Evaluation of the Combined Application of Ethanol-Fixed and Formaldehyde-Fixed Neutrophil Substrates for Identifying Atypical Perinuclear Antineutrophil Cytoplasmic Antibodies in Inflammatory Bowel Disease[▽]

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No clear guidelines for indirect immunofluorescence (IIF) detection and interpretation of antineutrophil cytoplasmic antibodies (ANCA) have been proposed for inflammatory bowel diseases (IBD). We evaluated the reliability of the combined use of ethanol- and formalin-fixed neutrophil substrates to identify atypical perinuclear ANCA (P-ANCA) by IIF under routine laboratory circumstances. A total of 204 IBD patients were assessed with four different fluorescent substrates in two distinct laboratories. Antibodies against myeloperoxidase, proteinase-3, and other specific granule proteins (elastase, lactoferrin, cathepsin G, lysozyme, and bactericidal permeability-increasing protein) were measured by an enzyme-linked immunosorbent assay. The combined application of ethanol- and formalin-fixed slides to detect atypical P-ANCA resulted in a lack of agreement between assays (κ, $\leq$0.39) in the interassay study and moderate agreement in the interobserver study (κ, 0.42). After atypical and typical P-ANCA patterns were combined, the consensus improved greatly. A total of 26.9% of patients were P-ANCA positive by at least two tests (44.3% of ulcerative colitis [UC] and 13.1% of Crohn's disease [CD] patients; $P < 0.0001$), while overall ANCA positivity was 22.5% to 34.8%. The combined application of ethanol-fixed and formaldehyde-fixed neutrophil substrates did not facilitate differentiation between P-ANCA and atypical P-ANCA, and the results were not consistent when substrates from different sources were used. Combining all P-ANCA ensures the highest sensitivity and specificity in differentiating UC from CD.

Inflammatory bowel diseases (IBD) are disorders affecting the gastrointestinal tract and include two major entities, Crohn's disease (CD) and ulcerative colitis (UC). Although the etiology of IBD is not fully understood, it is considered an immunologically mediated disease in genetically susceptible hosts (15).

Of the expanding panel of serological markers for IBD, anti-*Saccharomyces cerevisiae* antibodies (ASCA) and perinuclear antineutrophil cytoplasmic antibodies (P-ANCA) remain the most widely investigated (2, 12). Despite methodological difficulties, which significantly hamper the diagnostic potential of the test, the presence or absence of P-ANCA is often determined in IBD for its clinical value. P-ANCA are present in the sera of 40% to 80% of patients with UC and to a lesser extent in CD (5 to 25%). Several independent studies (8, 11, 14, 16, 21) have found that the combination of P-ANCA and ASCA may be helpful in distinguishing between CD and UC, with a specificity and a positive predictive value of >90%, albeit the sensitivity is low (7, 13, 20, 30, 31).

ANCA detected in IBD are called P-ANCA, although they differ substantially from the classical ANCA used to diagnose and monitor the inflammatory activity in primary small-vessel vasculitides. This failure to clearly distinguish vasculitis-associated typical P-ANCA reactivity from the atypical P-ANCA reactivity found in IBD or autoimmune liver diseases (27, 29) leads to confusion and renders the interpretation and comparison of different studies difficult.

The target antigens of atypical P-ANCA have not been definitively identified. Considerable evidence supports the notion that these are not cytoplasmic antigens like those for typical P-ANCA, but nuclear antigens, associated with the inner side of the neutrophils' nuclear membrane (50-kDa myeloid-specific protein) (1, 26). Some granular and nonhistone chromosomal proteins (HMG1 and HMG2) are also potential candidates (18, 19, 25, 32). Because of the variability and putative characteristics of the antigens, no sensitive and specific solid-phase assays are available, leaving indirect immunofluorescence (IIF) performed on ethanol-fixed human neutrophil granulocytes as the only widespread method for the detection of these antibodies.

According to the Consensus Statement on Testing and Reporting of Antineutrophil Cytoplasmic Antibodies (23), reporting of IIF results should distinguish among cytoplasmic ANCA (C-ANCA), atypical C-ANCA, P-ANCA, and atypical ANCA. Ideally, typical P-ANCA occur as perinuclear fluorescence with a nuclear extension, and usually with anti-myeloperoxidase (anti-MPO) specificity. Under nonvasculitic

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conditions, the IIF pattern is often characterized by a broad, nonhomogeneous rim-like staining of the nuclear periphery, without a nuclear extension (atypical P-ANCA). These two patterns are not easy to distinguish, and it should be noted that the Consensus Statement describes all perinuclear fluorescence as P-ANCA, mainly because the demonstration of nuclear extension depends on the type of substrate, the fluorescence intensity, and the observer's experience. Hence, perinuclear neutrophil fluorescence alone does not necessarily indicate vasculitis.

Some serological studies of IBD use ethanol-fixed neutrophils only, rendering them biased toward MPO-specific ANCA, antinuclear antibodies (ANA), and other non-IBD-associated antibodies (antilamin, anti-Golgi complex, and antiactin, etc.) (22). Recently, a reproducible, specific, and sensitive method was described by Terjung et al., who used the combination of ethanol- and formalin-fixed human neutrophil substrates and confocal laser scanning microscopy to distinguish between P-ANCA and atypical P-ANCA. By use of the cross-linking fixative formalin, typical P-ANCA diffusely labeled the cytoplasm, that is, they converted to a C-ANCA pattern. In contrast, sera containing atypical P-ANCA produced a fine "perinuclear" labeling with multiple intranuclear fluorescent foci. This pattern was obvious only with confocal laser scanning microscopy (28). When lower-resolution planar IIF microscopy is used, this labeling can be difficult to detect, and therefore the fluorescence is usually considered negative (3, 17). The goal of our study was to evaluate the reliability of the combined use of ethanol- and formalin-fixed neutrophil substrates for the identification of atypical P-ANCA in patients with IBD. We aimed to use tests and equipment available to routine laboratories. To assess the feasibility and reproducibility of this system, we conducted an interassay and an interobserver variability study, comparing the results obtained with four different commercially available fluorescent substrates and by two geographically distinct laboratories. Besides the evaluation of fluorescence patterns on ethanol- and formalin-fixed slides, our study included investigation of the presence of ANA and measurement of the levels of antibodies against MPO, proteinase-3 (PR3), elastase, lactoferrin, cathepsin G, lysozyme, and bactericidal permeability-increasing protein (BPI) by enzyme-linked immunosorbent assays (ELISA).

MATERIALS AND METHODS

Patients. Two hundred four well-characterized IBD patients (96 with UC and 108 with CD) were investigated. The diagnosis was based on the Lennard-Jones criteria (10). The disease phenotype (age at onset, duration, location, and behavior) was determined according to the Vienna classification (5).

Data were also collected from two non-IBD gastrointestinal-disease control groups with symptoms similar to those occurring in IBD. These groups consisted of patients with irritable bowel syndrome/diverticulosis without inflammation ($n = 64$) and patients with celiac disease ($n = 96$). The clinical data of the patient groups are presented in Table 1.

The study protocol was approved by the Ethical and Science Committee of the University of Debrecen. Each patient signed the informed consent.

IIF assay for ANCA. Serum samples were stored at $-80°C$ until analysis. Aliquots from the same samples were used to detect ANCA in interassay and interobserver examinations. Ethanol- and formalin-fixed human peripheral blood neutrophils were examined simultaneously for each patient, as suggested by Terjung et al. (28).

For the evaluation of interassay variability, four different commercially available immunofluorescent ANCA assays were used to test the serum samples of IBD patients (the Nova Lite ANCA [Inova Diagnostics, San Diego, CA],

TABLE 1. Clinical data for UC and CD patients and non-IBD gastrointestinal-disease controls

Group and characteristic	Value for group
UC patients ($n = 96$)	
Males/females	46/50
Age (yr) (mean ± SD)	39.8 ± 14.9
Disease location (%)	
Left-sided colitis	67.4
Extensive	23.1
Proctitis	9.5
Surgery (%)	5.2
Extraintestinal manifestations (%)	28.4
CD patients ($n = 108$)	
Males/females	43/65
Age (yr) (mean ± SD)	34.0 ± 12.2
Disease behavior (%)	
Nonstricturing, nonpenetrating (B1)	39.8
Stricturing (B2)	26.7
Penetrating (B3)	33.5
Disease location (%)	
Terminal ileum (L1)	22.8
Colon (L2)	25.1
Ileocolon (L3)	51.3
Upper gastrointestinal tract (L4)	0.9
Perianal disease (%)	29.2
Surgery (%)	40.9
Extraintestinal manifestations (%)	41.7
Non-IBD gastrointestinal-disease controls	
Irritable bowel syndrome/diverticulosis patients ($n = 64$)	
Males/females	30/34
Age (yr) (mean ± SD)	38.2 ± 15.1
Celiac disease patients ($n = 96$)	
Males/females	23/73
Age (yr) (mean ± SD)	35.7 ± 17.4

ImmuGlo [Immco Diagnostics, Inc., Buffalo, NY], and Granulocyte Mosaic [Euroimmun Medizinische Labordiagnostika AG, Lübeck, Germany] assays and the ANCA Test System [Immuno Concepts N.A. Ltd., Sacramento, CA]), and two tests were used for non-IBD controls (Inova and Euroimmun), with one experienced observer. Each assay includes ethanol- and formalin-fixed neutrophil substrates. Specimens were diluted to 1:20 (Inova, Immco, and Immuno Concepts assays) and 1:10 (Euroimmun assay) in phosphate-buffered saline. The assays were performed according to the manufacturers' instructions.

For evaluating interobserver variability, we used the Inova ANCA assay, and only the IBD cohort was evaluated. The determinations were performed in two distinct laboratories (Laboratory of Clinical Immunology, University of Debrecen, Debrecen, Hungary, and Inova Diagnostics, Inc., San Diego, CA) and were evaluated by observers experienced in the interpretation of ANCA IIF results. The same lot of ANCA slides was used in both laboratories in order to avoid any possible lot-to-lot variation.

The patterns were evaluated and classified under UV light using a Leica microscope (Leitz Wetzler, Germany) at a magnification of ×400 in both laboratories.

Interpretation of the immunofluorescence results was based on the behavior of the specimens on ethanol- and formalin-fixed slides and included the following patterns: (i) C-ANCA occurred as coarse, speckled cytoplasmic fluorescence with accentuation between the nuclear lobes on both ethanol- and formalin-fixed substrates; (ii) typical P-ANCA presented as fine, homogeneous fluorescent staining of the perinuclear cytoplasm with nuclear extension on ethanol-fixed neutrophils and as granular cytoplasmic staining on formalin-fixed cells; (iii) atypical P-ANCA were considered to be present if the specimen produced a broad, nonhomogeneous rim-like staining of the nuclear periphery or a combined pattern characterized by diffuse cytoplasmic staining, not accentuated between the nuclear lobes, along with broad, nonhomogeneous perinuclear fluorescence on ethanol-fixed substrates and weak perinuclear staining or a lack of fluorescence on formalin-fixed granulocytes.

TABLE 2. Results of the interassay study with four ANCA assays

Patient group and ANCA pattern	No. (%) of patients with ANCA patterns observed by the following assay:			
	Inova	Immco	Euroimmun	Immuno Concepts
ANA-negative UC patients (*n* = 79)				
Total ANCA	43 (54.4)	40 (50.6)	31 (39.2)	30 (38.0)
Atypical P-ANCA	34 (43.0)	5 (6.3)	23 (29.1)	23 (29.1)
P-ANCA	6 (7.6)	30 (38.0)	8 (10.1)	4 (5.1)
C-ANCA	3 (3.8)	5 (6.3)	0 (0.0)	3 (3.8)
ANA-negative CD patients (*n* = 99)				
Total ANCA	18 (18.2)	23 (23.2)	11 (11.1)	11 (11.1)
Atypical P-ANCA	16 (16.2)	9 (9.1)	8 (8.1)	9 (9.1)
P-ANCA	2 (2.0)	10 (10.1)	3 (3.0)	1 (1.0)
C-ANCA	0 (0.0)	4 (4.0)	0 (0.0)	1 (1.0)
Non-IBD gastrointestinal-disease control groups				
Irritable bowel syndrome/ diverticulosis patients without inflammation (*n* = 64)				
Total ANCA	2 (3.1)		3 (4.7)	
Atypical P-ANCA	1 (1.6)		1 (1.6)	
P-ANCA	1 (1.6)		2 (3.1)	
C-ANCA	0 (0.0)		0 (0.0)	
Celiac disease patients (*n* = 96)				
Total ANCA	5 (5.2)		4 (4.2)	
Atypical P-ANCA	2 (2.1)		2 (2.1)	
P-ANCA	3 (3.1)		2 (2.1)	
C-ANCA	0 (0.0)		0 (0.0)	

Examinations were performed in a blinded fashion without knowledge of patient diagnoses or other clinical information both in the interassay and in the interobserver variability testing.

Serum samples were screened for the presence of ANA on HEp-2 cells by IIF (Immco) according to the manufacturer's instructions. We also included a HEp-2 biochip in the Euroimmun ANCA assay (the Granulocyte Mosaic assay). If either of the tests was positive, the sample was considered ANA positive and was excluded from the final ANCA estimate.

Antigen-specific assays. Serum samples were tested separately for the presence of immunoglobulin G (IgG) antibodies against MPO, PR3, lactoferrin, BPI, cathepsin G, elastase, and lysozyme by ELISA (Orgentec Diagnostika GmbH, Mainz, Germany) according to the manufacturer's protocol. Quantitative results were obtained with anti-MPO and anti-PR3 (cutoff level, 5 U/ml), while qualitative values were determined for other antibodies.

Statistical methods. The agreement between the four immunofluorescent ANCA assays was estimated by a weighted kappa coefficient (κ). Correlations between the presence of antibodies and disease phenotypes were assessed by Fisher's exact test, and differences between antibody levels were assessed by the Mann-Whitney U test. SPSS software (version 13.0; SPSS Inc., Chicago, IL) was used for statistical analysis.

RESULTS

Interassay study. (i) Fluorescence patterns. ANCA examination was carried out on sera from 108 CD and 96 UC patients by IIF microscopy using both ethanol- and formalin-fixed neutrophil substrates. The prevalence of all ANCA patterns as determined by the four IIF assays ranged from 23.0% to 35.4% in the study population: 38.0 to 54.7% for UC patients and 11.1 to 23.2% for CD patients. The occurrence of total ANCA was similar in the two non-IBD gastrointestinal-disease control groups (3.1 to 5.2%).

Nonetheless, the ANA-positive samples were excluded from

TABLE 3. Concordance between four different immunofluorescent ANCA tests with regard to atypical P-ANCA

Test	Concordance[a] with the following assay:			
	Inova	Immco	Euroimmun	Immuno Concepts
Inova	1			
Immco	0.11	1		
Euroimmun	0.39	0.03	1	
Immuno Concepts	0.35	0.12	0.29	1

[a] Agreement was characterized by the κ coefficient.

the final ANCA estimate. Of the patients in the UC and CD groups, 17.7% (17/96) and 8.3% (9/108) were found ANA positive, respectively. The frequencies of different ANCA patterns among ANA-negative patients in the interassay study are summarized in Table 2.

According to the preestablished criteria (see Materials and Methods), for all but one assay, the ANCA patterns were mainly atypical P-ANCA both for UC (74.2 to 79.1% of all ANCA) and for CD (72.7 to 88.9% of all ANCA) patients. With the Immco assay, a relatively low proportion of the ANCA detected showed atypical P-ANCA patterns in UC and CD (12.5% and 39.1% of all ANCA, respectively); the prevalence of typical P-ANCA was high (75.0% and 43.5% of all ANCA, respectively). All assays rarely detected the C-ANCA pattern.

Altogether, the agreement among the four different assays ranged from 71.9 to 79.8%, 79.8 to 94.4%, and 93.2 to 98.3% for atypical P-ANCA, P-ANCA, and C-ANCA, respectively. The κ coefficients suggested poor to fair concordance between the different assays (κ, 0.11 to 0.39 for atypical P-ANCA) (Table 3). The κ coefficient was not calculated for P-ANCA and C-ANCA because of their very low prevalences. The results were similar when UC and CD patients were evaluated separately (data not shown).

Figure 1 shows the ANCA patterns from the sera of two patients with UC by all four different assays. For patient 104, all the tests yielded atypical P-ANCA patterns (Fig. 1A), while for patient 95, the various ANCA tests gave different results: the Inova and Immuno Concepts tests gave C-ANCA patterns, while the Immco and Euroimmun tests gave P-ANCA patterns (Fig. 1B). The latter sample was positive for anti-PR3 IgG (17.8 U/ml).

To further examine and compare the results obtained by different assays, we established consensus results. The fluorescent pattern was considered true atypical P-ANCA when three out of four tests produced identical results. Consensus was reached only in 12 cases (6.7% of all patients), clearly reflecting the lack of agreement. However, when typical and atypical P-ANCA patterns were combined (according to the Consensus Statement [23]), consensus was reached in 32 cases (18.0% of all patients). These data suggest that differentiation between typical and atypical P-ANCA using formalin-fixed slides is highly dependent on the commercial substrate utilized and that agreement can be improved if typical and atypical P-ANCA patterns are not differentiated. For the 32 consensus cases, the Inova, Immco, and Euroimmun assays detected similar percentages of positivity (93.8, 90.6, and 81.3%, respectively), while the Immuno Concepts assay underestimated the number
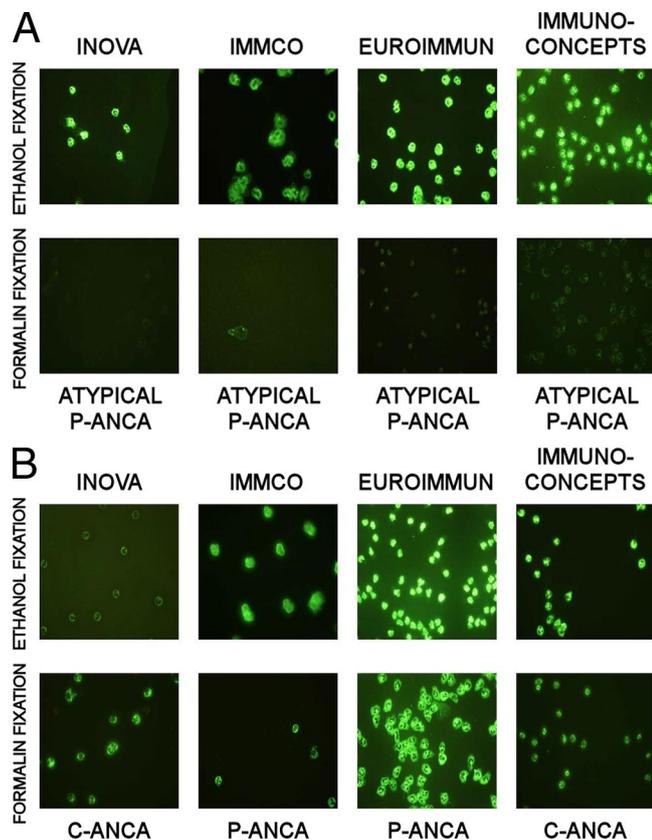
FIG. 1. Immunofluorescent staining patterns produced by four different ANCA assays, both on ethanol- and on formalin-fixed human neutrophils, for two different patients. Atypical P-ANCA present as broad, nonhomogeneous rim-like staining of the nuclear periphery on an ethanol slide and show little or no perinuclear or cytoplasmic staining on a formalin slide. P-ANCA (perinuclear staining) show a fine, homogeneous fluorescent staining of the perinuclear cytoplasm on an ethanol slide and diffusely label the cytoplasm (i.e., convert to C-ANCA) on a formalin slide. C-ANCA (cytoplasmic staining) yield a coarse, speckled cytoplasmic fluorescent pattern with accentuated fluorescence between the nuclear lobes on an ethanol slide, and this pattern becomes more pronounced on a formalin slide. (A) All four ANCA assays show atypical P-ANCA patterns for the serum sample of UC patient 104. (B) The four ANCA assays show distinct ANCA patterns for the serum sample of UC patient 95. Magnification, ×40.

of P-ANCA-positive specimens (62.5%). Therefore, we excluded the results obtained by the Immuno Concepts kit and established a new consensus using results only from the three former assays. In this case, consensus (two positive results out of the three results) was reached in 23 cases with regard to atypical P-ANCA (12.9% of all patients) and in 48 cases with regard to all P-ANCA (26.9% of all patients), including 35 UC (44.3%) and 13 CD (13.1%) patients (P < 0.0001 for the comparison between UC and CD). Considering the overall rate of ANCA positivity (24.2 to 34.8% with the Inova, Immco, and Euroimmun assays, a rate that also includes some C-ANCA patterns), these results suggest that by calculating all P-ANCA together, consensus was reached for the vast majority of samples. The three different assays detected similar percentages of the consensus cases (87.5, 89.6, and 71.9%, respectively).

(ii) Antigen specificity. We assessed the presence of IgG antibodies reacting with different cytoplasmic components of the neutrophil.

The prevalence and the level of antibodies against PR3 were higher than those for MPO, albeit both were low. In UC, anti-MPO and anti-PR3 antibodies were more prevalent than in CD (4.2% versus 0.9% [not significant {NS}] and 16.7% versus 3.7% [P = 0.002], respectively). Considering only positive results, the anti-PR3 level was higher in UC than in CD (25.9 U/ml [interquartile range, 15.8 to 38.1 U/ml] versus 9.6 U/ml [interquartile range, 7.9 to 13.4 U/ml]; NS). One UC patient had both anti-MPO and anti-PR3, and two CD and four UC patients had antibodies against lactoferrin and/or BPI besides the anti-MPO and anti-PR3. Of the 24 patients with different anti-MPO or anti-PR3 antibody levels, 21 (87.5%) showed some type of immunofluorescence on Immco test slides, and 13 (54.2%) on Inova, Euroimmun, and Immuno Concepts substrates. Excluding those specimens containing antibody levels lower than twice the cutoff, only 13 patients had considerable levels of anti-MPO or anti-PR3 (6.4% of all patients), and 8 to 10 of them were positive with IIF by the various assays.

We could not demonstrate anti-cathepsin G or antielastase antibodies in either group, and we detected antilysozyme antibodies in only one person in each patient group. The prevalences of antilactoferrin and anti-BPI antibodies were 10.4% versus 4.6% and 10.4% versus 7.4% in UC and CD, respectively (NS). One CD and three UC subjects had both antibodies. Furthermore, concomitant anti-MPO or anti-PR3 was present in six patients. Altogether, of the 31 cases (15.2% of all patients) with antilysozyme, antilactoferrin, and/or anti-BPI antibodies, 27 (87.1%) showed positive IIF results on the Immco slides, and 20 (64.5%), 13 (41.9%), and 11 (35.5%) showed positive IIF results on Inova, Euroimmun, and Immuno Concepts substrates. The distributions of the immunofluorescent patterns differed between the methods and included P-ANCA, atypical P-ANCA, and C-ANCA (Table 4).

The results obtained by ELISA were also analyzed with regard to clinical phenotype, and no statistically significant differences were detected between patients with or without these antibodies.

Interobserver study. In the interobserver study, the same test (the Nova Lite ANCA test; Inova) was performed in two independent laboratories, and the results were evaluated by two different experienced readers. For patients with UC, the prevalence of reported ANCA patterns was higher in the San

TABLE 4. Distribution of the immunofluorescent patterns detected by different assays in IBD patients with antibodies against lactoferrin, BPI, or lysozyme

Test	No. of cases[a]			
	Atypical P-ANCA	Typical P-ANCA	C-ANCA	Negative
Immco	7	14	6	4
Inova	16	4	0	11
Euroimmun	9	4	0	18
Immuno Concepts	9	1	1	20

[a] A total of 31 patients were tested. Four patients had antibodies against both lactoferrin and BPI, and six patients had antibodies against MPO or PR3 as well.

Diego laboratory than in the Debrecen laboratory (68.4% versus 54.4%), an outcome that was attributable mainly to atypical P-ANCA, the most frequent of the ANCA (62.0% versus 43.0%). In contrast, the occurrences of P-ANCA (3.8% versus 7.6%) and C-ANCA (2.5% versus 3.8%) were somewhat lower. In CD, the observers found nearly identical prevalence rates for the different ANCA patterns: 19.2% versus 16.2% for atypical P-ANCA, 2.0% versus 2.0% for P-ANCA, and 1.0% versus for 0.0% for C-ANCA (altogether, 22.2 versus 18.2%). The agreement rate for atypical P-ANCA in the whole study population was 74.1%, which corresponded to a κ coefficient of 0.42, suggesting moderate interobserver concordance. The κ coefficient was not calculated for P-ANCA and C-ANCA because of their very low prevalence rates.

When P-ANCA and atypical P-ANCA patterns were grouped together, the prevalence rates of P-ANCA in UC and CD were 62.0% versus 19.2% ($P < 0.0001$) and 50.6% versus 18.2% ($P < 0.0001$) in the San Diego and Debrecen laboratories, respectively. This reflects an agreement rate of 77.5% (κ, 0.51). These results reflect sensitivities and specificities (95% confidence intervals) for the determination of P-ANCA in UC of 62.0% (51.0 to 72.0%) and 80.8% (72.1 to 87.4%), respectively, in the San Diego laboratory and 50.6% (39.8 to 61.4%) and 81.8% (73.1 to 88.2%), respectively, in the Debrecen laboratory. The differences in sensitivity and specificity between the two laboratories were not significant.

DISCUSSION

In contrast to the Consensus Statement for vasculitis-associated ANCA (23), there are no clear guidelines for immunofluorescence detection and interpretation of ANCA patterns in IBD (24). Differentiating between atypical and typical P-ANCA on ethanol-fixed substrates by IIF is still challenging. Previous data regarding formalin-fixed neutrophils in the detection of atypical P-ANCA are scattered and controversial (4, 7, 31). Differences in formaldehyde fixation techniques and the resolution of the immunofluorescence microscope used might be responsible for these contradictions. However, the use of a formalin-fixed substrate was reported as a simple, useful technique for differentiating between ANA and typical P-ANCA, which may occur together (9). Simultaneous reactivity of atypical P-ANCA on formalin- and ethanol-fixed neutrophils and the feasibility of the new microscopic criteria suggested by Terjung et al. (28) have not been systematically studied in IBD (7, 31). ANCA systems that replace formalin-fixed neutrophils with an enzyme (DNase I) digestion step during IIF have also been developed but are rarely used (7, 31). Instead of the term "atypical P-ANCA," the term "DNase-sensitive P-ANCA" (i.e., not detectable on DNase-treated neutrophils) is generally used (31).

The poor agreement between various ANCA assays and different observers is well documented (6, 21). The remarkable differences are likely attributable to the distinctive parameters of the various manufacturers' kits, including differences in cell preparation affecting background fluorescence, fixation methodologies, buffers, and conjugates. The discrepancies are further increased by the use of different microscopes, variations in routine protocols, and the specific criteria used for pattern interpretation. An additional critical parameter contributing to

variations in the interobserver studies is the various levels of experience of the observers. Since IIF interpretation is subjective, consistent interpretation requires an experienced observer.

In the present study, we attempted to differentiate between P-ANCA and atypical P-ANCA patterns in IBD patients by using ethanol- and formalin-fixed slides and predetermined IIF criteria. The most frequent pattern, both in UC and in CD, was atypical P-ANCA. However, we found significant differences in the occurrence of atypical P-ANCA in patients with UC (6.3 to 43.0%) and CD (8.1 to 16.2%) according to different commercial ANCA assays. All possible pairwise comparisons demonstrated a statistically significant lack of agreement between the assays (κ, 0.11 to 0.39). Similar results were published in two earlier studies. Joossens et al. (6) assayed the presence of ANCA in 50 UC patients by four different assays (from Bio-Rad, The Binding Site, Immuno Concepts, and Inova). The prevalence of atypical P-ANCA ranged from 16% to 62% using both ethanol- and formalin-fixed neutrophil substrates, and κ coefficients indicated poor agreement (<0.2). In the paper of Sandborn et al. (21), the sensitivity of detection of P-ANCA for 162 IBD patients differed (0% to 63%) in different laboratories (Prometheus, Oxford, Wuerzburg, Mayo, and SmithKline Beecham). One assay in this study used the DNase digestion method, while the others used ethanol-fixed slides (21). In our study, when all the typical and atypical P-ANCA results were combined (i.e., the different IIF patterns on formalin-fixed substrates were ignored), consensus was reached for the vast majority of ANCA-positive specimens. This finding suggests that the differing performances of the various commercial formalin-fixed slides may be responsible for much of the variance observed in the interassay study.

The ANCA test alone was able to differentiate between non-IBD gastrointestinal-disease controls, UC cases, and CD cases with an acceptable specificity (85 to 96%) and positive predictive value (72 to 87%). However, the overall sensitivity was low. The use of this marker alone is insufficient for screening purposes for patients suspected to have IBD. However, the high positive predictive value supports its use in the confirmation of a diagnosis.

In the interobserver part of our study, we found better concordance for atypical P-ANCA (κ, 0.42), suggesting that the differences between the various commercial IIF ANCA assays contributed more to the observed discrepancies than the type of the microscope or the observers' experience. Again, when P-ANCA and atypical P-ANCA patterns were grouped together, the agreement improved (κ, 0.51), and there were no significant differences between the two laboratories with regard to the sensitivity and specificity of P-ANCA detection in UC (62.0% versus 50.6% and 80.8% versus 81.8%, respectively). These values are in accordance with our previously published observations (13).

Specific antibodies against lysozyme, lactoferrin, or BPI were detected in 15.2% of all patients, with no differences between UC and CD. Multiple positivities were prevalent. Some of these antibodies did not produce positive IIF patterns; the others presented as P-ANCA, atypical P-ANCA, or C-ANCA. No associations between particular antibody specificities and IIF patterns were detected, and the distribution of the patterns differed widely between the IIF assays. Moreover,

there was no obvious correlation between the presence of these various antibodies and the type and characteristics of IBD. Anti-MPO and anti-PR3 antibodies were present in the sera of 11.8% of all patients. Anti-PR3 antibodies were more prevalent in UC than in CD (16.7% versus 3.7%; $P = 0.002$). One UC patient had both anti-MPO and anti-PR3 antibodies, and two CD and four UC patients had antibodies against lactoferrin and/or BPI besides the anti-MPO or anti-PR3 antibody. Antibody levels were usually low. Considering only specimens containing antibody levels higher than twice the cutoff, 13 (6.4% of all patients) cases had considerable median levels of anti-MPO (13.9 U/ml) or PR3 (24.9 U/ml), and only 8 to 10 of them were positive by IIF with the various assays. The low levels of anti-MPO and anti-PR3, as well as the concomitant antibody positivity against other (sometimes multiple) granule components, suggest that the clinical significance and specificity of these antibodies are irrelevant. At the same time, the presence of anti-MPO and anti-PR3 at levels higher than twice the cutoff in the IBD population studied is remarkable, since these antigens are not considered autoantigens in IBD. We have no clinical explanation for this finding. None of these patients had vasculitis at the time of sampling or during the follow-up period.

In conclusion, our results demonstrated wide variations in the performance of commercial ANCA slides and less dramatic differences between different observers, in accordance with the findings of previous publications. Differentiation between P-ANCA and atypical P-ANCA by using formalin-fixed slides is neither reliable nor reproducible under routine laboratory circumstances using fluorescent microscopes. P-ANCA (regardless of the typical or atypical appearance on ethanol-fixed substrates) should be considered together in order to ensure the highest sensitivity and specificity in differentiating UC from CD.

ACKNOWLEDGMENTS

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