

## Monitoring Proteinase 3 Antineutrophil Cytoplasmic Antibodies for Detection of Relapses in Small Vessel Vasculitis

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Received 2 December 2002/Returned for modification 8 April 2003/Accepted 9 June 2003

**The clinical usefulness of antineutrophil cytoplasmic antibodies (ANCA) in the monitoring of patients treated for small vessel vasculitis is debated. A capture enzyme-linked immunosorbent assay (ELISA) based on anti-proteinase 3 (anti-PR3) monoclonal antibody 4A3 has previously been proven to be superior to indirect immunofluorescence (IIF) and standard ELISA for the diagnosis of vasculitis. The present study compared the effectiveness of the capture ELISA for the detection of disease relapse. Samples from patients with relapses and remissions (relapse and remission samples, respectively) were identified through the database of the Glomerular Disease Collaborate Network. Twenty-one relapse samples and 49 remission samples were analyzed by the capture PR3-ANCA ELISA from Wieslab AB, the standard PR3-ANCA ELISA from Inova, and IIF. A Medline search was performed to identify published data on ANCA status at relapse. The capture ELISA was positive for 21 instances of relapses in 14 patients, while the standard ELISA and IIF each failed to detect 2 relapses (*P* was not significant). By using a higher cutoff value, the capture ELISA correctly categorized 84% of the remission samples and 81% of the relapse samples. Similar degrees of discrimination could be achieved by IIF but not by the standard ELISA. In previously published series, the median proportions of patients positive at relapse were 100% by IIF (range, 75 to 100%) and 86% by standard ELISA (range, 38 to 100%). The corresponding values for a rise that accompanied or preceded a relapse were 75% (range, 20 to 100%) for IIF and 50% (range, 25 to 81%) for ELISA. The capture PR3-ANCA ELISA is a sensitive tool for the detection of relapses. Larger studies are needed to detect differences between methods. Negative results by tests for ANCA are rare during relapses.**

Measurement of antineutrophil cytoplasmic antibodies (ANCA) is an established tool for the diagnostic workup of patients with small vessel vasculitis. However, different views concerning the usefulness of serial ANCA measurements for the monitoring of patients prevail. In the original report linking ANCA to Wegener's granulomatosis in 1985, it was stated that ANCA titers are related to disease activity (29). Later it was claimed that treatments based on ANCA titers were more beneficial than treatments based only on clinical signs and symptoms (5). This notion has been challenged. For instance, a report from the National Institutes of Health found that changes in ANCA titers were poorly correlated with disease activity (7, 15).

On the basis of the antigen specificities of the autoantibodies, ANCA are divided into two major categories, proteinase 3 (PR3) ANCA (PR3-ANCA) and myeloperoxidase ANCA. ANCA can be detected either by indirect immunofluorescence (IIF) with normal neutrophils or by immunochemical methods, such as enzyme-linked immunosorbent assay (ELISA). Different methods do not yield identical results, and the correlations between the titers obtained by different assays are especially poor (30). One basis for these discrepancies is the presence of antigenic molecules other than PR3 and myeloperoxidase in the specimen used for the assay. For instance,

antibodies to bactericidal/permeability increasing protein can give rise to a classic ANCA (C-ANCA) pattern that is indistinguishable from the PR3 pattern. Discrepancies are also due to differences in the way in which the antigens are presented in different assays. Autoantigenic epitopes may be masked or enhanced by fixation and coating. During fixation for IIF, interactions with other granule constituents may mask epitopes and lower the sensitivity (24). During coating for standard ELISA, denaturation may alter the antigenicities of conformational epitopes on PR3. A capture assay reduces the problem with coating by immobilizing the antigen with a previously coated monoclonal antibody. However, this introduces the risk that the monoclonal antibody is directed to the same epitope region as the autoantibodies in the test sample. Under such circumstances PR3 will be unavailable for the autoantibodies in the test sample, yielding a false-negative result. Circulating immune complexes containing PR3 and anti-PR3 may also be detected by a capture assay but not by a standard ELISA.

After having defined three major nonoverlapping epitope regions on the PR3 molecule, a capture ELISA was developed (25). This assay has previously been shown to exhibit a higher degree of sensitivity and a similar degree of specificity for the detection of systemic vasculitis compared with the sensitivities and specificities of IIF and standard direct ELISA (2). In the study described in this report we studied the ability of this capture assay to detect relapses among patients with PR3-ANCA-associated small vessel vasculitis compared with those of a standard PR3 ELISA and IIF. We also compared our

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results with those of previously published investigations concerning the use of ANCA for the detection of relapses in patients with vasculitis.

## MATERIALS AND METHODS

**Patients and sera.** Patients with biopsy-proven PR3-ANCA-associated small vessel vasculitis with renal involvement were detected through the Glomerular Disease Collaborate Network database at the University of North Carolina at Chapel Hill. This cohort has been monitored prospectively since the day of biopsy, and remissions and relapses have been recorded as published previously (19). In short, a relapse was defined as the occurrence of one of the following: (i) a rapid rise in serum creatinine levels accompanied by active urine sediment, (ii) the detection of necrosis or crescent formation by renal biopsy or the identification of necrotizing vasculitis in other tissue, (iii) pulmonary hemorrhage or expanding nodules, (iv) the observation of active vasculitis in the gut by endoscopy, (v) iritis or uveitis, or (vi) new mononeuritis multiplex. The results of ANCA testing were not included in the criteria used to define relapses and remissions.

Serum samples were retrieved from the serum bank of the Nephropathology Laboratory at the University of North Carolina at Chapel Hill. Healthy blood donors were used as negative controls. From each instance of relapse noted in the database, a serum sample was retrieved from the serum bank, when such a sample was available. Serum samples were classified as "relapse samples" if they were drawn within a period of 30 days before and 60 days after the date of relapse noted in the database. If several serum samples were available from the same relapse episode, the one closest to the date of relapse was included in the study. Most of the samples included were drawn within 1 week of the recorded relapse. Samples were classified as "remission samples" if at least 3 months had passed since the last flare of vasculitis and if there was a documented disease-free period of at least 3 months after the sample was drawn. For each patient, no more than six remission samples drawn at least 1 month apart were included in the study. The study was performed in accordance with local ethical guidelines.

**Capture ELISA.** The capture PR3 ELISA kit was a gift from Wieslab AB (Lund, Sweden) and was used according to the instructions of the manufacturer. In short, purified PR3 at a concentration of 1 µg/ml in phosphate-buffered saline was added to microtiter plates precoated with monoclonal antibody 4A3, and the plates were incubated at room temperature for 60 min. Sera diluted 1:80 in phosphate-buffered saline with 0.1% bovine serum albumin and 0.05% Tween 20 were incubated in duplicate wells for 60 min at room temperature, and after washing of the plates, bound immunoglobulin G was detected with alkaline phosphatase-conjugated anti-human immunoglobulin G. To exclude nonspecific binding and rheumatoid factor binding, a control plate precoated with an unrelated monoclonal antibody of the same mouse immunoglobulin subclass was run in parallel with the plate with monoclonal antibody 4A3. For each well, the absorbance values for this plate were subtracted from the absorbance values for the anti-PR3-coated plate. The results of the capture PR3 ELISA were categorized as negative if the result was <20 ELISA units and positive if the result was ≥20 ELISA units, on the basis of the mean value for healthy blood donors + 3 standard deviations. The interassay variability was determined to be 7%, while the intra-assay variability by comparison of the results for individual wells was 8%. A significant increase was defined as a 50% or greater rise in the number of ELISA units. However, increases had to be at least 20 ELISA units in absolute numbers (the same value as the limit for a positive result) to be considered significant. The definition for a significant increase was made arbitrarily before any tests were performed.

**Standard ELISA.** A standard commercial PR3-ANCA ELISA kit (Inova) was used with modification. Instead of the secondary antibody supplied with the kit, alkaline phosphatase-conjugated anti-human gamma chain from Inova was used. Extensive evaluation of this assay system has shown that it has a sensitivity and a specificity comparable to or better than those of multiple commercial kits (17). Standard ELISA results were categorized as negative if the result was <15 ELISA units and positive if the result was ≥15 ELISA units. A significant increase was defined as a rise in the number of ELISA units of 50% or more, with a minimum increase of 15 units required (the same value as the limit for a positive result).

**IIF.** IIF was performed with ethanol-fixed granulocytes in accordance with internationally accepted guidelines (23). One experienced investigator (J.C.J.) analyzed all samples. The starting dilution was 1:20, and all samples were titrated until a negative finding was obtained. An increase of two titration steps or more was defined as a significant increase.

**Literature search.** The following search terms were entered into the Medline database: "ANCA and follow-up," "ANCA and serial," "ANCA and outcome," "ANCA and relapse," and "ACPA" (anticytoplasmic autoantibodies). All articles in the English language for which the abstract suggested that the article contained systematic evaluation of C-ANCA or PR3-ANCA values during disease relapses were selected for extraction of data. Single case reports, data concerning patients with transplants, and data obtained by methods that did not distinguish between C-ANCAs and perinuclear ANCAs were not included. Reports in which the results of ANCA testing were included in the definition of relapse or remission were also excluded.

## RESULTS

**Relapses.** Samples from 21 relapses in 14 individuals (11 men and 3 women) were analyzed by the capture ELISA, the standard PR3 ELISA, and IIF. The results are presented in Fig. 1. The median age of the patients at the time of the first relapse was 50 years (age range, 40 to 82 years). All 21 relapses were identified by the capture ELISA, while the standard ELISA and IIF each failed to recognize 2 relapses (not in the same patients). Even the lowest value detected by the capture ELISA during a relapse (69 ELISA units) was more than a factor of 3 above the normal range.

For 13 of the relapse episodes, the value for the relapse sample could be compared with the values for samples drawn during a disease-free interval prior to the relapse. A significant increase was documented for all 13 episodes by the capture ELISA, while significant increases were seen for 10 episodes each by IIF and the standard ELISA (Table 1). Only one of the relapses went undetected by both IIF and the standard ELISA, while four relapses were detected by one method but not the other.

**Remission.** A total of 49 samples from 20 patients in remission were allocated for this study. Among these 49 samples, 17 samples were from 10 patients who had never experienced a relapse during follow-up. Thirty-two samples were from 10 patients who subsequently developed at least one relapse. Many patients were ELISA positive during remission, but the titers were usually lower than the titers measured during relapse (Fig. 1). Among the 49 samples taken during remission, 14 were negative by capture ELISA, 26 were negative by IIF, and 7 were negative by standard ELISA. The best discrimination between relapse samples and remission samples for the capture ELISA was achieved by using 100 ELISA units as the upper limit. With this cutoff, 41 of 49 (84%) of the remission samples had values below the cutoff and 17 of 21 (81%) of the relapse samples had values above the cutoff. A similar result was obtained by IIF with a value of <1:80 as a limit (41 of 49 remission samples and 19 of 21 relapse samples had values above and below the cutoff, respectively). The relationship between the capture ELISA results and the IIF results is shown in Fig. 2. When the capture ELISA results were between 50 and 100 ELISA units, evaluation by capture ELISA in combination with IIF seemed to provide additional information, with no relapses observed among patients with <100 ELISA units by the capture assay in conjunction with a value of ≤1:80 by IIF. For the standard ELISA, no titer provided a clear distinction between relapses and remissions. Use of 40 ELISA units as the cutoff point for the standard ELISA resulted in the titers for 86% (18 of 21) of the relapse samples being above the cutoff but the titers for only 57% (28 of 49) of the remission samples titers being below the cutoff. The better discrimination

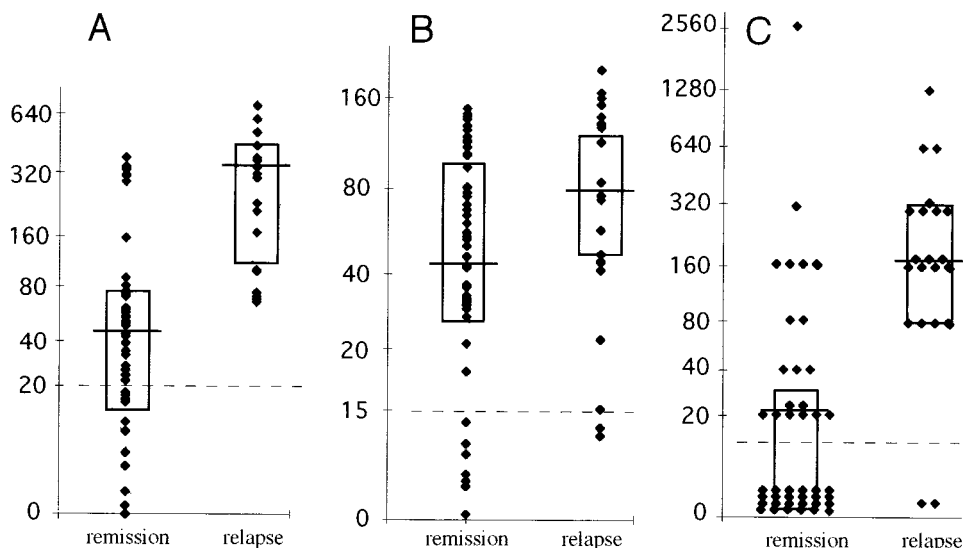


FIG. 1. Values of ANCA determinations for 49 remission samples from 20 patients and 21 relapse samples from 14 patients analyzed by capture PR3-ANCA ELISA (A), standard PR3-ANCA ELISA (B), and IIF (C). Horizontal bars indicate median levels, and boxes indicate the first and third quartiles. The discrimination between relapse and remission seems to be better by the IIF and capture techniques than by the standard ELISA.

between remission and relapse by IIF and capture ELISA than by the standard ELISA is shown in Fig. 1.

**Review of the literature.** Twenty articles were found to contain systematic data concerning ANCA measurements during follow-up. The data are summarized in Table 2 together with the data from the present study. Approximately 250 relapses have been investigated by IIF. Since relapses are defined in many different ways and the technique for IIF was not standardized, an arithmetic mean value for samples with positive results was not calculated. The median value of the results across all studies, including the present study, was 100% (range, 75 to 100%). Fewer data concerning the standard ELISA

were found in the literature, with eight articles reporting the results of the standard ELISA for approximately 125 relapses being found. The median proportion of positive results for the detection of ANCAs by the standard ELISA at clinical relapse was 86% (range, 38 to 100%). Most of the articles also summarized data regarding increases in ANCA titers that either preceded or accompanied relapses. The median proportions of rises in ANCA titers before or in conjunction with a relapse were 75% for IIF (range, 20 to 100%) and 50% for the standard ELISA (range, 25 to 81%).

A smaller number of studies also included data on the occurrence of increases in ANCA titer in the absence of clinical

TABLE 1. ANCA determinations for 10 patients with 13 relapses for which remission samples before the relapse could be compared with relapse samples

Patient sex/age (yr) <sup>a</sup>	Organ(s) <sup>b</sup>	IIF titer		ELISA units			
		Remission sample	Relapse sample	Standard ELISA		Capture ELISA	
				Remission sample	Relapse sample	Remission sample	Relapse sample
M/82	E, L, K	Neg	Neg	<b>2<sup>c</sup></b>	47	24	163
M/51	E, +	Neg	<b>1:80</b>	53	82	76	211
M/49	E, K	<b>1:20</b>	<b>1:320<sup>d</sup></b>	<b>114</b>	<b>184<sup>d</sup></b>	15	<b>78<sup>d</sup></b>
M/65	L, K, +	Neg	<b>1:320</b>	5	74	17	398
F/46	E, K, +	Neg	<b>1:160</b>	22	57	17	74
—/46	E	<b>1:20</b>	<b>1:160</b>	16	41	43	102
—/47	E	<b>1:40</b>	<b>1:160</b>	4	14	46	76
M/68	L	1:320	Neg	80	125	3	233
F/72	K	Neg	<b>1:1,280</b>	2	157	16	537
M/72	K, +	Neg	<b>1:160<sup>d</sup></b>	17	<b>123<sup>d</sup></b>	3	<b>358<sup>d</sup></b>
—/76	K, +	1:160	1:320	156	151	173	383
F/40	L	Neg	<b>1:320</b>	5	72	10	71
M/41	EI	<b>1:20</b>	<b>1:160</b>	28	22	77	210

<sup>a</sup> M, male; F, female; —, multiple relapses from the same patient, i.e., the patient for whom data is presented in the preceding row.

<sup>b</sup> Organ system involvement at time of relapse: E, upper respiratory tract; L, lower respiratory tract; K, kidney; +, other organ systems.

<sup>c</sup> Boldface indicates relapses accompanied by a significant increase.

<sup>d</sup> Data for the two patients from whom samples were drawn more than 2 weeks after the clinical diagnosis of relapse.

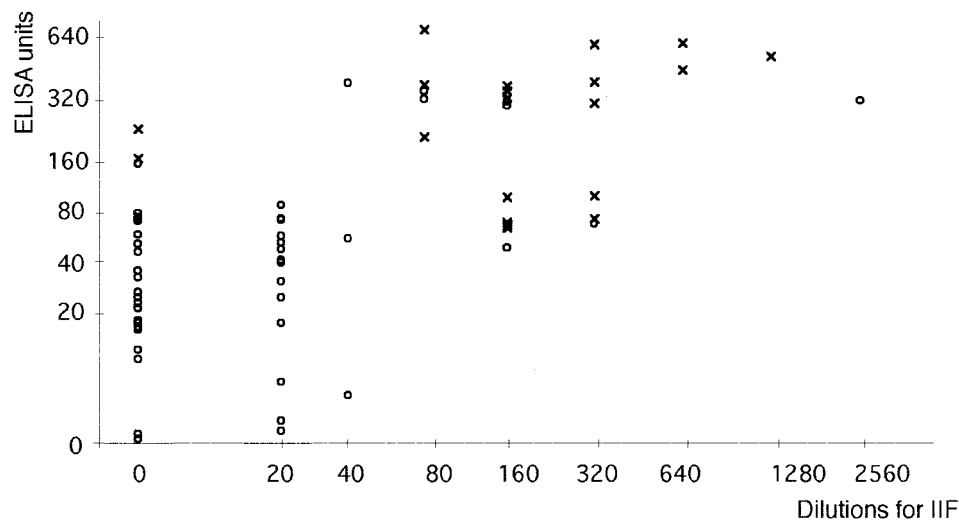


FIG. 2. Comparison between IIF titers and PR3-ANCA capture ELISA results from 49 remission samples (○) and 21 relapse samples (×). The capture assay detected all relapses, but a combination with an IIF titer above 1:80 decreases the likelihood of a false-positive result.

signs of disease activity (Table 2). The large discrepancies between different studies make interpretation of this association difficult. By IIF the proportion of significant increases in titers not associated with relapse ranged from 0 to 86%, and by ELISA this proportion ranged from 0 to 41%. Studies with larger sample sizes tended to report a higher proportion of false-positive tests (7).

## DISCUSSION

This study shows that the capture ELISA for PR3-ANCA based on monoclonal antibody 4A3 is a sensitive tool for the detection of relapses among patients with small vessel vasculitis. All instances of relapse included in this study yielded positive results by the capture ELISA. When remission samples drawn prior to the relapse were available for comparison, the capture assay detected significant changes in titer between the states of remission and relapse. Most samples were positive during remission, but the values were lower than those for samples taken during a relapse. To distinguish between remission and relapse by the capture ELISA a limit of 50 or 100 ELISA units instead of 20 ELISA units, which is the current borderline for positive versus negative results, must be used. Still, there was considerable overlap, and a diagnosis of relapse should not be made on the basis of a single result by the capture assay. Instead, the best clinical use of the capture assay is for the exclusion of relapses. If a patient has negative or unchanging low values, the probability of active vasculitis is likely to be low.

The performance of IIF was also good in this study. IIF was negative for only 2 of 21 relapses, and increasing titers were seen in 77% of the relapses. A larger study will be necessary to conclude if the difference between the capture ELISA and IIF is a consistent finding. A major disadvantage with the use of IIF is that the outcome is dependent on the experience of the person reading the slides. In this study we had optimal circumstances for evaluation by IIF, with all slides read by one investigator (J.C.J.) with a long track record assessing ANCA tests.

Other studies evaluating the clinical performance of IIF have shown less favorable results (7).

Previously published results concerning ANCA and relapses have mainly been performed by IIF. Many of the studies were performed before IIF for ANCA was standardized. Our results concerning IIF do not deviate from the results of others, in which positive results were seen for 75 to 100% of the published cases. Fewer data are available from studies that have used the standard ELISA. These data are even more difficult to compare because of the different methods applied. However, the results indicate that the standard ELISA might have a lower sensitivity for the detection of relapses than IIF and the capture ELISA. This and two recent studies (1, 20) are the only attempts that have been made to compare the abilities of different ELISA methods to detect relapses. There are substantial differences in the results, especially between the German study (20) and the present study. The reason for this discrepancy is not clear, but one major difference between the studies is how relapses were defined. In our study a minimum of 3 months of remission must have elapsed for the two disease periods to have been classified as separate relapses. Thus, minor fluctuations in disease activity during an exacerbation (which may render a change in the Birmingham vasculitis activity score) were not listed as separate relapses.

In the present study the capture assay was better than the standard ELISA method for discrimination between a relapse and a remission. Most ANCA are directed to conformational epitopes on PR3. Breaking up of disulfide bonds or boiling of the antigen diminishes or abolishes reactivity by Western blotting analysis (30). The most probable explanation for the good performance of the capture assay is that an epitope on PR3 that is sensitive and potentially relevant for disease activity is better preserved or presented by this method. This is of theoretical interest concerning the role of ANCA in the pathogenesis of vasculitis. The ANCA present during a remission may have a lower capacity to elicit intracellular signaling events. If so, it would also be possible, using recombinant



TABLE 2. Summary of the present study compared with data from previously published series for C-ANCA determinations by IIF and PR3-ANCA determinations by ELISA during relapses<sup>a</sup>

Assay and author, year (reference)	No. of relapses/no. of patients with relapses	Percent		
		Positive at relapse	Rise in assay value at relapse	Rise in assay value without relapse
<b>IIF</b>				
van der Woude et al., 1985 (29)	9/7	100 (9/9) <sup>b</sup>	NA <sup>c</sup>	NA
Specks et al., 1989 (26)	3	100 (3/3)	100 (3/3) <sup>b</sup>	43 (3/7) <sup>d</sup>
Cohen Tervaert et al., 1989 (6)	17/12	100 (17/17)	100 (17/17)	23 (5/22)
Halma, 1990 (11A)	8/6	75 (6/8)	50 (3/6)	86 (19/22)
Maclsaac et al., 1990 (18)	5/3	100 (5/5)	100 (5/5)	29 (2/7)
Gaskin et al., 1991 (10)	25/15	100 (25/25)	? (>6/25)	NA
Stegeman et al., 1993 (27)	18/16	100 (18/18)	83 (15/18)	NA
Dolman et al., 1993 (9)	8/8	100 (8/8)	75 (6/8)	0 (0/6)
Kerr et al., 1993 (14)	45/45	87 (39/45)	20 (9/45)	44 (7/16)
Jeffrey et al., 1994 (13)	7/5-7	100 (7/7)	100 (7/7)	63 (12/19)
Davenport et al., 1995 (7)	29/<17	72-100 (21-29/29)	48 (14/29)	83 (50/64)
Kyndt et al., 1999 (16)	32/13	100 (30/30)	27 (8/30)	43 (6/14)
Boomsma et al., 2000 (3)	33/33	100 (33/33)	52 (17/33)	43 (13/30)
Girard et al., 2001 (11)	16-20/16	62-85 (10/16-17/20)	NA	NA
Towbin et al., 2001 (28)	?/<14	NA	37	NA
Present study	21/14	86 (19/21)	77 (10/13)	NA
Median (range) for IIF		100 (75-100)	75 (20-100)	43 (0-86)
<b>Standard ELISA</b>				
Dolman et al., 1993 (9)	8/8	75 (6/8)	75 (6/8)	0 (0/6)
Power et al., 1995 (22)	4/4	75 (3/4)	25 (1/4)	NA
Pettersson and Heigl, 1992 (21)	7/5	86 (6/7)	75 (3/4)	40 (2/5)
Jayne et al., 1995 (12)	16/16	81-88 (13-14/16)	≈81 (≈13/16)	≈24 (≈4/≈17)
De'Oliviera et al., 1995 (8)	23/18	87 (20/23)	50 (10/20)	33 (5/15)
Kyndt et al., 1999 (16)	32/13	100 (30/30)	33 (10/30)	41 (7/17)
Boomsma et al., 2000 (3)	33/33	100 (33/33)	81 (27/33)	29 (11/38)
Nowack et al., 2001 (20)	?/<14	NA	32	NA
Arranz et al., 2001 (1)	8/5	38 (3/8)	38 (3/8)	NA
Present study	21/14	86 (19/21)	77 (10/13)	NA
Median (range) for standard ELISA		86 (38-100)	50 (25-81)	31 (0-41)
<b>Capture ELISA</b>				
Nowack et al., 2001 (20)	?/<14	NA	43	NA
Arranz et al., 2001 (1)	8/5	88 (7/8)	77 (6/8)	NA
Capture ELISA, present study	21/14	100 (21/21)	100 (13/13)	NA

<sup>a</sup> When the exact figures could not be extracted from the paper, the possible range of data are presented.

<sup>b</sup> Values in parentheses are number of relapse incidents assay positive at relapse/total number of relapses.

<sup>c</sup> NA, not available.

<sup>d</sup> Values in parentheses are the number of patients without a relapse but with a rise in assay value/total number of instances with a rise in assay value.

technology, to construct molecules harboring only this relevant epitope region and thus construct better assays in the future.

The small number of samples available from periods of remission hampered this study. This prevents us from doing a meaningful evaluation of the specificity of a rise in ANCA titers for the detection of relapses. Other studies that have focused on this issue claim that a rise in ANCA titer without a relapse is relatively common (7). According to the previously reported data, approximately half of all increases in ANCA titers are associated with clinically significant relapses. Our data cannot be used as an argument against this notion; instead, they support the concept that the best use of ANCA titers is for the exclusion of relapses.

In conclusion, the capture PR3 ELISA based on monoclonal antibody 4A3 is a sensitive tool for the detection of relapses in patients with systemic vasculitis. However, larger studies are needed to conclude whether it is superior to other methods in clinical practice.

**ACKNOWLEDGMENTS**

The work was supported by the Swedish Renal Foundation and the Swedish Medical Research Council.

We thank Jörgen Wieslander at Wieslab AB for donating material for the capture ELISA and Robin Tuttle, Department of Pathology, University of North Carolina at Chapel Hill, for extraordinary work in retrieving stored samples.

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