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Research paper

Evaluation of a multiplex flow cytometric immunoassay to detect PR3- and MPO-ANCA in active and treated vasculitis, and in inflammatory bowel disease (IBD)☆

Michelle Trevisin [a,b], Wendy Pollock [b,c], Wayne Dimech [d], Judy Savige [b,*]

[a] Pathology Department, Austin Health, Heidelberg, VIC 3084, Australia
[b] The University of Melbourne, Department of Medicine, Northern Health, Epping VIC 3076, Australia
[c] Gribbles Pathology, Clayton, VIC 3168, Australia
[d] National Serology Reference Laboratory, Fitzroy, VIC 3065, Australia

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ABSTRACT

This study compared the performance of a flow cytometric immunoassay for antineutrophil cytoplasmic antibodies (ANCA) directed against proteinase 3 (PR3) and myeloperoxidase (MPO), with indirect immunofluorescence (IIF) and ELISAs from 12 different manufacturers. Sera were from patients with active (n=55) or treated (n=68) small vessel vasculitis, or inflammatory bowel disease (IBD, n=22).

The immunoassay specificity was 88% compared with 96% for IIF and 94% (median, range 91–96%) for both ELISAs. Its sensitivity in treated disease was 82% compared with 84% for IIF and 69% (median, range 57–82%) for the ELISAs. The immunoassay's specificity was 88% which was the same as the median for both ELISAs (range 84–95%). The PR3- and MPO-ANCA immunoassay was almost as sensitive as IIF, and more sensitive than, but just as specific as, most ELISAs, in detecting ANCA in active and treated vasculitis. A major advantage of this assay is its ability to be further modified to simultaneously screen for a panel of autoantibodies relevant to vasculitis.

1. Introduction

The 'International Consensus Statement on Testing and Reporting Antineutrophil Cytoplasmic Antibodies (ANCA)' requires sera from patients with suspected small vessel vasculitis to be screened by indirect immunofluorescence (IIF) on ethanol-fixed neutrophils, and all IIF-positive sera to be confirmed in antigen-specific ELISAs (Savige et al., 1999). The IIF assay is highly sensitive for ANCA but also labour-intensive, expensive, subjective and lacking in specificity (Savige et al., 1998). The direct and capture ELISAs are less sensitive and more specific than IIF for small vessel vasculitis (Csernok et al., 2002; Csernok et al., 2004), but their performance is varied in treated disease where antibody levels are low (Trevisin et al., 2008). The usefulness of both IIF and the ELISAs is further limited by their inability to demonstrate antibodies directed against minor ANCA antigens and other vasculitis-associated antibodies, including antiglomerular basement membrane (GBM) antibodies. The early detection of both ANCA and antiGBM antibodies is critical in patients with rapidly progressive glomerulonephritis where renal function recovers with timely therapeutic intervention.

The multiplex flow cytometric immunoassay for vasculitis (FIDIS, Biomedical Diagnostics, France) uses a novel technique

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* Corresponding author. The University of Melbourne, Department of Medicine (Austin/Northern), The Northern Hospital, Epping VIC 3076, Australia. Tel.: +613 8405 8823; fax: +613 8405 8724.
E-mail address: jasavige@unimelb.edu.au (J. Savige).

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to detect and quantitate multiple autoantibodies simultaneously in a single reaction (Vercammen et al., 2007; Yiannaki et al., 2004; Tozzoli et al., 2006). This technology is fast, easy-to-use, and operator-independent. Microspheres covalently-conjugated with the major ANCA targets, proteinase (PR3), myeloperoxidase (MPO) (Goldschmeding et al., 1989; Falk and Jennette, 1988) are incubated with diluted patient serum and subsequently phycoerythrin-conjugated anti-human IgG, and the resulting fluorescence intensity measured in a flow cytometer (Luminex Corporation, Texas, USA).

This study compared the performance of the PR3- and MPO-ANCA flow cytometric immunoassays with IIF and 12 different PR3- and 12 MPO-ANCA ELISAs in the diagnosis of active small vessel vasculitis, and in detecting low ANCA levels in treated vasculitis and inflammatory bowel disease (IBD) (Saxon et al., 1990).

2. Patients and methods

2.1. Patients

2.1.1. Small vessel vasculitis

All sera were from patients with biopsy-proven Wegener's granulomatosis, microscopic polyangiitis, or necrotizing vasculitis that could not be classified further. 'Active' sera (A, $n=55$) were collected within 3 months of presentation ($n=36$, 65%) or relapse ($n=19$, 35%) from patients with Wegener's granulomatosis ($n=20$), microscopic polyangiitis ($n=26$) or necrotising vasculitis ($n=9$). 'Treated' or 'inactive' sera (T, $n=68$) were collected at least 3 months after presentation or relapse and in the absence of obvious ongoing clinical activity from patients with Wegener's granulomatosis ($n=38$), microscopic polyangiitis ($n=24$) or necrotising vasculitis ($n=6$). IIF-positive sera were mainly selected to compare the assays' abilities to detect positive and especially low-positive antibodies.

2.1.2. IBD

Sera were collected from patients diagnosed with IBD by their treating clinicians ($n=22$ for the PR3-ANCA and $n=23$ for the MPO-ANCA assays). Again IIF-positive sera were usually selected.

2.1.3. Controls

Controls included consecutive hospital patients referred for ANCA testing with suspected vasculitis who were subsequently shown to have a non-vasculitic disease ($n=34$), patients with IBD described above or with SLE diagnosed using the ARA criteria (Tan et al., 1982) and positive anti-double stranded DNA antibodies ($n=20$), as well as normal healthy blood donors ($n=33$).

All participants provided signed informed consent, and their sera were stored in aliquots at −70 °C, and thawed once only. This study was approved by the Human Research Ethics Committee of Austin Health.

2.2. Methods

2.2.1. IIF and PR3- and MPO-ANCA ELISAs

Results of testing sera in the immunoassay for PR3- and MPO-ANCA sera were compared with those seen with IIF and the 12 commercial and in-house direct and capture ELISAs described previously (Table 1) (Trevisin et al., 2008).

Table 1
PR3- and MPO-ANCA ELISAs

Kit	Kit name	Manufacturer
1	Bindazyme	The Binding Site, Birmingham, UK
2	ORG 518	ORGENTEC, Mainz, Germany
3	Immunoscan	Euro-Diagnostica, Malmo, Sweden
4	Varelisa	Pharmacia Diagnostics, Freiburg, Germany
5	Wielisa PR3 capture MPO direct	Wieslab, Lund, Sweden
6	Rainbow ELISA	Bio-Diagnostics Ltd, Worcestershire, UK
7	Euroimmun	Euroimmun, Seekamp, Germany
8	Kallestad	Bio-Rad Laboratories, California, USA
9	QUANTA Lite	INOVA Diagnostics Inc., California, USA
10	Aeskulisa	Aesku.Diagnostics GmbH, Wendelsheim, Germany
11	Medical Innovations	Analytica Ltd, New South Wales, Australia
12	In-house, PR3- and MPO-ANCA	In-house

2.2.2. Flow cytometric immunoassay

The PR3- and MPO-ANCA immunoassay (FIDIS vasculitis kit, Biomedical Diagnostics, Cedex, France) was performed according to the manufacturer's instructions using the reagents provided. The assay incorporated microspheres coated with PR3 and MPO, that were incubated with a 1/201 dilution of serum in phosphate-buffered saline Tween (PBS/T) buffer for 30 min at room temperature. Plates were washed twice with PBS/T and then incubated with prediluted phycoerythrin-labelled anti-human IgG for 30 min at room temperature, and the fluorescence intensity determined using a flow cytometer, the Luminex 100™ system (Luminex Corporation, Texas, USA).

All sera were tested in single wells, and dilution, washing, and dispensing of reagents were performed using an automated workstation (Biomedical Diagnostics, France). A positive and negative control, a single point calibrator and an external commercial QC control (ImmunoTrol I, Biomedical Diagnostics) were included in each run. Only assays where all controls behaved appropriately were included in the data analysis. Results were calculated using the single point calibrator and reported in units provided by the manufacturer (AU/ml). Binding less than 20 AU/ml was considered 'negative', 20–25 AU/ml 'borderline' and >25 AU/ml 'positive'. Borderline results were included in the 'positives'. All analyses except where indicated otherwise were performed using SPSS 13.0 (SPSS, Chicago, IL, USA).

2.2.3. Evaluation protocol

2.2.3.1. Precision. Within-run reproducibility was determined using 2 dilutions that resulted in low- and moderately-positive binding for each of PR3- and MPO-ANCA. These were each tested 30 times in a single run, and the coefficients of variation (CV) determined for each assay.

2.2.3.2. Binding characteristics and linearity

2.2.3.2.1. Binding levels. Binding levels of sera from active and treated Wegener's granulomatosis, microscopic polyangiitis and necrotising vasculitis were compared using one-way analysis of variance.