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Colocalization of ANCA-antigens and fibrinoid necrosis in ANCA-associated vasculitis

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Colocalization of ANCA-antigens and fibrinoid necrosis in ANCA-associated vasculitis. A variety of antineutrophil cytoplasmic auto-antibodies (ANCAs) are known to be associated with small vessel vasculitides such as Wegener's granulomatosis and microscopic polyangiitis. To visualize colocalization patterns of the fibrinoid necrotic lesions and ANCA-antigens more accurately, we have developed a double staining technique in which an immunohistochemical staining is followed by a histological staining. Instead of using sequential biopsy slides of histologically and immunohistochemically stained sections, which may lead to an underestimation of the number and size of the lesions, our technique permits the visualization of the colocalized patterns of fibrinoid necrosis with an ANCA-antigen in a single slide. The double staining procedure is presented in this Technical Note.

In 1985, antineutrophil cytoplasmic auto-antibodies (ANCAs) were first reported to occur in patients with Wegener's granulomatosis, one of the small vessel vasculitides [1]. Today, a variety of ANCA antigens are known, of which proteinase-3 (Pr3) is most closely associated with Wegener's granulomatosis, and myeloperoxidase (MPO) with other small vessel vasculitides such as microscopic polyangiitis [2]. The detection of ANCA in these syndromes heralded a new era of hypotheses on the etiology of systemic vasculitis. For several years it has been known that ANCA can stimulate primed polymorphonuclear cells (PMN) to degranulate and produce increased reactive oxygen species [3, 4]. This finding established it was only a small step to suggest that the necrotizing vasculitic lesion most likely would be the result of a disturbed neutrophil-endothelial interaction. The basic assumption is that somehow the vessel wall is damaged in the process of degranulation [5], and this requires the primed PMN with ANCA antigens on its surface to be at the site of the lesion. In line with this hypothesis, it was recently reported that if FcγRIIIb is activated on the surface of PMN by ANCA IgG, L-selectin expression is maintained, making it possible for the PMN to adhere to the endothelium in an activated state [6], leading to vessel damage.

In renal tissue fibrinoid necrosis mainly occurs as a segmental intraglomerular lesion, representative of vasculitis of the glomerular capillaries. Some years ago, it was shown that the amount of primed neutrophils and of human elastase (HE)-bearing cells, but not of Pr3 and MPO-bearing cells, correlated with the severity of renal impairment as measured by serum creatinine levels [7]. However, whether ANCA-antigens were actually present at the site of the necrotic lesion was left unquestioned. Following current theories on primed neutrophils bearing ANCA-antigens on their surface and causing vessel damage as depicted in Figure 1, colocalization of the ANCA-antigens with the necrotic lesion is to be expected. In traditional immunohistochemical techniques, antigens are detected against a hematoxylin background in which the necrotic lesion is barely visible. Using consecutive biopsy slides of histologically and immuhistochemically stained sections to solve the inadequate detection of fibrinoid necrosis in the hematoxylin background staining may lead to an underestimation of the colocalization rate of the necrotic lesion and the antigen, because the size of the fibrinoid necrotic lesion varies considerably between consecutive sections, and the lesion is so focal that it may even disappear from the glomerulus from one section to another [8]. With these techniques, testing the colocalization hypothesis is therefore not possible.

We now report a double staining procedure by which immunohistochemical detection of ANCA-antigens is followed by a histological staining in which fibrinoid ne-

Key words: Wegener's granulomatosus, small vessel vasculitides, proteinase-3, myeloperoxidase, microscopic polyangiitis, polymorphonuclear cells.

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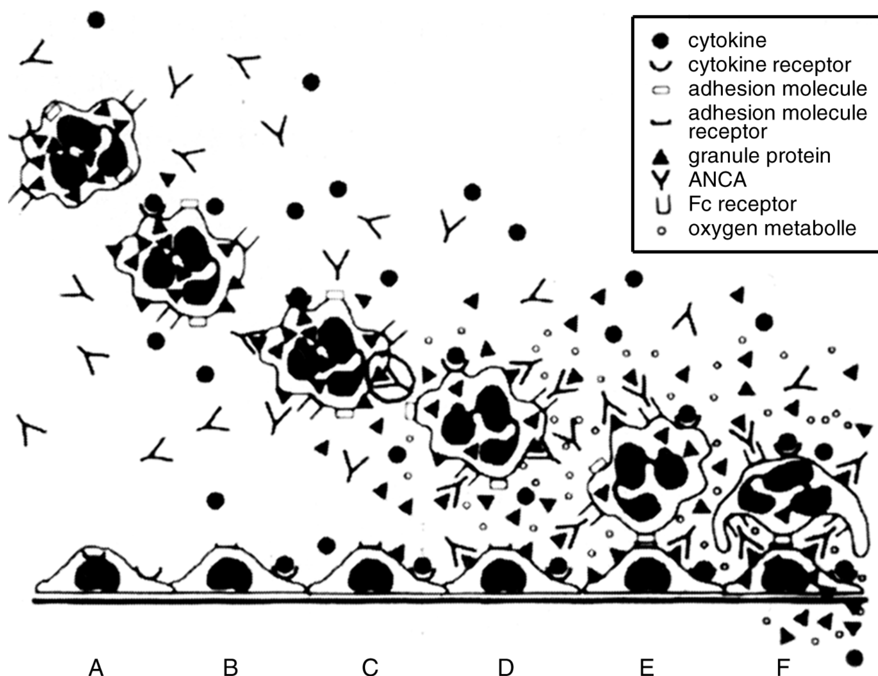


Fig. 1. Theoretical pathogenic scenario leading to antineutrophil cytoplasmic antibody (ANCA)-induced vasculitis. (*A*) Circulating quiescent neutrophils contain ANCA antigens not accessible to interaction with ANCA. (*B*) Priming of neutrophils by cytokines such as interleukin (IL)-1 and tumor necrosis factor (TNF), resulting in the expression of ANCA antigens at the cell surface. (*C*) ANCA bind to target antigens at the cell surface. (*D*) Neutrophils release toxic oxygen metabolites. (*E*) Neutrophils adhere to endothelial cells via adhesion molecules and ligands. (*F*) Vessel wall injury leading to fibrinoid necrosis (reproduced with kind permission of J.C. Jennette and R.J. Falk).

Table 1. Clinical data

Patient	M/F	Age *years*	Serum creatinine *μmol/L*	ANCA: IIF	ANCA: ELISA (anti-Pr3 or anti-MPO antibodies)
1	F	32	192	cANCA	anti-Pr3
2	M	49	710	cANCA	anti-Pr3
3	M	65	110	cANCA	anti-Pr3
4	M	54	1200	cANCA	anti-Pr3
5	F	53	77	cANCA	anti-Pr3
6	M	61	126	cANCA	both weakly positive
7	M	67	145	cANCA	both negative
8	F	66	190	cANCA	anti-MPO
9	F	75	207	pANCA	anti-MPO
10	M	57	113	pANCA	anti-MPO

crosis is clearly detectable, making it possible to visualize co-localization patterns of fibrinoid necrosis with the ANCA-antigens Pr3, MPO, HE, and lactoferrin (LF) in one single slide.

Renal biopsies were selected from patients with ANCA-associated systemic vasculitis with renal involvement. Their clinical data are listed in Table 1. An indirect immunofluorescence test for ANCA was performed by using an IgG-specific FITC-conjugate, and the results were scored as cytoplasmic (cANCA), perinuclear (pANCA), atypical, or negative staining. Sera were tested for the presence of anti-PR3 and anti-MPO antibodies in an ELISA format.

The monoclonal antibody against Pr3 was kindly provided by Dr. Hoidal (Department of Medicine, University of Minnesota, Minneapolis, MN, USA). The polyclonal antibodies against MPO, HE, and LF were kindly provided by Professor M.R. Daha (Department of Nephrology, Leiden University Medical Center, the Netherlands). Peroxidase-conjugated rabbit anti-mouse Ig (RAM) and swine anti-rabbit Ig (SWAR) were obtained from Dako (Glostrop, Denmark).

Four-micrometer sections of frozen renal biopsies were mounted on Starfrost glasses, air-dried at room temperature, and then fixed in acetone for three minutes. After three washings with phosphate-buffered saline (PBS) of five minutes each, the first antibody was incubated in a dark moisturized box for one hour. After three washings with PBS, the second antibody was applied: swine anti-rabbit IgG or rabbit anti-mouse IgG (Dako) with 10% human IgG as a blocking agent. All antibodies were diluted in bovine albumin solution (Boseral; Organon Technika) and PBS. The second step lasted 30 minutes, after which the sections were washed again with PBS. They were incubated with 3,3-diaminobenzidine (DAB; 2.5 mg; Fluka) in 50 mL 0.5 mol/L Tris-HCl at pH 7.6 for five minutes, briefly washed in PBS, and rinsed in running tap water for five minutes. As a negative control, an irrelevant isotype- and species-matched first step Ig (goat antiserum to human uromucoid; Organon), followed by labeled detection (second step) Ig. Also, RAM and SWAR were applied to all sections in the absence of primary antibodies. These controls gave negative test results on all biopsy specimens used in this study.

The Picro-Mallory staining method for paraffin sec-

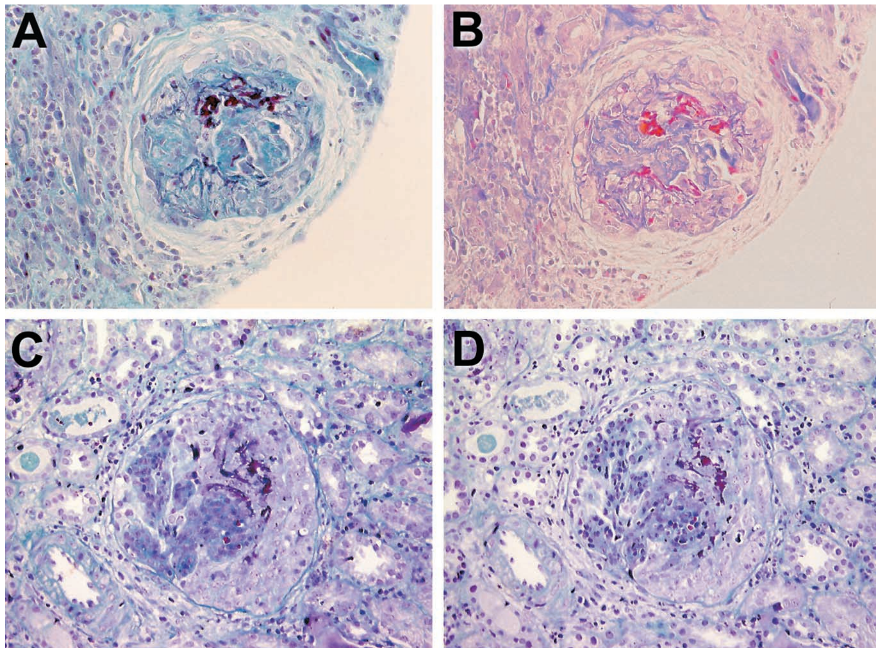


Fig. 2. (*A*) Glomerular fibrinoid necrosis in trichrome Gomori staining, and (*B*) consecutive section in the modified Mallory staining. (*C*) Modified Mallory staining preceded by a peroxidase/DAB procedure, and (*D*) modified Mallory staining on the consecutive section showing that the specificity for fibrinoid necrosis is unaltered (all magnifications ×40). The publication of the color illustrations was financially supported by the Dutch Arthritis Association and by the Dutch Friedrich Wegener Foundation (*www.vasculitis.nl*).

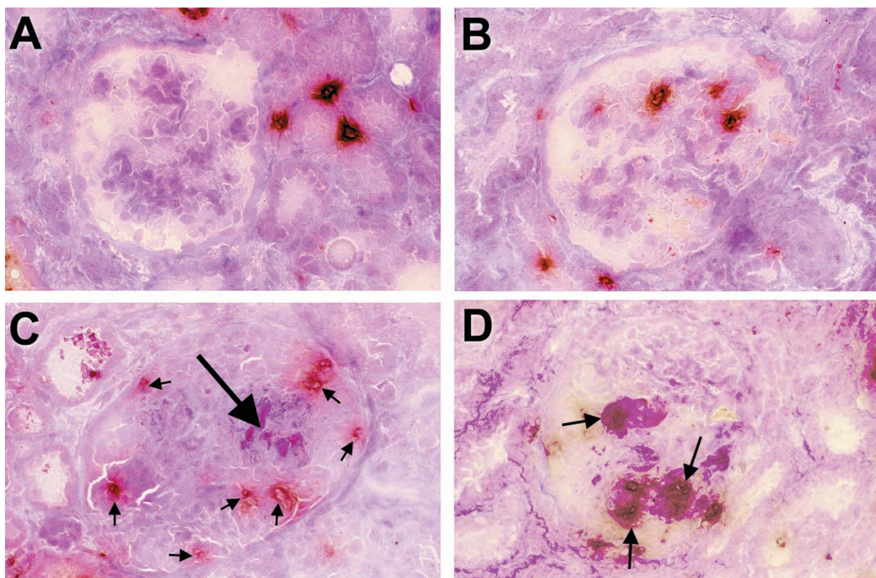
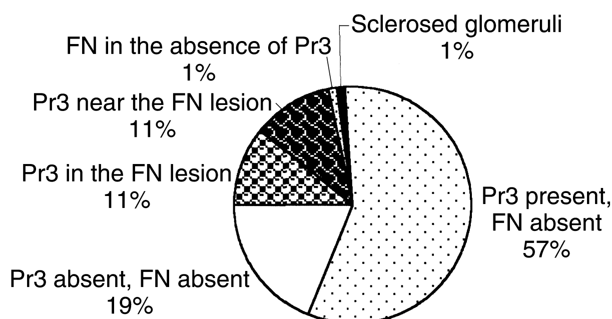
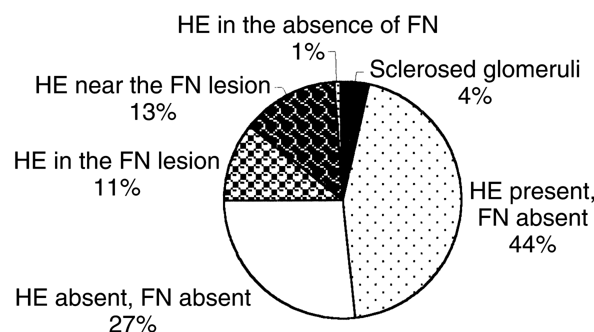
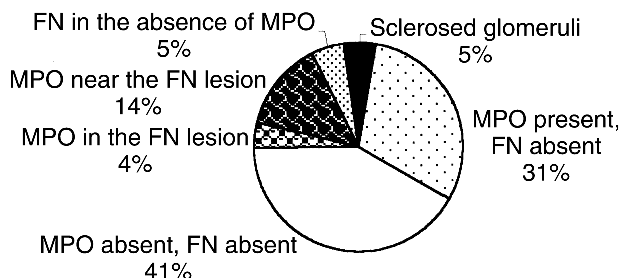


Fig. 3. (*A*) Glomerulus without fibrinoid necrosis, proteinase-3 (Pr3) present in the interstitium. (*B*) Glomerulus without fibrinoid necrosis, Pr3 present in the glomerulus. (*C*) Fibrinoid necrosis in a glomerulus (carmine red, big arrow) with Pr3 in its near vicinity (brown DAB staining, small arrows). (*D*) Pr3 present inside the fibrinoid necrotic lesion: necrosis stains carmine red, and brown DAB blots indicated by small arrows (all magnifications, ×40). The publication of the color illustrations was financially supported by the Dutch Arthritis Association and by the Dutch Friedrich Wegener Foundation (*www.vasculitis.nl*).

tions was originally described by Lendrum et al [9] and later modified for cryostat sections [10]. To make the staining adaptable as a background staining after an immunohistochemical procedure, each dye was dissolved in a separate solution and the concentrations were adjusted accordingly: 1.5 g Orange G (Merck, Darmstadt, Germany) in 150 mL aquadest; 0.5 g acid Fuchsin (Fluka Chemika, Germany) in 150 mL aquadest; 0.1 g Aniline Blue (Anilinblau, wasserlöslich; Fluka AG) in 150 aquadest; 0.75 g phosphotungsten acid (Wolframatophosphorsäure-Hydrat; Merck) in 150 mL aquadest. The slides were incubated in the Orange G solution for 15 minutes and then quickly washed in aquadest. After incubation in acid Fuchsin for five minutes, they were immediately put in the phosphotungsten acid solution for a few seconds and without washing incubated in the aniline blue solution for one minute. Then they were dehydrated in short washings of ethanol 70, 95, and 100%, dipped in xylol and mounted with mannitol. The various steps were monitored microscopically to achieve an optimal result. The Orange G stains the fibrinoid necrosis in a negative background (all other structures remain un-







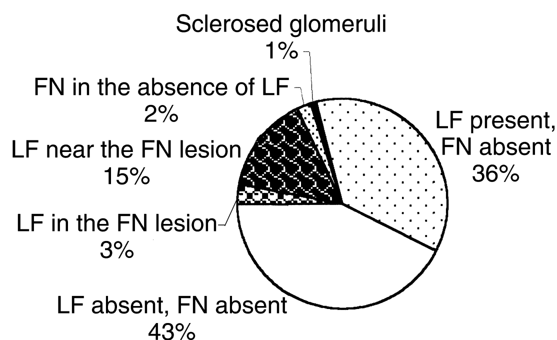


Fig. 4. Co-localization patterns of four ANCA antigens. Antigens were stained individually. For each staining, the total number of glomeruli is divided into: those without fibrinoid necrosis (FN) with the antigen either present or absent in the glomerulus; and those with fibrinoid necrosis with the antigen present inside the lesion or in its direct vicinity, or with the antigen absent. The number of sclerosed glomeruli is indicated separately. (*A*) Proteinase 3 staining. (*B*) Elastase staining. (*C*) Myeloperoxidase staining. (*D*) Lactoferrin staining.

stained). The acid Fuchsin highlights the fibrinoid necrosis in bright red while it stains the surrounding areas in a faint red. The phosphotungsten acid etches the Orange G with the acid Fuchsin at the site of the lesion, and washes away most of the acid Fuchsin in the surrounding areas. The Aniline Blue provides a light blue background. Figures 2 A and B illustrate that the modified Mallory staining has the same specificity for fibrinoid necrosis as traditional trichrome stainings on paraffin sections. Figures 2 C and D show that the specificity of the modified Mallory staining for detecting fibrinoid necrosis is not altered by the preceding peroxidase/DAB procedure.

For each of the antigen stainings, the glomeruli were categorized as follows: antigens present in the glomerular necrotic lesion, antigens present in the vicinity of the glomerular necrotic lesion, no antigens present in a glomerulus with fibrinoid necrosis, antigens present in a glomerulus without fibrinoid necrosis, and no antigens present in a glomerulus without fibrinoid necrosis. The presence of antigens in the interstitium was semiquantitatively scored as −, +, or ++. Arteries were separately scored on the presence of antigens in their walls. The distribution patterns were compared by means of the χ^2 test. A semiquantitative scoring was performed of intraglomerular DAB-positive dots, and a comparison was made between the median number of dots in glomeruli with and without fibrinoid necrosis by means of the Median test.

Figure 3 shows various combination patterns of ANCA-antigens in the presence or absence of fibrinoid necrosis. The frequency of occurrence of the various colocalization patterns of ANCA antigens with fibrinoid necrosis is demonstrated in Figure 4 for the total number of glomeruli in all biopsies. In the majority of glomeruli with fibrinoid necrotic lesions, ANCA antigens were present either inside the lesion or in its direct vicinity, but this colocalization pattern was significantly stronger for Pr3 and HE than for MPO and LF. Pr3 and HE, more than MPO and LF, occurred more frequently inside the lesion itself than in its vicinity (χ^2 test, $P < 0.05$). Also in the Pr3- and the HE-staining, the number of DAB-positive dots was significantly higher in glomeruli with fibrinoid necrosis than in glomeruli without fi-

Table 2. Median number of DAB-positive dots in glomeruli with and without fibrinoid necrosis

	Median number of positive cells		
Antigen	Glomeruli with fibrinoid necrosis	Glomeruli without fibrinoid necrosis	*P* value
Pr3	8	2	<0.001[a]
HE	6	2	<0.001[a]
MPO	2	1	NS
LF	4	2	NS

Abbreviations are: Pr3, proteinase-3; HE, human elastase; MPO, myeloperoxidase; LF, lactoferrin.
[a] Median test

Table 3. ANCA-antigens present in the interstitium and in arteries

	Interstitial score *number of biopsies*			Number of arteries with antigen in the vessel wall/total number of arteries present
Antigen	−	+	++	
Pr3	0	3	7	3/25
HE	2	5	3	0/30
MPO	1	9	0	0/18
LF	0	8	2	1/17

Abbreviations are in Table 2.

brinoid necrosis (median test, both $P < 0.001$; Table 2), whereas for MPO and LF this difference was statistically insignificant. Although all antigens were present in the interstitium, they were hardly ever encountered within vessel walls (Table 3).

DISCUSSION

In ANCA-associated systemic vasculitis, the endothelium is the target of primed granulocytes and monocytes expressing ANCA-antigens such as proteinase 3 (Pr3), myeloperoxidase (MPO), human elastase (HE), and lactoferrin (LF) on their surface, leading to the development of fibrinoid necrosis. The necrotizing vasculitic lesion is considered to result from a disturbed neutrophil–endothelial interaction [11], which requires primed inflammatory cells with ANCA antigens on their surface present at the lesion site. Recently, it was reported by Kocher et al that if the FcγRIIIb receptor located on the surface of polymorphonuclear cells (PMN) is activated by ANCA IgG, then L-selectin expression is maintained, making it possible for the PMN to adhere to the endothelium in an activated state [6]. This finding gives further evidence for the hypothesis that activated PMN are responsible for vessel wall damage in ANCA-associated vasculitis syndromes, characterized by the fibrinoid necrotic lesion.

Although it is generally accepted that ANCA-antigens such as Pr3 are expressed on the surface of neutrophils and monocytes, there has been controversy about the expression of these antigens on other tissues and cells, for example, on the endothelium. To some extent, this discussion remains outside our study, which investigates the expression of ANCA-antigens in the necrotic lesions they are assumed to induce. If, for instance, the antigens are expressed on the endothelium as well as on PMN, that should be detected by the antibodies used in this study. The aim of the present study was to provide a staining procedure by which patterns of ANCA-antigen expression in the necrotic lesion could be visualized.

The present study illustrates a double-staining procedure that makes it possible to envisualize the colocalization patterns of ANCA antigens and the vasculitic lesion they are assumed to induce. We investigated whether the ANCA antigens colocalized with the vasculitic lesion, bearing in mind that perhaps some, but not all, ANCA-antigens would do so, in view of previous publications on the variation in histopathological lesions in patients with either antibodies against Pr3 or MPO [12]. Our findings indicate that although Pr3, MPO, HE, and LF were all found either within or around the fibrinoid necrotic lesion, Pr3 and HE were the antigens most prominently present. They occurred more often inside the lesion than MPO and LF, and moreover, by a semi-quantitative scoring their presence was significantly higher in glomeruli with fibrinoid necrosis than in those without it. It is telling that of the ANCA-antigens known, Pr3 and HE are the ones that share considerable amino acid sequence homology with each other [13] and that in vitro, Pr3 and HE have been shown to induce detachment and cytolysis of umbilical vein endothelial cells [14].

A considerable number of necrotic lesions, however, appeared in the absence of any of the ANCA-antigens chosen for staining. Within the paradigm presented in Figure 1, the most likely explanation is that these are the somewhat older lesions that remain after degranulating PMN have caused the damage that led to necrosis of the vascular wall. That this is in fact the sequence of events can only be tested within experimental animal models, for which we advocate the usage of our double-staining method.

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