Anti–α-enolase Antibodies in Patients with Inflammatory Bowel Disease

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BACKGROUND: Patients with inflammatory bowel disease (IBD) carry autoantibodies such as perinuclear antineutrophil cytoplasmic antibodies (pANCA). α-Enolase has been proposed as a target antigen in IBD. We evaluated the prevalence and diagnostic value of anti–α-enolase antibodies in IBD and related disorders.

METHODS: We used a classic proteomic approach with extracts from granulocytes and pANCA-positive ulcerative colitis (UC) sera to confirm α-enolase as a target antigen. By means of Western blot analysis, we screened a cohort of 525 subjects for the presence of anti–α-enolase antibodies. We performed GeneArray experiments on RNA extracted from colonic mucosal biopsies from 35 IBD and 6 control patients.

RESULTS: We detected anti–α-enolase antibodies 49.0% of patients with UC, 50.0% of patients with Crohn’s disease, 30.5% of patients with primary sclerosing cholangitis, 37.8% of patients with autoimmune hepatitis, 34.0% of patients with ANCA-positive vasculitis, 31.0% of non-IBD gastrointestinal controls, and 8.5% of healthy controls. Gene array experiments showed a significant upregulation of α-enolase mRNA in colonic mucosal biopsies from patients with IBD, but not from controls. There was no association between the presence of pANCA and anti–α-enolase antibodies. Preabsorption with α-enolase did not eliminate the pANCA pattern on indirect immunofluorescence.

CONCLUSIONS: Anti–α-enolase antibodies are present in a substantial proportion of patients with IBD, patients with various inflammatory/autoimmune disorders, and non-IBD gastrointestinal controls. Therefore, anti–α-enolase antibodies are of limited diagnostic value for the diagnosis of IBD.

Ulcerative colitis (UC)5 and Crohn’s disease (CD), collectively known as inflammatory bowel disease (IBD), are chronic inflammatory disorders that affect the gastrointestinal tract (1). Although the cause is still unknown, it is believed that IBD is an immunologically mediated disorder in genetically predisposed individuals (2). One theory is that IBD results from an aberrant immune response to the intestinal flora, resulting in chronic inflammation of the gut (3). The immune response is characterized by the presence of different circulating antibodies. These antibodies are directed against microbial (e.g., anti-l2, anti–outer membrane protein C [anti-OmpC], anti–CBir1 flagellin [anti-CBir1], anti–Saccharomyces cerevisiae antibodies [ASCA]) and self (e.g., perinuclear antineutrophil cytoplasmic antibodies [pANCA]) antigens. pANCA are present in 50% to 80% of patients with UC and in a subgroup of patients with CD (5%–25%) (4–8). The origin of these antibodies and their target antigen is still unknown. Several proteins have been described as the potential target antigen of pANCA in UC (9). These candidates include nuclear proteins (histone H1, a 50-kDa myeloid cell–specific nuclear envelope protein, and HMG-1 and HMG-2, nonhistone chromosomal proteins), cytoplasmic proteins (α-enolase and catalase), and proteins present in the β- and alphafoophilic granules (lactoferrin, β-glucuronidase, lysozyme, elastase, cathepsin G, and bacterial/permeability-increasing [BPI] protein). None of these candidate antigens, however, has been proven to be the target antigen of pANCA in IBD.
In a search for the target antigen of pANCA in IBD, we studied differences in immune reactivity toward a neutrophil protein extract between serum from a pANCA-positive patient with UC and control serum. A strong and differential immune reactivity toward the 50-kDa α-enolase protein was detected in the patient with UC. This confirmed the findings of Roozendaal et al. (10), who described antibodies directed against α-enolase in 10% of 96 patients with UC and in 18% of 112 patients with CD. In the present study, we evaluated the importance of anti–α-enolase antibodies in patients with IBD. More specifically, we examined the prevalence and diagnostic value of anti–α-enolase antibodies in patients with IBD and other pANCA-related immune diseases. We also studied the expression of α-enolase mRNA in patients with IBD and in controls.

Materials and Methods

MATERIALS
We obtained buffy coat from the Blood Transfusion Centre of the Red Cross and the human promyelocytic leukemic HL60 cell line from American Type Culture Collection. We purchased recombinant glutathione S-transferase (GST)-tagged α-enolase from Abnova; polyvinylidene fluoride (PVDF) membranes, SDS-polyacrylamide gels (ExcelGel SDS homogeneous 12.5), immobilized pH gradient strips (Immobiline™ DryStrip, pH 3–10), and all specific solutions for protein electrophoresis from GE Healthcare Bio-Sciences AB; goat antihuman IgG from Sigma-Aldrich; peroxidase-conjugated rabbit anti-goat IgG from Dako A/S; and all chemical reagents from Sigma-Aldrich. Protein concentrations were determined by use of the Bio-Rad protein assay.

STUDY POPULATION
We studied a cohort of 525 subjects for the presence of anti–α-enolase antibodies. This included 100 patients with UC (female/male ratio 45/55, mean age 47 years, range 21–83 years), 100 patients with CD (female/male ratio 53/47, mean age 43 years, range 14–84 years), 105 healthy blood donors (HC) (female/male ratio 58/47, mean age 43 years, range 14–84 years), 105 gastrointestinal (GI) controls (43 with diverticulitis, 26 with culture-negative gastroenteritis/collitis, 10 Salmonella, 2 Clostridium, 1 Yersinia), 18 with culture-negative gastroenteritis, 6 with ischemic colitis, 2 with periappendicular abscess, 1 with chronic rectitis, 1 with gastroparesis, 1 with irritable bowel syndrome, 1 with chronic constipation, and 1 with postradiotherapy diarrhea (female/male ratio 51/49, mean age 61 years, range 22–99 years), 36 patients with primary sclerosing cholangitis (PSC) (female/male ratio 16/20, mean age 46 years, range 23–80 years), 37 patients with autoimmune hepatitis (AIH) (female/male ratio 27/10, mean age 61 years, range 27–94 years), and 47 patients with ANCA-positive vasculitis (30 with Wegener’s granulomatosis, 9 with microscopic polyangiitis, and 8 others) (female/male ratio 19/28, mean age 62 years, range 16–92 years).

The diagnoses of UC or CD (11), PSC (12), AIH (13), Wegener’s granulomatosis, and systemic vasculitis (14) were based on accepted clinical and endoscopic criteria. Serum and tissue samples were stored at −20 °C until use (15). Institutional review board approval was obtained from the Catholic University of Leuven, Belgium, and all individuals signed an informed consent.

INDIRECT IMMUNOFLUORESCENCE
ANCA was determined by indirect immunofluorescence (IIF) according to the manufacturer’s instructions, by means of substrates from Inova Diagnostics. Slides were incubated with sera at a 1:40 dilution and examined under UV using a Nikon Eclipse E400 microscope.

PROTEIN EXTRACTS
Granulocytes were isolated from peripheral blood buffy coat by density gradient centrifugation on Lymphoprep (Axis-Shield) and hypotonic lysis (140 mmol/L NH₄Cl) of the remaining erythrocytes. Human promyelocytic leukemic HL60 cells were grown and harvested after 3–4 weeks. To obtain protein extracts, both granulocyte sources were treated as described (16).

SDS-PAGE AND 2-DIMENSIONAL ELECTROPHORESIS
SDS-PAGE was performed according to Laemmli (17). Protein extracts were electrophoresed through a SDS-polyacrylamide gel on a Multiphor II electrophoresis system (GE Healthcare Bio-Sciences AB). For 2-dimensional gel electrophoresis, dry immobilized pH gradient polyacrylamide gel strips were rehydrated overnight with cell extract (after trichloroacetic acid precipitation and dissolution in rehydration solution). Subsequently, the strips underwent the first-dimension isoelectric focusing at 20 °C. Before the second-dimension run, the immobilized pH gradient strips were submerged in equilibration solution (SDS and iodoacetamide) and then drained for up to 10 min on a filter paper moistened with distilled water. Two equilibrated immobilized pH gradient strips were run si-
mutilaneously on ExcelGel™ 2-D SDS Homogenous 12.5, according to guidelines of the manufacturer. After finishing the polyacrylamide gel electrophoresis, the proteins underwent Western blot analysis or were visualized by Coomassie blue staining.

WESTERN BLOTTING
The proteins from SDS-PAGE and 2-dimensional gels were electroblotted onto a PVDF membrane with a semidry blotting apparatus. Subsequently, we incubated the membranes with 5% (wt/vol) bovine serum albumin at room temperature for 1 h to avoid nonspecific binding. Western blots were then incubated with patient or control sera (1:500) overnight. IgG binding was detected by goat antihuman IgG (1:5000) followed by peroxidase-conjugated rabbit antigoat IgG (1:5000). All antibodies were diluted in Tris-saline buffer (TSB) (10 mmol/L Tris, 150 mmol/L NaCl, and 0.1% [vol/vol] Triton X-100, pH 7.6). Before each transfer, an intermediate washing step was performed with three 10-min TSB washes. To detect positive protein–antibody interaction, 0.7 mmol/L 3,3′-diaminobenzidine-tetrahydrochloride dihydrate containing 0.01% (vol/vol) H2O2 was added as substrate. We quantified Western blot strips by use of a Hyrys densitometer. Antibody response was defined as the area under the peak corresponding to the specific band, identified by comparison with the molecular weight standard. The background was subtracted.

PROTEIN SEQUENCE ANALYSIS
Spot picking, trypsin digestion, and automated LC-MS analyses were run on an UltiMate Capillary/Nano LC System (LC Packings), with a Switchos Micro Column Switching module, on-line coupled to a Q-TOF mass spectrometer (nano-ESI-Q-TOF; Micromass) as described (18). LC-MS data were processed with the ProteinLynx automated routine and submitted to the Mascot MS/MS ions search engine with default parameters.

GENE ARRAY STUDY
We obtained colonic mucosal biopsies at routine colonoscopy from IBD and control patients. In patients with IBD, biopsies were taken from the inflamed area. Total RNA was extracted from the mucosa biopsy specimens with the RNeasy Mini Kit (Qiagen, Benelux B.V.), according to the manufacturer’s instructions. The isolated RNA was labeled and hybridized to an Affymetrix HGU133plus2.0 array. All steps were performed according to Affymetrix protocols. We performed statistical analysis of the raw data with the Bioconductor software package with the R environment. The procedure included the robust multichip average (RMA) method and the moderated t test (Limma) with a multiple testing correction (false discovery rate by Benjamin and Hochberg) (19, 20). A P value <0.05 was considered significant.

ABSORPTION STUDY
Absorption experiments were performed according to Seibold et al. (21). Strong pANCA-positive sera were absorbed with increasing amounts of the target antigen for 30 min, followed by centrifugation at 15000g for 10 min. The supernatants were tested by indirect immunofluorescence.

CRP DETERMINATION
We assessed inflammation by measuring serum C-reactive protein (CRP) concentrations, measured with a latex-enhanced immunoturbidimetric method (Tina quant; Roche Diagnostics) on a Modular P analyzer (Hitachi). The upper limit of normal serum CRP was 5 g/L.

Results
IDENTIFICATION OF α-ENOLASE AS A TARGET ANTIGEN OF AUTOANTIBODIES IN A PATIENT WITH IBD
In preliminary experiments, we confirmed α-enolase as a target antigen in IBD. We performed 1-dimensional gel electrophoresis of a cell extract prepared from isolated granulocytes or HL60 cells followed by Western blotting with serum obtained from a patient with UC atypical pANCA reactivity (titer 1:1280). This revealed a clear band at approximately 50 kDa, which was present when serum from the patient with UC was used but absent with serum from a healthy control individual. By means of Western blotting, performed after 2-dimensional gel electrophoresis with the same extracts and the same sera, we revealed several differential spots with molecular weights ranging between 30 and 66 kDa and an isoelectric point of 7. These spots were present when the blots were incubated with sera from the patient and absent in the control. The spots recognized by the antibodies were picked from the 2D gels loaded with the extract prepared from isolated granulocytes and with the extract prepared from HL60 cells. Protein identification by mass spectrometry revealed α-enolase as the target antigen, independent of the extract analyzed (Fig. 1).

To confirm the presence of anti–α-enolase antibodies, we performed Western blotting after SDS-PAGE with recombinant α-enolase as antigen source. We found that Western blotting with the patient’s serum, but not with the control serum, revealed a band at 70 kDa, which corresponded to the molecular weight of the recombinant GST-tagged α-enolase.
We screened sera from patients with IBD (UC and CD), diseased controls, and healthy controls for the presence of anti–α-enolase antibodies by Western blotting. Fig. 2 shows the measured densitometric areas reflecting antibody binding to α-enolase for the IBD patients and controls. Based on these results, a ROC curve was constructed for discriminating IBD patients from healthy controls (Fig. 3). The area under the curve was 0.789. A specificity of 95% corresponded to a sensitivity of 35.0% of anti–α-enolase antibodies for IBD (cutoff point 32). The optimal cutoff point, based on the Youden index (J = maximum [sensitivity + specificity – 1]), was 27 (22). The sensitivity and specificity at this cutoff point were 49.5% and 91.4%, respectively.

The prevalence of anti–α-enolase antibodies in the various groups of patients, calculated at the cutoff point with a specificity of 95% and at the optimal cutoff point, is summarized in Table 1. Differences between prevalences were evaluated by χ² test. Anti–α-enolase antibodies were detected in 49% of patients with UC (49/100; P < 0.0001 vs healthy controls [HC] and P = 0.014 vs GI controls) and 50% of patients with CD (50/100; P < 0.0001 vs HC and P = 0.0095 vs GI controls). Anti–α-enolase antibodies were found in 31% of GI controls (31/100) and 8.5% of healthy subjects (9/105). Because atypical pANCA has been described in patients with AIH and PSC, we also searched for anti–α-enolase antibodies in these patient groups. Anti–α-enolase antibodies were found in 38% of patients with AIH (14/37; P < 0.0001 vs HC and P = 0.58 vs GI controls) and 33% of patients with PSC (12/36; P = 0.0009 vs HC and P = 0.96 vs GI controls). We detected anti–α-enolase antibodies in 34% of patients with ANCA-positive vasculitis (16/47; P = 0.0002 vs HC and P = 0.85 vs GI controls).

Combined positivity of anti–α-enolase antibodies and pANCA was found in 28% of patients with UC (28/100; P < 0.0001 vs HC and P < 0.0001 vs GI controls) and 8% of patients with CD (8/100; P = 0.0094 vs HC and P = 0.1048 vs GI controls). Combined positiv-
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Dr. Smith et al. investigated the presence of anti–α-enolase antibodies and pANCA in patients with inflammatory bowel disease (IBD). They found that 2% of GI controls (2/100; $P = 0.4560$ vs HC) were positive for anti–α-enolase antibodies and pANCA, whereas none of the healthy subjects (0/100) showed positivity. Combined positivity of anti–α-enolase antibodies and pANCA was observed in 13.8% of patients with PSC (5/36; $P = 0.0008$ vs HC and $P = 0.0199$ vs GI controls), 21.6% of patients with AIH (8/37; $P = 0.0001$ vs HC and $P = 0.0004$ vs GI controls), and 8.5% of patients with ANCA-positive vasculitis (4/47; $P = 0.0131$ vs HC and $P = 0.1575$ vs GI controls).

**UPREGULATION OF α-ENOLASE IN COLONIC MUCOSA OF PATIENTS WITH IBD**

Whole genome screening was performed on colonic biopsies from 21 patients with UC, 14 patients with CD, and 6 GI control subjects. For α-enolase, 3 probe sets were present on the array. Significantly increased α-enolase mRNA concentrations were detected in patients with IBD compared with control subjects (average fold change 1.541) (Fig. 4). All probe sets gave similar results, with a fold change ranging between 1.643 and 1.854, and $P$ values between $2.88 \times 10^{-9}$ and $4.03 \times 10^{-6}$ (Limma $t$ test).

**α-ENOLASE IS NOT THE TARGET ANTIGEN OF ATYPICAL PANCA**

In the cohort of 525 patients, pANCA was detected in 161 (30.6%), and anti–α-enolase antibodies were detected in 180 (34.2%). Only 40.9% (66/161) of the pANCA-positive subjects were also positive for anti–α-enolase antibodies, and only 36.6% (66/180) of the patients positive for anti–α-enolase antibody were also positive for pANCA ($\chi^2 = 4.22, P = 0.040$). Next, sera were preabsorbed with α-enolase before indirect immunofluorescence. Such preabsorption did not result in the disappearance of the atypical pANCA staining.

Fig. 2. Densitometric areas of the anti–α-enolase band on Western blot for UC patients, CD patients, healthy controls, and GI controls.

Fig. 3. ROC curve of anti–α-enolase antibodies to discriminate between IBD patients and healthy controls. The area under the curve was 0.789.
pattern. In contrast, when sera were preabsorbed with the protein extract from HL60 cells, the atypical pANCA staining pattern was strongly reduced. This indicates that the target antigen of pANCA is present in the protein extract of HL60 cells, but that it is not α-enolase (see Supplemental Data Fig. 1).

ANTI–α-ENOLASE ANTIBODIES AND INFLAMMATION

The high prevalence of anti–α-enolase antibodies in gastrointestinal controls suggested that these antibodies were a hallmark of inflammation. To test this hypothesis, a cohort of 90 patients (60 UC and 30 GI controls) was tested for CRP. Thirty patients with UC were positive for anti–α-enolase antibodies. Seven of them (23.3%) were also CRP-positive. In the UC patients negative for anti–α-enolase antibody, 13 (43.3%) were CRP-positive. Thirteen GI controls were positive for anti–α-enolase antibodies. Ten of them (76.9%) were also CRP-positive. These results indicated that there was no association between increased CRP and anti–α-enolase antibodies (\(\chi^2 = 3.57, P = 0.058\)).

Discussion

Enolase is a glycolytic enzyme that catalyzes the interconversion of 2-phospho-D-glycerate (PGA) and phosphoenolpyruvate (PEP) (23). In addition to its role in the glycolytic pathway, the multifunctional protein enolase has also been described as a heat shock protein, a Myc-binding protein, and a plasminogen-binding protein (24–26). Therefore, enolase is highly abundant and preserved (27). In higher eukaryotes, 3 distinct isoforms of enolase (\(\alpha\), \(\beta\), and \(\gamma\)) are present. Whereas the \(\beta\)-isoform is restricted to muscle and the \(\gamma\)-isoform to neurons, \(\alpha\)-enolase is expressed in all tissues (28).

Antibodies against \(\alpha\)-enolase have previously been observed in 10% of 96 patients with UC and 18% of 112 patients with CD (10). We found a higher prevalence in UC (49%) and CD (50%). Anti–α-enolase antibodies have also been reported in various diseases, mainly chronic inflammatory and/or autoimmune disorders. In these reports, however, the number of patients studied was often limited (29). The prevalence of anti–α-enolase antibodies in our study seems to be higher in

Table 1. Prevalence of anti–α-enolase antibodies and pANCA in the study population.

<table>
<thead>
<tr>
<th>n</th>
<th>Anti–α-enolase antibodies (cutoff 32)</th>
<th>Anti–α-enolase antibodies (cutoff 27)</th>
<th>pANCA</th>
<th>Anti–α-enolase antibodies (cutoff 27) and pANCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>UC</td>
<td>100</td>
<td>34 (34.0)%</td>
<td>49 (49.0)</td>
<td>56 (56.0)</td>
</tr>
<tr>
<td>CD</td>
<td>100</td>
<td>36 (36.0)%</td>
<td>50 (50.0)</td>
<td>15 (15.0)</td>
</tr>
<tr>
<td>PSC</td>
<td>36</td>
<td>8 (22.2)%</td>
<td>12 (33.3)</td>
<td>16 (44.4)</td>
</tr>
<tr>
<td>AIH</td>
<td>37</td>
<td>11 (29.7)%</td>
<td>14 (37.8)</td>
<td>17 (45.9)</td>
</tr>
<tr>
<td>ANCA-positive vasculitis</td>
<td>47</td>
<td>15 (31.9)%</td>
<td>16 (34.0)</td>
<td>19 (40.4)</td>
</tr>
<tr>
<td>GI controls</td>
<td>100</td>
<td>17 (17.0)</td>
<td>31 (31.0)</td>
<td>5 (5.0)</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>105</td>
<td>5 (4.76)</td>
<td>9 (8.5)</td>
<td>5 (4.8)</td>
</tr>
</tbody>
</table>

Data are n (%).

* \(P < 0.05\) vs healthy controls.

** \(P < 0.05\) vs GI controls.

Fig. 4. mRNA expression in colonic mucosa biopsies of 21 patients with UC, 14 with CD, and 6 control subjects, with indication of median.

The elevation of \(\alpha\)-enolase mRNA in the patients with IBD compared with the control subjects was statistically significant (fold change 1.643; \(P = 2.88 \times 10^{-5}\)).
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almost every group of patients compared with the literature. The discrepancies between our study and previously published results could be due to the method used for detection (differences in limit of detection or the assigned threshold value for positivity). We found anti–α-enolase antibodies in 8.5% of 105 healthy subjects, which is consistent with the reported prevalence that varies between 0% and 11.8% (29). Finally, we examined a cohort of 100 patients with specific gastrointestinal diseases that are not IBD-related. To our knowledge, we are the first to describe a prevalence of 31% for antibodies against α-enolase in these GI controls.

α-Enolase is a heat shock protein. Ludwig et al. (30) and Winrow et al. (31) described an increased expression of the heat shock proteins HSP70 and HSP60 in colonic mucosa of patients with IBD, but not controls. The authors suggested that cytoprotective heat shock proteins were upregulated in response to conditions of chronic cellular stress, such as trauma, viral infection, or inflammation (32). Circulating antibodies against HSP60 and HSP70 have been detected in patients with IBD by some authors, but not by others (30, 33). Bene et al. (34) postulated that an immune response toward heat shock proteins expressed by pathogens results in an autoimmune response through the mechanism of molecular mimicry, but other mechanisms have been suggested (35, 36).

We found (a) an upregulation of the mRNA of α-enolase in the colonic mucosa in IBD patients and (b) the presence of circulating antibodies against α-enolase in patients with IBD. The upregulation is consistent with earlier findings that heat shock proteins are upregulated in inflammatory conditions (32). α-Enolase can be released after necrosis or apoptosis and taken up by tissue-resident antigen-presenting cells (APCs) (29, 37). The upregulation of α-enolase as a heat shock protein may enhance its uptake by APCs.

Lopez-Alemany et al. (38) have shown that antibodies against α-enolase abrogate 84% to 90% of cell surface–mediated plasminogen activation and thereby reduce fibrinolysis. This reduction and the accompanying increased concentrations of fibrinogen and fibrin increase the production of proinflammatory cytokines and chemokines and, consequently, increase the inflammatory response (39). This could be an explanation for the high prevalence of anti–α-enolase antibodies in inflammatory controls. A possible association of anti–α-enolase antibodies with increased concentrations of CRP was examined in a cohort of 90 UC and GI patients. In this cohort, we did not find such an association, possibly due to a modest CRP response in UC (40).

We found no association between the presence of anti–α-enolase antibodies and the pANCA status of patients with IBD. Absorption with α-enolase before indirect immunofluorescence did not eliminate the pANCA staining pattern, which is consistent with the idea that α-enolase is not the target antigen of pANCA.

In summary, anti–α-enolase antibodies are found in 50% of patients with IBD. An increased expression of α-enolase mRNA is found in patients with IBD. Anti–α-enolase antibodies are likely to be induced by aberrant expression of the α-enolase self antigen. Although these antibodies may be important in the pathogenesis of the disease, they have only a very limited potential as diagnostic markers for IBD.


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References


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