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Research paper

Multiplex serology of paraneoplastic antineuronal antibodies

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ABSTRACT

Paraneoplastic neurological syndromes (PNS) are devastating neurological disorders secondary to cancer, associated with onconeural autoantibodies. Such antibodies are directed against neuronal antigens aberrantly expressed by the tumor. The detection of onconeural antibodies in a patient is extremely important in diagnosing a neurological syndrome as paraneoplastic (70% is not yet known to have cancer) and in directing the search for the underlying neoplasm. At present six onconeural antibodies are considered 'well characterized' and recognize the antigens HuD, CDR62 (Yo), amphiphysin, CRMP-5 (CV2), NOVA-1 (Ri), and Ma2. The gold standard of detection is the characteristic immunohistochemical staining pattern on brain tissue sections combined with confirmation by immunoblotting using recombinant purified proteins. Since all six onconeural antibodies are usually analyzed simultaneously and objective cut-off values for these analyses are warranted, we developed a multiplex assay based on Luminex technology. Reaction of serial dilutions of six onconeural standard sera with microsphere-bound antigens showed lower limits of detection than with Western blotting. Using the six standard sera at a dilution of 1:200, the average within-run coefficient of variation (CV) was 4% (range 1.9–7.3%). The average between-run within-day CV was 5.1% (range 2.9–6.7%) while the average between-day CV was 8.1% (range 2.8–11.6%). The shelf-life of the antigen coupled microspheres was at least two months. The sensitivity of the multiplex assay ranged from 83% (Ri) to 100% (Yo, amphiphysin, CV2) and the specificity from 96% (CV2) to 100% (Ri). In conclusion, Luminex-based multiplex serology is highly reproducible with high sensitivity and specificity for the detection of onconeural antibodies. Conventional immunoblotting for diagnosis of onconeural antibodies in the setting of a routine laboratory may be replaced by this novel, robust technology.

1. Introduction

Paraneoplastic neurological syndromes (PNS) are defined as neurological syndromes that often associate with cancer (Posner, 1995). The discovery that many PNS are associated with antibodies that are directed against neural antigens expressed by the tumor (onconeural antibodies) has suggested that most PNS are immune-mediated (Darnell and Posner, 2003; de Beukelaar and Sillevis Smitt, 2006; Iorio and Lennon, 2012). The detection of onconeural antibodies is extremely useful in indicating the presence and type of an underlying tumor and in diagnosing a neurological syndrome as paraneoplastic (Graus et al., 2004). In 2004, an international panel of neurologists suggested two levels of evidence ('definite' and 'possible') to define a neurological syndrome as paraneoplastic, based on the presence or absence of cancer, and the definitions of 'well characterized' onconeural

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antibodies and 'classical' clinical syndrome (Graus et al., 1997). According to this set of criteria, in the absence of a detected cancer, only 'well characterized' onconeural antibodies can be used to classify the associated disorder as 'definite' PNS (Graus et al., 2004). The 'well characterized' onconeural antibodies are those for which there exist recognizable patterns on routine immunohistochemistry and for which immunoblotting on recombinant proteins must be used to confirm their specificity (Graus et al., 2004). Other criteria are (2) the number of cases associated with tumors; (3) the description of well characterized neurological syndromes associated with them; (4) the unambiguous identification of the antibodies among different studies; and (5) the frequency in patients without cancer. Based on these five criteria there are at present six 'well characterized' onconeural antibodies, directed against the antigens HuD, CDR62 (Yo), amphiphysin, CRMP-5 (CV2), NOVA-1 (Ri), and Ma2 (Graus et al., 2004).

Immunoblotting, required for confirmation of the specificity of the onconeural antibodies, was originally performed by Western blotting with the purified recombinant proteins as substrate (Manley et al., 1995). This technology has been more and more replaced by commercially available dot blots that combine the purified recombinant onconeural antigens produced either in *Escherichia Coli* (EUROIMMUN AG, Lübeck, Germany) or insect cells (Ravo Diagnostika, Freiburg, Germany) on a single strip, allowing the detection of all six different autoantibodies in a single assay (Willison et al., 2000; Jarius et al., 2008; Vigliani et al., 2011). More recently, an in vitro transcription–translation and immunoprecipitation technique has been described that is potentially more sensitive but also more laborious (Monstad et al., 2009; Storstein et al., 2010).

Since different onconeural antibodies can be associated with the same clinical findings (Shams'ili et al., 2003) and the same antibody can occur with different clinical syndromes (Graus et al., 2001; Sillevis Smitt et al., 2002), all six well characterized onconeural antibodies are generally tested simultaneously (Pittock et al., 2004). A relatively new 'multiplexing' technology uses color-coded polystyrene beads (xMAP, Luminex, Austin, TX) as a solid support for the target protein. The beads are filled with 2 fluorescent dyes in various ratios to produce potentially 100 different bead sets that can be distinguished by their internal color. The Luminex analyzer resembles a flow cytometer with two lasers. Excitation with the red laser allows the identification of the bead set while the green laser is used to excite the reporter fluorescent label bound to each bead (Waterboer et al., 2005). This technology provides quantitative results with predefined objective cut-off scores while immunohistochemistry, Western blotting and dot blots require visual analysis by an experienced observer.

In this paper we develop a multiplex assay based on Luminex xMAP technology to provide more objective criteria to separate background from low signal and compare results with Western blotting for the confirmation of six onconeural antibodies using sera from 119 patients with 'definite' PNS, 40 autoimmune control samples and 100 blood bank controls.

We found that multiplex serology is highly reproducible with a high sensitivity and specificity for the detection of onconeural antibodies. In addition, the shelf-life of beads with covalently linked onconeural antigens was at least 2 months.

2. Methods

2.1. Human sera

From our database we selected 119 patients with a diagnosis of 'definite' paraneoplastic neurological syndrome (Graus et al., 2004). All patients had onconeural antibodies directed against HuD (54), Yo (23), amphiphysin (25), CV2 (16), Ri (12) or Ma2 (11). 19 patients had multiple onconeural antibodies: anti-Hu and anti-CV2 in 9; anti-Hu and anti-amphiphysin in 5; anti-Hu and anti-Ri in 2; anti-Hu, anti-amphiphysin and anti-CV2 in 1; anti-Hu, anti-Yo and anti-amphiphysin in 1; and anti-amphiphysin and anti-CV2 in 1. IgG titers of the paraneoplastic antibodies were determined by endpoint titration on rat cerebellar sections using peroxidase immunohistochemistry as described before (Moll and Vecht, 1995). From all patients clinical information was retrieved from the treating neurologist.

A syndrome was classified as encephalomyelitis (EM) when 2 or more of the following structures were affected: the limbic system, basal ganglia, brainstem, medulla, basal ganglia, cerebellum, optic nerves and peripheral nerves (Graus et al., 2004). Syndromes with unifocal affection were classified according to the affected area, including limbic encephalitis (LE), brainstem encephalitis (BE), cerebellar ataxia (CA), opsoclonus-myoclonus (OM), sensory neuronopathy (SN), peripheral neuropathy (PNP), stiff-person syndrome (SPS) and Lambert–Eaton myasthenic syndrome (LEMS). Characteristics of patients and sera are depicted in Table 1. All sera were aliquoted and stored at −80 °C until use. The study was approved by the Erasmus MC institutional review board. Negative control samples included 100 blood bank controls while autoimmune control samples were collected from 20 patients with rheumatoid arthritis, 10 patients with SLE and 10 patients with Sjögren's syndrome.

2.2. Recombinant antigen production and purification

cDNAs encoding the antigens HuD (Manley et al., 1995), CDR62 (Yo) (Fathallah-Shaykh et al., 1991), CRMP-5 (CV2) (Yu et al., 2001), NOVA-1 (Ri) (Buckanovich et al., 1993) and Ma2 (Voltz et al., 1999) were ligated into pET-21b vector (Novagen/Merck4Biosciences, Darmstadt, Germany). This vector encodes a N-terminal T7 tag while a polyhistidine (6×His) region is added to the C-terminus. Amphiphysin (De Camilli et al., 1993) was ligated into the pTrcHisC vector (Novagen/Merck4Biosciences) that has 6×His at the N-terminus. All vectors were transfected and induced in *E. coli* strain BL21(DE3)pLysS (Novagen) as described before (Manley et al., 1995). After *E. coli* lysis, His-tag purification was performed on HisTrap columns (HisTrap FF, GE Healthcare, Diegem, Belgium) using a 20–500 mM imidazole gradient in a buffer containing 8 M urea, 0.1 M sodium phosphate and 0.3 M sodium chloride (pH 8.0). Elution fractions containing the recombinant proteins were analyzed for purity by SDS-PAGE and Colloidal Coomassie Blue staining (Novex®, Invitrogen, Carlsbad, CA). Subsequently, Western blot detection with an anti-T7 monoclonal antibody (Merck Millipore, Darmstadt, Germany) was performed. Elution fractions with the highest concentration of recombinant protein were subjected to further purification by ion exchange chromatography on HiTrapQ HP columns (GE Healthcare) using a buffered pH gradient of 12 to 2.5 in a buffer containing 8 M urea, 0.01 M