Anti-*Saccharomyces cerevisiae* antibody titers are stable over time in Crohn’s patients and are not inducible in murine models of colitis

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**AIM:** To investigate ASCA production over time in CD and murine colitis in order to further our understanding of their etiology.

**MATERIALS AND METHODS:** Sixty-six CD patients were compared to ulcerative colitis (UC) and irritable bowel syndrome patients with respect to ASCA production as measured by ELISA. ASCA IgG or IgA positivity as well as change in titers over a period of up to 3 years (ΔIgG/A) was correlated with clinical parameters such as CD activity index (CDAI) and C-reactive protein levels (CRP). Moreover, two murine models of colitis (DSS and IL-10 knock out) were compared to control animals with respect to ASCA titers after oral yeast exposure.

**RESULTS:** ASCA IgG and IgA titers are stable over time in CD and non-CD patients. Fistular disease was associated with a higher rate of ASCA IgA positivity (P = 0.014). Ileal disease was found to have a significant influence on the ΔIgG/A of ASCA (P = 0.032). There was no correlation found between ASCA positivity or ΔIgG/A and clinical parameters of CD: CDAI and CRP. In mice, neither healthy animals nor animals with DSS-induced or spontaneous colitis exhibited a marked increase in ASCA titers after high-dose yeast exposure. On the other hand, mice immunized intraperitoneally with mannann plus adjuvant showed a marked and significant increase in ASCA titers compared to adjuvant-only immunized controls (P = 0.014).

**CONCLUSION:** The propensity to produce ASCA in a subgroup of CD patients is largely genetically predetermined as evidenced by their stability and lack of correlation with clinical disease activity parameters. Furthermore, in animal models of colitis, mere oral exposure of mice to yeast does not lead to the induction of marked ASCA titers irrespective of concomitant colonic inflammation. Hence, environment may play only a minor role in inducing ASCA.

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**Key words:** Crohn’s disease; Anti-*Saccharomyces cerevisiae* antibodies; Colitis

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**INTRODUCTION:** Much attention has been focused on serologic markers in inflammatory bowel disease (IBD). The anti-*Saccharomyces cerevisiae* antibody (ASCA) is one such marker, which possesses an intermediate sensitivity and a high specificity for Crohn's disease (CD)\(^{[1-3]}\). Antibodies to *S cerevisiae* in CD were first described by Main et al\(^{[4]}\), using whole killed yeast cells as antigens. Sendi et al\(^{[5]}\) demonstrated greater diagnostic value for CD with *S cerevisiae* Su1, a strain of baker's yeast, and identified the antigenic oligomannosidic epitopes of this organism. Subsequent work demonstrated that CD patients develop antibodies to a variety of baker's and brewer's yeast strains\(^{[6]}\). The antigen reacting with ASCA is a phosphopeptidomannan, a component of the *S. cerevisiae* cell wall\(^{[7]}\).

Aside from the diagnostic role performed by ASCA, uncertainty remains as to whether they possess a pathophysiologic significance. One can argue for a genetic origin due to ASCA presence in 20-25% of unaffected first-degree family members\(^{[7-10]}\). Healthy monozygotic twins of CD patients also demonstrate increased IgA, IgG, and IgM ASCA levels\(^{[11]}\). In one study of non-IBD families,
ASCA were found to be familial with a vertical transmission pattern\textsuperscript{[9,10].} ASCA stability over time and independence from disease activity further indicate a genetic link\textsuperscript{[11,12].} Moreover, we have shown that T cells from ASCA-positive patients proliferated upon stimulation with \textit{mbL} \textsuperscript{[13].} We were able to show that \textit{mbL} binding lectin (MBL) deficient patients were significantly more frequently ASCA-positive and showed an enhanced T cell proliferation upon \textit{mbL} stimulation compared to \textit{mbL} wildtype patients\textsuperscript{[14].} These results further support the importance of genetic determination of ASCA. Nevertheless, some of the familial studies have yielded conflicting data. For example, one group showed increased ASCA production in familial vs sporadic CD\textsuperscript{[15],} but others have shown equal or increased ASCA prevalence for sporadic CD\textsuperscript{[16–18].} Thus, the case for an environmental etiology for ASCA has been articulated as well. For instance, a decline in ASCA levels has been observed post-surgically in a pediatric CD population\textsuperscript{[19].} Another group demonstrated lower ASCA titers in CD patients taking mesalazine than in CD patients not taking mesalazine\textsuperscript{[20].} Additionally, both breeding and baking strains of \textit{S cerevisiae} provoke an antibody response in CD, implicating dietary antigens in disease pathogenesis\textsuperscript{[21].} One group has noted higher ASCA IgG antibody levels in patients with small bowel Crohn’s disease vs those with colonic disease\textsuperscript{[22].} This same study found high levels of ASCA IgG but not IgA in celiac disease, indistinguishable from levels seen in CD. It was, thus, concluded that ASCA may result from a mucosal permeability defect. However, Vermeire \textit{et al.}\textsuperscript{[23]} were not able to show a correlation between ASCA and intestinal permeability.

In this study, our aim was to characterize ASCA over time in patients with IBD in order to further our understanding of their etiology. Furthermore, we assessed the possibility to induce an ASCA response in animal models.

**MATERIALS AND METHODS**

**Patients**

Sixty-six Crohn’s disease (CD) patients, 29 ulcerative colitis (UC) patients, and 10 irritable bowel syndrome (IBS) patients with informed consent were enrolled in the study, and the study was approved by the ethical committee of the local authorities. Patient serum samples were drawn during routine outpatient visits (Gastroenterology clinic, University Hospital, Bern, Switzerland) for ASCA IgG and IgA analysis. Diagnosis of CD and UC was established by endoscopic, histological, and clinical criteria. Diagnosis of IBS was established by the Rome criteria\textsuperscript{[24]}.

A subgroup of CD (73 sera from 29 patients), UC (26 sera from 12 patients) and IBS (22 sera from 10 patients) patients, whose serum samples were drawn on at least 2 consecutive (maximally 4 consecutive) routine out-patient visits, were retrospectively selected for ASCA IgG and IgA titer analysis. An assessment of the Crohn’s disease activity index (CDAI) was obtained at each CD patient’s visit to the clinic\textsuperscript{[25].} The serum CRP levels were obtained at each clinic visit as well. Three patients with stoma were excluded from the CDAI analysis. One patient was excluded from the clinical analysis, since this patient’s CDAI and CRP could not be obtained.

**Mice**

Four to six-weeks-old female interleukin-10 knockout mice (IL10\textsuperscript{0/0}; henceforth abbreviated as IL-10\textsuperscript{0/0}) on a C3H/HeJBir background were obtained from the University of Bern breeding facility (Bern, Switzerland) and housed under specified pathogen-free conditions. Four to six-week-old BALB/c mice were obtained from Harlan (Horst, The Netherlands). All procedures involving animals were performed in compliance with the local authorities on the use of animals in research.

**ASCA ELISA for mouse and human sera**

Investigators were blinded to disease status when performing the ELISA. Phosphopeptidomannans from baker’s yeast (Hefe Vital Gold, Deutsche Hefewerke, Nürnberg, Germany) were extracted as previously described\textsuperscript{[8,23].} Ninety-six well ELISA plates (MaxiSorb, Nunc, Wiesbaden, Germany) were coated with 100 µL of 0.25 µg/mL phosphopeptidomannans in carbonate-bicarbonate buffer, pH 9.6 and incubated overnight at 4 °C. Serum diluted 1/2 000 was applied to the coated plates in triplicates. The plates were incubated for 1 h at 37 °C. Secondary antibody was added and plates were incubated for 1 h at RT. The secondary antibodies (Sigma, Buchs Switzerland) were as follows: goat anti-mouse polyvalent immunoglobulin peroxidase conjugate at 1/1 000; goat anti-human IgA (alpha-chain specific) peroxidase conjugate; and goat anti-human IgG (Fc specific) peroxidase conjugate, both at 1/5 000. The plates were developed using TMB substrate (Sigma) and the reaction was stopped with sulfuric acid. The absorbance at 450 nm (reference filter = 490 nm) was read by an EL 800 microplate reader (Bio-Tek Instruments, Winoski, Vermont). The mean absorbance value for the healthy control sera plus two standard deviations was used to discriminate between positive and negative subjects. Absorbance units equal to or above this value were considered positive for ASCA antibodies. A patient was considered ASCA\textsuperscript{+}, when positive for ASCA IgG, IgA or both.

For the human ELISA, one serum highly positive for ASCA was used to obtain a standard curve at dilutions ranging from 1/100 to 1/102 400. The curve was fitted using a four parameter logistic function to the logarithmically scaled dilutions. Human titers are expressed as dilution units from this curve. We computed the average change of IgG and IgA (Δ\textit{IgG/A}) in human ASCA titer as our main variable of interest. An increase of Δ\textit{IgG/A} represents a decrease in ASCA levels and vice versa. Of the CD patients, two patient’s IgG titer and one patient’s IgA titer could not be reliably calculated from the standard curve after two repeated assays and thus were excluded from the IgG/A titer analysis. The murine ASCA titers are expressed as optical density (OD).

**Preparation of yeast solution**

Baker’s yeast (Hefe Vital Gold, Deutsche Hefewerke, Nürnberg, Germany) was freshly grown in YPD broth.
(Becton Dickinson, Le Pont de Claix, France) for 12 h at 30 °C in a shaker at 200 r/min. The yeast solution was centrifuged at 2 000 r/min for 5 min. The cellular pellet was washed twice, resuspended in sterilized water and sonicated at 100 W for 2 min. Protein concentration was determined using the Bradford reagent (Sigma, Buchs, Switzerland).

Induction of chronic dextran sulfate sodium-induced (DSS) colitis, oral yeast administration and histological assessment

The previously described model of chronic DSS colitis was adapted from Mähl er et al. Briefly, Balb/c mice were given three 5-day cycles of 3% DSS (DSS salt, MW= 36 000-50 000, ICN Biomedicals, Inc., Ohio), each interrupted by the 7th day without DSS. All mice received yeast ad libitum at a protein concentration of 100 or 500 µg/mL for the entire experiment. Murine serum samples were drawn on d 0 and the day of killing (d 36) for ASCA immunoglobulin analysis.

Mice were euthanized via CO2 asphyxiation. Colon were harvested, fixed in 4% phosphate-buffered formaldehyde, embedded in toto and H&E-stained sections were examined by a pathologist (Mähl er) blinded to the code. The sections were scored on a previously described scale ranging from 0 (no inflammation) to 4 (severe inflammation). A total colonic inflammation score was obtained by averaging the individual scores of the proximal, middle, and distal colon.

Immunizations

Balb/c mice from our central animal facility were intraperitoneally injected with 50 µg phosphopeptidomannans or ovalbumin (Sigma, Gaithersburg, MD) mixed 1:1 (v/v) with GERBU adjuvant (GERBU, Gaiberg, Germany). Injection was repeated after two weeks and mice were killed for analysis one week later. Serum samples were obtained before primary immunization by tail tip bleeding and after euthanization by puncture of the caval vein.

Statistical analysis

We analyzed the CD population in regard to ASCA IgG and IgA positivity to test for a significant influence of the following patient variables: age, gender, anatomical location of disease, medication, fistulae, and extra-intestinal manifestations. Significance of each variable was computed via unpaired, two-tailed Student’s t-test assuming equal variances if the additional F-test was non-significant. We analyzed the titer subgroup in respect to ASCA IgG and IgA positivity to test for a significant influence of the above-mentioned variables and additionally CD and ASCA IgG positivity to test for a significant influence of the above-mentioned variables and additionally CD and ASCA IgG positivity to test for a significant influence of the additional

RESULTS

ASCA positivity in the study population

Thirty-five of sixty-six CD patients, 4 of 29 UC patients and 3 of 10 IBS patients were ASCA positive. The sensitivity, specificity, and positive predictive value of the ASCA assay in distinguishing CD from UC are 53%, 86%, and 90%, respectively.

Fistular disease is associated with higher rate of ASCA IgA positivity

The baseline clinical characteristics of the patients and controls can be seen in Table 1. None of the following clinical characteristics had a significant effect on ASCA IgG and IgA positivity in the Crohn’s disease patients: age, gender, anatomical locations of disease, medication, and extra-intestinal manifestations. Patients with fistular disease were significantly more likely to possess ASCA IgA positivity (P = 0.014, 71% vs 32%) than patients with non-fistular disease.

ASCA IgG and IgA titers are stable over time in CD and non-CD patients

ASCA IgG and IgA titers in the CD subgroup, expressed as logarithmically (ln) scaled dilutions of a high positive control, have been plotted over a time-period of up to 3 years (Figure 1). The change in titers did not vary significantly as can be seen in the Gaussian distribution in the histogram, which is centered close to zero for all patient groups with respect to both IgG and IgA titers (Figure 2). The means, standard deviations and 95% confidence intervals of ASCA IgG and IgA positivity in the study population

Anatomic location of disease significantly influences the ∆ASCA of ASCA

Of the clinical parameters assessed upon entry, ileal disease was found to have a significant influence on the ∆ASCA of ASCA (Table 3). The mean ∆ASCA value for patients with ileal disease vs those without ileal disease was 0.13 and -0.34, respectively. This suggests that patients without ileal
Table 1 Baseline characteristics of the study populations tested for anti-Saccharomyces cerevisiae IgG and IgA antibodies. Unless otherwise indicated values are numbers of patients (%).

| Study population | Crohn’s disease (n = 66) | Ulcerative colitis (n = 29) | Healthy controls (n = 18) | IBS patients (n = 10) |
|------------------|---------------------------|-----------------------------|--------------------------|----------------------|
| Age, yr          | 38±16                     | 37±15                       | 40±13                    | 57±13                |
| Mean±SD          |                            |                             |                          |                      |
| Range            | 14-82                      | 16-78                       | 17-74                    | 41-81                |
| Male sex         | 32 (48)                   | 15 (52)                     | 10 (56)                  | 6 (60)               |
| Disease location |                            |                             |                          |                      |
| Colonic          | 58 (88)                   | 29 (100)                    |                          |                      |
| Ileal            | 45 (68)                   |                             |                          |                      |
| Upper gastrointestinal involvement | 3 (5) | 1 (3) | | |
| Presence of fistulae | 15 (23) | 1 (3) | | |
| Extraintestinal manifestations | 4 (6) | 0 (0) | | |
| Medical therapy  |                            |                             |                          |                      |
| Azathioprine     | 22 (33)                   | 9 (31)                      |                          |                      |
| Infliximab       | 5 (8)                     | 3 (10)                      |                          |                      |
| 5-ASA compounds  | 4 (6)                     | 11 (38)                     |                          |                      |
| Methotrexate     | 4 (6)                     | 0 (0)                       |                          |                      |
| Oral steroids    | 29 (44)                   | 15 (52)                     |                          |                      |
| None of the above| 19 (29)                   | 7 (24)                      |                          |                      |

Table 2 Mean, standard deviation (SD) and the 95% confidence interval of the mean of the change in ASCA IgG and IgA titers

|         | ΔIgG  |         | ΔIgA  |         |
|---------|-------|---------|-------|---------|
|         | Mean  | SD      | 95%CI | Mean  | SD      | 95%CI |
| CD      | 0.01  | 0.56    | (-0.20, 0.22) | -0.04 | 0.25    | (-0.13, 0.05) |
| UC      | 0.05  | 0.15    | (-0.03, 0.14) | 0.16  | 0.24    | (0.02, 0.29)  |
| IBS     | 0.05  | 0.08    | (-0.003, 0.09) | -0.01 | 0.12    | (-0.08, 0.06) |

Figure 1 Anti-Saccharomyces cerevisiae (A) IgG and (B) IgA antibody titers in ASCA-positive patients with Crohn’s disease expressed as a logarithmically (in) scaled dilution unit of a highly positive control over a time period of up to 3 years.

Figure 2 Histogram of change in ASCA IgA and IgG titers (ΔIgG/A) in patients with Crohn’s disease (CD), ulcerative colitis (UC) and irritable bowel syndrome (IBS). CD patients demonstrate higher variability compared to UC and IBS.

disease were more likely to experience an increase in ASCA level as opposed to patients with ileal disease. All other tested clinical parameters did not significantly influence the ΔIgG/A of ASCA. Regarding the clinical parameters of disease, no strong correlation was found between the ΔCDI and ΔIgG (r = 0.086) and ΔIgA (r = 0.127). Moreover, no strong correlation was found between the ΔIBS and ΔIgG (r = 0.424) and ΔIgA (r = 0.245).

Anti-S cerevisiae antibodies are not efficiently induced upon oral yeast administration, irrespective of concomitant induction of colitis in Balb/c or IL-10 knockout mice

All mice given yeast lysate and unfed controls exhibited slightly increased ASCA titers at the end of the experimental period compared with values obtained at the beginning of the experiment. A slight increase in ASCA titers was observed in mice exposed to 500 µg/mL yeast lysate in their drinking water when compared with mice exposed to 100 µg/mL (Figure 3A). Concomitant DSS-
OD at 450 nm representing relative levels of polyclonal anti-Ovalbumin µ antibody (ASCA) (DSS-treated Balb/c group, 0±0 for the non-DSS-treated

The observation of clinical signs such as soft stools and increase in ASCA titers. Development of colitis was evidenced by treatment (Figure 3A) or onset of colitis in IL-10  group.

**ASCA titers are induced in mice after immunization with yeast mannann**

Groups of Balb/c mice were immunized with adjuvant alone, or with adjuvant plus yeast mannann or, as a positive control, ovalbumin (Figure 3B). Mannan-immunized mice showed an average two-fold increase in ASCA titers when compared with mice receiving adjuvant only (P = 0.014). Mannan was poorly immunogenic compared to ovalbumin (P = 0.038) since specific serum antibody titers after ovalbumin plus adjuvant-immunization increased about seven-fold over adjuvant-only injected controls (P = 0.015).

**DISCUSSION**

Antibodies to the *S cerevisiae* cell wall mannans have been widely studied as a diagnostic tool to distinguish CD from UC. In this study, the rates of CD patient ASCA positivity correlate well with our previous results[8] as well as those cited in the literature[7,10,13]. Of all the clinical variables tested, fistular Crohn’s disease was found to be associated with ASCA IgA positivity. This study is not the first to demonstrate such an association. Others have shown that the subgroup of CD patients with high levels of IgG and IgA ASCA has more aggressive, small-bowel fistulizing disease[25].

Our study was the first to assess time-period streamlined ASCA titers. We found no association between the change in ASCA titers and the following clinical characteristics: age, gender, presence of fistulae, medical therapy, CDAI or CRP levels. Of the clinical parameters tested, patients without ileal disease were more likely to encounter an increase in their ASCA titers over time as compared with CD patients with ileal disease. There is no good explanation why anatomic location of CD should provoke an increase or decrease in ASCA titers. It should be noted that Giaffer *et al*[18] noted increased ASCA IgG positivity association with small bowel disease as compared to

| Clinical characteristics | ASCA IgG | ASCA IgA | ∆IgG | ∆IgA |
|--------------------------|----------|----------|------|------|
| Correlation with age     | c = -0.140 | c = 0.198 | c = -0.340 | c = -0.197 |
| Male vs female           | 0.881    | 0.881    | 0.089 | 0.571 |
| Disease Location         |          |          |      |      |
| Colonic vs non-colonic   | 0.667    | 0.169    | 0.203 | 0.289 |
| Ileal vs non-ileal       | 0.285    | 0.285    | 0.032 | 0.395 |
| Fistular vs Non-fistular disease | 0.152 | 0.075 | 0.499 | 0.907 |
| Medical therapy          |          |          |      |      |
| Azathioprine             | 0.881    | 0.881    | 0.749 | 0.375 |
| Infliximab               | 0.233    | 0.233    | 0.870 | 0.444 |
| 5-ASA compounds          | 0.806    | 0.233    | 0.903 | 0.491 |
| Oral steroids            | 0.812    | 0.385    | 0.475 | 0.531 |
| None of the above medical therapy vs medical therapy | 0.285 | 0.577 | 0.438 | 0.117 |

*P<0.05.
colonic disease. Nevertheless, this study does not explain why these patient’s titers were more likely to change over time. Also of special interest is the lack of association between ∆CDAI and ∆IgG/A and ΔCRP and ΔIgG/A. ASCA stability over time in relation to the changes in CDAI was demonstrated by Landers et al\[9\] utilizing a time period of at least 4 mo. Recently, Teml et al\[10\] established ASCA stability during a course of steroid or 5-ASA therapy. Our data further strengthen this stability and lack of association with CDAI or medical therapy with the addition of CRP as a serologic marker of disease. This lack of association with clinical activity leads others and us away from thinking of ASCA as a subclinical marker of disease activity as it does not follow CD clinical status variations.

In line with the results obtained with human sera, the murine ASCA titers were rather stable, at least during the time of the experimental procedure (36 d). This is the first study to investigate murine ASCA production in the setting of DSS and IL-10\[11\] colitis. After oral treatment with a high antigen dose (500 µg/mL), we observed a slight increase of ASCA titers compared to untreated controls or mice treated with 100 µg/mL. Interestingly, we observed slightly lower ASCA titers in young untreated mice compared to untreated mice at the end of the experiment. It is very likely that ASCA titers in mice may slightly vary between the breeder company and our animal facility as a consequence of differential exposure to yeast-antigens depending on the hygiene status of the animal facility and/or the composition of food pellets. We did not, however, observe a further increase upon induction of colitis. Therefore, the impairment of mucosal barrier during inflammation and/or the enhanced presence of immune competent cells alone does not lead to markedly enhanced ASCA generation.

In contrast, systemic immunization of mice with phosphopeptidomannans in the context of appropriate adjuvant leads to a significant increase in ASCA titers. However, the increase of ASCA titers is much less pronounced than that of anti-ovalbumin antibodies after ovalbumin immunization. Thus, these data show that mannans are not very efficient antigens when used for immunization, further indicating that normally mice and human beings are tolerant to mannans. Local inflammation during colitis is not sufficient to break this tolerance. In our recent study we were able to show that ASCA positivity was associated with MBL deficiency[13]. Furthermore, family studies show an increased incidence of ASCA in healthy family members[14-20]. Thus, generation of ASCA in a subgroup of CD patients and healthy family members may be mainly a result of a genetic predisposition, although biochemical and genetic analyses supporting this notion are not currently available.

In conclusion, we have demonstrated the stability of the ASCA titers over time and their lack of association with clinical activity in the form of CDAI and CRP. These findings are in line with the observation that ASCA are only marginally inducible in mice via high dose oral exposure, and are not further influenced by concomitant induction of colitis. Hence, we speculate that the propensity to produce ASCA in a subgroup of CD patients is largely genetically predetermined and, thus, may reflect a predisposition of distinct genetic backgrounds to establish immune responses against normally tolerated antigens.

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