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EVALUATION OF ANTINEUTROPHIL CYTOPLASMIC ANTIBODY SEROCONVERSION INDUCED BY MINOCYCLINE, SULFASALAZINE, OR PENICILLAMINE

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***Objective*. Case reports have suggested that minocycline, sulfasalazine, and penicillamine are associated with antineutrophil cytoplasmic antibody (ANCA)–positive vasculitis. This study evaluated ANCA seroconversion due to these agents in serum samples prospectively collected in randomized, double-blind, controlled trials.**

***Methods*. The sources of study sera were 3 clinical trials: 1) a 48-week trial of minocycline for early rheumatoid arthritis, with 64 patients receiving minocycline compared with 68 receiving placebo; 2) a 37-week trial of sulfasalazine for rheumatoid arthritis, with 51 receiving sulfasalazine compared with 38 receiving placebo; and 3) a 104-week trial of penicillamine for early systemic sclerosis, with 15 undergoing high-dose penicillamine treatment versus 12 receiving low-dose penicillamine. ANCA were measured in the baseline and study-end serum samples by indirect immunofluorescence (IIF) for perinuclear ANCA (pANCA) and cytoplasmic ANCA (cANCA) patterns, and by antigen-specific enzyme-linked immunosorbent assay (ELISA) for antibodies to myeloperoxidase (anti-MPO) and proteinase 3 (anti-PR3). Laboratory personnel were blinded to the group identity of the samples. ANCA results were interpreted using an ANCA scoring system that combines the results of IIF and ELISA testing.**

***Results*. No patient in any of the active study drug groups demonstrated ANCA seroconversion according to the final interpretation of the combined IIF and ELISA results. Twelve of the 248 patients (5%) were positive for anti-MPO with pANCA at baseline. No subject was positive for anti-PR3 with cANCA. There were no findings suggestive of vasculitis in any of these patients.**

***Conclusion*. From our study results, there was no suggestion of ANCA seroconversion induced by minocycline, sulfasalazine, or penicillamine. However, these findings do not rule out the possibility of rare, sporadic cases of either ANCA seroconversion or true drug-induced vasculitis with these drugs.**

The triggers that induce antineutrophil cytoplasmic antibody (ANCA)–positive vasculitis (APV) are largely unknown. However, there have been a number of cases reported of APV associated with prior use of certain medications. The 2 drugs that have been most often implicated in the induction of APV are hydralazine and propylthiouracil (1–5). In addition, there have been cases linked to other drugs, including minocycline, sulfasalazine, and penicillamine (2,6–16). Drug-associated APV can result in the same life-threatening vasculitic features as observed in idiopathic cases (1). Given the clinical severity of APV, even low rates of drug-induced disease would have important medical implications.

Seroconversion from ANCA negativity to ANCA positivity may occur even in the absence of clinical vasculitis in patients who are exposed to certain drugs. This process would be similar to that seen with antinuclear antibody seroconversion and would suggest a parallel between the pathogenetic steps of drug-associated lupus and vasculitis. ANCA seroconversion by drugs in the absence of clinical vasculitis would, if a real phenomenon, be a cause of false-positive ANCA findings (i.e., nonvasculitis condition with drug-induced ANCA posi-

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Table 1. Source of study subjects and summary of drug exposures*

Source trial, treatment arm (dosage)	Duration of drug exposure, mean ± SD weeks (range)	Number of subjects (n = 248)†
MIRA RA trial		
Minocycline (200 mg/day)	51 ± 7.1 (10–72)	64
Placebo	50 ± 6.7 (6–56)	68
CSSRD RA trial		
Sulfasalazine (2,000 mg/day)	31 ± 11 (4–39)	51
Placebo	30 ± 12 (2–49)	38
Scleroderma trial		
High-dose penicillamine (750–1,000 mg/day)	58 ± 4 (51–69)	15
Low-dose penicillamine (62.5 mg/day)	61 ± 13 (50–100)	12

* MIRA = Minocycline in Rheumatoid Arthritis; RA = rheumatoid arthritis; CSSRD = Cooperative Systematic Studies of the Rheumatic Diseases.
† Total number of paired sera = 496.

tivity). Nevertheless, ANCA seroconversion might provide an important means of identifying patients at risk for developing drug-induced APV. No published report has addressed this important issue in a systematic way.

To evaluate ANCA seroconversion in patients exposed to minocycline, sulfasalazine, or penicillamine, we performed blinded ANCA testing of prospectively collected, paired pre- and postexposure serum samples from patients who were enrolled in therapeutic clinical trials for rheumatic diseases.

PATIENTS AND METHODS

Clinical trials and study groups. Three double-blind, randomized trials provided prospectively collected sera for ANCA testing in our study: the Minocycline in Rheumatoid Arthritis (MIRA) trial (17), the Cooperative Systematic Studies of the Rheumatic Diseases (CSSRD) comparative study of sulfasalazine, injectable gold, and placebo (18), and a trial of penicillamine for early systemic sclerosis (scleroderma trial) (Table 1) (19). In the MIRA trial, patients with early rheumatoid arthritis (RA) received 200 mg of minocycline or placebo daily for 48 weeks. We used sera from both MIRA groups for this study. In the CSSRD trial, patients with RA received 2,000 mg of sulfasalazine, placebo, or gold sodium thiomalate daily for 37 weeks. Sera from the first 2 groups were used for this study. In the penicillamine trial, patients with early systemic sclerosis received penicillamine at 750–1,000 mg (high dose) or 62.5 mg (low dose) daily for 2 years. We used the sera from both of these groups.

Only sera that were paired, i.e., both a baseline (0 weeks) and a trial-end serum sample were available, were tested for ANCA. Thus, 132 paired MIRA sera (from 64 patients receiving minocycline and 68 placebo), 89 paired CSSRD sera (from 51 patients receiving sulfasalazine and 38 placebo), and 27 paired scleroderma trial sera (from 15 patients receiving high-dose penicillamine and 12 low-dose penicillamine) were tested. The total number of study subjects was 248 and total number of paired sera was 496 (Table 1).

Control groups and blinding. To ensure the quality of ANCA testing in our laboratory, various groups of control sera were also tested: samples from 15 patients with vasculitis that tested positive for antimyeloperoxidase antibodies (anti-MPO) and perinuclear ANCA (pANCA), samples from 15 patients with vasculitis that tested positive for anti–proteinase 3 (anti-PR3) and cytoplasmic ANCA (cANCA), and samples from 30 normal blood donors. All study sera were assigned new, randomized identification numbers. To keep laboratory personnel blinded to the study group identity, samples were realiquoted into identical tubes labeled only with study identification numbers. The group identification of the sera was kept masked until all of the ANCA assay results were collected for the final interpretation and the analysis was ready to begin.

ANCA assays by indirect immunofluorescence (IIF). IIF tests were done with the use of ethanol-fixed neutrophils as described elsewhere (20). The results of staining were classified as having 1 of 4 patterns: cANCA, pANCA, atypical (neither cytoplasmic nor perinuclear), or negative. Given the subjective nature of scoring of the results of IIF for ANCA, we utilized a comprehensive interpretation system as described in our previous work (20). Briefly, the interpretation was decided by agreement of initial independent readings by 2 investigators (MCS and JLN) or by resolution of their discrepancy by taking the majority as final after obtaining a third investigator's (CAW) reading. IIF staining was repeatedly performed to resolve discrepant readings. IIF and enzyme-linked immunosorbent assay (ELISA) results were determined independently.

ANCA assay by ELISA. Each sample was tested by direct antigen-specific ELISAs that used highly purified antigens to detect anti-MPO and anti-PR3 antibodies, as well as a sandwich anti-PR3 ELISA. These ANCA assays have been extensively characterized in prior reports from our laboratory (20–23).

Final interpretation by combining IIF and ELISA ANCA results. A final interpretation of ANCA testing was determined by combining the IIF and ELISA results. Samples were considered ANCA-positive when positive for both anti-PR3 and cANCA or when positive for anti-MPO and pANCA or atypical ANCA. Therefore, samples with IIF positivity without respective ELISA positivity or those with ELISA positivity without respective IIF positivity were interpreted as negative in our final interpretation. This is consistent with our previously published methods (20).

Statistical analysis. Comparisons between groups were analyzed by Fisher's exact test for categorical variables. All tests used a 2-tailed significance level of 0.05. We derived 95% confidence intervals (95% CI) using exact methods for proportions. Analysis was performed using the Stata software package (Stata Corporation, College Station, TX).

Table 2. ANCA test results*

Study group, time point	n	ANCA method: Indirect immunofluorescence cANCA	pANCA	Atypical ANCA	ELISA: Direct anti-PR3	Sandwich anti-PR3	Anti-MPO ELISA	Final interpretation: Anti-PR3	Anti-MPO
MIRA RA trial									
Minocycline									
Start	64	0 (0)	2 (3.1)	26 (40.6)	0 (0)	0 (0)	3 (4.7)	0 (0)	3 (4.7)
End	64	0 (0)	0 (0)	25 (39.1)	0 (0)	0 (0)	2 (3.1)	0 (0)	2 (3.1)
Placebo									
Start	68	0 (0)	1 (1.5)	35 (51.5)	0 (0)	1 (1.5)	3 (4.4)	0 (0)	3 (4.4)
End	68	0 (0)	1 (1.5)	29 (42.6)	0 (0)	1 (1.5)	2 (2.9)	0 (0)	2 (2.9)
CSSRD RA trial									
Sulfasalazine									
Start	51	0 (0)	2 (3.9)	23 (45.1)	0 (0)	1 (2.0)	4 (7.8)	0 (0)	4 (7.8)
End	51	0 (0)	3 (5.9)	19 (37.3)	0 (0)	0 (0)	3 (5.9)	0 (0)	3 (5.9)
Placebo									
Start	38	0 (0)	3 (7.9)	18 (47.4)	0 (0)	1 (2.6)	1 (2.6)	0 (0)	1 (2.6)
End	38	0 (0)	2 (5.3)	19 (50.0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Scleroderma trial									
High-dose penicillamine									
Start	15	0 (0)	0 (0)	1 (6.7)	1 (6.7)	0 (0)	1 (6.7)	0 (0)	1 (6.7)
End	15	0 (0)	1 (6.7)	2 (13.3)	0 (0)	0 (0)	1 (6.7)	0 (0)	1 (6.7)
Low-dose penicillamine									
Start	12	0 (0)	0 (0)	1 (8.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
End	12	0 (0)	0 (0)	1 (8.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Control groups									
Normal blood donors	30	0 (0)	0 (0)	2 (6.7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Anti-MPO+ vasculitis	15	0 (0)	11 (73.3)	3 (20.0)	0 (0)	0 (0)	14 (93.3)	0 (0)	14 (93.3)
Anti-PR3+ vasculitis	15	14 (93.3)	0 (0)	1 (6.7)	13 (86.7)	15 (100)	0 (0)	15 (100)	0 (0)

* Note that conversion from negative to positive *or* positive to negative could occur over the course of the study for each ANCA test. Values are the number (%) of antineutrophil cytoplasmic antibody (ANCA)–positive patients. ELISA = enzyme-linked immunosorbent assay; cANCA = cytoplasmic ANCA; pANCA = perinuclear ANCA; anti-MPO = antimyeloperoxidase antibodies; anti-PR3 = anti–proteinase 3 antibodies (see Table 1 for other definitions).

RESULTS

Results of ANCA testing among all study groups are summarized in Table 2.

ANCA seroconversion by final combined interpretation. No patient in any of the active study drug groups demonstrated ANCA seroconversion according to the final combined interpretation. Serum from 1 patient receiving placebo in the minocycline trial converted from ANCA negative to ANCA positive, with a very low titer of anti-MPO and an atypical IIF ANCA pattern. Twelve other patients were positive for ANCA at baseline (by final combined interpretation), but 5 of these were found to be anti-MPO negative at their end-study time point. All 13 anti-MPO–positive patients were positive for pANCA or showed an atypical ANCA IIF pattern, with 2 of the patient serum samples changing their IIF pattern from atypical to pANCA and 2 changing from pANCA to atypical. No study patient was positive for combined anti-PR3/cANCA at any time.

ANCA seroconversion by IIF alone. When the results of ANCA testing by immunofluorescence were analyzed separately from the ELISA data, seroconversions were found only in those samples with atypical ANCA. Nineteen of the 248 serum samples (8%) seroconverted for atypical ANCA (3 receiving sulfasalazine, 2 high-dose penicillamine, 7 minocycline, 1 placebo in the CSSRD trial, 6 placebo in the MIRA trial). No patient in any group showed a seroconversion for the pANCA or cANCA patterns by IIF alone.

ANCA seroconversion by ELISA alone. None of the patient sera seroconverted for anti-MPO in the absence of IIF positivity. One patient was anti-PR3 negative at baseline, but positive for anti-PR3 by sandwich ELISA alone at followup, representing a seroconversion by ELISA only. One patient was positive for anti-PR3 by anti-PR3 direct ELISA alone at baseline, but negative at followup. Three additional patients were anti-PR3 positive by anti-PR3 sandwich ELISA alone at baseline, but negative at followup.

Disease classification of ANCA-positive patients at baseline. *RA trials.* Among the patients in the 2 RA source trials, 6 patients with early RA in the minocycline

study were positive for ANCA at baseline (3 in the minocycline group and 3 in the placebo group), while 5 patients with RA in the CSSRD trial were positive for ANCA at baseline (4 in the sulfasalazine group and 1 in the placebo group). Of these 11 RA patients with ANCA positivity at baseline, 7 had a borderline positive anti-MPO ELISA titer and were positive for perinuclear or atypical IIF at baseline. The remaining 4 had high anti-MPO titers with perinuclear or atypical IIF patterns at baseline. There were no clinical findings suggestive of vasculitis beyond the features of RA (meeting the study entry criteria of source trials) in any of these patients. This false-positive ANCA testing rate (for the diagnosis of vasculitis) among patients with RA (5.4%) was not significantly different from our previous results (20).

Scleroderma trial. One patient in the high-dose penicillamine group was ANCA positive at baseline. This was the only patient in the study with a high titer of ANCA. This patient had diffuse scleroderma with lung and gastrointestinal involvement. He tolerated 1,000 mg of penicillamine throughout the trial. The anti-MPO ELISA titer rose from 78 to 2,061 units (normal <2.8 ELISA units) after 14 months. No findings suggestive of vasculitis were observed, even after 4 years of clinical followup. The finding that only 1 of 27 patients with scleroderma (3.7%) was positive for anti-MPO with pANCA represented a rate that was also not significantly different from our prior data (20).

Control groups. Of the 60 control sera (15 with anti-PR3/cANCA, 15 with anti-MPO/pANCA, and 30 normal blood donors), 59 sera were correctly classified. One sample from an anti-MPO/pANCA–positive patient was interpreted as ANCA negative by our combined scoring method (false negative), whereas the other 14 samples were positive for anti-MPO/pANCA or atypical ANCA. All anti-PR3/cANCA–positive samples were confirmed positive, and all blood donor samples were ANCA negative.

DISCUSSION

The current study evaluated potential ANCA seroconversion by minocycline, sulfasalazine, and penicillamine in prospectively collected sera from 3 major clinical trials on rheumatic disorders. The results do not support ANCA seroconversion induced by these 3 drugs.

Our ability to demonstrate drug-induced ANCA seroconversion might have been affected by a number of factors: 1) the dosages of the study medications, 2) the duration of drug exposures, and 3) the potential frequency of ANCA seroconversion. The dosages of medications used in source trials were similar to those used in previously published cases that implicated these drugs as a cause of ANCA-positive vasculitis (6–11). The duration of drug exposure was close to that reported in the previously published cases associated with minocycline (12 months versus 12–36 months) (6). However, the duration was shorter than in the reported cases associated with sulfasalazine (7 months versus 18–40 months) (7,8) or penicillamine (14 months versus 17–70 months) (9–11).

The lower bounds of the frequency of potential seroconversion were limited in this study by the number of paired sera available. Thus, this study does not rule out ANCA seroconversion rates <1.6% for minocycline (95% CI 0–5.8%), <1.7% for sulfasalazine (95% CI 0–7.2%), or <3.7% for penicillamine (95% CI 0–13.7%). In order to further investigate the possibility of rare ANCA seroconversion and drug-associated APV, case–control studies may be more feasible than prospective cohort studies; these would require large sample sizes with long periods of followup to demonstrate potential positive results.

There are several strengths in our study. By testing ANCA in pre- and postexposure serum samples, we were able to eliminate the potential false impression of ANCA seroconversion that might be presumed by administering the drugs to patients who were already positive for ANCA at baseline. Among the reported cases implicating minocycline, sulfasalazine, or penicillamine in inducing ANCA-associated syndromes (6–11), we are aware of only 1 case of APV associated with penicillamine in which a negative anti-MPO ANCA status was documented prior to drug initiation (9). Thus, it remains possible that the remaining cases described in the literature (6–11) could have had positive ANCA titers before the respective drug therapy was started, as demonstrated in our current study. In addition, the blinding of laboratory personnel to sample group assignment helped eliminate the potential bias involved in the subjective nature of the interpretation of immunofluorescence results (20). Another strength of our study is the extensive clinical and research experience of our ANCA testing laboratory (20,21).

Our combined ANCA testing system that utilizes both ELISAs and IIF is highly specific (20,21) and minimizes the potential false impression of ANCA seroconversion that could result from testing sera by IIF alone. Although we differentiate atypical from perinuclear patterns, IIF readings are subjective, and other laboratories may not read the slides the same way nor they may not have the samples read by at least 2 qualified

personnel. The apparent rate of seroconversion among study subjects exposed to the test drugs would have been as high as 5% if determined by IIF alone, rather than the 0% found with the more comprehensive combined IIF and ELISA scoring method used.

In conclusion, our study results do not support ANCA seroconversion by minocycline, sulfasalazine, or penicillamine, which raises a possibility that previous reports of retrospective cases could have been premature in claiming the causality between these drugs and ANCA. However, these data do not rule out the possibility of rare, sporadic cases of either ANCA seroconversion or true drug-induced vasculitis with these drugs.

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