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Research paper

EUROPLUS™ ANCA BIOCHIP mosaic: PR3 and MPO antigen microdots improve the laboratory diagnostics of ANCA-associated vasculitis

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ABSTRACT

Introduction: The consensus on anti-neutrophil cytoplasmic antibody (ANCA) testing requires screening with indirect immunofluorescence (IIF) and confirmation in MPO- and PR3-ANCA specific assays. The EUROPLUS™ system combines in one incubation the conventional cell substrates with microdots of single antigens, i.e., MPO and PR3. We evaluated the diagnostic applicability of this new system for ANCA-associated vasculitis (AAV).

Methods: To assess the diagnostic performance of the EUROPLUS™ Granulocyte Mosaic, sera from 249 AAV patients, 85 disease controls and 27 healthy controls were analysed. Results were compared with a reference multi-testing algorithm based on IIF with ethanol-fixed granulocytes, direct and capture ELISAs for both MPO- and PR3-ANCA.

Results: Based on the reference multi-testing algorithm 123 AAV patients were defined as having PR3-ANCA and 68 AAV patients as having MPO-ANCA (diagnostic sensitivity: 76.7%). For the EUROPLUS™ MPO and PR3 microdots the diagnostic sensitivity was 77.1% in the same AAV cohort. The concordance between both methods for PR3- and MPO-ANCA was 96.8% and 99.2%, respectively. In the control cohort the diagnostic specificity was 99.1% for the multi-testing algorithm and 98.2% for the EUROPLUS™ microdots.

Conclusions: The combination of conventional cell substrates and single MPO and PR3 antigen microdots greatly facilitates the identification of ANCA reactivity clinically relevant for AAV. Since our results obtained after a single incubation in the EUROPLUS™ system are highly concordant with the reference multi-testing algorithm (based on IIF, direct and capture ELISAs) the EUROPLUS™ system is advocated as an efficient test system.

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1. Introduction

Idiopathic small vessel vasculitides, including Wegener's granulomatosis (WG), microscopic polyangiitis (MPA), and the Churg-Strauss syndrome (CSS), are strongly associated with the presence of anti-neutrophil cytoplasmic antibodies (ANCA) (Jennette and Falk, 1997; Bosch et al., 2006). Detection of ANCA by indirect immunofluorescence (IIF) on ethanol fixed neutrophils reveals two relevant ANCA patterns, i.e. a granular cytoplasmic pattern with central interlobular accentuation (C-ANCA) and a perinuclear pattern, often with nuclear extension (P-ANCA). The C-ANCA and P-ANCA found

Abbreviations: AAV, ANCA-associated vasculitis; ANA, anti-nuclear antibodies; ANCA, anti-neutrophil cytoplasmic antibody; CSS, Churg-Strauss syndrome; ELISA, enzyme-linked immunosorbent assay; FEIA, fluorescent-enzyme immuno-assay; IIF, indirect immunofluorescence; MPA, microscopic polyangiitis; MPO, myeloperoxidase; PR3, proteinase 3; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome, WG, Wegener's granulomatosis.

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in vasculitis patients are mostly directed against proteinase 3 (PR3) or myeloperoxidase (MPO), respectively (Savige et al., 2000; Rutgers et al., 2003). Both enzymes are located in the azurophilic granules in the cytoplasm of neutrophilic granulocytes. The P-ANCA pattern therefore is an artefact due to relocalization of the MPO upon ethanol fixation. Indeed, a P-ANCA pattern due to MPO-ANCA shows a C-ANCA pattern when formalin-fixed granulocytes are used as substrate. ANCA as detected by IIF are also apparent in several other inflammatory conditions. In these cases, however, ANCA are directed against other proteins than PR3 and MPO, and hence ANCA as detected by IIF is not specific for ANCA associated vasculitis (AAV) (Kallenberg et al., 1992; Merkel et al., 1997). Therefore, ANCA should be demonstrated by using a combination of IIF and assays specific for PR3 and MPO (Savige et al., 1999, 2003). This combination has been shown to have high diagnostic value (Hagen et al., 1998; Choi et al., 2001).

Since adequate ANCA detection requires distinct technologies that are most often performed step-wise, the diagnostic work-up of a new patient can be time-consuming and delayed. In case of rapidly progressive disease manifestations, like pulmonary alveolar haemorrhage or glomerulonephritis, several rapid screening assays are available (Westman et al., 1997; Rutgers et al., 2004). However, these assays are based only on the detection of PR3- and MPO-ANCA. Therefore, the advent of the EUROPLUS™ system, that combines IIF and antigen specific tests in a single incubation, may be of great help in the diagnosis of AAV.

In the current study, we have evaluated the diagnostic performance of the EUROPLUS™ Granulocyte Mosaic. We determined the diagnostic characteristics in patients with AAV ($n=249$), as well as healthy ($n=27$) and disease controls ($n=85$). The performance of this new assay was compared with a reference multi-testing algorithm based on a combination of IIF with ethanol-fixed granulocytes, direct and capture ELISAs for both MPO- and PR3-ANCA, as described before (Damoiseaux et al., 2005). To confirm the observed specificity, an additional 496 samples of patients with other systemic autoimmune diseases were analysed by the EUROPLUS™ system.

2. Materials and methods

2.1. Human sera

Sera of 3 distinct AAV cohorts (renal biopsy-proven AAV patients, out-patient AAV patients, and WG patients) and 3 distinct control cohorts (out-patient cohort with other vasculitides, disease controls with systemic autoimmune diseases, and healthy controls) were used. This study was performed in accordance with the 1997 Declaration of Helsinki of the World Medical Association. From healthy controls informed consent was obtained. For the analyses on patient material serum was obtained for diagnostic and follow-up purposes. Since rest-serum was used in an anonymous way, ethical approval was not necessary according to the Dutch guide lines.

Consecutive patients with renal biopsy-proven AAV ($n=115$) were retrospectively identified in our renal biopsy registry as diagnosed with pauci-immune NCGN between 1989 and 2006 (Van Paassen et al., 2004). Sera were obtained at the time of kidney biopsy, i.e., at the time of diagnosis. The out-patient AAV cohort ($n=86$) was registered with AAV (including WG, MPA, CSS, and the renal limited form of AAV) in the out-patient clinic of the Department of Internal Medicine, Division of Clinical and Experimental Immunology, Maastricht University Medical Centre (MUMC). Twelve samples of this out-patient AAV cohort were also included in the renal biopsy-proven AAV cohort and therefore were excluded from further analysis. The 74 included serum samples were diagnostic samples ($n=49$), or follow-up samples ($n=25$). Finally, sera of 60 consecutive, new onset WG patient were collected at the Department of Rheumatology, University Hospital of Schleswig-Holstein (kindly provided by Dr. E. Csernok). These samples were previously used in a multicenter study for detection of PR3-ANCA in WG (Csernok et al., 2004). For all AAV patients, the clinical diagnosis was established according to the definitions of the 1992 Chapel Hill Consensus Conference (Jennette et al., 1994).

As control samples from healthy controls ($n=27$) and from patients with RA ($n=30$) were included; these samples were also part of the previously mentioned multicenter study (Csernok et al., 2004). In addition, the out-patient cohort with other vasculitides (non-AAV) included patients ($n=55$) that were registered in the out-patient clinic of the Department of Internal Medicine, Division of Clinical and Experimental Immunology, MUMC. The diagnoses included: Henoch-Schönlein purpura ($n=9$), cutaneous polyarteritis nodosa ($n=8$), giant-cell arteritis ($n=7$), cryoglobulinaemia ($n=5$), idiopathic leucocytoclastic vasculitis of the skin ($n=5$), Buerger's disease ($n=3$), Behçet's disease ($n=3$), idiopathic retinal vasculitis ($n=3$), Goodpasture's disease ($n=3$), Takayasu's disease ($n=2$), and miscellaneous forms of vasculitis ($n=7$). Finally, we included samples (cross-sectional) from patients with rheumatoid arthritis (RA; $n=200$), systemic lupus erythematosus (SLE; $n=100$), and Sjögren's syndrome (SS; $n=196$). All these control patients were diagnosed at the Department of Rheumatology and Clinical Immunology, Charité University of Medicine Berlin, according to internationally recognised criteria (Arnett et al., 1988; Hochberg, 1997; Vitali et al., 2002). All these sera were kindly provided by Prof. F. Hiepe (Charité University of Medicine Berlin).

2.2. Reference multi-testing algorithm

Sera used for the evaluation of the EUROPLUS™ Granulocyte Mosaic were tested for ANCA by IIF, direct and capture ELISA for both PR3- and MPO-ANCA. Routine IIF for ANCA detection was performed on ethanol-fixed granulocytes (INOVA Diagnostics, San Diego, CA) according to manufacturer's instruction and as described before (Damoiseaux et al., 2005). Three staining patterns were considered ANCA positive: perinuclear (P-ANCA), fine granular cytoplasmic (C-ANCA), and diffuse homogeneous cytoplasmic (atypical ANCA) staining of the neutrophils. The finding of homogeneous nuclear staining was considered inconclusive in terms of ANCA pattern due to the interference of anti-nuclear antibodies (ANA). The presence of PR3- and/or MPO-ANCA was determined and quantified using commercially available assays. Before October 2005 ELISAs were performed (Euro-Diagnostica, Malmö, Sweden) and since October 2005 ANCA

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