CLINICAL RESEARCH

Partial overlap of anti-mycobacterial, and anti-\textit{Saccharomyces cerevisiae} mannan antibodies in Crohn's disease

Stefan Müller, Thomas Schaffer, Alain M Schoepfer, Annamarie Hilty, Thomas Bodmer, Frank Seibold

Abstract

\textbf{AIM:} To test whether humoral immune reaction against mycobacteria may play a role in anti-\textit{Saccharomyces cerevisiae} antibodies (ASCA) generation in Crohn's disease (CD) and/or whether it correlates with clinical subtypes.

\textbf{METHODS:} The dominant ASCA epitope was detected by \textit{Galanthus nivalis} lectin (GNL)-binding assay. ASCA and IgG against mycobacterial lysates [\textit{M avium, M smegmatis, M chelonae, M bovis BCG, M aviumssp. paratuberculosis} (MAP)] or purified lipoarabinomannans (LAM) were detected by ELISA. ASCA and anti-mycobacterial antibodies were affinity purified to assess cross-reactivities. Anti-mycobacterial IgG were induced by BCG-infection of mice.

\textbf{RESULTS:} GNL bound to different extents to mycobacterial lysates, abundantly to purified mannose-capped (Man) LAM from \textit{M tuberculosis}, but not to uncapped LAM from \textit{M smegmatis}. Fifteen to 45\% of CD patients but only 0\%-6\% of controls were seropositive against different mycobacterial antigens. Anti-mycobacterial IgG correlated with ASCA ($r = 0.37$-$0.64; P = 0.003-P < 0.001$). ASCA-positivity and deficiency for mannan-binding lectin synergistically associated with anti-mycobacterial IgG. In some patients, anti-mycobacterial antibodies represent cross-reactive ASCA. Vice-versa, the predominant fraction of ASCA did not cross-react with mycobacteria. Finally, fistulizing disease associated with antibodies against \textit{M avium, M smegmatis} and MAP ($P = 0.024, 0.004$ and $0.045$, respectively).

\textbf{CONCLUSION:} Similar to ASCA, seroreactivity against mycobacteria may define CD patients with complicated disease and a predisposition for immune responses against ubiquitous antigens. While in some patients anti-mycobacterial antibodies strongly cross-react with yeast mannan; these cross-reactive antibodies only represent a minor fraction of total ASCA. Thus, mycobacterial infection unlikely plays a role in ASCA induction.

© 2008 The WJG Press. All rights reserved.

\textbf{Key words:} Crohn’s disease; Anti-mycobacterial antibodies; Anti-\textit{Saccharomyces cerevisiae} antibodies; Cross-reactivity; Mannan; Lipoarabinomannan

Peer reviewer: Dr. Daniel R Gaya, Gastrointestinal Unit, Molecular Medicine Centre, School of Molecular and Clinical Medicine, University of Edinburgh, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, United Kingdom

Müller S, Schaffer T, Schoepfer AM, Hilty A, Bodmer T, Seibold F. Partial overlap of anti-mycobacterial, and anti-\textit{Saccharomyces cerevisiae} mannan antibodies in Crohn's disease. \textit{World J Gastroenterol} 2008; 14(23): 3650-3661 Available from: URL: http://www.wjgnet.com/1007-9327/14/3650.asp DOI: http://dx.doi.org/10.3748/wjg.14.3650

INTRODUCTION

Crohn’s disease (CD) is a multifactorial disease that affects genetically susceptible hosts. The exact pathogenesis is still largely unknown. However, it is generally accepted that the disease, once established,
is driven by antigens of the intestinal flora, reflecting a loss of tolerance against commensal microorganisms\[8,9\]. The hypothesis that genetic predisposition, together with unfavorable environmental and commensal triggers cause CD with its various phenotypes contradicts the highly controversial idea of a single infectious origin of the disease\[9\].

A number of serological markers have been detected that have a certain degree of specificity and sensitivity for CD\[5-9\]. Of the most intriguing antibodies are those directed against outer cell wall mannans of the baker’s yeast \textit{Saccharomyces cerevisiae} (anti-\textit{Saccharomyces cerevisiae} antibodies, ASCA)\[9,9\]. These antibodies are found in more than 50% of CD patients, but rarely in healthy controls or patients with ulcerative colitis (UC)\[9\]. Yeasts are ubiquitous and ingested on a daily basis. Why an organism that, with a few reported exceptions of virulent mutants\[9,9\], is not adapted to live or even grow in the human body elicits a strong IgG response in CD patients has not yet been conclusively answered. A recent report presented experimental data supporting the idea that the facultative opportunistic pathogen \textit{Candida albicans} may be the inducer of ASCA\[9\]. However, our recent study showed that ASCA and anti-\textit{C albicans} antibodies correlate to a lower degree than ASCA with antibodies to mannans from other ubiquitous yeasts\[13\]. Thus, whether \textit{C albicans} infection may indeed represent the dominant trigger for ASCA cannot be definitively answered so far and there may be other cross-reactivities that play a role in ASCA induction. Potential candidates are mycobacteria since their cell wall contains lipoarabinomannans with similar mannose side chains as the cell wall mannans of yeast. The exact epitope recognized by ASCA has been demonstrated to be an \(\alpha\)-1,3 mannose-(\(\alpha\)-1,2 mannose)n with \(n = 2\) or 3 by two independent studies\[9,9\]. Similar or equal oligo-mannose motives are found in other yeasts, as well as in the mannosylated side chains of mycobacterial lipoarabinomannans (LAM)\[14,14\]. Part of this motif, the terminal \(\alpha\)-1,3 linked mannose, can be detected by the \textit{Galanthus nivalis} lectin (GNL)\[16-17\] and has been shown to be present in the lipo(arabino)mannan of \textit{M chelonei}\[18\]. Two other publications have demonstrated presence of the GNL-reactive motif in some mycobacterial species, including \textit{M bovis}, \textit{M avium} and \textit{Mycobacterium avium} ssp. paratu-berculosis (MAP)\[19,20\]. Hence, we were interested whether ASCA-positive CD patients may also more frequently contain antibodies against distinct mycobacterial strains and specifically against LAM, and whether these antibodies would be of cross-reactive nature.

In the case of MAP studies have shown a very high (77%-87%) prevalence of seroreactivity against the MAP antigens p35 and p36 in CD\[21,22\]. While this is intriguing, the interest in the possible relationship between CD and MAP mainly comes from the fact that Johnes’ disease in cattle which is caused by MAP infection in some aspects resembles CD.

The acute phase reactant mannose-binding lectin (MBL) specifically binds to mannose residues and is an important first line of defense innate immune effector molecule\[23-24\]. We have previously shown that deficiency for MBL associates with the ASCA-positive subgroup of CD patients\[27,28\]. Thus, it was of interest, if in CD, deficiency for MBL might associate with elevated levels of anti-mycobacterial IgG as well. Finally, we correlated our findings regarding anti-mycobacterial antibodies with different clinical CD phenotypes.

### MATERIALS AND METHODS

#### Patients and sera

Sera from 105 patients with CD, 45 patients with UC and 35 healthy controls were obtained with informed consent and with the approval of the local ethical authorities. Diagnosis of CD and UC was established by endoscopic, histological and clinical criteria. CD: 54 women and 51 men, mean age 40 years (19-73); UC: 22 women and 23 men, mean age 38 years (20-65); healthy controls: 21 women and 14 men, mean age 37 years (18-67). Disease activity was graded for CD according to the CD activity index (CDAI), or for UC according to the Mayo-score\[28,29\]. At the time of blood collection, CD patients were treated as follows: no medical treatment \((n = 29)\), 5-ASA \((n = 4)\), steroids \((n = 8)\), antibiotics \((n = 3)\), infliximab \((n = 4)\), or immunomodulators such as 6-mercaptopurine or azathioprine \((6\text{-MP}/aza, n = 47)\), or methotrexate \((n = 20)\). Some patients had combined medication: infliximab + methotrexate \((n = 1)\), steroids + methotrexate \((n = 4)\), steroids + 6-MP/aza \((n = 3)\), infliximab + 6-MP/aza \((n = 2)\). CDAI ranged between 75 and 380 (mean: 138 points). UC patients were treated as follows: no medical treatment \((n = 9)\), 5-ASA \((n = 15)\), steroids \((n = 6)\), immunomodulators 6-MP/aza \((n = 20)\), or tacrolimus \((n = 2)\). Some of these patients had combined medication: 5-ASA + 6-MP/aza \((n = 4)\), steroids + 6-MP/aza \((n = 3)\). CD patients were grouped according to the Montreal classification\[31\] into a UC-like (purely inflammatory, non-stenosing, non-fistulizing), a stenosing, or a fistulizing phenotype. The latter two are summarized as complicated disease phenotype.

#### Mycobacteria, yeast and mannan

Mycobacterial strains used in this study were \textit{M avium}, \textit{M smegmatis}, \textit{M chelonei}, MAP (all strains are patients’ isolates and property of the Institute of Infectious Diseases, University of Bern), and BCG (commercial vaccination strain). Strains were grown in Middlebrook 7H9 medium (Difco\textsuperscript{TM} BBL\textsuperscript{TM}, BD Biosciences, San Jose, CA, USA) with 10% OADC enrichment (Difco\textsuperscript{TM} BBL\textsuperscript{TM}, BD Biosciences), 1 mg/mL casein peptone (Merck, Glattbrugg, Switzerland) and 0.5% glycerin, and were collected during logarithmic growth phase. For MAP cultures, growth medium was supplemented with 2 mg/mL Mycobactin J (Synbiotics Europe SAS, Munich, Germany). Bacteria were heat-inactivated for 1 h at 80°C and then lysed in the presence of trypsin inhibitor and PMSF (Sigma, Buchs, Switzerland) by needle sonication (2 \(\times\) 1 min. with 100 watts, on ice). The lysate was cleared by centrifugation and protein concentration determined.
Mannose-capped lipoarabinomannan (ManLAM) from M tuberculosis and (non-mannose-capped) phospho-myoinositol-capped LAM (PILAM, subsequently termed LAM) were kindly provided by J. Belisle, Colorado State University. S cerevisiae Vita Gold was obtained from Deutsche Hefewerke, Nürnberg, Germany. The yeast was grown in 5% yeast peptone D-glucose (YPD, Sigma) and harvested during logarithmic growth. Mannan was extracted according to Kocourek and Baloul[34]. Briefly, cells were resuspended in 20 mmol/L Na-citrate buffer (pH 7.0) and autoclaved for 3 h at 125℃. The supernatant was cleared by centrifugation and the pellet resuspended in Na-citrate buffer (1.5 × initial volume). After centrifugation, the supernatants were pooled and cleared from residual debris by centrifugation. The mannans were complexed with equal volumes of Fehling’s solutions (Fehling I: 6.93 g CuSO₄·5 H₂O/100 mL in H₂O; Fehling II: 34.6 g Cu₂H₂KNaO₄·4H₂O + 12 g NaOH/100 mL in H₂O) and pelleted by centrifugation. The pellet was resolved in 3 mol/L HCl before precipitating the mannans off the CuSO₄ complex with a methanol/acidic solution (8:1 v/v) while stirring. The precipitate was pelleted and washed repeatedly with methanol/acidic solution (8:1 v/v until the supernatant was colorless and clear and finally washed twice with methanol alone and dried in a desiccator at 4℃.

**ELISA**

ELISA was performed in Nunc-Immuno™ Maxisorp 96-well plates (Nunc, Wiesbaden, Germany). For mycobacteria-specific ELISA, 50 µL of mycobacterial lysates, purified LAM or ManLAM at 5 µg/mL PBS was added per well and dried over night at 37℃. The next day plates were blocked with 1% skim milk in PBS. For ASCA, tetanus toxoid (TT) or galanthus nivalis lectin (GNL)-binding ELISA, plates were coated with the respective antigen at 0.25 µg/mL carbonate-bicarbonate (25 mmol/L Na₂CO₃, 25 mmol/L NaHCO₃) coating buffer pH 9.6, overnight at 4℃. On the next day, plates were blocked with PBS + 0.5% BSA (PBS-BSA). Plates were washed and patients’ sera added 1/500 (mycobacteria) or 1/1000 (ASCA, TT) PBS-BSA. Plates were incubated 1.5 h room temperature (mycobacteria) or overnight at 4℃ (ASCA, TT). For the GNL-binding ELISA, biotinylated GNL (Vector Laboratories, Burlingame, CA, USA) was added at 5 µg/mL to the blocked plates. Plates were washed with PBS-BSA + 0.05% Tween 20 (PBST-BSA). For ASCA and TT ELISA peroxidase-coupled anti-human IgG (Sigma) was added 1/5000 in PBST-BSA and plates incubated for 1 h at room temperature. The plates incubated with biotinylated GNL were further incubated with peroxidase-coupled streptavidin (BD Biosciences). After washing ELISA was developed with TMB substrate (Sigma) for 15-30 min. in the dark. Reaction was stopped with 0.5 mol/L sulphuric acid and plates read at 450 nm. A cut-off value discriminating between negativity and positivity for anti-mycobacterial IgG was defined using the average extinction values for the healthy population (without the clearly positive individuals as defined by A₉₀ ≥ 0.3) and addition of 3 standard deviations. To determine the MBL oligomer concentration sera were diluted 1/100 and assessed in an MBL-oligomer ELISA kit according to the manufacturer’s instructions (The Antibodyshop, Gentofte, Denmark).

**Affinity purification of ASCA**

S cerevisiae mannan was separated on an 8% polyacrylamide gel. The separated mannan was transferred to Hybond-ECL nitrocellulose membrane (Amersham GE HealthCare Europe, Otelfingen, Switzerland) and the ASCA-reactive material was localized by Western blot on a small section of the membrane. The ASCA-reactive region of the remaining membrane was cut into 8 equal pieces and the pieces blocked in TBS + 2% BSA.

Eight highly ASCA-positive sera from Crohn’s patients were diluted 1/5 in TBS + 2% BSA and incubated with one piece of the membrane overnight at 4℃, on a rocking platform. After incubation the membranes were thoroughly washed with TBS and 1× with H₂O and bound antibodies eluted with 0.2 mol/L glycine, pH 2.8. The eluate was neutralized with Tris-base pH 8.0 and the eluted antibodies stabilized with 0.1% BSA.

**Affinity purification of anti-M smegmatis antibodies**

1 cm x 1 cm pieces of M smegmatis lysate and sequentially incubated overnight at 4℃ and for 2 h at rt. Membranes were rinsed and blocked in TBS + 2% BSA. Incubation with sera and elution of bound antibodies was performed as described above.

**Immunization of mice**

C57BL/6 mice were reared in individually ventilated cages (IVC) under specified pathogen-free conditions. Housing and experimental procedures were in accordance with the European regulations on animal experimentation (FELASA). BCG culture was adjusted to a density of A₆₀₀ = 0.5 MacFarland (10⁶ cells per mL) with 0.9% sterile NaCl and 100 µL (10⁵ viable bacteria) injected into the tail vein per mouse. Mice were 8 wk old and sex-matched. Viability of the mycobacteria was confirmed by reculturing the remaining bacterial suspension. Four weeks after infection serum was prepared from whole blood samples after clipping the tail tips. By week 5, the mice were boosted by iv with the same amount of mycobacteria and sera collected 4 and 13 wk later. Anti-BCG and ASCA IgM and IgG were determined by ELISA. To that end, sera were diluted 1/250 and anti mouse IgM and IgG HRP antibodies (Sigma) 1/1000 and 1/500, respectively.

**Statistical analysis**

Raw data were imported into a statistical package program (STATA Version 9.0, Texas). Results of numerical data are presented as mean ± SE. Categorical data are summarized as the percentage of the group total. Two-sided Fisher's exact test (n < 20) or the Chi square test (n ≥ 20) was used to explore associations of
categorical data in 2 independent groups. The Wilcoxon rank sum test was used to explore associations of numerical data in 2 independent groups. A $P < 0.05$ was considered statistically significant. Associations between numerical data were evaluated using the Spearman rank correlation coefficient.

**RESULTS**

The dominant ASCA epitope terminal $\alpha$-1,3 linked mannose is differentially present in lysates of different mycobacterial species

Phosphopeptidomannan with $\alpha$-1,3 mannose ($\alpha$-1,2 mannose $\alpha$-1,2 mannose) ($\alpha$-1,1) sugar residues represent a prominent epitope recognized by ASCA from CD patients\cite{16,17}. The snowdrop lectin *Galanthus nivalis* agglutinin or lectin (GNL) has been shown to have high specificity for such terminal mannose sugar residues\cite{16,17} and has been used to detect ASCA epitopes from microorganisms directly or in infected tissue\cite{16,18}. Marked binding was further observed with lysates from BCG and MAP followed by *M avium* and *M smegmatis*. While no binding was observed to purified LAM, GNL strongly bound to ManLAM (Figure 1).

**Figure 1** Reactivity of *Galanthus nivalis* lectin (GNL) with mycobacterial lysates and purified LAM or ManLAM. ELISA plates were coated with yeast mannan, mycobacterial lysates, purified LAM or ManLAM or coating buffer alone without antigen (-), and binding of biotinylated GNL to these antigens assessed by further incubation with peroxidase-coupled streptavidin followed by TMB substrate reaction. Shown are mean values ± SE of 3 individual experiments.

*Higher frequencies of anti-mycobacterial IgG-positive individuals among patients with IBD compared to healthy controls*

$A_{450}$ extinction values of the anti-mycobacterial ELISA obtained with sera from the healthy population were used to define cut-off values for anti-mycobacterial IgG as described in the materials and methods section. According to these cut-off levels, between 13% (anti-LAM) and 45% (anti-*M smegmatis*) of CD patients were designated anti-mycobacterial IgG positive while in the group of healthy controls only 0 (anti-ManLAM) to 6% (anti-*M chelonae*, anti-MAP) were positive (Figure 2). Between 2% (anti-LAM) and 36% (anti-BCG) of UC patients were anti-mycobacterial IgG positive. Despite similar age groups, only one of the 35 healthy volunteers was tested positive for anti-BCG IgG while 42% of CD patients and 36% of UC patients were above the cut-off. Furthermore, while none of the healthy controls reached positivity for anti-ManLAM IgG, 19% of CD and 11% of UC patients did. Using the Chi-square test, differences in frequencies of positive individuals between the group of CD patients and healthy controls were significant for IgG against all mycobacterial lysates tested and purified ManLAM. Differences between UC patients and controls were significant for *M chelonae*, BCG and ManLAM. Finally, compared to UC patients, CD patients were significantly more frequently positive for anti-*M avium* and anti-*M smegmatis* IgG (Figure 2).

**Anti-mycobacterial IgG correlate with ASCA**

To test whether seroreactivity against mycobacterial antigens in general and mycobacterial mannans in particular may correlate with that against yeast mannans, we grouped CD patients into ASCA-negative and ASCA-positive categories. ASCA-positive CD patients showed the highest frequencies of seropositivities against all mycobacterial antigens tested. Between 21% (anti-LAM) and 65% (anti-*M smegmatis*) of ASCA-positive CD patients were anti-mycobacterial IgG positive, while only 5% (anti-LAM) to 29% (anti-*M smegmatis*) of ASCA-negative CD patients had anti-mycobacterial IgG titers above the cut-off (Figure 3A). Compared to healthy controls, even the proportion of anti-LAM-positive individuals was significantly elevated in ASCA-positive CD patients (21% vs 4%). In addition, ASCA-positive CD patients were also significantly more frequently positive for IgG against *M smegmatis*, *M chelonae*, BCG, MAP and purified ManLAM when compared to ASCA-negative CD patients. On the other hand, ASCA-negative CD patients were significantly more frequently positive than healthy controls only for anti-*M smegmatis* IgG. When ASCA-positive CD patients were further grouped according to the levels of their ASCA titers, we observed a weak trend of increasing titers of IgG against mycobacterial lysates with increasing ASCA titers (data not shown). In contrast, seropositivity against purified LAM or ManLAM was almost exclusively and strongly elevated in the highest ASCA-positive subgroup. While less than 20% of ASCA-negative, or weakly positive CD patients were positive for anti-LAM
and anti-ManLAM IgG, 50% and 70% of the highest ASCA-positive CD patients were positive for anti-LAM ($P \leq 0.0362$, vs ASCA-low or ASCA-negative patients) and anti-ManLAM IgG ($P \leq 0.0047$, vs ASCA-low or ASCA-negative patients; Figure 3B), respectively. To better visualize the extent of correlations between anti-mycobacterial IgG and ASCA, we plotted the extinction values obtained in the mycobacterial ELISA against those of the ASCA ELISA (Figure 4). All correlations were highly significant and ASCA correlated best with
IgG against *M. smegmatis* (*r* = 0.64, *P* < 0.0001), followed by anti-BCG and anti-ManLAM IgG (*r* = 0.53, *P* < 0.0001).

**ASCA-positivity and MBL-deficiency synergistically associate with anti-mycobacterial IgG**

Since deficiency for MBL associates with the ASCA-positive subgroup of CD patients 27, 28 we asked whether deficiency for MBL might also associate with elevated levels of anti-mycobacterial IgG. We found that similar to ASCA-positivity, MBL-deficiency was associated with higher proportions of anti-mycobacterial IgG-positive individuals for some but not all mycobacterial antigens tested (Figure 5), intriguingly, MBL-negativity synergistically contributed to increased frequencies of anti-mycobacterial IgG-positive individuals in the ASCA-positive/MBL-negative as compared to the ASCA-/MBL-double positive subgroup of CD patients. The synergistic effect that MBL-negativity and ASCA-positivity had regarding increased frequencies of anti-mycobacterial IgG-positive individuals was most obvious when the ASCA-positive/MBL-negative group was compared with the ASCA-negative/MBL-positive group of CD patients: between 2.7-fold (from 13.0% to 35.3% for anti-*M. chelonae*) and 7.4-fold (from 8.7% to 64.7% for anti-*M. avium*) increased proportion of anti-mycobacterial IgG-positive individuals.

**Anti-mycobacterial antibodies bind to yeast mannan**

To determine potential cross-reactivities between anti-mycobacterial antibodies and ASCA, we affinity-purified eight highly ASCA-positive sera from CD patients on yeast mannan and compared original sera with the corresponding affinity purified antibodies for reactivity with yeast mannan (positive control), mycobacterial lysates and purified LAM and ManLAM. A tetanus toxoid-specific ELISA confirmed that affinity-purified serum antibodies were virtually free of contaminating, non-yeast mannan-specific IgG. Affinity-purified ASCA from individual patients showed individual reactivity patterns. While three of eight patients’ ASCA reacted markedly with all mycobacterial preparations (patients No. 1, 4 and 7), 5 patients showed restricted reactivity (Figure 6A). On average, affinity-purified antibodies showed the highest degree of reactivity with LAM (73% ± 34% of original sera) and lowest with MAP lysate (35% ± 16% of original sera). The same 8 highly ASCA-positive sera were also affinity purified on *M. smegmatis* lysate and assessed for reactivity with yeast mannan and tetanus toxoid. All sera showed strongly reduced reactivity with yeast mannan after affinity-purification (Figure 6B).

**Infection of mice with BCG leads to transient production of immunoglobulins that cross-react with *S. cerevisiae* mannan**

To test, whether experimentally induced antibodies...
against mycobacteria may cross-react with \textit{S. cerevisiae} mannan, we infected mice with $10^7$ live BCG, followed by an equal booster injection after 5 wk. Figure 7 shows that infection with BCG induced marked levels of BCG-lysate-specific IgM and moderate levels of specific IgG after 4 wk and up to 13 wk after the booster injection. Intriguingly, BCG infection led to transiently elevated titers of ASCA IgM and IgG which declined to background or near background levels within 2 mo post booster injection.

**Higher frequency of anti-mycobacterial IgG-positive individuals in the subgroup of CD patients with fistulizing disease**

In order to address the possible significance of anti-mycobacterial IgG in CD, patients were grouped according to the Montreal classification into UC-like (purely inflammatory), or stenosing or fistulizing disease phenotype (the latter two summarized as complicated disease). Sera from these groups of CD patients were compared for anti-mycobacterial IgG and ASCA by ELISA. ASCA were included because earlier reports indicate that ASCA associate with complicated disease phenotype (the latter two summarized as complicated disease). There was a weak tendency for patients with complicated disease to more often express anti-mycobacterial IgG compared to patients with UC-like disease (data not shown). On the other hand, a markedly higher frequency of patients expressed antibodies against mycobacterial lysates, but not purified LAM or ManLAM, when only the subgroup of patients with fistulizing disease was compared with UC-like disease (Figure 8). These differences were statistically significant for anti-\textit{M. avium} ($P = 0.024$), anti-\textit{M. smegmatis} ($P = 0.004$) and anti-MAP IgG ($P = 0.045$). In contrast, the proportion of patients with stenoses expressing IgG against mycobacterial lysates was not markedly elevated compared to those with UC-like disease. Both subgroups with complicated disease had a markedly higher proportion of individuals being positive for ASCA compared to UC-like disease. Here, the difference was statistically significant for the subgroup with stenoses compared to UC-like disease ($P = 0.008$, Figure 8). Finally, compared to patients with UC-like disease or stenoses, patients with fistulizing disease on average showed more individual seroreactivities per patient against the mycobacterial antigens tested ($2.5 \text{ vs } 1.5/1.6$, $P = 0.050$, Figure 8B).

**DISCUSSION**

In the present study we have chosen a number of mycobacterial strains with more or less ubiquitous occurrence and - with the exception of BCG-originally isolated from patients suffering from mycobacterial infections to serve as antigens for ELISA with IBD patients’ and control sera in order to assess a possible relationship between anti-mycobacterial antibodies and ASCA in CD. In addition, we used purified lipoarabinomannans with or without a richly mannosylated arabinose moiety (LAM from \textit{M. smegmatis} and ManLAM from \textit{M. tuberculosis}, respectively) because of similar oligomannose side chains as found in mannan from \textit{S. cerevisiae}, the specific antigen for ASCA. In agreement with published data\cite{19,20}, terminal $\alpha$-1,3 linked mannose which is part of the dominant ASCA epitope was present at different extents in our mycobacterial preparations. In particular this epitope was strongly present in ManLAM from \textit{M. tuberculosis} while it was completely absent in LAM from \textit{M. smegmatis}. In accordance with that, \textit{M. smegmatis} lysate showed the weakest binding to GNL among all lysates tested. With the exception of non-mannose-capped LAM, we found significantly higher proportions of anti-mycobacterial IgG-positive individuals in CD compared to the healthy control group. We focused on IgG because initial screenings generally showed much lower levels...
or absence of specific IgA (data not shown). Previous studies have mainly focused on seroreactivities against selected antigens from MAP\cite{22,36,37}. Naser et al. have observed that a large proportion of CD, but neither UC patients nor controls had antibodies reactive with two recombinant antigens (75% and 89%, respectively) from their MAP genomic library\cite{22}. In contrast, less than 30% of our CD patients showed broad MAP-specific seroreactivity. Our results are comparable to those described by Polymeros et al., who found that sera of 42% of their small cohort of CD patients reacted with one or more MAP-derived peptides\cite{37}. In our study UC patients showed - although less marked than CD patients - enhanced seroreactivity compared to healthy controls, in particular against some of the crude lysates. This is not surprising as enhanced seroreactivities against unusual,
untypical or commensal antigens is not only a hallmark of CD but of IBD in general[10,11].

While Polymeros et al.[37] addressed a potential self-cross-reactive nature of anti-MAP antibodies in CD patients, in the present study, we were focusing on potential cross reactivities of CD patients’ anti-mycobacterial antibodies with mannan from S. cerevisiae. We could clearly demonstrate that the frequent seroreactivity against cell wall mannan from S. cerevisiae (ASCA-positivity) in CD patients significantly correlated with seroreactivity not only against MAP, but also against antigens from all other mycobacteria that we tested. Importantly, correlations were best for M. smegmatis lysate and purified ManLAM. The M. tuberculosis-derived ManLAM used in the present study is characterized by highly mannosylated arabinose (similar to the ManLAM from BCG). While Stokes et al[40] have also observed binding of GNL to M. tuberculosis[39], ManLAM from M. tuberculosis should theoretically not contain the GNL-reactive α-1,3 linked mannose residues according to biochemical analyses[39,40]. On the other hand, the phospho-myoinositol-capped LAM of M. smegmatis has no rich mannose-cap on its arabinose moiety[41,42] and shows no binding to GNL. Collectively, the good correlations between seroreactivities against S. cerevisiae mannan and mycobacterial antigens shown in the present study do not solely depend on the presence of either the terminal α-1,3 linked mannose residues or a rich mannose-cap. M. chelonae is one of the rare mycobacterial strains with truly uncapped arabinose moieties on their LAM, also termed AraLAM or CheLAM[19]. On the other hand, this strain’s lysate is very well recognized by GNL which may explain the increased reactivity with ASCA-positive CD patients’ IgG.

Even in the absence of the dominant ASCA epitope terminal α-1,3 linked mannose there was a good and significant correlation of ASCA-positivity with seroreactivities against purified LAM, most strikingly if only those patients with high ASCA titters were considered.

Because in our cohort of CD patients MBL-deficiency associates with positivity for ASCA[27,28], we wondered whether generation of anti-mycobacterial antibodies may associate with this deficiency as well, and whether such an association may depend on certain strains with differential presence of the terminal α-1,3 linked mannose motive and/or distinctly capped LAM. Indeed, we found an association of MBL deficiency with the prevalence of anti-mycobacterial antibodies. However, this association was not confined to strains with mannose-capped LAM and was not apparent for antibodies to BCG with rich mannose caps or M. chelonae with the best binding to GNL apart from yeast mannan. It is known that MBL facilitates the entry of mycobacteria into host cells[43,44]. Therefore, in MBL-deficient persons, a stronger systemic immune response may be expected since the mycobacterial (cell wall) antigens are not rapidly eliminated by phagocytes.

The strong association of anti-mycobacterial antibodies with high ASCA titters with the observed synergistic effect of MBL-deficiency may be explained in different ways. First, it may be that these individuals have, due to a genetic predisposition, an increased reactivity to environmental (mannosylated) antigens. Alternatively there could be a true antibody cross-reactivity between the mannan antigens of S. cerevisiae and mycobacteria. To address this question we affinity-purified ASCA from highly ASCA-positive CD patients and could show that these purified antibodies exert variable degrees of cross-reactivities between yeast mannan and mycobacterial lysates or purified (Man) LAM. On the other hand, affinity-purification of antibodies against M. smegmatis lysate - the lysate that showed the highest degree of binding with affinity-purified ASCA-led to strongly reduced reactivity with yeast mannan compared to the original serum. Thus, while in some patients anti-mycobacterial IgG are mainly due to cross-reactive ASCA, in others, ASCA and anti-mycobacterial antibodies have separate specificities. In either case, the fact that affinity-purified anti-M. smegmatis IgG only show weak or no reactivity with yeast mannan suggests that such cross-reactive antibodies only account for a minor fraction of total ASCA. This constellation makes it very unlikely that mycobacterial antigens play a role in the induction of ASCA. Since our purified LAM does not contain the terminal α-1,3 mannose motif but shows a high degree of reactivity with affinity-purified ASCA, it has to be discussed whether the spectrum of antigens recognized by CD patients’ ASCA goes beyond terminal α-1,3 linked mannose side chains and may encompass other antigens such as peptide epitopes from the protein part of the mannan. In this context, it is of interest that according to a recent study CD patients contain antibodies that cross-react to an individual extent between β2-glycoprotein I and yeast phosphopeptidomannans[45].

Our experimental mouse model was to test whether mycobacterial infection is theoretically able to trigger induction of ASCA. For infection of mice we have chosen BCG because it is a commercial vaccination strain and is well established for infection and immunization studies in mice. Our finding that ASCA were only transiently expressed and declined by 2 mo post-booster
infection while BCG-specific IgM and IgG remained high, supports our conclusion from the results of the affinity-purification study that an immune reactivity to mycobacterial antigens is unlikely to trigger the induction of a stable phenotype of ASCA-positivity.

We were interested whether seroreactivity to mycobacterial antigens may associate with a certain disease phenotype. We observed that more patients with complicated disease (stenosing or fistulizing) expressed antibodies against mycobacterial lysates compared to patients with purely inflammatory disease. Since we observed strong associations of anti-mycobacterial antibodies with ASCA-positivity and ASCA have been shown to associate with complicated disease, our findings were not unexpected. However, the fact that this trend was clearly confined to the subgroup with fistulizing disease, while ASCA-positivity even better associated with stenoses, is surprising. Possibly, ingested mycobacteria more easily gain access to systemic immune compartments for priming if the bowel wall is transmurally damaged. Regarding ASCA, the origin of this unusual immune response is still unknown and whatever it is may actually be involved in the progression to more severe CD phenotypes. Finally, patients with fistulizing disease more frequently showed seroreactivities against multiple mycobacterial antigens compared to those with UC-like disease. This observation fits to data published by others showing a more severe phenotype of disease in patients with increasing numbers of seroreactivities to various intestinal (commensal) antigens.

In conclusion, we were able to demonstrate that ASCA-positive patients had significantly more immune reactivities to mycobacterial antigens. In a subgroup of ASCA-positive CD patients, anti-mycobacterial immunoglobulins at least partially represent cross-reactive ASCA, while in others there seem to be separate ASCA and anti-mycobacterial antibodies that do not cross-react. Furthermore, purified anti-\textit{M. smegmatis} IgG showed low or no binding to yeast mannan. Therefore, we postulate that our results reflect more the predisposition of CD patients to develop increased immune reactivities to various ubiquitous antigens in general and mannosylated antigens in particular, rather than a role of mycobacteria in the induction of ASCA.

**ACKNOWLEDGMENTS**

The authors would like to thank Martin Sauter for technical assistance with purification and characterization of \textit{S. cerevisiae} mannan, Beatrice Flogerzi for lab-technical assistance, Irène Garcia for help with mycobacterial infection, and Linda Bolzern for carefully reading the manuscript.
A subgroup of patients with Crohn's disease (CD) develops antibodies against Saccharomyces cerevisiae cell wall mannan (ASCA). The mechanism of ASCA generation is still unclear. It is possible that some opportunistic or pathogenic infectious microorganism may be the initial inducer of this unusual antibody response because similar (cell wall) mannans also occur in other microorganisms such as mycobacteria with their lipopiraminomannan (LAM).

**Research frontiers**

It has been shown that the opportunistic pathogen Candida albicans is able to experimentally induce ASCA (Standaert-Vitse et al 2006) and we have shown that Crohn's patients' ASCA cross-react with cell wall mannans from different yeast strains including C albicans (Schaffer et al 2007). In contrast to yeast, there exists a highly controversial debate on a possible role for mycobacteria in the etiopathogenesis of Crohn's disease.

**Innovations and breakthroughs**

Our study clearly shows that (1) mycobacterial infection is very unlikely the origin of ASCA since anti-mycobacterial antibodies and ASCA in an individual patient are either non-overlapping or the former only represents a minor part of all antibodies recognizing yeast mannans; and (2) the correlation of antibodies against mycobacterial antigens with those against yeast mannans reflects - apart from pure cross-reactivity in some patients - increased predisposition for adaptive immune responses against ubiquitous antigens, especially observed in patients with a severe disease phenotype.

**Applications**

The findings in the present study represent an important basis for further research on the role of antimicrobial immune responses in the pathogenesis of Crohn's disease. Furthermore, antimicrobial antibody patterns may define distinct subgroups of Crohn's patients requiring individual treatment approaches.

**Peer review**

Similar to ASCA, seroreactivity against mycobacteria may define CD patients with complicated disease and a predisposition for immune responses against ubiquitous antigens. Mycobacterial infection does not likely play a role in ASCA induction.

**REFERENCES**

1. Duchmann R, Kaiser I, Herrmann E, Mayet W, Ewe K, Meyer zum Buschenfelde KH. Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease (IBD). Clin Exp Immunol 1995; 102: 448-455
2. Sartor RB. Targeting enteric bacteria in treatment of inflammatory bowel diseases: why, how, and when. Curr Opin Gastroenterol 2003; 19: 358-365
3. Greenstein RJ. Is Crohn's disease caused by a mycobacterium? Comparisons with leprosy, tuberculosis, and Candida albicans. Lancet Infect Dis 2003; 3: 507-514
4. Sandborn WJ. Serologic markers in inflammatory bowel disease: state of the art. Rev Gastroenterol Disord 2004; 4: 167-174
5. Reumaux D, Sendid B, Poulain D, Duthilleul P, Dewit O, Colombel JF. Serological markers in inflammatory bowel diseases. Best Pract Res Clin Gastroenterol 2005; 17: 19-35
6. Main J, McKenzie H, Yeaman GR, Kerr MA, Robson D, Pennington CR, Parratt D. Antibody to Saccharomyces cerevisiae (bakers' yeast) in Crohn's disease. BMJ 1988; 297: 1105-1106
7. Seibold F. ASCA: genetic marker, predictor of disease, or marker of a response to an environmental antigen? Gut 2005; 54: 1212-1213
8. Seibold F, Stich O, Hufnagl R, Kamil S, Scheurlen M. Anti-Saccharomyces cerevisiae antibodies in inflammatory bowel disease: a family study. Scand J Gastroenterol 2001; 36: 196-201
9. Sendid B, Colombel JF, Jacquinot PM, Faille C, Fruit J, Cortot A, Lucidarme D, Camus D, Poulain D. Specific antibody response to oligomannosidic epitopes in Crohn's disease. Clin Diagn Lab Immunol 1996; 3: 219-226
10. Wheeler RT, Kupiec M, Magnelli P, Abejon C, Fink GR. A Saccharomyces cerevisiae mutant with increased virulence. Proc Natl Acad Sci USA 2003; 100: 2766-2770
11. Standaert-Vitse A, Jouault T, Vandewalle P, Mille C, Schedik M, Sendid B, Mallet JM, Colombel JF, Poulin D. Candida albicans is an immunogen for anti-Saccharomyces cerevisiae antibody markers of Crohn's disease. Gastroenterology 2006; 130: 1764-1775
12. Schaffer T, Muller S, Fluggeri B, Seibold-Schmid B, Schoepfer AM, Seibold F. Anti-Saccharomyces cerevisiae mannan antibodies (ASCA) of Crohn's patients crossreact with mannan from other yeast strains, and murine ASCA IgM can be experimentally induced with Candida albicans. Inflamm Bowel Dis 2007; 13: 1339-1346
13. Young M, Davies MJ, Bailey D, Gradwell MJ, Smestad-Paulsen B, Wold JK, Barnes RM, Hounsell EF. Characterization of oligosaccharides from an antigenic mannan of Saccharomyces cerevisiae. Glycoccon 1998; 15: 815-822
14. Chatterjee D. The mycobacterial cell wall: structure, biosynthesis and sites of drug action. Curr Opin Chem Biol 2003; 7: 579-588
15. Nigou J, Gilleron M, Puzo G. Lipoarabinomannans: from structure to biosynthesis. Biochimie 2003; 85: 153-166
16. Shibuya N, Goldstein IJ, Van Damme EJ, Peumans WJ. Binding properties of a mannose-specific lectin from the snowdrop (Galanthus nivalis) bulb. J Biol Chem 1988; 263: 729-734
17. Kaku H, Goldstein IJ, Oscarson S. Interactions of five D-mannose-specific lectins with a series of synthetic branched trisaccharides. Carbohydr Res 1991; 213: 109-116
18. Guerardel Y, Maes E, Elss E, Leroy Y, Timmerman P, Besra GS, Locht C, Streeker G, Kremer L. Structural study of lipomannan and lipopirabinomannan from Mycobacterium chelonae. Presence of unusual components with alpha 1,3-mannopyranoside side chains. J Biol Chem 2002; 277: 30635-30648
19. Michell SL, Whelan AO, Wheeler PR, Panico M, Easton RL, Etienne AT, Haslam SM, Dell A, Morris HR, Reason AJ, Herrmann JL, Young BJ, Hewinson RG. The MPB83 antigen from Mycobacterium bovis contains O-linked mannosyl and (1--3)-mannobioside moieties. J Biol Chem 2003; 278: 16423-16432
20. Mpofo CM, Campbell BJ, Subramanian S, Marshall-Clarke S, Hart CA, Roberts CL, McGoldrick A, Edwards SW, Rhodes JM. Microbial mannan inhibits bacterial killing by macrophages: a possible pathogenic mechanism for Crohn's disease. Gastroenterology 2007; 133: 1487-1498
21. Shafran I, Piromalli C, Decker JW, Sandoval J, Naser SA, El-Zaatari FA. Seroreactivities against Saccharomyces cerevisiae and Mycobacterium avium subsp. paratuberculosis p35 and p36 antigens in Crohn's disease patients. Dig Dis Sci 2002; 47: 2079-2081
22. Naser SA, Halten K, Graham DY, El-Zaatari FA. Specific seroreactivity of Crohn's disease patients against p35 and p36 antigens of M. avium subsp. paratuberculosis. Vet Microbiol 2000; 77: 497-504
23. Turner MW. Mannose-binding lectin (MBL) in health and disease. Immunology 1999; 199: 227-339
24. Vasta GR, Quesenberry M, Ahmed H, O'Leary N. C-type lectins and galectins mediate innate and adaptive immune functions: their roles in the complement activation pathway. Dev Comp Immunol 1999; 23: 401-420
25. Jack DL, Klein NJ, Turner MW. Mannose-binding lectin: targeting the microbial world for complement attack and opsonophagocytosis. Immunol Rev 2001; 180: 86-99
26. Dommett RM, Klein NJ, Turner MW. Mannose-binding lectin in innate immunity: past, present and future. Tissue Antigens 2006; 68: 193-209
27. Seibold F, Konrad A, Fluggeri B, Seibold-Schmid B, Arni S, Juliger S, Kun JF. Genetic variants of the mannann-binding lectin are associated with immune reactivity to mannans in...
Crohn's disease. *Gastroenterology* 2004; 127: 1076-1084

28 Seibold F, Boldt AB, Seibold-Schmid B, Schoepfer AM, Flögerzi B, Muller S, Kun JF. Deficiency for mannan-binding lectin is associated with antibodies to Saccharomyces cerevisiae in patients with Crohn's disease and their relatives. *Gut* 2007; 56: 152.

29 Best WR, Becktel JM, Singleton JW, Kern F Jr. Development of a Crohn's disease activity index. National Cooperative Crohn's Disease Study. *Gastroenterology* 1976; 70: 439-444.

30 Schroeder KW, Tremaine WJ, Ilstrup DM. Coated oral 5-aminosalicylic acid therapy for mildly to moderately active ulcerative colitis. A randomized study. *N Engl J Med* 1987; 317: 1625-1629.

31 Satsangi J, Silverberg MS, Vermeire S, Colombel JF. The Montreal classification of inflammatory bowel disease: controversies, consensus, and implications. *Gut* 2006; 55: 749-753.

32 Kocourek J, Ballou CE. Method for fingerprinting yeast cell wall mannans. *J Bacteriol* 1969; 100: 1175-1181.

33 Vasiliauskas EA, Kam LY, Karp LC, Gaiennie J, Yang H, Targan SR. Marker antibody expression stratifies Crohn's disease into immunologically homogeneous subgroups with distinct clinical characteristics. *Gut* 2000; 47: 487-496.

34 Dassopoulos T, Frangakis C, Cruz-Corrales L, Talor MV, Burek CL, Datta L, Nouvet F, Bayless TM, Brant SR. Antibodies to saccharomyces cerevisiae in Crohn's disease: higher titers are associated with a greater frequency of mutant NOD2/CARD15 alleles and with a higher probability of complicated disease. *Inflamm Bowel Dis* 2007; 13: 143-151.

35 Forcione DG, Rosen MJ, Kisiel JB, Sands BE. Anti-Saccharomyces cerevisiae antibody (ASCA) positivity is associated with increased risk for early surgery in Crohn's disease. *Gut* 2004; 53: 1117-1122.

36 Olsen I, Wiker HG, Johnson E, Langeggen H, Reitan LJ. Elevated antibody responses in patients with Crohn's disease against a 14-kDa secreted protein purified from Mycobacterium avium subsp. paratuberculosis. *Scand J Immunol* 2001; 53: 198-203.

37 Polymeros D, Bogdanos DP, Day R, Arioli D, Vergani D, Forbes A. Does cross-reactivity between mycobacterium avium paratuberculosis and human intestinal antigens characterize Crohn's disease? *Gastroenterology* 2006; 131: 85-96.

38 Bossuyt X. Serologic markers in inflammatory bowel disease. *Clin Chim* 2006; 52: 171-181.

39 Stokes RW, Norris-Jones R, Brooks DE, Beveridge TJ, Doosee D, Thorson LM. The glycan-rich outer layer of the cell wall of Mycobacterium tuberculosis acts as an antiphagocytic capsule limiting the association of the bacterium with macrophages. *Infect Immun* 2004; 72: 5676-5686.

40 Chatterjee D, Lowell K, Rivoire B, McNeil MR, Brennan PJ. Lipooarabinomannan of Mycobacterium tuberculosis. Capping with mannosyl residues in some strains. *J Biol Chem* 1992; 267: 6234-6239.

41 Chatterjee D, Khoo KH. Mycobacterial lipooarabinomannan: an extraordinary lipoheteroglycan with profound physiological effects. *Glycobiology* 1998; 8: 113-120.

42 Vercellone A, Nigou J, Puzo G. Relationships between the structure and the roles of lipooarabinomannans and related glycoconjugates in tuberculosis pathogenesis. *Front Biosci* 1998; 3: e149-e163.

43 Soborg C, Madsen HO, Andersen AB, Lillebaek T, Kok-Jensen A, Garred P. Mannose-binding lectin polymorphisms in clinical tuberculosis. *J Infect Dis* 2003; 188: 777-782.

44 Bonar A, Chmiela M, Rudnicka W, Rozalska B. Mannose-binding lectin enhances the attachment and phagocytosis of mycobacteria in vitro. *Arch Immunol Ther Exp (Warsz)* 2005; 53: 437-441.

45 Krause I, Blank M, Cervera R, Font J, Matthias T, Pfeiffer S, Wies I, Fraser A, Shoenfeld Y. Cross-reactive epitopes on beta2-glycoprotein-I and Saccharomyces cerevisiae in patients with the antiphospholipid syndrome. *Ann N Y Acad Sci* 2007; 1108: 481-488.

46 Arnott ID, Landers C, Nimmo EJ, Drummond HE, Smith BK, Targan SR, Satsangi J. Sero-reactivity to microbial components in Crohn's disease is associated with disease severity and progression, but not NOD2/CARD15 genotype. *Am J Gastroenterol* 2004; 99: 2376-2384.