Abstract

To assess whether dietary antigens play a role in inflammatory bowel disease, 25 monozygotic twin pairs and 32 healthy controls were investigated for serum antibodies to ovalbumin and betalactoglobulin in non-twin siblings of patients with ulcerative colitis, Crohn's disease, or both diseases. The response was found in patients with ulcerative colitis and Crohn's disease and the two ulcerative colitis. The results of the study were supported by the findings of the present investigation. The study was approved by the Local Ethical Committee, Orebro Medical Center, Orebro, Sweden.

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Antibody (IgG, IgA, and IgM) to baker's yeast (Saccharomyces cerevisiae), yeast mannan, gliadin, ovalbumin and betalactoglobulin in monozygotic twins with inflammatory bowel disease

The response was specific for healthy controls. The response was specific for healthy controls, and the response was specific for healthy controls.
be very accurate, and in monozygotic twins a correct classification is obtained in 99% in comparison with serological methods.  

**CONTROLS**

One healthy control for each twin was chosen from members of the staff or blood donors, all without any history of gastrointestinal disease. In total, 52 persons were matched for sex and age within two years.

**BLOOD SAMPLING**

Venous blood was obtained and allowed to clot at room temperature and centrifuged before withdrawal of serum. Aliquots of serum were frozen at −70°C until analysed.

**DETERMINATION OF ANTIBODIES TO FOOD ANTIGENS**

Protein antigen preparations were obtained from Sigma Chemical Co (St Louis, Mo, USA) – namely, ovalbumin (A 5503), beta-lactoglobulin (L 0130), gliadin (G 3375), and mannan (M 3640). Whole yeast cell antigen was prepared in the following way. The cells of ordinary baker's yeast, *Saccharomyces cerevisiae*, were boiled for one hour, diluted to 25 µg/ml and usually used directly, or stored at −20°C until use.

To prepare antigen solutions, ovalbumin and beta-lactoglobulin were diluted in phosphate buffered saline, pH 7-3 to 25 µg/ml, and yeast cell wall mannann to 100 µg/ml, whereas gliadin was dissolved in 70% ethanol and diluted with phosphate buffered saline to 100 µg/ml. Whole yeast extract was used at 25 µg/ml.

As antisera, horseradish-peroxidase conjugated rabbit antihuman immunoglobulins were used (Dakopatts, Stockholm, Sweden) – that is, anti-IgG (type P 214), anti-IgA (P 216), and anti-IgM (P 215). Two hundred microtitre plate wells were added to each well of the microtitre-plate (96 well plate, Haager Plastics, Oslo, Norway), incubated for two hours at 37°C, and incubated overnight in a moist chamber. The microtitre plates were then washed three times with 250 µl phosphate buffered saline with Tween 20 (0-05%).

Patients' sera were diluted 1:25 in phosphate buffered saline-Tween and heat inactivated in a water bath for 30 minutes at 56°C. Then, 200 µl of each serum sample was added to three separate wells for each antigen and incubated for hour at 37°C; thereafter, the wells were washed three times with phosphate buffered saline-Tween and incubated with 200 µl horseradish-peroxidase conjugates (diluted 1:200 with phosphate buffered saline-Tween), washed again with phosphate buffered saline-Tween, and followed by substrate solution. The plates were light protected until they were read, and the reaction was stopped after 30 minutes by adding 50 µl 12.5% sulphuric acid. The substrate containing 100 µl phosphate buffered saline, 0.1 µl hydrogen peroxide (2%), and 10 µl 1,4-phenylenediamine dissolved in methanol (10 mg/ml) was freshly prepared and kept in the dark before use. Absorbance was read with an automatic ELISA reader (Immu-no-Reader NJ-2000, Nippon Intermed KK, Japan).

To obtain a positive control in each plate, serum from a volunteer with measurable levels of anti ovalbumin, anti beta-lactoglobulin, and anti gliadin of all immunoglobulin classes was used. For negative controls, sera with negligible specific antibody activity to these antigens was used in addition to phosphate buffered saline-Tween alone. The reference positive control serum was obtained during the period of investigation, frozen at −20°C in aliquots suitable for one day of experiments. When a deviation >10% was noted between a positive or negative control measurement, and accumulated median of corresponding controls the whole determination was repeated. To further reduce the influence of methodological errors, the median value of the three samples was used for calculations, and all analyses were performed by the same technician with the same equipment throughout the study. The results were expressed in arbitrary absorbance units per millimetre (absorbance units/ml) without subtraction of background, which was ≤0.07, 0.06, and 0.06 absorbance units/ml for IgG, IgA, and IgM, respectively. The overall reproducibility over the whole measurement range was within 2% for triple samples.

**STATISTICAL ANALYSIS**

When comparing Crohn's disease and ulcerative colitis disease twins, healthy twins, and controls, Mann-Whitney U test and Student's t test were used. Wilcoxon's sign rank test was used when comparing pairs, where only one individual had developed the disease.

**Results**

**PATIENT CHARACTERISTICS**

Patients with Crohn's disease had a mean age at diagnosis of 28-5 years (range 20-45), and the actual mean age was 42-9 years (range 34-63). In patients with ulcerative colitis the mean age at diagnosis was 27-7 years (range 17-45), and the actual mean age was 49-1 years (range 24-74). Two patients with Crohn's disease (Crohn's disease twin) had mild diarrhoea and slightly increased serum C-reactive protein and orosomucoid levels, which was treated with sulphasalazine. The other patients were inactive and the only therapy was vitamins or loperamide (Imodium, Janssen Pharmaceutica, Beere, Belgium). Two patients were on a lactose reduced diet, but only one had verified lactose intolerance. Eight patients were on a fat reduced diet. One of the two patients with increased disease activity was on tube feeding with elemental diet (Reabilan, Roussel Nordiska AB, Stockholm, Sweden) plus milk. All patients with ulcerative colitis (ulcerative colitis twin) were in clinical remission and had normal levels of haemoglobin, C-reactive protein and serum orosomucoid. Six were treated with sulphasalazine. One healthy twin was treated with prednisolone (5 mg on alternate days) for chronic hepatitis. None of the ulcerative colitis twins nor their healthy twins were on a special diet. A
thorough interview did not reveal symptoms suggesting inflammatory bowel disease in the healthy twins. They had remained healthy for an average 14·9 years (range 7–31) and 21·4 years (range 8–40) after diagnosis in the Crohn's disease and ulcerative colitis groups, respectively.

Sigmoidoscopy was performed in the subjects, except in five patients with ulcerative colitis and three patients with Crohn's disease who had had a proctocolectomy and in one Crohn's disease twin pair where both had severe perianal disease preventing sigmoidoscopy. Patients as well as healthy twins showed an inactive rectal mucosa on macroscopic and microscopic assessment.

**ANTIBODY LEVEL**

**General**

There was a considerable individual variation in antibody response, especially for IgG to all tested antigens but also the level of IgA and IgM to yeast cell wall mannan and whole yeast (*Saccharomyces cerevisiae*) varied much. For this reason a non-parametric method, Mann-Whitney U test, as well as a parametric method (Student's *t* test) were used to analyse the results. A few general observations can be made based on the material presented in the Figure and the Table. In ulcerative colitis twins the antibody response (IgA, IgG, IgM) to tested antigens were similar to that of the healthy twin of patient with ulcerative colitis twins and controls with a few exceptions. In contrast, Crohn's disease twins had higher titres of IgA, IgG, and IgM to yeast cell wall mannan and IgG to whole yeast (*Saccharomyces cerevisiae*), but not to the other dietary antigens.

Disease location did not have any influence on antibody response with one exception. Crohn's disease patients with small bowel disease only displayed higher IgG to whole yeast (*Saccharomyces cerevisiae*) than those with combined small and large bowel disease (*t* = 2·469, *p* = 0·024).

**Figure 1**: Mean (SEM) serum IgG, IgA and IgM antibody-response (AU/ml) against (A) yeast cell wall mannan (MN), (B) whole yeast (*Saccharomyces cerevisiae*) preparation (YT), (C) betalactoglobulin (BLG), (D) gliadin (GL), and (E) ovalbumin (OA) in patients with ulcerative colitis (UC twin), healthy twins, matched controls (UC-C), and in patients with Crohn's disease (CD twin), healthy monozygotic twins (HCD) and matched controls (CD-C).
TABLE Summary of statistical evaluations of serum levels in healthy twins, twins with inflammatory bowel disease and healthy control. Only statistically significant differences are depicted

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CD: Twin with Crohn’s disease, HCD: Healthy twin of patient with Crohn’s disease, CD-C: Healthy control of Crohn’s disease and HCD, UC: Twin with ulcerative colitis, HUC: Healthy twin of patient with ulcerative colitis, UC-C: Healthy control of UC and HUC.

Significances: < and > (p<0.05), <<< and >> (p<0.01), << < and >> (p<0.001).

*Indicates that Wilcoxon’s sign rank test was used instead of Students t test.

SPECIFIC

Crohn’s disease

As seen in the Table, Crohn’s disease patients had significantly higher levels of all immunoglobulin classes towards yeast cell wall mannan. The healthy twins had only higher IgA and the difference was not striking (p<0.05). When comparing Crohn’s disease twins and healthy Crohn’s disease twins using Wilcoxon’s sign rank test, the diseased twins had slightly higher IgA and IgM against yeast cell wall mannan, but the difference was barely significant (p=0.069 for both). IgA against whole yeast (Saccharomyces cerevisiae) was higher in the diseased and healthy twins (p<0.001 and p=0.011, respectively).

The proportion of patients who had higher IgG, IgA, and IgM against yeast cell wall mannan compared with controls were 12 of 19, 18 of 19, and 14 of 19 respectively. The corresponding figures for whole yeast (Saccharomyces cerevisiae) were eight of 19, 15 of 19 and eight of 19.

The antibody levels towards betalactoglobulin, gliadin, and ovalbumin were similar in the three groups. In a few cases controls displayed higher antibody levels – for example, IgM to betalactoglobulin (Table).

The two patients on lactose reduced diet had similar in antibody response to betalactoglobulin compared with the Crohn’s disease patient group.

Ulcerative colitis

Although the differences between ulcerative colitis twins, healthy twin of patient with ulcerative colitis and healthy control of twins concordant and discordant for ulcerative colitis were in general small, the following observations could be made on a group basis. IgM antibodies against yeast cell wall mannan and whole yeast (Saccharomyces cerevisiae) were higher in ulcerative colitis twins compared with healthy twin of patient with ulcerative colitis twins (p=0.014 and p=0.026 respectively) using Wilcoxon’s sign rank test (Table).

Both diseased and healthy twins were found to have higher IgA response to yeast cell wall mannan compared with healthy control of twins concordant and discordant for ulcerative colitis (p=0.046 and p=0.031 respectively, Table).

The most striking finding was for IgA to gliadin, where both healthy twin of patient with ulcerative colitis and ulcerative colitis twins had higher levels than healthy control of twins concordant and discordant for ulcerative colitis (p=0.001 and p=0.012 respectively). The healthy twins had even higher IgG than ulcerative colitis twins when analysing with Wilcoxon’s sign rank test. The antibody response to betalactoglobulin and ovalbumin is shown in the Table. The twins reacted similarly to controls.

Discussion

Earlier studies of food antigens in inflammatory bowel disease have shown conflicting results. The findings in the present study do not support an aetiologic role in inflammatory bowel disease of cow’s milk proteins as reflected by antibody titres to betalactoglobulin. Neither could we find evidence supporting the importance of the potent egg allergen ovalbumin.

High IgA titres against gliadin were found in healthy and diseased ulcerative colitis twins. This could indicate a subclinical and/or genetically determined gluten intolerance. An increased prevalence of coeliac disease has been observed in patients with inflammatory bowel disease, especially ulcerative colitis. Clinical experience does not strongly support such an aetiologic link, however, and so far no good epidemiological studies of gluten intolerance in inflammatory bowel disease exist.

More important is the fact that Crohn’s disease patients had increased antibody titres to yeast cell wall mannan for all tested immunoglobulin classes and the difference was statistically strong when compared with controls. Crohn’s disease patients also had high IgA levels to whole yeast (Saccharomyces cerevisiae). These observations are remarkable as almost all patients had inactive disease. Also healthy monozygotic twins of Crohn’s disease diseased patients had a raised level of IgA to yeast cell wall mannan and whole yeast (Saccharomyces cerevisiae) compared with controls but the significance was weaker (Table).

This is in accordance with Main et al. who found an increase in IgG and IgA antibody levels to a crude yeast preparation in Crohn’s disease patients compared with ulcerative colitis patients, and healthy controls, which was regarded as a reaction to Saccharomyces
Antibody to dietary antigens in inflammatory bowel disease twins

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