Correlations between IF and ELISA tests for ANCA, MPO, PR3

Among IF + patients		Among ELISA + patients	
Anti-PR3+if cANCA+	42% (24 of 57)	cANCA+if anti-PR3+	80% (24 of 30)
Anti-MPO+ if cANCA+	7% (4 of 57)	pANCA+if anti-PR3+	7% (2 of 30)
Anti-MPO+ if pANCA+	24% (11 of 45)	cANCA+ if anti-MPO+	25% (4 of 16)
Anti-PR3+ if pANCA+	4% (2 of 45)	pANCA+ if anti-MPO+	69% (11 of 16)
Either anti-PR3+ or anti-MPO+	40% (41 of 102)	Either cANCA+or pANCA+	89% (41 of 46)

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Test Characteristics of Immunofluorescence and ELISA Tests in 856 Consecutive Patients with Possible ANCA-Associated Conditions

John H. Stone, Monica Talor, Justin Stebbing, Misty L. Uhlfelder, Noel R. Rose, Kathryn A. Carson, David B. Hellmann, and C. Lynne Burek

Objective. *To examine the test characteristics of immunofluorescence (IF) and enzyme-linked immunosorbent assays (ELISA) in a consecutive series of patients under evaluation for anti-neutrophil cytoplasmic antibody (ANCA)–associated vasculitis (AAV).*

Methods. *Using stored sera, we performed a cross-sectional study on 856 consecutive patients tested prospectively for ANCA by IF. Based on guidelines from the 1994 Chapel Hill Consensus Conference (CHCC), we determined each patient's underlying diagnosis by a medical records review without regard to their ANCA status (the CHCC guidelines do not require ANCA as a prerequisite for diagnosis). We grouped patients with forms of vasculitis commonly associated with ANCA into one of 4 types of AAV: Wegener's granulomatosis (n = 45), microscopic polyangiitis (n = 12), Churg-Strauss syndrome (n = 4), and pauci-immune glomerulonephritis (n = 8). We also classified patients without clinical evidence of AAV (92% of all patients tested) into 5 predefined categories of disease (including "other") and an additional category for no identifiable disease. In a blinded fashion, we then performed ELISAs on the stored serum for antibodies to proteinase-3 (PR3) and myeloperoxidase (MPO) and calculated the test characteristics for both ANCA assay techniques.*

Results. *Sixty-nine of the 856 patients (8.1%) had clinical diagnoses of AAV based on CHCC guidelines. The positive predictive value (PPV) of ELISA for AAV was superior to that of IF, 83% versus 45%. For patients with both positive IF tests and positive ELISA tests, the PPV increased to 88%. Both IF and ELISA had high negative predictive values (97% and 96%, respectively). Positive ELISA tests were associated with higher likelihood ratios (LR) than IF (54.2 [95% CI = 26.3, 111.5] versus 9.4 [95% CI = 6.9, 12.7]). The LR of both a positive IF and a positive ELISA was 82.1 (95% CI = 33.3, 202.5).*

Conclusions. *Compared with IF, an ELISA test for ANCA was associated with a substantially higher PPV and LR for AAV. This fact, combined with the greater sensitivity of IF, suggests that an effective testing strategy is to perform ELISA tests only on samples that are positive for ANCA by IF.*

Key words. *ANCA; Vasculitis; Immunofluorescence; Enzyme-linked immunoassay.*

INTRODUCTION

Anti-neutrophil cytoplasmic antibodies (ANCA) were first reported in 1982 in a group of patients

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John H. Stone, MD, MPH, Justin Stebbing, MD, Misty L. Uhlfelder, BA, and David B. Hellmann, MD, Department of Medicine, Johns Hopkins University Vasculitis Center, Baltimore, Maryland; Monica Talor, MS, Noel R. Rose, MD, PhD, and C. Lynne Burek, PhD, Department of Pathology, Johns Hopkins University Vasculitis Center, Baltimore, Maryland; and Kathryn A. Carson, ScM, Department of Oncology, Johns Hopkins University Vasculitis Center, Baltimore, Maryland.

Address correspondence to John H. Stone, MD, MPH, Johns Hopkins Vasculitis Center, 1830 E. Monument Street, Suite 7500, Baltimore, MD 21205.

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with segmental necrotizing glomerulonephritis (1). These findings were ascribed initially to arbovirus infections, but within several years ANCA had been linked to certain forms of idiopathic systemic necrotizing vasculitis (2). The first association between ANCA and vasculitis was established with Wegener's granulomatosis (WG), but associations with other types of vasculitis were recognized later, including microscopic polyangiitis (MPA) (3–5), the Churg-Strauss syndrome (CSS) (6,7), and pauci-immune glomerulonephritis (GN) (8).

Two types of ANCA tests, immunofluorescence (IF) and enzyme-linked immunosorbent assays (ELISA), are now in common use. With IF, 3 principal patterns of fluorescence are recognized: cytoplasmic (cANCA), perinuclear (pANCA), and atypical. The cANCA pattern, which often correlates with antibodies to proteinase-3 (PR3), has been strongly linked to WG (9). The pANCA pattern, which corresponds strongly to the presence of anti-myeloperoxidase (MPO) antibodies in patients with vasculitis, is found frequently in MPA, CSS, and pauci-immune GN (and sometimes in WG). Atypical ANCA may occur in association with a wide variety of diseases, including inflammatory bowel disease (10) and connective tissue disorders such as systemic lupus erythematosus (11). Forms of vasculitis with similar clinical and pathological features that are commonly accompanied by ANCA are known collectively as "ANCA-associated vasculitides" (AAV), even though not all patients with these disorders have ANCA. The precise role of ANCA in the pathogenesis of these diseases remains unclear (12).

The reported sensitivities and specificities of ANCA tests range widely, depending not only on the test characteristics of the assays used but also on the population under study. IF was initially believed to have sensitivities and specificities both in excess of 90% for WG (2,13–16). Frequently, however, these studies were performed in selected patient groups (e.g., subjects with previously defined AAVs and healthy controls) rather than in series of patients with undiagnosed illnesses whose clinical presentations suggested vasculitis. Subsequent studies, most of which have focused on the more widely available IF technique, have shown less favorable results. Although a major prospective study of IF confirmed the high specificity of cANCA for the diagnosis of WG (i.e., most patients who did not have WG were cANCA−), the sensitivity and positive predictive value of cANCA for WG were only 28% and 50%, respectively (17).

Recently, advantages of ELISA techniques to measure ANCAs have been reported. Two rigorous studies (using selected disease and control subject populations) demonstrated significant improvement of ELISA testing over IF in terms of specificity (11,18). When confronted with a potential case of vasculitis in clinical practice, however, clinicians must interpret the results of ANCA assays in the context of patients' overall presentations. In this setting, knowledge of the positive and negative predictive values (PPV and NPV, respectively) of diagnostic tests may be more useful than sensitivities and specificities, because the latter pertain to situations in which the diagnosis is already known. Few data exist about PPV and NPV in unselected groups of patients undergoing both IF and ELISA tests for AAV.

Another useful test characteristic is the likelihood ratio (LR). LRs—the ratio of the probability of a given test outcome in patients with a disease to the probability of the same test outcome in patients without the disease—permit direct calculations of posttest probabilities from estimates of the pretest likelihood of disease. LRs are most useful in patients who have intermediate probabilities of a given disease (e.g., the patient demonstrating some features of a multiorgan system inflammatory illness but not a "classic" AAV phenotype). For such patients, a positive test known to be associated with a high LR may shift a clinician's thinking dramatically toward AAV and direct attention to the proper steps for diagnostic confirmation and treatment.

In this study, we examined the predictive values and LR of both IF and ELISA among 856 consecutive patients who underwent serologic testing at our center as part of evaluations for ANCA-associated conditions.

PATIENTS AND METHODS

This study was approved by the Joint Committee on Clinical Investigation of the Johns Hopkins Medical Institutions. Between January 1, 1995, and April 1, 1998, IF was the standard ANCA assay technique at our institution. The Johns Hopkins Immunologic Disorders Laboratory (IDL) performs ANCA for inpatients and for patients from the attached outpatient clinics at our institution. During the study period, 856 patients underwent testing for ANCA. These sera were submitted to the IDL through the core laboratory facility at Johns Hopkins Hospital. The samples came from inpatients from the hospital, from various outpatient clinics contiguous to the hospital, and from satellite outpatient clinics of Johns Hopkins Medical Institutions. Thus, the phy-

Table 1. Diagnoses in 856 patients tested for ANCA during the study period*

Diagnosis	Number (%)
ANCA-associated vasculitis	69 (8.1%)
Wegener's granulomatosis	45 (5.3%)
Microscopic polyangiitis	12 (1.4%)
Churg-Strauss syndrome	4 (0.5%)
Pauci-immune glomerulonephritis	8 (0.9%)
Inflammatory bowel disease	22 (2.6%)
Undifferentiated disease process, possibly ANCA-associated	27 (3.2%)
Other vasculitic or renal disorder	105 (12.3%)
Other disease	477 (55.7%)
No identifiable disease	156 (18.2%)

* ANCA = anti-neutrophil cytoplasmic antibodies.

sicians ordering the tests were a diverse group of generalists and subspecialists who suspected AAV in their patients. The samples were collected sequentially and were selected for this study only because an ANCA was requested. If multiple samples were available for an individual patient over time, only the patient's first sample was used. Serum samples of the specimens tested for ANCA as part of routine clinical care were frozen at −20°C.

Disease classification. Using the computer-based medical records system at our institution (Electronic Patient Record, EPR), supplemented by medical chart reviews and discussions with patients' treating physicians when necessary, 2 investigators (either JHS or JS) determined each patient's diagnosis without regard to their ANCA status (Table 1). Followup on the patients from the time of the original ANCA assay ranged from 6 to 46 months. We defined 6 disease categories: 1) AAV (WG, MPA, CSS, or pauci-immune GN); 2) inflammatory bowel disease; 3) undifferentiated disease process, possibly ANCA-associated; 4) other vasculitic or renal disorder; 5) other disease; and 6) no identifiable disease. Classification of the AAVs (i.e., the patients' clinical diagnoses) was based on guidelines from the 1994 Chapel Hill Consensus Conference (CHCC) (19). These guidelines do not require positive ANCA tests for the diagnosis of an AAV. The diagnosis of pauci-immune GN required a renal biopsy and a tissue immunofluorescence study showing few immunoreactants.

For WG, the largest subset of AAV patients (n = 45), we recorded the patients' treatment at the time of the original IF assay. We also recorded the presence or absence of active disease, defining activity as the presence of any clinical, radiologic, or pathologic evidence of ongoing inflammation attributable to WG (20,21).

ANCA testing by IF. All samples were screened in an identical manner on 3 types of slides: 1) ethanol-fixed slides prepared in our laboratory, 2) commercially prepared slides fixed with ethanol (INOVA Diagnostics, San Diego, CA), and 3) commercially prepared, formalin-fixed slides (INOVA Diagnostics, San Diego, CA). We screened with all 3 types of slides both to optimize the sensitivity of our IF assay and to mitigate any potential artifacts that may occur due to preparation of either the laboratory-prepared or the commercial slides. If a positive sample was identified using any of these methods, we titrated the sample on the laboratory-prepared slides.

For the laboratory-prepared slides, neutrophils were derived from human group O blood. Briefly, the blood (collected with heparin) was mixed with 3% Dextran in normal saline and sedimented for 20 minutes. The leukocyte-rich plasma was centrifuged and the red cells lysed. The cells were washed with normal saline. Final resuspension to approximately 10^5 cells/ml was done in tissue culture medium with 10% Nuserum IV (Collaborative Research, GIBCO, Rockville, MD). The cells were cytocentrifuged onto glass slides, air dried, and then fixed in cold ethanol for 5 minutes. After drying, the slides were stored at −80°C.

The cells were overlaid with serum diluted in phosphate-buffered saline (PBS) (pH 7.4), incubated in a humid chamber for 30 minutes at room temperature, washed in PBS for 30 minutes, and placed into the humid chamber again. The cells were then incubated with goat anti-human IgG (h+1 chains) conjugated to DTAF (Jackson Laboratories, West Grove, PA) and mixed with Rhodamine-B BSA as counterstain (DIFCO, Detroit, MI). After another wash period, the slides were coverslipped with buffered polyvinyl alcohol in glycerol as mounting medium and were examined using a Zeiss fluorescence microscope for pattern of ANCA staining and end point titer. A separate slide with rodent liver as substrate was used to assess the possibility of concurrent antinuclear antibodies (ANA) (which may interfere with evaluation of perinuclear IF). If ANAs were found, the patient's serum was titrated on formalin-treated cells.

Four IF results were recognized: cytoplasmic (cANCA), perinuclear (pANCA), atypical, and negative. The cANCA pattern on ethanol-fixed slides has a diffuse, granular staining of the neutrophil cytoplasm. In contrast, the pANCA pattern appears around the nucleus on ethanol-fixed slides and

Table 2. Test characteristics of IF and ELISA tests in 856 consecutive patients with possible ANCA-associated conditions*

	IF+	cANCA+	pANCA+	ELISA+	PR3+	MPO+	IF+ and ELISA+
Sensitivity: patients with positive tests who have AAV/patients with AAV	67% (46/69)	42% (29/69)	25% (17/69)	55% (38/69)	35% (24/69)	20% (14/69)	52% (36/69)
Specificity: patients with negative tests without AAV/patients without AAV	93% (731/787)	96% (759/787)	96% (759/787)	99% (779/787)	99% (781/787)	99.7% (785/787)	99% (782/787)
Positive predictive value: patients with AAV who have positive tests/ patients with positive tests	45% (46/102)	51% (29/57)	38% (17/45)	83% (38/46)	80% (24/30)	88% (14/16)	88% (36/41)
Negative predictive value: patients without AAV who have negative tests/ patients with negative tests	97% (731/754)	95% (759/799)	94% (759/811)	96% (779/810)	95% (781/826)	93% (785/840)	96% (782/815)
Likelihood ratio of a positive test: sensitivity/ (1-specificity)	9.4	11.8	6.9	54.2	45.6	79.8	82.1
Likelihood ratio of a negative test: (1-sensitivity)/specificity	0.36	0.60	0.78	0.45	0.66	0.80	0.48

* IF = immunofluorescence; ELISA = enzyme-linked immunosorbent assays; ANCA = anti-neutrophil cytoplasmic antibodies; cANCA = cytoplasmic ANCA; pANCA = perinuclear ANCA; PR3 = proteinase-3; MPO = myeloperoxidase; AAV = ANCA-associated vasculitis (Wegener's granulomatosis, microscopic polyangiitis, Churg-Strauss syndrome, or pauci-immune glomerulonephritis).

sometimes covers the entire nucleus. Atypical ANCA appear as a "rim" pattern on the nuclear membrane with an area of central clearing. Whereas "true" pANCA IF demonstrates a cytoplasmic staining on formalin-fixed slides, atypical ANCA samples are negative on the formalin-fixed substrate. Based on previous experience in our laboratory with IF techniques for the detection of ANCA, titers of 1:20 or greater were considered positive (we confirmed the validity of this cutoff point with receiver operating characteristic [ROC] curves in this study). For the purposes of analysis in this study, atypical ANCA patterns were considered negative.

ANCA testing by ELISA. In preparation for the ELISA portion of this study, we performed a small pilot study (22). We tested 3 commercially available ELISA kits on the sera of 20 patients with either WG or MPA and chose 1 set of kits (for antibodies to PR3 and MPO) based on consistency of test results for use in the larger study. Using the stored serum specimens, we then performed the ELISA tests according to the manufacturer's specifications (INOVA Diagnostics, San Diego, CA). These ELISA kits are approved for commercial use by the Food and Drug Administration. The PR3 and MPO antigens used in the assay are purified and bound to polystyrene wells using conditions to ensure that the antigens remain in the native state. Controls were prediluted and consisted of a high positive, low positive, and negative control sample. In both the anti-PR3 and the anti-MPO assays, IgG antibodies were targeted.

Statistical analysis. For both IF and ELISA, we calculated the sensitivity, specificity, PPV, NPV, and LR for positive and negative results. Definitions of these test characteristics are provided in Table 2, column 1. To illustrate the use of LR in populations similar to ours, we used the prevalence of AAV in our sample as the pretest probability and computed the posttest probabilities of AAV using the formula:

$$(\text{pretest odds of AAV}) \times (\text{LR}) = \text{posttest odds of AAV}$$

We compared the sensitivities and specificities of the IF and ELISA tests using appropriate tests for paired data. Because the total of discordant pairs was less than 20, we used STATA (StataCorp, College Station, TX) to compute a 2-sided exact binomial test

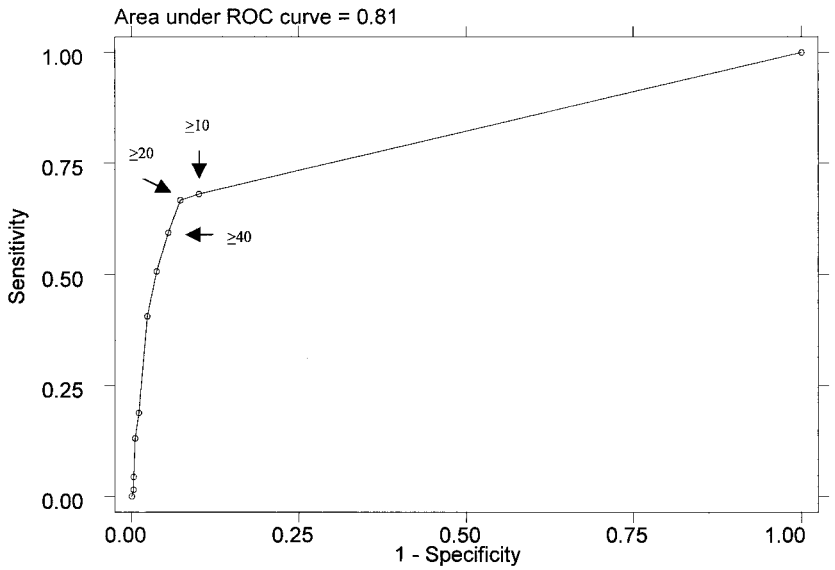

Figure 1. Receiver operating characteristic (ROC) curve for immunofluorescence assays. At titers <1:20, there is minimal gain in sensitivity at a point on the curve where specificity begins to fall off dramatically. Thus, 1:20 appears to be the optimal cutoff for a positive test.

of the difference in sensitivities. McNemar's test was used to test the difference in specificities of the IF and ELISA tests. We considered *P* values less than 0.05 to be significant, and we calculated 95% confidence intervals (CI) for LR according to the formula described by Dujardin and colleagues (23). We also constructed ROC curves for the IF and ELISA tests, using STATA. ROC curves plot 1-specificity (X-axis) versus sensitivity (Y-axis), illustrating the rates of true- and false-positive results at all possible choices of cutoff points for positive tests.

Finally, we examined the correlations between ANCA positivity by IF testing and ANCA positivity by ELISA. For the subset of patients with WG, we calculated the sensitivities of IF and ELISA in the group with active disease at the time of the assay (n = 29) and in the group with active disease who were receiving no treatment at the time of the assay (n = 18).

RESULTS

Among the 856 patients who underwent testing for ANCA at our center during the period of this study, 45 had WG, 12 had MPA, 4 had CSS, and 8 had pauci-immune GN. Thus, 69 of the 856 patients tested for ANCA (8.1%) had forms of vasculitis that are commonly associated with these antibodies. The categories of diagnosis and the number of patients in each category are listed in Table 1.

Test characteristics. The test characteristics of IF and ELISA are displayed in Table 2. IF was superior to ELISA in terms of sensitivity: 67% (46 patients with positive tests, among 69 with AAV) versus 55% (38 of 69), respectively ($P < 0.05$, exact binomial 2-sided test). Whereas 10 of the 69 patients with AAV were IF+ but ELISA−, only 2 were ELISA+ and IF−. The converse was true with regard to specificity, with ELISA being superior to IF: 99% versus 93%, respectively (McNemar's test = 42.7; $P < 0.001$). Of the 787 patients who did not have AAV, 51 were IF+ but ELISA−, whereas only 3 were ELISA+ but IF−.

The greater specificity of ELISA was reflected in substantial improvements over IF in terms of PPV and the LR of a positive test. The PPV for ELISA was 83%, compared with 45% for IF. The LR of a positive ELISA test was 54.2 (95% CI = 26.3, 111.5), compared with 9.4 for IF (95% CI = 6.9, 12.7). When the information from both tests was combined, a positive IF plus a positive ELISA test corresponded to a PPV of 88% and an LR of 82.1 (95% CI = 33.3, 202.5).

The ROC curve for the IF tests is shown in Figure 1. Although lowering the cutoff point for a positive test from 1:20 to 1:10 increased the sensitivity of IF

Table 3. Correlations between IF and ELISA tests*

Among IF+ patients		Among ELISA+ patients	
Anti-PR3+ if cANCA+	42% (24 of 57)	cANCA+ if anti-PR3+	80% (24 of 30)
Anti-MPO+ if cANCA+	7% (4 of 57)	pANCA+ if anti-PR3+	7% (2 of 30)
Anti-MPO+ if pANCA+	24% (11 of 45)	cANCA+ if anti-MPO+	25% (4 of 16)
Anti-PR3+ if pANCA+	4% (2 of 45)	pANCA+ if anti-MPO+	69% (11 of 16)
Either anti-PR3+ or anti-MPO+	40% (41 of 102)	Either cANCA+ or pANCA+	89% (41 of 46)

* IF = immunofluorescence; ELISA = enzyme-linked immunosorbent assays; PR3 = proteinase-3; ANCA = anti-neutrophil cytoplasmic antibodies; cANCA = cytoplasmic ANCA; MPO = myeloperoxidase; pANCA = perinuclear ANCA.

by 1.4% (from 66.7% to 68.1%), it decreased the specificity of the test by 2.8% (from 92.9% to 90.1%). Alternatively, increasing the cutoff for a positive test to 1:40 lowered the sensitivity by 7.3%, while increasing the specificity by only 1.8%. The cutoff points of 1:10, 1:20, and 1:40, indicated by arrows on Figure 1, validated our empiric use of 1:20 as the cutoff for a positive IF assay. ROC curves for the anti-PR3 and anti-MPO ELISA assays confirmed the validity of the manufacturer's recommendations regarding the cutoff for positive tests (data not shown).

Correlations between IF and ELISA tests. The correlations between IF and ELISA testing are summarized in Table 3. On ELISA testing, more than half of the cANCA+ patients (29 of 57; 51%) and nearly three quarters of the pANCA+ patients (32 of 45; 71%) did not have antibodies to either PR3 or MPO. In contrast, 87% of the anti-PR3+ patients and 94% of the anti-MPO+ patients were either cANCA+ or pANCA+. Four patients had IF patterns that were atypical. Two of these patients had inflammatory bowel disease, one had scleritis, and the fourth had no known disease. None of the 4 patients with atypical IF patterns had positive ELISA tests for anti-PR3 or anti-MPO antibodies.

Correlations between IF/ELISA results and AAV. Twenty-eight of the 57 cANCA+ patients (49%) did not have clinical diagnoses of AAV. The diagnoses of these patients are displayed in Table 4. The median titer of these "false-positive" tests was 1:40, compared with 1:160 for all patients with AAV. Only 2 of the 28 cANCA+ patients without AAV had antibodies to PR3.

Twenty-eight of the 45 pANCA+ patients (62%) did not have clinical diagnoses of AAV. The diagnoses of these patients are displayed in Table 5. The median titer of these "false-positive" tests was 1:80. Only 2 of the 28 pANCA+ patients without AAV had antibodies to MPO.

Six of the 30 anti-PR3+ patients (20%) did not have AAV. Their diagnoses were poliomyelitis, multiple sclerosis and asthma, Sjögren's syndrome, hepatitis C and end-stage renal disease, intraventricular brain hemorrhage, and possible WG.

Two patients of the 16 anti-MPO+ patients (13%) did not have AAV. One of these patients had thrombotic thrombocytopenic purpura. The other had polymyalgia rheumatica and renal insufficiency of unclear etiology (no clinical signs of glomerulone-

Table 4. Non–"ANCA-associated diseases" associated with positive cANCA assays (n = 28)*

Multiple sclerosis, asthma (>1:2560)†
Eosinophilia and mesenteric adenopathy (undifferentiated disease process) (1:320)
Inflammatory bowel disease (1:160)
Sjögren's syndrome (1:160)†
Dementia and aspiration pneumonia (1:160)
Cystic fibrosis (1:160)
Inflammatory bowel disease (1:80)
Idiopathic pulmonary hemosiderosis (1:80)
Post-streptococcal GN (1:80)
Alport's syndrome (1:80)
Hermansky-Pudlak syndrome (1:80)
Morbid obesity, proteinuria (1:40)
Poliomyelitis (1:40)†
HIV/HCV infection (1:40)
Idiopathic pulmonary fibrosis (1:40)
Behçet's disease (1:40)
Endocarditis (1:40)
Focal segmental GN (1:40)
Immune complex–mediated GN (1:40)
HIV nephropathy (1:40)
Viral infection with pustulo-vesicular rash (1:20)
Cocaine-induced pharyngeal necrosis (1:20)
Dilated cardiomyopathy and sinusitis (1:20)
Giant cell arteritis (1:20)
Cryoglobulinemic vasculitis (2° to HCV) (1:20)
HIV and anterior uveitis (1:20)
Idiopathic pulmonary fibrosis (1:20)
Polyarteritis nodosa (1:20)

* ANCA = anti-neutrophil cytoplasmic antibodies; cANCA = cytoplasmic ANCA; GN = glomerulonephritis; HIV = human immunodeficiency virus; HCV = hepatitis C virus. Titers of ANCA by immunofluorescence are in parentheses.
† Also had antibodies to proteinase-3.

Table 5. Non–"ANCA-associated diseases" associated with positive pANCA assays (n = 28)*

Inflammatory bowel disease (7 patients)†
SLE, urticarial vasculitis (>1:2560)
Polymyalgia rheumatica, renal insufficiency (1:320)‡
Pyoderma gangrenosum (1:320)
Renal cell carcinoma (1:320)
Antiphospholipid antibody syndrome (1:160)
Scleritis (1:160)
Inflammatory brain mass (1:160)
Immune complex-mediated GN (1:160)
Thrombotic thrombocytopenic purpura (1:80)‡
Myasthenia gravis, nephrotic syndrome (1:80)
Polyarteritis nodosa (1:80)
Focal sclerosing GN (1:80)
Chronic sinusitis (1:40)
Fibromyalgia (1:40)
No known disease (1:40)
Idiopathic subglottic stenosis (1:20)
Focal sclerosing GN (1:20) (2 patients)
Chronic lymphocytic leukemia (1:20)
Paraproteinemia, Alzheimer's disease (1:20)
Undifferentiated inflammatory arthritis (1:20)

* ANCA = anti-neutrophil cytoplasmic antibodies; pANCA = perinuclear ANCA; SLE = systemic lupus erythematosus; GN = glomerulonephritis. Titers of ANCA by immunofluorescence are in parentheses.
† Titers ranged from 1:20 to 1:640.
‡ Also had antibodies to myeloperoxidase.

phritis). Because of profound depression, she was unable to consent to renal biopsy. The clinical impression at the time of her evaluation and in followup discussions with her clinicians was that her renal insufficiency was not secondary to AAV.

Sensitivity in WG. Table 6 displays the test results for patients with WG, subdivided by the presence of active disease and by whether or not they were under treatment at the time of the assay. Among the subgroup of WG patients with active disease who were not on treatment, 72% were positive for ANCA by both IF and ELISA. Patients with active disease (regardless of treatment status) were more likely to have ANCA by IF than those with inactive disease (24/29 versus 8/16; $P = 0.02$ [95% CI = 0.05, 0.60]). This same comparison did not achieve statistical significance for the ELISA tests (19/29 versus 7/16; $P = 0.16$ [95% CI = −0.08, 0.52]).

Table 6. Sensitivities of IF and ELISA tests in Wegener's granulomatosis*

Test	All (n = 45)	Active disease (n = 29)	Active disease and no treatment (n = 18)
IF+	71%	83%	89%
cANCA+	53%	59%	72%
pANCA+	18%	24%	17%
ELISA+	58%	66%	78%
Anti-PR3+	51%	59%	72%
Anti-MPO+	7%	7%	6%
IF+ and ELISA+	56%	62%	72%

* IF = immunofluorescence; ELISA = enzyme-linked immunosorbent assays; ANCA = anti-neutrophil cytoplasmic antibodies; cANCA = cytoplasmic ANCA; pANCA = perinuclear ANCA; PR3 = proteinase-3; MPO = myeloperoxidase.

DISCUSSION

In the past decade, the increasing availability of ANCA testing has provided an important tool for the evaluation of patients with possible systemic vasculitis. This study is the first to examine both PPV and LR for IF and ELISA tests in an unselected patient population. Thus, these results provide data for a current, evidence-based approach to the use of these assays. The major findings of this study were: 1) the ELISA technique for the assay of ANCA had substantial advantages over IF in terms of both PPV and the LR of a positive test; 2) antibodies to PR3 or MPO were detected in fewer than half of the patients who tested positive for ANCA by IF; and 3) even though our study was conducted at a tertiary care center, 55% of all patients in our population with positive IF assays did not have AAV.

In this study, all patients originally had ANCA ordered by their physicians because of suspicion of an ANCA-associated condition. Using stored sera from these prospectively collected patients, we tested all of the patients by ELISA in a blinded manner. The PPV of IF for AAV in our population was only 45%. In contrast, the PPV of ELISA was 83%. The LR of a positive IF test (9.4) boosted the odds of an AAV, but a positive ELISA was associated with a substantially larger increase in the odds of AAV (54.2). When combined, positive IF and positive ELISA tests yielded an LR that was higher still (82.1). Understanding the differences in the PPV and LR for these two types of ANCA assay is essential for the proper clinical use of these tests. The results of this study in a consecutively tested group of patients with possible AAV strongly support the performance of ELISA tests for antibodies to PR3 and MPO in all patients who are positive for ANCA by IF.

For most of this decade, IF has been the gold standard for ANCA testing. However, the initial enthusiasm about high sensitivities and specificities of

IF has yielded to the realization that IF testing for ANCA has important shortcomings. IF assays are not antigen-specific; their interpretation depends upon the sometimes subjective analysis of fluorescence patterns, requiring experience and a high degree of skill (11). Substantial variability even among expert ANCA labs has been reported (24). "False-positive" tests have been reported in a variety of conditions, including infections (25–27), drug-induced vasculitis (28,29), other systemic autoimmune diseases (30,31), malignancies (32), and disorders not associated with vascular inflammation (10,33). Similarly, negative tests are known to occur in a significant subset of patients whose clinical and histopathological features are consistent with "ANCA-associated" vasculitis (34,35).

The high number of "false-positive" cANCA results in our study was striking and provides a contrast with studies from more selected patient populations (11,13–16). Merkel and colleagues (11), for example, reported high specificities of cANCA (no reproducible cANCA occurred in any of the 664 non-AAV study subjects). In our study, nearly half of all positive cANCA tests (28 of 57) occurred in patients *without* AAV and thus were considered false-positive results with respect to this group of disorders. In test populations such as ours, which have a high prevalence of multiorgan system diseases that may easily mimic AAV (yet have treatments that are quite different), this is a sobering reality. Confirming all positive IF tests by ELISA testing would dramatically reduce the number of false-positive ANCA results in patient populations similar to ours (from 28 of 57 to 2 of 57, in the case of cANCA). The findings in our study are extrapolated most easily to other tertiary medical centers. However, in populations with lower prevalences of AAV, such confirmation may have even greater value.

We found that IF had a significantly higher sensitivity than ELISA (67% versus 55%). We anticipated this finding, because more than one type of antibody specificity may demonstrate the cANCA and pANCA patterns on IF. Both IF and ELISA had excellent specificities for AAV, even though that of ELISA was statistically higher (99% versus 93%).

In a prospective examination of IF ANCA tests, Rao and colleagues (17) reported a sensitivity of 28% for cANCA, substantially lower than the sensitivities for both IF and ELISA in this study. Several explanations for this discrepancy are apparent. First, Rao and colleagues focused only on cANCA results (to the exclusion of pANCA) and only on WG (as opposed to the entire group of AAV). As others have reported, patients who meet current guidelines for the diagnosis of WG (including tissue biopsies with granulomatous inflammation) may demonstrate pANCA staining patterns by IF (36,37). Second, for the purpose of diagnosis, Rao and colleagues used the American College of Rheumatology criteria for the classification of WG (38). The shortcomings of these criteria in diagnosis are well described (39). Finally, Rao and colleagues excluded patients with known diagnoses of WG from their study, which presumably decreased their reported sensitivity of cANCA for WG. The PPV of cANCA for WG reported by Rao and colleagues (50%) is similar to our result for IF related to all AAV (45%).

Over the past few years, European investigators have conducted a series of studies designed to standardize ANCA testing methodology (18,24,40). In a 14-center study involving 169 newly diagnosed and 189 historical patients with idiopathic systemic vasculitis or pauci-immune GN (i.e., "ANCA-associated vasculitis"), as well as 184 controls with other diseases and 740 healthy controls, the investigators reported sensitivities and specificities very similar to ours. For example, the sensitivity of cANCA for WG was 64%, and that of pANCA 21% (compared with 53% and 18%, respectively, in our population). In contrast to our data, the European group reported higher sensitivities and lower specificities for ELISA tests compared with IF (e.g., 64% versus 66% sensitivity for cANCA and PR3 in WG, and 95% versus 87% specificity). We suspect that this discrepancy between the 2 studies is due to differences in the performance of IF assays rather than to differences in characteristics of the ELISA tests used or to patient selection. In the multicenter European study, which employed a selected patient population rather than a consecutively evaluated one such as ours, the PPV, NPV, and LR of ANCA tests were not calculated.

The cutoff point for positive IF assays was established empirically by our previous experience with this test. The validity of this cutoff point (and that recommended by the manufacturer for the ELISA kits we used) was confirmed by our ROC curves. It must be noted that, just as the expertise with use of the IF technique varies from center to center, ELISA kits are also of varying quality (41). Clinicians who order ANCA assays on a frequent basis should familiarize themselves with the test characteristics of the assay used at their centers.

Our study has certain weaknesses. First, some of the results considered to be false-positive tests may reflect incomplete knowledge about the precise contribution of ANCA to many disease states. It is currently considered that there are a handful of "ANCA-associated" conditions defined by a set of similar

clinical and pathological characteristics, even though not all patients demonstrating these characteristics have demonstrable ANCA. Thus, the designation of an ANCA-associated condition is not based on knowledge of the true role of ANCA in disease pathogenesis (if any), and some false-positive test results might be reclassified in the light of future knowledge.

Second, test sensitivity in our study may have been lowered by the fact that some patients with AAV had inactive disease at the time of the assay, and many were also on treatment. Even among the untreated WG patients with active disease, however, both IF and ELISA were positive in only 72% of patients. Thus, even under optimal testing conditions, some patients with "ANCA-associated" vasculitis do not have demonstrable ANCA. Moreover, this does not alter the principal finding of our study: when applied in a blinded fashion to the same group of patients, ELISA tests performed substantially better than IF assays.

Use of ANCA tests is increasing. From 1996 to 1998, for example, the annual number of ANCA serologies requested at our center nearly doubled, from 319 to 609. Increased usage of these tests will result inevitably in the occurrence of greater numbers of false positive results and will lead to greater difficulty in test interpretation. The increased ordering of ANCA assays calls for use of a rational strategy in ANCA testing. IF tests are cheaper and probably somewhat more sensitive than ELISA in actual clinical settings. On the other hand, ELISA tests, though more expensive and labor-intensive, are directed against antigens that may be relevant to disease pathophysiology (42–44). In our study, ELISA tests also had superior specificity for AAV, and positive tests were associated with a substantially higher PPV and LR. The combined characteristics of IF and ELISA make them highly complementary in clinical practice. At our center, we now perform IF testing initially on all patients whose physicians order ANCA serologies, reserving ELISA tests for patients with positive IF assays. In the current study, this strategy would have resulted in failure to detect positive ELISA tests in only 2 patients with AAV (out of 856 total subjects).

In conclusion, we have demonstrated that confirmation of all positive IF tests with anti-PR3 or anti-MPO ELISA assays substantially strengthens the likelihood that a patient with such a positive test result actually has AAV. Nevertheless, because of the significant number of ANCA− patients who meet the current guidelines for these conditions, the diagnosis of these conditions remains, in the final analysis, a clinicopathological one.

REFERENCES

1. Davies D, Moran J, Niall J, Ryan G. Segmental necrotising glomerulonephritis with antineutrophil antibody: possible arbovirus aetiology. BMJ 1982;285:606.
2. Van der Woude F, Rasmussen N, Lobatto S, Wiik A, Permin H, van Es LA, et al. Autoantibodies against neutrophils and monocytes: tools for diagnosis and marker of disease activity in Wegener's granulomatosis. Lancet 1985;1:425–9.
3. Gaskin G, Savage C, Jones R, Rees A, Lockwood C, Pusey C. Anti-neutrophil cytoplasmic antibodies and disease activity during long-term follow-up of 70 patients with systemic vasculitis. Nephrol Dial Transplant 1991;6:689–94.
4. Bosch X, Mirapeix E, Font J, Cervera R, Ingelmo M, Khamashta M, et al. Anti-myeloperoxidase antibodies in patients with necrotizing glomerular and alveolar capillaritis. Am J Kidney Dis 1992;20:231–9.
5. Falk R, Hogan S, Carey T, Jennette J, and the Glomerular Disease Network. Clinical course of anti-neutrophil cytoplasmic autoantibody–associated glomerulonephritis and systemic vasculitis. Ann Intern Med 1990;11:656–63.
6. Cohen Tervaert J, Kallenberg C. Anti-myeloperoxidase antibodies in Churg Strauss syndrome. J Neurol 1993; 240:449–52.
7. Guillevin L, Visser H, Noel L, Pourrat J, Vernier I, Gayraud M, et al. Antineutrophil cytoplasm antibodies in systemic polyarteritis nodosa with and without hepatitis B virus infection and Churg-Strauss syndrome: 62 patients. J Rheumatol 1993;20:1345–9.
8. Falk R, Jennette J. Anti-neutrophil cytoplasmic autoantibodies with specificity for myeloperoxidase in patients with systemic vasculitis and idiopathic necrotizing and crescentic glomerulonephritis. N Engl J Med 1988;318:165–7.
9. Jenne D, Tschopp J, Ludemann J, Utecht B, Gross W. Wegener's autoantigen decoded. Nature 1990;346:520.
10. Mulder AHL, Broekroelofs J, Horst G, Limburg PC, Nelis GF, Kallenberg CGM. Anti-neutrophil cytoplasmic antibodies (ANCA) in inflammatory bowel disease: characterization and clinical correlates. Clin Exp Immunol 1994;95:490–7.
11. Merkel P, Polisson R, Chang Y, Skates S, Niles J. Prevalence of antineutrophil cytoplasmic antibodies in a large inception cohort of patients with connective tissue disease. Ann Intern Med 1997;126:866–73.
12. Hoffman G, Specks U. Antineutrophil cytoplasmic antibodies. Arthritis Rheum 1998;41:1521–37.
13. Gross WL, Ludemann J, Kiefer G, Lehmann H. Anticytoplasmic antibodies in Wegener's granulomatosis. Lancet 1986;1:806.
14. Specks U, Wheatly C, McDonald T, Rohrbach M, De-

Remee R. Anticytoplasmic autoantibodies in the diagnosis and follow-up of Wegener's granulomatosis. Mayo Clin Proc 1989;64:28–36.
15. Nolle B, Specks U, Ludemann J, Rohrbach M, DeRemee R, Gross W. Anticytoplasmic autoantibodies: their immunodiagnostic value. Ann Intern Med 1989; 111:28–40.
16. Cohen Tervaert J, van der Woude F, Fauci A, Ambrus J, Velosa J, Keane WF, et al. Association between active Wegener's granulomatosis and anticytoplasmic antibodies. Arch Intern Med 1989;149:2461–5.
17. Rao J, Allen N, Feussner J, Weinberger M. A prospective study of antineutrophil cytoplasmic antibody (c-ANCA) and clinical criteria in diagnosing Wegener's granulomatosis. Lancet 1995;346:926–31.
18. Hagen E, Daha M, Hermans J, Andrassy K, Csernok E, Gaskin G, et al. Diagnostic value of standardized assays for anti-neutrophil cytoplasmic antibodies in idiopathic systemic vasculitis. Kidney Int 1998;53:743–53.
19. Jennette JC, Falk RJ, Andrassy K, Bacon PA, Churg J, Gross WL, et al. Nomenclature of systemic vasculitides: proposal of an international consensus conference. Arthritis Rheum 1994;37:187–92.
20. Hoffman JS, Leavitt RY, Kerr GS, Fauci AS. The treatment of Wegener's granulomatosis with glucocorticoids and methotrexate. Arthritis Rheum 1992;35: 1322–9.
21. Sneller MC, Hoffman GS, Talar-Williams C, Kerr GS, Hallahan CW, Fauci AS. An analysis of forty-two Wegener's granulomatosis patients treated with methotrexate and prednisone. Arthritis Rheum 1995;38: 608–13.
22. Talor M, Stone J, Rose N, Burek C. Anti-neutrophil cytoplasmic antibody (ANCA) detection correlating three different commercially-available ELISA kits with indirect immunofluorescence testing. Abstract presented at: Association of Medical Laboratory Immunologists; 1998; Montreal, Canada.
23. Dujardin B, van den Ende J, van Gompel A, Unger J, van der Stuyft P. Likelihood ratios: a real improvement for clinical decision making? Eur J Epidemiol 1994;10:29–36.
24. Hagen E, Andrassy K, Csernok E, Daha M, Gaskin G, Gross W, et al. The value of indirect immunofluorescence and solid phase techniques for ANCA detection: a report on the first phase of an international cooperative study on the standardization of ANCA assays. J Immunol Methods 1993;159:1–16.
25. Wagner J, Andrassy K, Ritz E. Is vasculitis in subacute bacterial endocarditis associated with ANCA? Lancet 1991;337:799–800.
26. Efthimiou J, Spickett G, Lane D, Thompson A. Antineutrophil cytoplasmic antibodies, cystic fibrosis, and infection. Lancet 1991;337:1037–8.
27. Galperin C, Shoenfeld Y, Gilburd B, Esterre P, Meroni P, Del Papa N, et al. Anti-neutrophil cytoplasmic antibodies in patients with chromomycosis. Clin Exp Rheumatol 1996;14:479–83.
28. Eklayam O, Yaron M, Caspi D. Minocycline induced arthritis associated with fever, livedo reticularis, and pANCA. Ann Rheum Dis 1996;55:769–71.
29. Nassberger L, Sjoholm A, Jonsson H, Sturfelt G, Akesson A. Autoantibodies against neutrophil cytoplasm components in systemic lupus erythematosus and in hydralazine-induced lupus. Clin Exp Immunol 1990; 81:380–3.
30. Targan S, Landers C, Vidrich A, Czaja A. High-titer antineutrophil cytoplasmic antibodies in type-1 autoimmune hepatitis. Gastroenterol 1995;108:1159–66.
31. Haagsma E, Mulder A, Gouw A, Horst G, Meerman L, Sloof M, et al. Antineutrophil cytoplasmic autoantibodies after liver transplantation in patients with primary sclerosing cholangitis. J Hepatol 1993;19:8–14.
32. Savige J, Smith C, Chang L, Duggan J. Anti-neutrophil cytoplasmic antibodies (ANCA) in myelodysplasia and other haematologic disorders. Aust N Z J Med 1994;24:282–7.
33. Saxon A, Shanahan F, Landers C, Ganz R, Targan S. A distinct subset of antineutrophil cytoplasmic antibodies is associated with inflammatory bowel disease. J Allergy Clin Immunol 1990;86:202–10.
34. Rao J, Weinberger M, Oddone E, Allen N, Landsman P, Feussner J. The role of antineutrophil cytoplasmic antibody (c-ANCA) testing in the diagnosis of Wegener's granulomatosis. Ann Intern Med 1995;123:925–32.
35. Hagen E, Ballieux E, vas Es L, Daha M, van der Woude F. Antineutrophil cytoplasmic autoantibodies: a review of the antigens involved, the assays, and the clinical and possible pathogenic consequences. Blood 1993;81:1996–2002.
36. Cohen Tervaert JW, Goldschmeding R, Elema JD, Limburg PC, van der Giessen M, Huitema MG, et al. Association of autoantibodies to myeloperoxidase with different forms of vasculitis. Arthritis Rheum 1990;33: 1264–72.
37. Sinico R, Radice A, Pozzi C, Ferrario F, Arrigo G. Diagnostic significance and antigen specificity of antineutrophil cytoplasmic antibodies in renal disease: a prospective multicentre study. Nephrol Dial Transplant 1994;9:505–10.
38. Leavitt RY, Fauci AS, Bloch DA, Michel BA, Hunder GG, Arend WP, et al. The American College of Rheumatology 1990 criteria for the classification of Wegener's granulomatosis. Arthritis Rheum 1990;33:1101–7.
39. Hunder G. The use and misuse of classification and diagnostic criteria for complex diseases. Ann Intern Med 1998;129:417–8.
40. Hagen E, Andrassy K, Csernok E, Daha M, Gaskin G, Gross W, et al. Development and standardization of solid phase assays for the detection of anti-neutrophil cytoplasmic antibodies (ANCA): a report on the second phase of an international cooperative study on the standardization of ANCA assays. J Immunol Methods 1996;196:1–15.

41. Wang G, Csernok E, deGroot K, Gross WL. Comparison of eight commercial kits for quantitation of anti-neutrophil cytoplasmic antibodies (ANCA). J Immunol Methods 1997;208:203–211.
42. Jennette J, Falk R. Small vessel vasculitis. N Engl J Med 1997;337:1512–23.
43. Csernok E, Ernst M, Scmitt W, Bainton D, Gross W. Activated neutrophils express proteinase 3 on their plasma membrane in vitro and in vivo. Clin Exp Immunol 1994;98:244–50.
44. Falk R, Terrell R, Charles L, Jennette J. Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals in vitro. Proc Natl Acad Sci U S A 1990;87:4115–9.