG2-producing cells in untreated adult coeliac disease compared with food allergy

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SUMMARY The subclass distribution of IgG-producing immunocytes was studied by two colour immunohistochemistry with monoclonal antibodies in jejunal biopsy specimens from 10 adults with untreated coeliac disease, 11 coeliac disease patients on a gluten free diet, and seven patients with established food allergy. Paired immunofluorescence staining was performed with subclass specific murine monoclonal antibodies in combination with polyclonal rabbit antibody reagent to total IgG; the proportion of cells belonging to each subclass could thereby be determined. The ratio of IgG2 immunocytes was significantly higher ($p < 0.05$) in untreated coeliac disease patients (median, 35.2%; range, 26.7-65.2%) than in those on a gluten free diet (median, 7.3%; range, 0-31.9%) or those having food allergy (median, 12.5%; range, 0-36.5%). The disparity in the local IgG2 response between patients with untreated coeliac disease and those with food allergy might be due to differences in the nature of the antigenic stimuli, dissimilar genetic 'make-up' of the subjects, or both.

Previous studies in our laboratory have indicated altered local homeostasis of the mucosal immune system in several intestinal disorders.¹⁻⁵ In these conditions luminal antigens or mitogens apparently penetrate the mucosa and induce overstimulation of the local B-cell system; altered homeostasis is reflected by a raised proportion of mucosal IgG-producing immunocytes.¹⁻³ Furthermore, in coeliac disease there are increased levels of IgA and IgG antibodies to various dietary antigens in serum.⁶

It was recently reported that the local IgG- and IgA-subclass response is different in ulcerative colitis and Crohn's disease of the colon.^{4,5,7,8} Such disparity could signify an interplay between genetic and

immunological factors and might be of great interest for the understanding of disease processes.

A recent study in the United States⁹ suggested that persistently raised serum antibody concentrations to gliadin in adult coeliac disease after treatment with a gluten free diet (GFD), occurred only in patients with the chromosome 14-encoded IgG2 heavy-chain allotype marker G2m(n). This observation could not be confirmed by others, however.¹⁰ In addition to such genetically determined variation, it has been shown that various viral and bacterial antigens or mitogens may stimulate B cells to produce different IgG subclasses both *in vitro*¹¹ and *in vivo*.¹²

The purpose of the present study was to determine the IgG-subclass distribution of mucosal immunocytes in untreated coeliac disease compared with that found in treated coeliac disease patients and in patients suffering from food allergy. The distribution of the IgG heavy-chain marker (Gm) in the patients

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and in a control group of healthy blood donors was also determined.

Methods

PATIENTS AND TISSUE SPECIMENS

Twenty eight subjects with adverse reactions to food were studied. They included 10 patients with coeliac disease who were untreated and 11 coeliac disease patients on gluten free diet. The coeliac disease diagnosis was based on clinical investigation, intestinal biopsy¹³ (Table 1), and levels of serum antibodies to gluten^{6,14} (Table 2). All coeliac disease patients responded to gluten restriction, and follow up examinations did not disclose other conditions associated with villous atrophy.

Seven patients with abdominal complaints who had a positive history of food allergy (Table 3) and a sufficient number of IgG-producing immunocytes for differential subclass-counting in the biopsy specimens were also included. Five of the latter had positive skin prick test for relevant foods and five had IgE positive mucosal mast cells (Table 3) detected by immunofluorescence staining of tissue sections.^{15,16}

Table 1 Clinicopathological information about the patients with coeliac disease

Untreated patients	Age (yr)	Sex	Time on GFD* (yr)	Histological characteristics†	Clinical symptoms‡
1§	25	F	–	II	1
2	33	F	–	IV	2
3	52	F	–	IV	3
4	27	F	–	IV	2
5	31	M	–	IV	2
6	59	F	–	IV	3
7	39	F	–	IV	2
8	73	F	–	IV	2
9	81	F	–	IV	3
10	19	F	–	IV	2
Patients on gluten free diet					
11	47	M	2.5	I	1
12	63	F	0.5	I	1
13	39	F	0.75	I	1
14	58	F	0.25	II	2
15	35	F	3	I	1
16	21	F	18	I	1
17	41	F	1	I	1
18	39	M	14	I	1
19	40	M	0.5	III	1
20	62	F	5	II	1
21	46	F	0.5	II	2

*GFD, gluten free diet; †Histological characteristics: I, normal; II, partial villous atrophy; III, subtotal villous atrophy; IV, total villous atrophy; ‡Clinical symptoms: 1, no symptoms 2, moderate symptoms 3, severe symptoms; §This patient had ingested mainly fruit and vegetables before biopsy.

Table 2 Concentrations of serum IgG and IgA antibodies to gluten determined by ELISA, mucosal IgG and IgG2 immunocyte density, and G2m(n) type of various patient categories

Untreated coeliac disease	ELISA values (od at 405 nm)		Mucosal immunocytes number/grid area*		G2m(n) positivity
	IgG	IgA	IgG	IgG2	
1	1.15	1.55†	29	9	+
2	0.68	1.50	39	14	+
3	nd	nd‡	30	8	+
4	1.67	1.58	54	35	–
5	0.95	1.17	19	6	+
6	1.45	1.40	28	10	+
7	0.80	0.17	36	12	–
8	0.89	0.98	24	6	–
9	1.62	2.55	18	5	+
10	2.19	1.28	20	8	+
Treated coeliac disease					
11	0.32	0.11	15	5	–
12	nd	nd	8	1	nd
13	0.70	0.13	9	3	+
14	1.15	0.14	36	1	+
15	0.12	0.27	23	1	+
16	0.59	0.04	16	3	+
17	0.21	0.00	3	1	+
18	0.11	0.02	7	0	nd
19	0.16	0.05	26	3	+
20	1.21	3.48	24	2	nd
21	1.02	0.24	16	1	–
Food allergy					
22	0.62	0.00	9	1	+
23	0.46	0.00	40	0	–
24	1.43	0.01	11	2	+
25	1.38	0.08	7	1	–
26	0.14	0.15	6	1	+
27	1.31	0.53§	17	6	+
28	0.62	0.33	6	1	+

*Each grid area represent 0.32 mm² lamina propria; †Pathologically raised levels (14) are underlined; ‡nd, not determined; §This patient had positive RAST for wheat.

Table 3 Clinicopathological information about the food allergy patients

Patients	Age (yr)	Sex	Symptoms	Positive history	Skin prick test to relevant foods	IgE positive mucosal mast cells
22	21	F	Diarrhoea	egg	–	+
23	39	F	Diarrhoea	egg, milk, hazelnut	+	+
24	16	F	Asthma	milk, chocolate	–	–
25	39	M	Diarrhoea	milk	+	+
26	31	F	Diarrhoea	egg	+	–
27	55	F	Diarrhoea	Quincke's oedema	+	+
28	56	M	Vomiting	egg, hazelnut, shellfish	+	+

IMMUNOHISTOCHEMISTRY

Small mucosal specimens were extracted in cold phosphate buffered (pH 7.6) isotonic saline for 48 h before ethanol fixation and paraffin embedding.¹⁷ The prewashing procedure was crucial to avoid interstitial background staining caused by extracellular IgG.

Five serial sections were cut at 6 µm from each tissue block. One section was stained with haematoxylin and eosin for histological evaluation, whereas each of the remaining was subjected to paired immunofluorescence staining for one of the four IgG subclasses and for total IgG. These sections were first incubated with murine monoclonal antibody (ascites, 1:800) to IgG1 (Clone 2C7), IgG2 (Clone GOM2), IgG3 (Clone CB1-AH7), or IgG4 (Clone R74) and subsequently with a mixture of fluorescein isothiocyanate labelled rabbit antimouse IgG and rhodamine B sulphonyl chloride labelled antihuman IgG. The sources of monoclonal antibodies,¹⁸ the characteristics of the fluorochrome conjugates,¹⁹ and other details of this two-colour staining procedure⁴ have been reported previously.

MICROSCOPY AND CELL COUNTING

Enumeration of IgG subclass producing cells

Immunocytes with distinctly cytoplasmic fluorescence were counted in a Leitz Orthoplan microscope equipped with a ×10 immersion objective, a ×10 ocular, and a Ploem-type vertical illuminator with interference filters for selective observation of fluorescein (green) or rhodamine (red) emission colours. The cells were counted within the outer frame of a square ocular grid (Leitz code no 519902), representing an area of 0.32 mm². The median evaluated number of grids in each specimen was five, (range, 1–10 grids), and the median counted number of cells with cytoplasmic red fluorescence was 140 (range 100–1000 cells). In each section these red cells represented the internal reference for the total number of IgG-producing immunocytes, and they were all examined for concomitant green (subclass) fluorescence. The proportion of immunocytes positive for one of the four subclasses was calculated

Fig. 1 Scatter diagram of the percentage distribution of IgG1-, IgG2-, IgG3- and IgG4-producing cells in duodenal mucosa of three patient categories: untreated coeliac disease (CD), CD patient on gluten free diet (GFD), and patients with food allergy.

in relation to the total number of IgG-producing cells present in the evaluated area of the actual section. In a previous study the intraobserver and interobserver reproducibility of such cell enumerations showed good correlation ($r=0.96$ and 0.89 , respectively) and no systematic error.⁴

Comparison of monoclonal subclass specific antibodies and polyclonal anti-IgG

Tissue sections from two of the patients were first incubated with a mixture of the four monoclonal antibodies (each at a final dilution of 1:800) and then with the mixed fluorochrome conjugates as described above. Red and green cells were counted in altogether 23 square grids (15 from one section and eight from the other). To avoid bias, these enumerations were performed without checking for double staining.

SERUM ANTIBODIES TO GLUTEN

Serum IgG and IgA activities to gluten proteins were measured by an ELISA method.^{6,20}

Figures 2(a-d) Paired immunofluorescence staining for IgG subclasses in four serial sections of duodenal mucosa from a patient with untreated coeliac disease. Red fluorescence indicates total IgG-producing cells and green indicates (a) IgG1, (b) IgG2, (c) IgG3, or (d) IgG4. In these double exposed pictures the respective subclass-producing cells are identified by mixed (yellow) colour. Note that purely green cells are extremely rare (arrow). (e) Paired immunofluorescence staining with polyclonal anti-IgG conjugate (red) and mixture of monoclonal antibodies (green) to the four IgG-subclasses. In this double exposed picture yellow colour indicates true identification of subclass producing cells. (f) Relation between results of cell enumerations performed in control experiment depicted in Figure e. The number of fluorescein-positive (green) cells per grid unit represented all those reacting with the four monoclonal antibodies to IgG subclasses, whereas the number of rhodamine positive (red) cells per unit represented those reacting with the polyclonal anti-IgG conjugate ($r=0.996$). The identity line is indicated.

IgG HEAVY CHAIN MARKERS

Gm typing was performed by haemagglutination inhibition as described elsewhere²¹ in the patients studied and in 50 healthy blood donors.

STATISTICAL ANALYSIS

Correlation analyses were performed by the Pearson *r*-test. Differences between median proportions of IgG subclass producing cells and serum antibody

activities among the three patients' groups were determined by Wilcoxon's two-tailed test for unpaired samples. Possible differences in Gm marker frequencies were estimated by the χ^2 test.

Results

SUBCLASS DISTRIBUTION OF IgG-PRODUCING CELLS

The median proportion of IgG2-producing cells in untreated coeliac disease patients (35.2%) was significantly higher ($p < 0.05$) than in coeliac disease patients on a gluten free diet (7.3%) or patients with food allergy (12.5%) (Figs 1 and 2a-d). There were no statistically significant differences between the medians of the other IgG-subclasses in the three patient categories.

The sum of the four IgG subclass percentages in serial sections from each biopsy specimen was usually close to 100% (median, 99.9%; range, 90.4%–113.5%). These results attested to the reliability of the subclass determinations.

COMPARISON OF MONOCLONAL SUBCLASS SPECIFIC ANTIBODIES AND POLYCLONAL ANTI-IgG

In this control experiment virtually all positive immunocytes showed both red and green fluorescence (Fig. 2e). The coefficient of correlation (r) was 0.996 (Fig. 2f). Purely green or red cells were extremely rare. This result showed that the paired staining procedure used for individual IgG subclasses was highly reliable.

LOCAL IgG RESPONSE, SERUM ANTIBODIES TO GLUTEN, AND Gm TYPING

Untreated coeliac disease patients had significantly ($p < 0.05$) increased serum IgG or IgA ELISA activities to gluten compared with both treated coeliac disease patients and patients with food allergy (Table 2). A positive correlation ($r = 0.45$, $p = 0.05$) was found between the density of jejunal IgG2 subclass producing cells and the serum concentrations of IgG gluten antibodies in treated and untreated coeliac disease patients. No significant correlation was found between the density of total IgG cells and the serum levels of gluten IgG antibodies (Table 2).

The frequency of the Gm marker n was 72% in the coeliac disease patients tested (six treated and seven untreated), 71% in the patients with food allergy, and 70% in the healthy blood donors. Thus, these frequencies and also those of the other IgG heavy chain allotype markers (data not shown), were not different.

Discussion

A mucosal IgG response has previously been suggested to be of pathogenic importance in coeliac disease,^{1,2} and it was of interest to study the subclass pattern of this response. Here we report that the proportion of duodenal IgG2-producing cells was found to be significantly increased in untreated coeliac disease patients compared with treated ones and with patients who had food allergy.

Because of the scarcity of IgG immunocytes in healthy gut mucosa,¹ we were unable to evaluate the subclass pattern in normal control specimens.

Our results indicated that the mucosal immune response in coeliac disease includes – in addition to IgA, IgM and IgG, in that order¹ a preferential local expansion of the IgG2 cell population. The proportion of mucosal IgG2 cells found in untreated coeliac disease was similar to that seen in Crohn's disease of the colon.⁴ Conversely, in ulcerative colitis we observed a disproportionate expansion of the IgG1 cell population.⁴ In normal nasal mucosa we often found a preference of IgG3- over IgG2-producing cells,²² thus contrasting that seen in inflamed intestinal mucosa – probably because of differences in the nature of antigenic stimulation at the two mucosal sites.

The relatively prominent jejunal IgG2 response in untreated coeliac disease may hence be directly related to gluten ingestion. A possible role of IgG2 in the pathogenesis of coeliac disease, however, remains obscure. As IgG2 has relatively little complement activating capacity²³ and shows poor Fc receptor binding,²⁴ it is unlikely that this subclass is important in the perpetuation of coeliac disease as we previously suggested for IgG antibodies on the basis of our observations of the total mucosal IgG response.^{1,2} IgG2 may, instead, be involved in local protective mechanisms in concert with the stimulated secretory IgA and IgM system.^{1,2}

The present finding of a prominent mucosal IgG2 response is of interest in light of the conflicting results reported on the IgG subclass distribution of gliadin antibodies in serum. Such antibodies have been described in all of the IgG subclasses, but mainly in the IgG1 subclass.²⁵⁻²⁷

The relatively weak but significant correlation between local IgG2 cell response and serum concentrations of IgG antibodies to gluten seen in our study, suggested a possible contribution of serum IgG from mucosal production. Regional lymph nodes seeded by gluten stimulated mucosal immunocytes, however, may be an even more important source of such antibodies. We are currently evaluating the serum IgG subclass response to gluten by ELISA.

The raised proportion of mucosal IgG2 producing

cells observed in untreated coeliac disease may signify an interplay between genetic and environmental factors. Association between immunoglobulin genetic markers and levels of antibody production has been reported.³⁸ Thus, in American caucasian adult coeliac disease patients the IgG2 allotype marker G2m(n) seems to be associated with a persistent IgG response to gluten in treated coeliac disease patients.⁹ Conversely, neither our study nor that of Finnish coeliac disease children¹⁰ has been able to confirm that this marker is associated with persistently raised serum concentrations of IgG gluten antibodies after introduction of gluten free diet. Moreover, we found no difference in the G2m(n) distribution between coeliac disease patients on gluten free diet with a relative high or a low mucosal IgG2 response. Our study supports previous reports^{7,10} of a high and equal frequency of G2m(n) in coeliac disease patients and controls.

Little is known about the biological significance of the various IgG subclasses, and further studies are clearly necessary to elucidate the role of the IgG subclass response in coeliac disease. Longitudinal studies of the same patients will be of great interest and should include both the local IgG response pattern and monitoring of serum concentrations of dietary antibodies.

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