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Elevated Serum anti-Saccharomyces Cerevisiae, anti-I2 and anti-OmpW Antibody Levels in Patients with Suspicion of Celiac Disease

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Abstract

Objectives—Expression of anti-Saccharomyces Cerevisiae antibodies (ASCA) identifies patients and individuals at risk for Crohn's disease and has also been reported in 40–60% of Celiac disease (CD) cases, suggesting a role of host response to enteric microbiota in the development of inflammatory lesions. In this prospective study in patients with suspicion of CD, we evaluate the frequency and association of ASCA to serological responses for other host microbial targets formally associated with Crohn's disease, including the *P.fluorescens* associated sequence I2 and a *Bacteroides caccae* TonB-linked outer membrane protein, OmpW.

Methods—Small bowel mucosal biopsies were taken from 242 patients with CD suspicion, their sera were tested for antibodies to tissue transglutaminase (tTG), ASCA, I2 and OmpW. 80 adult healthy blood donors were used as controls.

Results—The diagnosis of CD was confirmed on biopsy in 134 cases. The occurrence of ASCA and I2 positivity was significantly higher in adult CD patients as compared to patients with non-CD disease. Anti-I2 levels in the sera were significantly higher in adult CD patients compared to non-CD disease or the controls and anti-OmpW levels in CD and non-CD patients when compared to controls. Positive seroreactivity to OmpW seemed to increase with the age. 90% of CD patients were seropositive for at least one microbial antigen tested.

Conclusions—This study demonstrates a mosaic of disease-related serological responses to microbial antigens in patients with CD. Immune responses to commensal enteric bacteria may play a role in the small intestine mucosal damage in CD.

Keywords

tissue transglutaminase; I2; OmpW; ASCA; serology; Celiac disease

INTRODUCTION

Celiac disease (CD) is a gluten-induced disease with small bowel mucosal damage manifesting with villous shortening, crypt hyperplasia and inflammation usually recovering on a gluten-free diet (GFD).^{1–3} Both CD and inflammatory bowel diseases (IBD) are characterized by the presence of distinct autoantibodies. Immunoglobulin A (IgA) autoantibodies to tissue transglutaminase (tTG) are commonly used for screening and diagnosing of CD.^{4–6} Anti-Saccharomyces Cerevisiae antibodies (ASCA) have suggested to be a specific marker of Crohn's disease, as several studies have provided evidence of seroreactivity against the Saccharomyces cerevisiae both in adult and paediatric Crohn's disease patients^{7–12}. Recently, ASCA positivity has also been detected in up to 40–60% of CD cases.^{13–14} Consistent evidence of linkage to IBD3 (6p21.1-23), an area which encompasses the human leucocyte antigen (HLA) complex, has been demonstrated in IBD, both in Crohn's disease and in ulcerative colitis.¹⁵ A strong HLA association with CD is also evident. Furthermore, chromosomal region 5q31 has demonstrated to link with both CD and Crohn's disease in genome-wide studies.^{16–18}

Commensal luminal bacteria are inducing dysregulated mucosal immune responses which may contribute to Crohn's disease pathogenesis.¹⁹ Indeed, a number of microorganisms and their products have been implicated in Crohn's disease shown in studies of proving their presence in mucosal lesions and the occurrence of serologic anti-microbial host responses (e.g., enteroadhesive *E. coli*, MAP, *P. fluorescens*, *B. caccae*, ASCA).^{7–12, 20–25} For example, high prevalence of antibodies to *P. fluorescens* associated sequence I2 has been found in adult and pediatric patients with Crohn's disease (54%, 60%, respectively).^{12, 20} In experimental mouse models, mechanistic evidence for the role of *Pseudomonas fluorescens* in the pathogenesis of Crohn's disease has been reported.^{12, 21}

The presence of anaerobic bacteria has thought to be critical in the development of inflammatory lesions.²⁶ Serum antibodies to a *Bacteroides caccae* TonB-linked outer membrane protein, OmpW, immunoglobulin (Ig) class A levels have reported to be elevated in patients with Crohn's disease.^{12, 22} Among these microbial candidates, the ensemble of anti-microbial specificities is a unique and stable phenotype with a substantial genetic contribution.²⁷ Previously mentioned observations support the concept of a mosaic of host traits controlling microbial colonization and regulating inflammation. Accordingly, heterogeneity in such traits is predicted to confer selective susceptibility to distinct microorganisms for disease pathogenesis among different patients.

The occurrence of ASCA also in CD patients raises the possibility that the biology similar to that in IBD may contribute to disease risk in this small bowel disorder. To test this, it is necessary to address the serological responses to a broader panel of microbial targets that have proved informative in Crohn's disease, especially at the earliest stages of disease onset. This study is evaluation to assess prospectively the frequency of ASCA seropositivity in children and adults with suspicion of CD. In addition, it provides data of host serologic responses to additional bacterial targets typical for IBD (the *Pseudomonas fluorescens*-associated sequence I2 and to a *Bacteroides caccae* TonB-linked outer membrane protein, OmpW) in this CD population.

MATERIALS AND METHODS

Patients

During periods, September 2003 – March 2005 (children) and 1995–2004 (adults), 242 clinically symptomatic patients with CD suspicion referred to the Department of Pediatrics

and Gastroenterology and Alimentary Tract Surgery, Tampere University Hospital were evaluated in a prospective manner. Eighty adult healthy blood donors were served as a healthy control group. Upper gastrointestinal endoscopies with multiple biopsies from duodenum were performed to all 242 patients. CD diagnosis was based on severe partial or subtotal villous atrophy with crypt hyperplasia and on the response to a GFD (1). At the same time sera were drawn for antibody testing.

Serum Antibody tests

Sera from patients were tested for antibodies to tTG (Celikey tTG IgA, Phadia, Freiburg, Germany) and/or endomysium antibodies (Ema)²⁸ and titres 1:5 were considered as positive. An enzyme immunoassay (EIA) kit (QUANTA Lite™ ASCA, INOVA Diagnostics Inc., San Diego, CA) was used for the determination of anti-Saccharomyces cerevisiae antibodies (ASCA) of both IgG and IgA isotypes from the sera. The kit included positive and negative controls and was used according to the manufacturer's instructions. Quantitative results in arbitrary EIA units were obtained from standard curves defined by the manufacturer, but the results were statistically handled as qualitative. Equivocal/borderline results were interpreted as negative. Results exceeding 25 U for IgG and/or IgA ASCA were regarded as positive.

Sera for the determination of anti-I2 and anti-OmpW IgA levels were stored at -70°C until testing. In our laboratory, *E. Coli* XL-1-blue and *E. Coli* BL-21 (Stratagene, La Jolla, California, USA) strains were used for all cloning and recombinant expression experiments. I2-GST and OmpW were produced by using previously reported antigen purification techniques.^{21, 22} Sera were analysed with IgA enzyme-linked immunosorbent assays (ELISA) to I2 and OmpW.¹² The cut-off level for positivity in the IgA-class ELISA test was set at 0.5 (for I2-GST) and 0.6 and 1.0 (for OmpW in children and adults, respectively).^{12, 20} (see also Results)

Biopsy Specimen Processing and Immunohistochemical Staining

Two to three biopsy specimens were processed by routine histological methods and sections of formalin-fixed and paraffin-embedded specimens were stained with haematoxylin and eosin. Small bowel mucosal morphology (ratio of villous height and crypt depth) was determined in 162 adult patients with CD suspicion as previously described.²⁹

Statistical analysis

Optical densities of antibody ELISA tests were expressed by means with 95% confidence interval of the mean. Other continuous variables were expressed by medians with range due to the skewed distribution. The differences between categorical variables were tested using the Pearson Chi-Square test or Fisher's exact test. Statistical calculations were carried out with SPSS for Windows (version 14.0.2; SPSS INC., Chigaco IL). The analyses for specificity and sensitivity values were performed.

Ethical considerations

The study protocol was approved by the Ethical Committee of Tampere University Hospital and informed consent was obtained from the parents and also from the children when appropriate.

RESULTS

Biopsy-proven CD was confirmed in 134 patients (86 adults and 48 children). One hundred and eight patients (87 adults and 21 children) were excluded for CD on biopsy. These cases served as disease-controls. The diagnosis of children with non-CD disease were dolores

abdominis NUD (n=10), anorexia nervosa (n=1), food allergy with gastrointestinal symptoms (n=1) and positive CD antibodies (tTG antibodies) (n=9). Adult non-CD cases were diagnosed to have gastroesophageal reflux disease and dyspepsia. From all CD cases positive tTG antibody titers were detected in 127/134 (95%), in 10/108 (9%) of non-CD patients and none (0%) of healthy controls. Demographic data and age of study population are shown in Table I.

Sixty-two out of 134 (46%) of those having CD were ASCA (IgA and/or IgG class) positive at the time of diagnosis. Frequency of ASCA positivity was significantly elevated in adult CD patients when compared to the disease-controls (16/108, 15%) or to healthy persons (0/80, 0%)(Table II).

Ninety-two out of 131 (70%) biopsy-proven CD patients, 65/107 (61%) of non-CD patients and 25/80 (31%) of healthy controls showed positive titres of I2 (specificity 59%, sensitivity 79%). Specificity and sensitivity of the I2 antibodies for adult CD cases were 82%, 75% and for pediatric CD cases 67%, 42%, respectively. Frequencies of I2 positivity in different study groups are presented in Table II. Anti-I2 levels in the sera were significantly higher in adult CD patients than in those excluded for the disease or in healthy controls ($p<0.001$, $p<0.001$; median of absorbances) (Figure 1a).

Positive seroreactivity to OmpW antibodies seemed to increase along the age (Figure 2). The cut-off levels for OmpW antibodies were set based on age and on the ability of the tests to distinguish patients with CD from the healthy control persons and according to the information of the previous data.¹² Eighty out of 131 CD patients (61%), 65/106 (61%) of non-CD patients and 19/80(24%) of healthy controls were positive for OmpW when the cut-off level for seropositivity was set at values 0.6 (pediatric cases) (specificity 81%, sensitivity 62%,) and 1.0 (adult cases) (specificity 62%, sensitivity 50%). When focusing on the whole CD study group, sensitivity for OmpW antibodies was 81%, specificity 54%. Frequency of positive serological response for OmpW was similar in patients with CD and disease controls excluded for CD. Table II Serum anti-OmpW levels were significantly higher in CD patients in comparison to the healthy control group ($p<0.001$; (Figure 1b). Ninety per cent of CD patients were seropositive toward at least one of microbial antigens tested here. Furthermore, many CD patients had immune reactivity towards more than one of the microbial antigens examined in the present study. (Figure 3)

Data on small bowel mucosal morphology (ratio of villous height and crypt depth) were available in 79 adult CD patients. A significant association between the frequency of ASCA positivity and the mucosal morphology was seen ($p=0.027$). No significant association between the serological responses to I2 and OmpW and villous atrophy was observed ($p=0.847$ and $p=0.711$, respectively).

DISCUSSION

This study demonstrated a mosaic of disease-related serological responses to microbial antigens in children and adults with CD, some of which are also associated with duration of the disease. Here these findings in relation to CD predisposition and pathogenesis are discussed. The understanding of such serologic microbial signatures in IBD is also assessed.

As expected, in this study, 95% of biopsy-proven CD patients were positive for tTG antibodies, a distinct characteristic for CD but not IBD. 4, 30 ASCA has been described as a specific marker of Crohn's disease in adults and children^{7-12, 24, 31} ASCA positivity has reported in proportion of adult CD patients^{13, 32-35}. Here we demonstrate an increased ASCA both in adult (58%) and pediatric (17%) patients with CD. The higher prevalence of ASCA seroreactivity found here in adult CD patients is notable compared to children, and to

previous adult studies. Whether this might reflect a longer duration of disease in our adult CD patients. In IBD, ASCA positivity is associated with an increased intestinal permeability.³⁶ In CD gliadin induces zonulin release and zonulin-dependent pathway of increased intestinal permeability.^{37–38} Thus, increased ASCA in CD adults might be due their longer exposure to gluten, recurrent damage to the small bowel mucosa, and more numerous episodes of microbial challenge all resulting in increased permeability.

Alternatively, the high ASCA seropositivity in this adult CD cohort may reflect additional traits related to Finnish heritage. Also in IBD, ASCA positivity may be a marker of genetic subsets of disease with distinct clinical traits, and serve as a preclinical trait in those subsets.^{36, 39–41} Elevated intestinal permeability is observed in CD patients and their relatives.^{42–45} As suggested Granito et al ASCA positivity may also be associated with silent CD as it occurs in proportion of CD patients before they develop any clinical symptoms.⁴⁶ It may be fruitful to evaluate whether ASCA would be able to distinguish cohorts with distinct disease risk or course in CD.

We also report for the first time high prevalence of antibodies to the *Pseudomonas fluorescens*-associated sequence I2 and to a *Bacteroides caccae* TonB-linked outer membrane protein, OmpW in CD. I2 and OmpW were commonly found to be elevated in biopsy-proven CD patients (70% and 61%, respectively). Previously, high prevalence of anti-I2 and anti-OmpW antibodies has been reported in children and adults with Crohn's disease.^{12, 17, 47} Interestingly, ninety per cent of CD patients showed positive seroreactivity at least one of microbial antigens (ASCA, I2, OmpW) tested in the present study. These findings indicate that there is a shared mosaic of anti-microbial seroresponse in CD and IBD. As in IBD, this suggests that immune responses to commensal enteric bacteria may play a role in inducing mucosal damage in CD. If this is a case, microbial seroreactivity may reflect with immune reaction and inflammation of small intestine in CD patients, or in patients with other upper gastrointestinal symptoms. Altered permeability in the small intestine might explain the frequent detection of seroreactivity for different microbial antigens in patients with CD suspicion.^{36–37, 42–45} In addition, increasing age associated with positive seroreactivity for anaerobic bacterial antigen in this study. This may be caused by exposure to different environmental factors (food, medications) or to genetic background and the age.

Recently, it has been shown that seroreactivity to microbial components in Crohn's disease is associated with disease severity, progression and the frequency of complications.^{11, 48–49} In contrast to the previous study, we showed here that, the frequency of ASCA positivity in CD patients was significantly associated to the grade of small bowel mucosal morphology.¹⁵ A larger study would be required for adequate power to better ascertain relationship between CD-associated cellular responses, and the microbial seroresponses.

In conclusion, this study reveals a mosaic of serological bacterial responses in CD, notably a new identification of the *Pseudomonas fluorescens*-associated sequence I2 and to a *Bacteroides caccae* TonB-linked outer membrane protein, OmpW. Current findings provide important evidence for a contribution of such responses to the pathogenic mechanisms of inflammation in CD and possibly in those of other upper intestinal diseases. These serologic specificities might provide new tools for the detection and differentiation of patients with such gastrointestinal diseases.

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Figure 1A

Figure 1B

Figure 1.

Serological responses to I2 (1A) and OmpW (1B) in patients with celiac disease (CD) suspicion. Patients are divided with their final diagnosis to CD and to non-CD patients. Horizontal lines denote the cut-off value for seropositivity (see text). P values compare median absorbances between the CD and non-CD groups, using the Mann-Whitney U-test.

Figure 2A

Figure 2B

Figure 2. Serum anti-I2 (2A) and anti-OmpW (2B) levels according to age in the study population. Horizontal lines denote the cut-off value for seropositivity (see text). P values compare the median absorbances between celiac disease (CD) and non-CD groups using the Mann-Whitney U-test.

Figure 3. Concordance in celiac disease patients of serum antibodies to microbial antigens (*Saccharomyces cerevisiae*, ASCA; *Pseudomonas fluorescens*-associated sequence, I2; *Bacteroides caccae* TonB-linked outer membrane protein, OmpW). The percentage of patients positive for each marker, any combination of two or all markers is presented (n=131).

Table 1

Demographic data of the study population (celiac disease; CD, non-celiac disease; non-CD and healthy controls).

	CD adults	CD children	Non-CD adults	Non-CD children	Healthy controls
N	86	48	87	21	80
Sex (F/M)	67/19	28/20	60/27	11/10	28/52
Age median	44.5	10.2	42.0	10.3	41.0
Range (yrs)	21.0–73.0	1.9–15.4	18.0–72.0	4.3–16.4	19.0–64.0

Table II

The Frequency of Positive Seroreactivity to Anti-Saccharomyces Cerevisiae (ASCA), I2 and OmpW Among Patients (242) with Celiac Disease Suspicion at the Time of Diagnosis.

	ASCA		I2		OmpW			
	n	%	n	%	n	%	p1	p2
All CD cases (n=134)*	62	46	92	70	80	61	0.968	<0.001
Non-CD cases (n=108)*	16	15	65	61	65	61		<0.001
Healthy controls (n=80)	0	0	25	31	19	24		
Adult CD cases (n=86)	50	58	74	86	49	57	0.598	<0.001
CD Children (n=48)*	12	25	18	40	31	69	0.656	<0.001
Adult Non-CD (n=87)*	15	17	58	67	53	61		<0.001
Non-CD children (n=21)*	1	5	7	33	12	63		0.001

CD, Celiac Disease; ASCA, anti- Saccharomyces cerevisiae antibodies; I2, anti-I2; antibodies to *P. fluorescens* associated sequence I2; OmpW, a *Bacteroides caccae* TonB-linked outer membrane protein. For p values, patient groups were compared to the disease controls (non-CD disease) (p1) and healthy adult control group (p2).

* I2 analyses were available from 131 CD cases (86 adults and 45 children) and from 107 non-CD cases (86 adults and 21 children).

* OmpW analyses were available from 131 CD cases (86 adults and 45 children) and from 106 non-CD cases (87 adults and 19 children).