

Analysis of Serum Antibodies in Patients Suspected of Having Inflammatory Bowel Disease

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Inflammatory bowel disease (IBD) is the general term used for a heterogeneous group of intestinal disorders, including Crohn's disease (CD) and ulcerative colitis (UC). Serological markers such as anti-*Saccharomyces cerevisiae* antibodies (ASCA) and atypical perinuclear antineutrophilic cytoplasmic antibody (atypical pANCA) have proven useful in the diagnosis and differentiation of CD and UC. Immunoglobulin A (IgA) antibody directed against the outer membrane protein C (OmpC) of *Escherichia coli* is said by one group to have clinical utility in diagnosing IBD, specifically in ASCA-negative CD patients. Our objective in this study was to compare the results obtained from two separate laboratories offering similar IBD tests using sera from suspected IBD patients. One hundred ninety-seven sera received for IBD testing were included in the study. The agreement between the two laboratories was 93.4% for ASCA IgA, 90.9% for ASCA IgG, and 87.8% for atypical pANCA IgG. There were 25 sera with ASCA-negative/OmpC-positive results reported by one laboratory. Thirteen of these 25 (52.0%) ASCA-negative/OmpC-positive sera were also atypical pANCA positive (9 as determined by both laboratories, 3 by one, and 1 by the other). Atypical pANCA antibody is found primarily in IBD patients with UC and colon-limited CD (Crohn's colitis). We conclude that the ASCA and atypical pANCA assays showed good agreement between the two laboratories, but the data for ASCA-negative/OmpC-positive sera suggest that many (52.0%) of these patients were more likely to have had UC or Crohn's colitis based on the presence of an atypical pANCA.

Crohn's disease (CD) and ulcerative colitis (UC) are the two major forms of inflammatory bowel disease (IBD). Both CD and UC are chronic, affecting children and adults (men and women almost equally), and are most common in northern Europe and North America. The onset of CD and UC is usually between the ages of 15 and 30, with a second, smaller peak of incidence between the ages of 50 and 70 (4, 23). Approximately 20% of individuals with CD have a biological relative with some form of IBD, and several reports have noted an increase in the prevalence of CD and UC in various geographic regions (2, 17, 24, 36).

Although there are many theories about the etiology of CD and UC, none have been proven. Many of the symptoms of CD and UC are similar, and diagnosis is often difficult, time-consuming, and invasive. Since CD and UC are treated differently, correct diagnosis and differentiation are medically important.

Two serological markers have been found to have clinical utility in diagnosing IBD and aiding in the differentiation of CD from UC: anti-*Saccharomyces cerevisiae* antibody (ASCA) (immunoglobulin A [IgA] and/or IgG), using enzyme immunoassay (EIA) techniques, and antineutrophil cytoplasmic antibody (ANCA) (IgG) that demonstrates atypical perinuclear staining (pANCA), using indirect fluorescent-antibody assay (IFA) techniques. ASCA is directed against mannose sequences in the cell wall of *Saccharomyces cerevisiae* (26, 35) and is significantly more prevalent in patients with CD than in those with UC and healthy controls (15, 18, 19, 25, 31, 32, 35).

ASCA IgA is found in 35 to 50% of patients with CD but in <1% of patients with UC. ASCA IgG is found in 50 to 80% of CD patients but only 20% of UC patients. Multiple studies have reported the simultaneous presence of ASCA IgA and IgG to be highly specific for CD (3, 31, 33). ASCA antibodies are currently identified using commercial or in-house-developed EIAs. Independent studies have shown differences in sensitivity and specificity between some of the ASCA EIAs that are currently available (13, 16, 20, 41).

The atypical pANCA (ethanol-positive/formalin-negative) IFA pattern seen in patients with UC and autoimmune hepatitis is directed against an antigen(s) on the inner side of the nuclear membrane of the neutrophil (37–39). One group has suggested that histone H1 is the target antigen associated with atypical pANCA (5), but histone H1 is not specific to neutrophils and histone H1 as a target is not supported by other researchers (37–39).

Atypical pANCA is found in 70% of patients with UC but in only 20% of patients with CD (8–10). Atypical pANCA has also been reported to be present in a subgroup of CD patients with colon-limited disease (12, 40). The “typical” pANCA pattern observed in vasculitis patients, using ethanol-fixed neutrophils, will convert to a cytoplasmic ANCA (cANCA) pattern on formalin-fixed neutrophils. These antibodies are usually directed against myeloperoxidase (MPO). In contrast, the “atypical” pANCA pattern found in IBD patients will not convert to a cANCA pattern on formalin-fixed neutrophils but rather exhibits an essentially negative pattern. ANCA systems that replace formalin-fixed neutrophils with an enzyme (DNase I) digest step are rarely used outside the group (the Cedars Sinai IBD group of Stephen R. Targan) that developed this method.

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TABLE 1. Comparison of results from two different laboratories for ASCA IgA by EIA in 197 patient sera

Prometheus ASCA IgA result	No. with ARUP ASCA IgA result ^a	
	Positive	Negative
Positive	29	11
Negative	2	155

^a Agreement, 93.4%.

Detection of IgA antibody against the outer membrane porin C (OmpC) of *Escherichia coli* has been promoted as a way to identify patients with Crohn’s disease that are seronegative for ASCA (14). Thus far, independent studies have reported poor sensitivity for OmpC IgA in IBD, and false positives are common (6, 43).

Our objectives in this study were to compare the results obtained from two different reference laboratories that offer similar IBD tests using sera from suspected IBD patients.

MATERIALS AND METHODS

Clinical samples. One hundred ninety-seven consecutive sera from patients suspected of having IBD, sent to our laboratory for IBD testing, were included in the study. All patient sera were processed according to the University of Utah Institutional Review Board approved protocol no. 13433 and meet the Health Information Portability and Accountability Act patient confidentiality guidelines. Physicians of patients having ASCA-negative/OmpC-positive results as determined by Prometheus Laboratories were contacted by phone in an attempt to obtain the diagnosis.

IBD immunoassays by ARUP Laboratories. ASCA IgA and IgG were detected using EIAs purchased from INOVA Diagnostics (San Diego, CA). These assays were performed at Associated Regional and University Pathologists (ARUP) Laboratories (Salt Lake City, UT), an esoteric reference laboratory owned by the University of Utah, according to the manufacturer’s product insert. These assays have been approved by the Food and Drug Administration (FDA) for in vitro diagnostic use with patients suspected of having Crohn’s disease.

Atypical pANCA IgG was detected using ANCA IFA substrate slides purchased from INOVA Diagnostics. This assay utilizes the standard IFA method (ethanol- and formalin-fixed neutrophils) for detecting ANCA and is the most accepted and widely used method in detecting atypical pANCA (1, 11, 21, 22, 28–30, 34, 37–39). This assay has been approved by the FDA for in vitro diagnostic use with patients suspected having systemic vasculitis.

Ethanol- and formalin-fixed neutrophils are run in parallel for each patient, and all steps are performed at room temperature. Patient serum is diluted 1:20 in phosphate-buffered saline and allowed to incubate with fixed neutrophils for 30 min in a moist chamber. Slides are then rinsed, submerged in phosphate-buffered saline for 5 min, and then incubated with fluorescent conjugate for 30 min in a moist chamber. Slides are then washed again as described above, and coverslips are applied with mounting medium. Neutrophils were observed at a magnification of ×400 using a Nikon Eclipse E200 fluorescence microscope. Sera demonstrating a perinuclear (pANCA) pattern with 1+ or greater fluorescence on ethanol-fixed neutrophils but negative on formalin-fixed neutrophils were considered positive for atypical pANCA, which is characteristic of patients having ulcerative colitis.

Additional neutrophil and nuclear immunoassays by ARUP Laboratories. EIAs detecting IgG antibody against MPO and proteinase 3 (PR3) were purchased from The Binding Site, Inc. (San Diego, CA). EIAs detecting IgG antibody against ribonucleoprotein (RNP), histone, and chromatin were purchased from INOVA Diagnostics, as was an IFA for detecting IgG antibody against double-stranded DNA (dsDNA). These assays were performed at ARUP Laboratories according to the manufacturer’s product insert, and all have been approved by the FDA for in vitro diagnostic use for various autoimmune diseases. These assays, which have been validated for clinical use, were utilized to assess atypical pANCA discrepant sera for the presence of additional autoantibodies against other known neutrophil/nuclear antigens.

IBD immunoassays by Prometheus Laboratories. Samples were sent to Prometheus Laboratories (San Diego, CA) for IBD testing, which is offered under various test panel names (IBD First Step, IBD Diagnostic System, IBD Confir-

TABLE 2. Comparison of results from two different laboratories for ASCA IgG by EIA in 197 patient sera

Prometheus ASCA IgG result	No. with ARUP ASCA IgG result ^a	
	Positive	Negative
Positive	14	0
Negative	18	165

^a Agreement, 90.9%.

matory System) and includes the following assays: ANCA IgG, ASCA IgA and IgG, and OmpC IgA (all by EIA) and pANCA IgG (by IFA) (excluded from IBD First Step). These assays were, apparently, developed in-house at Prometheus Laboratories and have not, to our knowledge, been approved by the FDA. The ANCA IFA method used at Prometheus replaces formalin-fixed neutrophils with an enzyme (DNase I) digest step. Although Prometheus does not use the term “atypical,” pANCA-positive sera that were “DNase sensitive” were considered positive for atypical pANCA. We were unable to obtain any further information on the protocols for these assays.

Anti-OMP IgA testing by EIA. Sera reported to be ASCA negative/OmpC positive by Prometheus were sent to INOVA Diagnostics (San Diego, CA) for anti-outer membrane protein (OMP) IgA testing by EIA. This prototype EIA uses a proprietary blend of OMPs from two strains of colonic bacteria isolated from patients with Crohn’s disease. Results were compared to those obtained from OmpC IgA testing performed at Prometheus.

RESULTS

The agreement between the two laboratories for the ASCA IgA was 93.4%, 90.9% for ASCA IgG and 87.8% for atypical pANCA IgG (Tables 1 to 3). The ASCA IgA assay performed at Prometheus gave positive results for 11 additional sera compared to the ASCA IgA performed at ARUP (Table 1). Nine of these 11 sera (81.8%; no. 57, 65, 68, 103, 158, 189, 195, 210, and 212) gave negative results for ASCA IgG, as determined by both laboratories (Table 4). There were two sera that were ASCA IgA negative as determined by Prometheus and that gave positive results as determined by ARUP (Table 2). One (no. 133) of these two sera was also positive for ASCA IgG, as determined by ARUP (Table 4). The Prometheus ASCA IgG gave negative results for 18 of the 32 positive sera (56.3%) detected by the ASCA IgG assay performed at ARUP (Table 2). In addition, eight (no. 14, 30, 113, 136, 138, 172, 193, and 194) of these 18 sera (44.4%) were ASCA IgA positive, as determined by both laboratories (Table 4).

The DNase digest method detected atypical pANCA (DNase sensitive) in 22 sera in addition to the 33 that were in positive agreement with the standard IFA method (Table 3). The standard IFA method detected atypical pANCA (ethanol positive/formalin negative) in two sera that were negative by the DNase digest method (Table 3). We assessed these 24 discrepant atypical pANCA sera for IgG antibodies against

TABLE 3. Comparison of results from two different laboratories for atypical pANCA IgG by IFA in 197 patient sera

Prometheus DNase I digest method result	No. with ARUP standard method result ^a	
	Positive	Negative
Positive	33	22
Negative	2	140

^a Agreement, 87.8%.

TABLE 4. ASCA results for 29 discrepant sera from two different laboratories

Sample no.	Result ^a			
	ASCA IgA		ASCA IgG	
	ARUP	Prometheus	ARUP	Prometheus
14	+	+	+	-
26	-	-	+	-
30	+	+	+	-
47	-	-	+	-
49	-	-	+	-
57	-	+	-	-
65	-	+	-	-
68	-	+	-	-
70	-	-	+	-
99	-	-	+	-
103	-	+	-	-
113	+	+	+	-
118	-	+	+	+
133	+	-	+	-
135	+	-	-	-
136	+	+	+	-
138	+	+	+	-
148	-	-	+	-
158	-	+	-	-
162	-	+	+	-
168	-	-	+	-
172	+	+	+	-
189	-	+	-	-
193	+	+	+	-
194	+	+	+	-
195	-	+	-	-
200	-	-	+	-
210	-	+	-	-
212	-	+	-	-

^a Samples 133 and 162 carry double discrepancies.

other known specific neutrophil/nuclear antigens (MPO, PR3, dsDNA, RNP, histone, and chromatin). Eleven of the 22 atypical pANCA discrepant sera by the DNase digest method (50.0%) gave positive antibody results for one or more of the six antigens tested (Table 5). The two atypical pANCA discrepant sera by the standard IFA method gave negative antibody results for all six antigens (Table 5). Cytoplasmic (cANCA) staining was not observed with any of these 24 discrepant sera.

Thirty-eight of 197 sera tested by Prometheus (19.3%) were positive for OmpC IgA. Twenty-five of these 38 sera (65.8%) were ASCA negative (IgA and IgG) as determined by Prometheus, while only 22 of 38 (57.9%) were ASCA negative as determined by ARUP. Three (no. 49, 133, and 135) of the 25 ASCA-negative/OmpC-positive sera detected by Prometheus (12.0%) were ASCA positive (one IgA, one IgG, and one IgA and IgG) by ARUP (Table 6). Twelve of the 25 (48.0%) ASCA-negative/OmpC-positive sera, as determined by Prometheus, were also positive for atypical pANCA (DNase sensitive), 9 of which (69.2%) were in agreement with the standard IFA performed at ARUP (Table 6), suggesting that these patients were more likely to have UC or Crohn's colitis. Four of six (66.7%) patients in this group for whom clinical and biopsy information was available actually had UC (Table 7).

Of the 197 sera tested for OmpC IgA by Prometheus, only 9 (4.6%) had a positive OmpC result in the absence of other antibodies in IBD, and 8 of these had very low antibody content (16.8 to 22.8 EIA units [EU]/ml) (Table 6). The majority (19 of 25; 76.0%) of sera with ASCA-negative/OmpC-positive results as determined by Prometheus gave very low values

TABLE 5. Assessment of 24 atypical pANCA discrepant sera for additional IgG autoantibodies against other known neutrophil/nuclear antigens

Sample no.	Prometheus pANCA ^a	Result						
		pANCA	MPO	PR3	ARUP			
					dsDNA	RNP	Histone	Chromatin
10	+	-	-	-	-	+	+	-
12	+	-	-	-	-	-	-	-
15	-	+	-	-	-	-	-	-
21	+	-	-	-	-	-	-	-
38	+	-	-	-	-	-	+	-
50	+	-	-	-	-	-	+	+
68	+	-	-	-	-	-	-	-
73	+	-	-	-	-	-	+	-
82	+	-	-	-	-	-	+	-
93	+	-	-	-	-	-	+	+
96 ^b	+	-	-	+	-	-	+	-
99	+	-	-	-	-	-	-	-
104	+	-	-	-	-	-	-	-
105	+	-	-	-	-	+	-	-
106	+	-	-	-	-	-	-	-
113	+	-	-	-	-	-	+	-
123	+	-	-	-	-	-	-	-
129	+	-	-	-	-	-	+	+
133	+	-	-	-	-	-	+	-
139	+	-	-	-	-	-	-	-
153	-	+	-	-	-	-	-	-
188	+	-	-	-	-	-	-	-
212	+	-	-	-	-	-	-	-
213	+	-	-	-	-	-	-	-

^a (S), DNase sensitive.

^b Cytoplasmic pattern (cANCA) absent, as determined by both laboratories.

TABLE 6. Results for 25 sera that were ASCA negative/OmpC positive as determined by Prometheus Laboratories

Sample no.	Result							
	Prometheus OmpC IgA (EU/ml) ^a	INOVA OMP IgA ^b	ARUP ASCA IgA	ARUP ASCA IgG	ARUP pANCA IgG	Prometheus pANCA IgG ^c		
6	20.0	EQV	-	-	+	+	(S)	
16	19.7	-	-	-	-	-	-	
24	18.0	EQV	-	-	-	-	-	
49	37.7	+	-	+	-	-	-	
55	20.4	EQV	-	-	-	-	+	(R)
69	27.9	+	-	-	+	+	+	(S)
79	26.3	+	-	-	+	+	+	(S)
80	21.3	-	-	-	-	-	-	-
82	20.2	-	-	-	+	+	+	(S)
89	16.8	-	-	-	-	-	-	-
93	40.2	+	-	-	-	-	+	(S)
102	22.8	-	-	-	-	-	-	-
107	25.9	-	-	-	+	+	+	(S)
108	41.4	+	-	-	+	+	+	(S)
111	18.1	-	-	-	-	-	-	-
116	16.9	EQV	-	-	-	-	+	(R)
133	>111.0	+	+	+	-	-	+	(S)
135	20.6	-	+	-	+	+	+	(S)
137	23.2	EQV	-	-	+	+	+	(S)
150	19.3	-	-	-	-	-	-	-
152	20.1	-	-	-	+	+	+	(S)
153	18.6	+	-	-	+	-	-	-
166	54.0	+	-	-	-	-	-	-
188	60.6	+	-	-	-	-	+	(S)
205	17.0	QNS	-	-	-	-	-	-

^a 16.5 EU/ml or greater is considered positive.
^b EQV, equivocal; QNS, quantity not sufficient.
^c (S), DNase sensitive; (R), DNase resistant.

(<30.0 EU/ml) for OmpC IgA (Table 6). The OMP IgA (prototype EIA) performed at INOVA Diagnostics gave 9 positive, 5 equivocal, and 10 negative results for 24 of these 25 sera (Table 6) (the quantity was not sufficient for sample 205).

DISCUSSION

The ASCA and pANCA assays showed good agreement between the two reference laboratories (Tables 1 to 3). The Prometheus ASCA IgG assay, however, detected only 14 of the 32 positives (43.8%) detected by ARUP (Table 2). These findings are in agreement with a study from Belgium by Vermeire et al. (41), which showed the INOVA ASCA IgG assay to be 23% more sensitive than the ASCA IgG assay performed at Prometheus. Discord between these ASCA EIAs is most likely due to differences in their cutoff values, as was demonstrated in the study by Vermeire et al. using clinically defined sera (41).

Of the three assays compared between the two laboratories, the atypical pANCA IgG IFA had the most discrepancies. The DNase I digest method detected atypical pANCA in 22 additional sera, while the standard IFA method detected it in only 2 additional sera. Further assessment of these atypical pANCA discrepant sera for autoantibodies against other known neutrophil/nuclear antigens (MPO, PR3, dsDNA, RNP, histone, and chromatin) showed 50.0% of the DNase IFA discrepant sera to contain other autoantibodies against one or more of the specific antigens tested (Table 5). In contrast, the two discrepant sera by the standard IFA method gave negative results for

TABLE 7. Diagnosis based on clinical and biopsy data for ASCA-negative/OmpC-positive patients for whom information was available^a

Sample no.	Age (yr)	Gender	Diagnosis
55	75	Female	Ulcerative colitis
80	30	Female	Ulcerative colitis
93	38	Male	Ulcerative colitis
137	54	Male	Crohn's disease
152	45	Male	Ulcerative colitis
166	61	Female	Crohn's disease

^a See Table 6 for IBD testing results.

all six additional markers (Table 5) and are more likely to be true atypical pANCA. The presence of antinuclear autoantibodies in patients with CD (18%) or UC (43%) has previously been reported (7). In addition, Reumaux et al. (27) detected IgG autoantibodies against several histone peptides primarily in patients with CD.

Since Prometheus utilizes a nonspecific ANCA (neutrophil) EIA for the screening of atypical pANCA, the presence of other autoantibodies as mentioned above could result in a positive ANCA EIA test that may affect the subsequent subjective neutrophil IFA pattern interpretation. Sera with strong homogeneous antinuclear reactivity could be misinterpreted as pANCA positive. If these specimens were then subjected to DNase digestion, sera with reactivity to dsDNA, histone, or chromatin (as shown to be the case for some of the atypical pANCA discrepant sera) would be interpreted as DNase sensitive. These results would be interpreted as supporting a diagnosis of ulcerative colitis when they are actually more likely to be false positives due to the presence of antibodies to other specific neutrophil/nuclear antigens. A serum that was positive for dsDNA antibody was a control (it was DNase sensitive) used in the study first describing the DNase IFA methodology for detecting atypical pANCA (42).

The reports for IBD testing from Prometheus indicate that the purpose of their ANCA (neutrophil) EIA is to validate positive pANCA IFA sera, meaning that both tests must be positive before the result is reported to be "IFA perinuclear pattern detected." For sera that are ANCA EIA positive/pANCA IFA negative, "elevated levels" of ANCA IgG are reported. Sixteen of 140 pANCA IFA-negative sera were ANCA EIA positive, giving the Prometheus ANCA EIA a false-positive rate of 11.4%. These patients must then continue with the Prometheus "IBD Diagnostic System" or "IBD Confirmatory System" to determine if the "IBD First Step"-positive ANCA EIA result is actually due to atypical pANCA. Six of 57 (10.5%) Prometheus pANCA IFA-positive sera (all six DNase sensitive) were reported to be "IFA perinuclear pattern not detected" since they were ANCA EIA negative. Thus, the IBD reports from Prometheus indicate that false positives occur often (10.5% in this study) with their DNase digest IFA method for detecting pANCA.

Currently, the OmpC IgA assay is performed only at Prometheus, whose reports indicate that this assay detects an additional 21% of CD patients who are seronegative for ASCA (14). It should be noted that only 56% of their CD patients were found to be ASCA seropositive (14). IBD test reports

from Prometheus show a positive predictive value of 85% for CD if OmpC IgA is the only positive result in their panel. If the results show OmpC positive/pANCA positive (DNase sensitive), the report states "results suggestive of ulcerative colitis." Studies performed at Prometheus indicate that this pattern of results (ASCA negative/OmpC positive/pANCA positive) occurred two times more frequently in patients with UC than in those with Crohn's disease. In view of this information and the data generated from this study, OmpC IgA appears to be more prevalent in UC, since many of the patients having ASCA-negative/OmpC-positive results were also atypical pANCA positive (Table 6). Of the limited number of patients who were ASCA negative/OmpC positive and from whom we were able to obtain clinical data (Table 7), 66.7% actually had ulcerative colitis, suggesting the limited diagnostic value of OmpC IgA in Crohn's disease.

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