Dissociation between systemic and mucosal humoral immune responses in coeliac disease

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Abstract

We examined humoral immunity in coeliac disease as expressed in serum (systemic immunity), and in saliva, jejunal aspirate, and whole gut lavage fluid (mucosal immunity). The aims were to define features of the secretory immune response (IgA and IgM concentrations and antibody values to gliadin and other food proteins measured by enzyme linked immunosorbent assay (ELISA)) in active disease and remission, and to establish whether secretions obtained by relatively noninvasive techniques (saliva and gut lavage fluid) can be used for indirect measurements of events in the jejunum. Serum, saliva, and jejunal aspirate from 26 adults with untreated coeliac disease, 22 treated patients, and 28 immunologically normal control subjects were studied, together with intestinal secretions obtained by gut lavage from 15 untreated and 19 treated patients with coeliac disease and 25 control subjects. Jejunal aspirate IgA and IgM and gut lavage fluid IgM concentrations were significantly raised in patients with untreated coeliac disease; the lavage fluid IgM concentration remained higher in patients with treated coeliac disease than in controls. Serum and salivary immunoglobulin concentrations were similar in the three groups. Patients with untreated coeliac disease had higher values of antibodies to gliadin compared with treated patients and control subjects in all body fluids tested; these were predominantly of IgA and IgG classes in serum, and of IgA and IgM classes in jejunal aspirate and gut lavage fluid. Values of salivary IgA antibodies to gliadin were significantly higher in untreated coeliacs, though antibody values were generally low, with a large overlap between coeliac disease patients and control subjects. In treated patients, with proved histological recovery on gluten free diet, serum IgA anti- gliadin antibody values fell to control values, though serum IgG antibody values remained moderately raised. In contrast, there was persistence of secretory anti- gliadin antibody in treated patients (particularly IgM antibody) in both jejunal aspirate and gut lavage fluid. Antibody responses to betalactoglobulin and ovalbumin were similar to those for gliadin, including persistence of high intestinal antibody values in patients with treated coeliac disease. There was a positive correlation between antibody values in jejunal aspirate and gut lavage fluid, but not between saliva and jejunal aspirate; thus salivary antibodies do not reflect intestinal humoral immunity.

Numerous studies have established that patients with untreated coeliac disease have high values of circulating antibodies to wheat derived proteins such as gliadin, and that antibody values fall after a period of treatment with a gluten free diet. Estimation of serum IgA anti-gliadin antibody is now routinely used both as a screening test for coeliac disease and as a means of assessing dietary compliance.

In contrast, information on mucosal immunity in coeliac disease is patchy. There have been many studies of mucosal lymphoid cells and there is circumstantial evidence of a local cell mediated immune response to gluten. Several studies have carefully mapped the numbers of Ig producing plasma cells in the jejunal mucosa of patients with untreated and treated coeliac disease, showing that untreated patients have increased numbers of IgA and IgM (and to a lesser extent, IgG) jejunal plasma cells. In the 1970s, the presence of intestinal antibodies to food antigens was recognised by a relatively insensitive precipitin technique, but there are only two studies published, both in children, on the isotype of antibodies to dietary antigens in intestinal secretions, and these give conflicting results.

The general objectives of this study were twofold. In relation to coeliac disease, our aim was to characterise, in vivo, intestinal humoral immunity. Total immunoglobulins and specific antibodies to gliadin and to two antigens which are not toxic in coeliacs were measured in three different mucosal secretions. Untreated and treated patients with coeliac disease were studied to determine whether abnormalities of secretory immunity are permanent and intrinsic to the coeliac diathesis or are only present in active disease. Separately, and of relevance to the clinical investigation of mucosal immunity, we studied the relations between systemic and intestinal antibodies, and we examined our data to establish whether patterns of immunoglobulins and antibodies in jejunal fluid are mirrored in other secretions which can be obtained without intubation. The salivary glands are considered part of the common mucosal immune system, and we therefore studied pure parotid saliva. We also used a whole gut lavage technique for the non-invasive collection of intestinal secretions.

Methods

PATIENTS STUDIED AT THE TIME OF JEJUNAL BIOPSY

Specimens of saliva, jejunal aspirate, and serum were collected at the same time as jejunal biopsy on 76 occasions in 69 patients. There were 41 patients with coeliac disease (seven studied twice), 23 women and 18 men; median age 42 years, range 15–78 and 28 control patients (14
women, 14 men, median age 35 years, range 14–75). Control subjects had jejunal biopsy to exclude coeliac disease – jejunal histology in these patients was normal, no other significant pathology was found, and a final diagnosis of functional bowel disease was made. Twenty six of the patients with coeliac disease were untreated and histological examination of the jejunal biopsy specimens showed subtotal or severe partial villous atrophy. Seven of these and a further 15 patients with treated coeliac disease (all with previous diagnostic biopsy specimens) underwent biopsy again while on a gluten free diet. The median period on gluten free diet was three years (range 3 months – 17 years). Eleven had entirely normal jejunal histology (all of these had been taking a gluten free diet for at least two years), and 11 had minor histological changes – for example increased intraepithelial lymphocytes (most patients in this group had been taking a gluten free diet for less than one year).

PATIENTS STUDIED BY WHOLE GUT LAVAGE

Gut lavage was carried out in 15 untreated coeliac disease patients, 19 with treated disease, and 25 control patients. These included 10, eight, and two patients respectively from each group who had also had collection of jejunal aspirate. The median period on a gluten free diet in patients with treated coeliac disease undergoing gut lavage was eight years (range 3 months – 19 years). Eleven of the patients on a gluten free diet had in the past shown a clinical and histological response to the diet, but did not undergo biopsy again at the time of this study. Gut lavage was carried out in 25 control patients (16 women and nine men, median age 52, range 21 – 92 years). These subjects were either healthy volunteers or patients with functional bowel disorder.

SPECIMEN COLLECTION AND PROCESSING

Saliva: parotid salivary flow was stimulated with 5% citric acid sublingually in four 0.5 ml aliquots over five minutes, and collected via a Carlsson-Crittenden cup placed over the parotid duct, with gentle aspiration to maintain position and suction. We collected stimulated saliva only.

Jejunal aspirate: samples were collected from a point just distal to the duodenal–jejunal junction, through the tubing of the Crosby capsule, before taking the biopsy specimen. The protease inhibitor phenylmethyl sulphonfluoride (PMSF, Sigma) 100 mM in 95% alcohol (20 μl per ml of aspirate) was added before aliquoting. Serum was obtained from all patients.

Gut lavage: the lavage fluid used was a polyethylene glycol (PEG) based electrolyte lavage solution (Golytely). After an overnight fast, patients drank this solution at a rate of 250 ml every 15 minutes for a period of four hours, making the total volume consumed four litres. Specimen collection began once the material passed per rectum became liquid, clear, and free of faecal material. Approximately 200 ml was collected and filtered into 50 ml polypropylene tubes; specimens were centrifuged and treated with protease inhibitors as described by Gaspari et al.[15]

All the above specimens were aliquoted and stored at –70°C.

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Reference materials and reporting of results

For assays of immunoglobulins in the various secretions, serial twofold dilutions of a standard preparation were used to produce a standard curve. For example, for IgA assays dilutions ranging from 1250–19.35 ng/ml of a human colostal IgA standard (Sigma) were used in each test run. Serial dilutions of test samples (varying in initial dilution depending on the type of specimen) were also assayed. Only when the optical density results of at least two of these sample dilutions fell within the range of the standard curve was the assay considered technically satisfactory. The IgA content of the sample was then determined by taking the mean IgA content of these two sample dilutions. For total IgM and IgG in secretions human reference serum (Protein Reference Unit, Sheffield) was used as a standard.

In the assays of specific antibodies, experiments were carried out to define optimal test conditions for each antigen, isotype, and secretion. Serum from a patient with untreated coeliac disease, previously recognised as having high titres of antibodies of all isotypes to a wide variety of dietary antigens, was used as a reference standard. The reference specimen and test specimens were studied at suitable dilutions, varying for the different assays, and the plates were read when the optical density for the standard reached 1.0. Results for test specimens are expressed as optical density readings, % of this standard. Results are thus expressed as non-parametric data; antibody values are not directly proportional to the antigen binding capacity of the sample. This is a feature of all such assays.

Immunoglobulins (jejunal aspirate, gut lavage fluid, and saliva)

Assays were performed in 96 well microtitre ELISA plates (Dynatech). All reagents were added in volumes of 0.125 ml per well and all washes were done three times using saline with 0.05% Tween-80 added. For the assay of total IgA, wells were coated with 100 ng/ml affinity purified goat antihuman IgA (Northeast Labs) in 0.1 M carbonate buffer, pH 9.6, and incubated overnight at 4°C and washed. After washing, serial twofold dilutions of standards and samples (initial sample dilution 1/100) were added to the coated wells. Plates were incubated overnight at 4°C and washed. Goat antihuman IgA conjugated with alkaline phosphatase (Northeast Labs) diluted (in saline with 1% fetal calf serum and 0.05% Tween-80) to a predetermined optimal value was added and plates were incubated for three hours at 20°C. After washing, paranitrophenylphosphate (PNPP, Sigma) 1 mg/ml in 10% diethanolamine (DEA) buffer, pH 9.8, was added. Plates were read at optical density 405 in an MR580 microELISA reader (Dynatech). The IgA content of any given sample was determined as described above.
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The method used to determine total IgM and IgG in secretions was similar; initial sample dilution was 1:25.

Serum immunoglobulins were measured by autoanalyser using an immunoturbidimetric method.

**Food antibodies**

The assay was similar to that described above. Immunol 2 (129B) ELISA plates (Dynatech) were used. Wells were coated with antigen (gliadin, betalactoglobulin and ovalbumin) at concentration of 5 μg/ml. Betalactoglobulin and ovalbumin were supplied by Sigma; gliadin was supplied by Dr Stefan Strobel. Reference standard and samples were added in duplicate dilutions to the coated wells. The following sample dilutions were used: serum: 1/100 (IgA and IgM) and 1/200 (IgG); jejunal aspirate: 1/10; gut lavage fluid: 1/2; saliva: 1/2. An appropriate dilution of standard was included in each assay; these dilutions gave optimal optical density readings.

We established that these antibodies were specific by incubating samples with the relevant antigen and showing that the antibody was specifically absorbed out. In a study of 20 samples, the within plate optical density coefficient of variation was 7-3%, and the between plate optical density variation was 11-1%. If the optical densities of the duplicate sample dilutions varied by >15%, the assay was repeated.

**STATISTICAL METHODS**

Differences in antibody values and immunoglobulin content were analysed using the Mann-Whitney U test. For correlations, Spearman’s test was used.

**Results**

**IMMUNOGLOBULIN CONCENTRATIONS (Table I)**

**Serum**

No significant differences were observed.

**Jejunal aspirate**

Untreated coeliac disease patients had significantly higher jejunal aspirate concentrations of IgA, IgM, and IgG compared with controls, and higher IgM compared with patients with treated coeliac disease. Values for treated coeliac disease patients were not significantly different from those of control subjects.

**Gut lavage fluid**

IgM content was significantly higher in both untreated and treated coeliac disease patients than in control subjects.

**Saliva**

No significant differences were observed.

**ANTIBODIES TO FOOD PROTEINS**

**Serum (Table II)**

Untreated coeliac disease patients had high values of serum IgA anti-gliadin antibody, with values for treated coeliac disease patients similar to control values. High values of serum IgG anti-gliadin antibody were found in both untreated and treated coeliac disease patients, with significantly higher values in the untreated patients. There were no significant differences between patient groups in values of serum IgM antibodies. Patterns of serum antibodies to OVA and betalactoglobulin were generally similar to those for anti-gliadin antibody, as detailed, with statistical information in Table II. Untreated patients had high values for IgA and IgG anti-betalactoglobulin and IgA anti-ovalbumin antibody. Serum IgA anti-betalactoglobulin and IgG anti-ovalbumin antibody values were higher in treated patients than in control subjects.

**Saliva**

No significant differences were observed.

**Jejunal aspirate (Table III)**

There was very little antibody detected in jejunal fluid.
aspirates from control subjects, but as detailed in Table III, for all three isotypes and all three antigens studied, antibody values were significantly higher in jejunal aspirates from untreated patients than from control subjects (p values all <0.02). For antigliadin antibodies, values in treated coeliac patients were intermediate between untreated coeliac disease patients and control subjects and significantly different from both. When antibody values in the 11 treated patients with entirely normal jejunal histology were compared with those in control subjects, IgA antigliadin antibody values were not significantly higher. Conversely, IgM antigliadin antibody values remained significantly raised (p<0.005) in this group. Antibodies to betalactoglobulin and ovalbumin showed a greater overlap between values in coeliac disease patients and control subjects, but again high IgM antibody values persisted in the treated patients.

Jejunal aspirate IgA and IgM antigliadin antibody values are shown in Figure 1.

**Gut lavage fluid (Table IV)**

As in jejunal aspirate, high values of IgA and IgG antibodies to gliadin were found in both untreated and treated coeliac disease patients compared with control subjects, with significantly higher antibody values in the untreated compared with the treated patients. High values of IgA and IgM antibodies to betalactoglobulin and ovalbumin were found in untreated patients; high values of IgA and IgM anti-ovalbumin and IgM anti-betalactoglobulin antibodies persisted in the treated coeliac disease patients.

Gut lavage fluid IgA and IgM antigliadin antibody values are shown in Figure 2.

**Saliva (Table V)**

Salivary antibody values were generally low, with large overlaps between patient groups. Untreated coeliac disease patients had higher values of IgA and IgG antigliadin antibodies compared with control subjects; only IgA antigliadin antibody values were higher than in the treated patients. Higher values of IgA anti-betalactoglobulin antibody were found in both untreated and treated coeliac disease patients compared with control subjects.

**Serial studies in coeliac disease patients**

Seven coeliac disease patients who had a clinical and histological response to a gluten free diet were studied before and after treatment. Serial changes in serum IgA antigliadin antibody and jejunal aspirate IgA and IgM antigliadin antibodies are shown in Figure 3. Whereas serum antibody values fell significantly (p<0.05) with treatment, there was no significant change in the values of intestinal antibody despite histological
Figure 3: Serial changes in values of serum anti-gliadin antibody and jejunal aspirate IgA and IgM anti-gliadin antibodies in 7 coeliac disease patients before and after treatment with gluten free diet. ND= normal diet; GFD = gluten free diet.

Discussion
Intestinal anti-gliadin antibodies in coeliac disease patients were mainly in the IgA and IgM classes, and significant amounts of IgM antibody persisted in the secretions of treated coeliac disease patients with entirely normal jejunal histology. Secreted IgA antibody was detected only in the subgroup of treated coeliac disease patients with minor histological abnormalities, most of whom had had less than a year's treatment with a gluten free diet. It is possible that the intestinal IgA anti-gliadin antibody and minor histological changes could both be due to continued ingestion of small amounts of gluten. Conversely, intestinal IgM antibody values remained higher than in control subjects, even in patients with completely normal jejunal mucosa who had been taking a gluten free diet for some years. It is likely that minute amounts of gluten (complete compliance to a gluten free diet is difficult to achieve in adults) maintains a local immune response rather than a systemic one. Our finding of a persistent IgM antibody response parallels the finding of a relatively high fraction of IgM plasma cells in treated coeliac disease patients.

We found high values of serum IgA and IgG anti-gliadin antibody in untreated coeliac disease patients. In the treated patients, serum IgA anti-gliadin antibody values were similar to those in controls but serum IgG anti-gliadin antibody values, though significantly lower than in the untreated coeliac disease patients, remained significantly higher than in control subjects. These findings agree with those of other reports. 

Concentrations of IgA, IgM, and IgG were all high in jejunal aspirates from untreated coeliac disease patients. Counts of jejunal plasma cells and in vitro immunoglobulin production in coeliac disease are higher than in control subjects for all isotypes, supporting the view that the immunoglobulins (at least IgA and IgM) in coeliac intestinal secretions are produced locally.

<table>
<thead>
<tr>
<th>TABLE VI</th>
<th>Correlation between IgA anti-gliadin antibody values in serum, jejunal aspirate, and saliva</th>
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</thead>
<tbody>
<tr>
<td>Group</td>
<td>Serum and saliva</td>
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<tr>
<td>----------</td>
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</tr>
<tr>
<td>Untreated coeliac disease</td>
<td>r=0.021 (NS)</td>
</tr>
<tr>
<td>(n=26)</td>
<td></td>
</tr>
<tr>
<td>Treated coeliac disease</td>
<td>r=0.392 (NS)</td>
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<tr>
<td>(n=22)</td>
<td></td>
</tr>
<tr>
<td>Control subjects</td>
<td>r=0.593 (p&lt;0.001)</td>
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<td>(n=28)</td>
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NS = not significant.
It is likely that most of the jejunal IgG is plasma derived as the numbers of IgG secreting plasma cells are low even in untreated coeliac disease. We should point out that immunoglobulin measured in jejunal aspirate was total immunoglobulin and not specific secretory IgA and IgM. It is possible that at least some of the jejunal IgG and IgM is serum derived (coeliac disease is a protein losing disorder). We are currently characterising jejunal immunoglobulins and antibody in terms of molecular weight and percentage of total Ig which contains a secretory component. An increase in the intestinal fluid immunoglobulin content was not accompanied by equivalent changes in serum immunoglobulins; in fact, the serum IgM content was noticeably low in some untreated coeliac disease patients.

We have not yet assessed the contribution of specific antigliadin antibody to the increase in intestinal immunoglobulin content in coeliac disease. Falchuk and Strober, using an affinity chromatography technique, reported that approximately half of the net increase in IgA and IgM synthesis (in an in vitro model of gluten challenge) was due to synthesis of antigliadin antibody. Conversely, in a more recent report of in vitro secretion of antigliadin antibody by coeliac jejunal mucosal biopsy specimens, Ciclitira et al calculated that antigliadin antibody accounted for 2-1%, 12-1%, and 4-1% of the total concentrations of IgA, IgM, and IgG respectively.

In any event, enhanced intestinal antibody production in coeliac disease is not limited to gluten derived proteins: we found high values of intestinal antibody to betalactoglobulin and ovalbumin in untreated coeliac disease patients, with persistence of IgM antibody to these proteins in treated patients. Antibodies to these food proteins were less specific to coeliac disease than antigliadin antibodies. It has been suggested that high values of serum antibody to these proteins in untreated coeliac disease is simply the result of increased intestinal permeability to antigens.

Although ELISA is now accepted as the standard assay technique for measuring antibodies to food proteins in coeliac disease, several different ELISA methods have been described, and different reference preparations are used by each group of investigators. The method used is essentially similar to that of Savilahiti et al, and we used crude gliadin as antigen for the ELISA (rather than say, alphagliadin), as Skerritt et al have shown that sera and intestinal aspirates from coeliac patients contain antibodies which bind to several different gliadin subunits. We have not ascribed levels of 'positivity' or 'negativity' to antibody values; such arbitrary designations are of some value in screening tests used in clinical practice but not in prospective research investigations.

Although untreated coeliac disease patients had statistically higher values of salivary IgA and IgG antigliadin antibodies compared with controls, salivary antibodies were generally low with a large overlap between patients and controls. Furthermore, there was no correlation between jejunal aspirate and salivary antibody values. Our results do not suggest that antibody tests on saliva have any diagnostic or screening potential.

On the other hand, gut lavage offers a relatively non-invasive alternative to intubation for the collection of intestinal secretions for immunoglobulin and antibody studies. Antibody findings in gut lavage fluid were broadly similar to those in jejunal aspirate, with a positive correlation for IgA antigliadin antibody values in those patients studied by both techniques. Immunoglobulin concentrations, however, differed considerably in jejunal aspirate and gut lavage fluid; all three immunoglobulin isotypes were raised in jejunal aspirate in the untreated coeliac disease patients, whereas only IgM was significantly raised in gut lavage fluid. Gut lavage fluid is likely to contain secretions from not only the small bowel but also gastric juice, bile, pancreatic secretions, and colonic secretions. In this respect, it is not a homogeneous fluid, unlike jejunal aspirate, which reflects events in the jejunum only, and is thus more likely to represent accurately local immune phenomena in coeliac disease. Find that rate of antigen and antigenic factor that may influence IgG values in exocrine secretions; this may partly account for differences in findings in jejunal aspirate and gut lavage fluid.

The relevance of gluten reactive intestinal B cells and antibodies to the pathogenesis of coeliac disease is uncertain; the presence of antibodies to gluten derived proteins in patients with coeliac disease may be merely an epiphenomenon in the context of a T cell mediated enteropathy with expansion of the relevant populations of T helper as well as effector cells. It is certainly possible that mucosal IgM antibody is immunopathogenic, for example by fixing complement in the immediate vicinity of enterocytes.

This study shows the dissociation of systemic and intestinal humoral immune responses in patients with coeliac disease, and is evidence that for the study of immunopathology of intestinal disease, direct investigation of the gut is mandatory.

Mrs J Johnstone and Mr N Anderson provided technical assistance. This work was funded by grants from the Scottish Hospitals Endowment Research Trust (SHERT), the National Association for Coeliac and Crohn's disease (NACC), the Sandzoe foundation, and Fisons pharmaceuticals. We thank the nursing staff of the GI investigation suite, Western General Hospital.

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