Prospective evaluation of neutrophil autoantibodies in 500 consecutive patients with inflammatory bowel disease

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Antineutrophil cytoplasmic antibodies (ANCA) have been useful as a diagnostic aid in patients with necrotizing vasculitis and glomerulonephritis (1,2). Recently a specific subclass of ANCA was also recognized in patients with inflammatory bowel disorders, particularly in those with idiopathic ulcerative colitis (3-5). While assays for ANCA are simple, inexpensive and reproducible (5), their utility in large series of patients with inflammatory bowel disease (IBD) still requires definition.

Subclasses of ANCA were initially based on the pattern of antibody response to neutrophil cytoplasmic antigens. A particular subclass, the so-called ‘atypical’ (perinuclear) p-ANCA type, occurs in the majority of patients with ulcerative colitis. The purpose of this prospective study was to assess, in a blinded fashion, this ‘subclinical’ serological marker in a consecutive series of IBD patients. Five hundred patients were evaluated, including 247 patients with ulcerative colitis and 253 with Crohn’s disease involving the small and/or large intestine. Overall, 194 (38.8%) of all patients with IBD were positive, including 164 (66.3%) with ulcerative colitis and 30 (11.9%) with Crohn’s disease. Except for coexistent sclerosing cholangitis, no other clinical or laboratory variable had an effect on the rate of ANCA detection. This is the largest single study of ANCA in patients with IBD and the only study to provide data solely from a single Canadian centre. Results emphasize the immunopathological differences between ulcerative colitis and Crohn’s disease, and indicate that both disorders are heterogeneous inflammatory disease processes.

Key Words: Antineutrophil cytoplasmic antibody, Atypical perinuclear antineutrophil cytoplasmic antibody, Inflammatory bowel disease, Ischemic/infectious colitis, Serum antibody, Serum marker

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Received for publication May 27, 1996. Accepted July 30, 1996
of indirect immunofluorescence using alcohol-fixed neutrophils. These included a diffuse cytoplasmic pattern (c-ANCA) and a perinuclear pattern (p-ANCA). p-ANCA is thought to be due to redistribution of antigen (myeloperoxidase and, possibly, other neutrophil granule components) towards the nucleus during alcohol fixation. A third subclass of ANCA, described as an ‘atypical’ form of p-ANCA not reactive with myeloperoxidase (5,6), occurs mainly in patients with idiopathic ulcerative colitis and primary sclerosing cholangitis (7,8); different studies have documented a wide range of positive results – from about 25% to more than 80% (6-21).

Although detection of atypical p-ANCA may have diagnostic value in IBD patients, p-ANCA has only been examined in limited numbers of patients in each reported series. In some studies, serum samples freeze-stored for variable periods were used, possibly altering results. Some of the earlier investigations provided data on ANCA serology from several countries. Except for some initial studies with a limited number of patients done in Los Angeles and including patient sera from Calgary (6,12), this report is not only the largest in the literature, but provides data solely from a Canadian population centre. In the present evaluation, ‘blinded’ fresh serum samples from 500 consecutive patients with ulcerative colitis or Crohn’s disease were examined for ANCA and, specifically, atypical p-ANCA. The results emphasize the immunopathological differences between ulcerative colitis and Crohn’s disease.

PATIENTS AND METHODS

Patient groups: Patients had an established diagnosis of idiopathic ulcerative colitis or Crohn’s disease in the small and/or large intestine based on clinical, radiologic, endoscopic and histological criteria, as well as based on negative microbiological studies (22).

There were 247 ulcerative colitis patients. Diagnosis was based on colonoscopic and histological studies demonstrating a diffuse mucosal inflammatory process in the rectum extending proximally within the colon. Radiologic studies were also often done to exclude small intestinal, particularly ileal, disease. Regarding disease extent and site, colitis was classified as, first, ‘distal disease’ if inflammatory changes extended from the anus but were localized in the distal 60 cm alone based on colonoscopic evaluation and mucosal biopsy; or, second, ‘extensive disease’ if inflammatory changes were over 60 cm. In all patients with distal disease that was confined to the distal 60 cm, biopsies were done to document the presence of normal proximal colonic mucosa.

There were 253 Crohn’s disease patients. Diagnosis was usually based on colonoscopic and/or histological studies showing a segmental or patchy inflammatory process within the colorectum, distal small intestine or both, often with histological features of a focal and/or granulomatous inflammatory process. In addition, endoscopic mucosal biopsy, radiologic evaluation or both of the upper gastrointestinal tract were usually done. Radiologic and endoscopic data, as well as histological changes of inflammatory disease, were used.

When available, surgical materials were also reviewed from patients with either ulcerative colitis or Crohn’s disease. All patient serological samples for this prospective study were collected in a consecutive fashion with no exclusions or refusals from October 1994 to December 1995; over 95% of samples were from out-patients rather than hospitalized patients. The clinician investigator was blinded to the results of serological studies for ANCA.

Control groups: Thirty-two additional patients, between 20 and 50 years old, served as controls for this study. Controls included 16 with abdominal pain and diarrhea; in these, endoscopic, radiologic and histological evaluation of the upper and lower gastrointestinal tracts were normal and microbiological studies were negative. Another 15 controls had infectious colitis; in all, typical endoscopic and histological features of a self-limited form of colitis were present, with a positive microbiological study for an established enteric pathogenic bacteria (Campylobacter and Yersinia species). A single patient with a focal segment of ischemic large intestine associated with cocaine use was included. The segment was resected.

Laboratory studies: For each patient, all blood samples were collected into vacutainer glass tubes (Becton Dickinson, New Jersey) by a laboratory technologist without knowledge of the patient’s clinical history, investigation results or diagnosis. Blood was also obtained for hematological studies (hemoglobin, white blood cell count and platelet count), an erythrocyte sedimentation rate, antinuclear antibodies, liver chemistry tests (aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase), serum iron studies and serum proteins, including serum albumin.

Finally, blood samples were collected into vacutainer glass tubes, allowed to clot at room temperature and used for detection of ANCA via two methods: ANCA immunofluorescence and ELISA. Serological studies for ANCA were done by a single laboratory technologist blinded to the clinical details and diagnosis. If ANCA immunofluorescence was positive, ANCA ELISA was done for autoantibodies to myeloperoxidase and serine protease 3 (PR3). As reported elsewhere with coded sera examined in a blinded fashion (3), excellent agreement was present between immunofluorescence and ELISA results.

ANCA immunofluorescence: Indirect immunofluorescence for c-ANCA, p-ANCA and atypical p-ANCA were detected with a standardized fluorescent antibody detection method (6,7,23), using a proprietary kit purchased from a commercial supplier (Inova Diagnostics Inc, California). Slides were supplied with an adherent layer of cultured human neutrophils. The culture conditions were designed to ensure stability and strong expression of the primary cytoplasmic granules. The adherent neutrophils were fixed by the manufacturer with either ethanol or formalin. The primary screen for c-ANCA or p-ANCA involved incubation of serum at a 1:20 dilution in phosphate-buffered saline with ethanol-fixed slides for 25 mins at room temperature followed by a 5 min wash with phosphate-buffered saline. This was followed by another 25 min incubation using affinity purified
antihuman immunoglobulin (Ig) G with a fluorescent tag and a
further 5 min wash with phosphate-buffered saline. Coverslips
were then applied to the slides and these were examined with
a fluorescent microscope at 500x magnification.

Positive ANCA cellular fluorescence was recorded and
the distribution pattern of fluorescence designated as
c-ANCA, p-ANCA or atypical p-ANCA (as shown in
Figure 1). c-ANCA-positive sera were also titrated. Sera posi-
tive for either p-ANCA or atypical p-ANCA were further
evaluated using formalin-fixed slides because this destroys
nuclear antigens and atypical p-ANCA. In addition, for-
malin fixes both c-ANCA and p-ANCA antigens in the cyto-
plasm so that a false positive pattern will not be observed.
Antinuclear antibody-positive sera were excluded because
these may mimic ANCA.

ELISA assays: ANCA ELISA assays were performed with a
standardized method (6,7) using commercial kits (Quanta-
Lite MPO and PR3 ELISA, Inova Diagnostics Inc). The test
kits utilize microtiter strips containing wells coated with
either proteinase-3, a primary antigen related to the c-
ANCA pattern, or myeloperoxidase, a primary antigen asso-
ciated with the p-ANCA pattern. Diluted serum was applied
and incubated. If specific antibodies are present, binding to
the wells occurs. Unbound material is initially removed by
washing, and bound antibody detected by adding enzyme-
labelled antihuman IgG, followed by a second washing, then
incubated with a nitrophenol substrate. Wells with
proteinase-3 or myeloperoxidase antibodies are quantitated
by a colourimetric method.

RESULTS

Control subjects: All 16 patients in this evaluation with no
detectable gastrointestinal disease, and all 15 patients with
confirmed infectious colitis as well as a single patient with
cocaine-associated ischemic colitis, had no neutrophil auto-
 antibodies detected based on either immunofluorescent or
ELISA assay detection methods.

IBD patients: As shown in Table 1, 194 of 500 patients with
IBD (38.8%) had atypical p-ANCA detected in serum sam-
plies with ELISA confirmation. A total of 164 of 247 patients
with ulcerative colitis (66.4%) were positive for atypical p-
ANCA, and 30 of 253 patients with Crohn’s disease (11.9%)
were positive for atypical p-ANCA.

Specific historical and clinical variables in patients with
either ulcerative colitis and Crohn’s disease had no apparent
effect on the rate of detection of atypical p-ANCA, includ-
ing age at the time of initial diagnosis, sex, prior surgery,
prior medication use or medical treatment, type of medica-
tion used, duration of disease and familial history of IBD (ei-
ther ulcerative colitis or Crohn’s disease). However, in this
study, all patients with concomitant primary sclerosing
cholangitis, radiologically defined, had a positive atypical
p-ANCA (7,8,10,14,15,21,23). Finally, no correlation with
other laboratory test results were detected including hemo-
gram (level of hemoglobin, white blood cell count and plate-
let count), erythrocyte sedimentation rate, serum iron or
protein levels, including serum albumin.

Figure 1) Representative immunofluorescent photomicrograph showing ‘atypical’ perinuclear antineutrophil cytoplasmic autoantibodies from a patient with ulcerative colitis. The patient serum in this alcohol-fixed slide appears completely negative on a formalin-fixed slide. Reproduced with permission from Inova Diagnostics Inc, San Diego, California

TABLE 1
Antineutrophil cytoplasmic autoantibody (ANCA) serology in inflammatory bowel disease

<table>
<thead>
<tr>
<th>Disease/site</th>
<th>ANCA-negative</th>
<th>ANCA-positive (%)</th>
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</thead>
<tbody>
<tr>
<td>Ulcerative colitis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distal disease</td>
<td>48</td>
<td>83 (63.4)</td>
</tr>
<tr>
<td>Extensive disease</td>
<td>35</td>
<td>81 (69.8)</td>
</tr>
<tr>
<td>Total</td>
<td>83</td>
<td>164 (66.4)</td>
</tr>
<tr>
<td>Crohn’s disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small bowel alone</td>
<td>65</td>
<td>4 (5.8)</td>
</tr>
<tr>
<td>Large bowel alone</td>
<td>43</td>
<td>10 (18.9)</td>
</tr>
<tr>
<td>Total</td>
<td>115</td>
<td>16 (12.2)</td>
</tr>
<tr>
<td>Total All patients</td>
<td>306</td>
<td>194 (38.8)</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No disease</td>
<td>16</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Infectious colitis</td>
<td>15</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Ischemic colitis</td>
<td>1</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Total</td>
<td>32</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

Location or extent of disease within the colon of patients
with ulcerative colitis had no significant effect on the rate
detection of atypical p-ANCA (Table 1). Patients with colo-
nic mucosal disease limited to the distal 60 cm or less had
a 65.3% detection rate for atypical p-ANCA. In contrast, pa-
patients with more severe and extensive colitis, ie, over 60
 cm of continuous mucosal disease extending proximally from
the anal verge, had an atypical p-ANCA detection rate of
69.8%. In addition, the nature of the pathological process in
ulcerative colitis, ie, presence or absence of endoscopically
defined inflammatory pseudopolyps, had no significant ef-
fect on the rate of ANCA detection.

Results from patients with Crohn’s disease are also shown in
Table 1. Most were negative for atypical p-ANCA. For the
30 Crohn’s disease patients with a positive test result, 26 had
colonic involvement; however, four had disease localized to
the small bowel alone. In these patients, the histologi cal
TABLE 2
Percentage positive perinuclear antineutrophil cytoplasmic autoantibodies in ulcerative colitis (UC) or Crohn’s disease (CD)

<table>
<thead>
<tr>
<th>Country</th>
<th>UC</th>
<th>CD</th>
<th>Control</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>USA</td>
<td>68</td>
<td>12</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>USA, Canada</td>
<td>61</td>
<td>6</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>USA</td>
<td>68</td>
<td>8</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>Norway</td>
<td>27</td>
<td>0</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>Germany</td>
<td>83</td>
<td>25</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Germany</td>
<td>59</td>
<td>10</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>France</td>
<td>50</td>
<td>8</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Sweden</td>
<td>50</td>
<td>8</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Netherlands</td>
<td>79</td>
<td>13</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>Greece</td>
<td>30</td>
<td>0</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Denmark</td>
<td>50</td>
<td>24</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Hong Kong</td>
<td>32</td>
<td>0</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Canada</td>
<td>66.3</td>
<td>11.8</td>
<td>0</td>
<td>*</td>
</tr>
</tbody>
</table>

*Present study

A significant number of ulcerative colitis patients, about 30%, did not have detectable ANCA in this series. Further studies are needed to determine whether this is indicative of different forms or causes of ulcerative colitis. Yang et al (26) have suggested that ulcerative colitis is a ‘genetically heterogeneous’ disease process, and the presence of ‘ANCA-positive’ and ‘ANCA-negative’ patients may reflect this heterogeneous inflammatory process in ulcerative colitis. Clearly, if this hypothesis were true, then evaluation and especially treatment of patients with ulcerative colitis may be made more complex. Clinical trials of new medications, particularly those that have been engineered to alter the inflammatory response, may be made more difficult to interpret if patients are not from a single ‘reagent grade’ category of ulcerative colitis (27).

Although the observations in earlier studies and the present report suggest the possibility of a blood test to support a diagnosis in IBD patients, the utility of this serological marker in patients with diarrheal diseases appears to be very limited. If positive, the test may be helpful in persuading the clinician to follow a patient carefully after an initial presentation with acute colitis, especially if an infectious or acute self-limited colitis is suspected. Our experience appears to confirm data reported elsewhere (6) that the ANCA test appears to be uniformly negative in patients with culture-positive enteric bacterial enteritis or colitis. In patients with proven IBD, the clinician must still depend on clinical and pathological features to differentiate ulcerative colitis from Crohn’s disease, especially if surgical treatment for colonic disease is being contemplated (eg, such as pelvic pouch surgery). In the present study, many patients with ulcerative colitis were ANCA-negative; in addition, a significant number of patients with Crohn’s disease, including Crohn’s disease involving the colon alone, were ANCA-positive. If other reasons for detection of this serological marker have been excluded, such as concomitant primary sclerosing cholangitis, a concordant assay result might then be especially reassuring for an established diagnosis of ulcerative colitis or Crohn’s disease. Conversely, a discordant assay result might lead to re-evaluation, but not necessarily exclusion, of the patient considering colectomy and creation of a pelvic pouch. Defining serological markers to differentiate patients with ulcerative colitis from Crohn’s disease or to identify potential genetic risk factors, such as the microsatellite loci for different tumour necrosis factors (28), may eventually prove to be very useful for therapeutic studies in patients with IBD.

REFERENCES

6. Duerr RH, Targan SR, Landers CJ, Sutherland LR, Shanahan F.


