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Review article

Detection of autoantibodies in central nervous system inflammatory disorders: Clinical application of cell-based assays

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ABSTRACT

The identification of autoantibodies in central nervous system (CNS) inflammatory disorders improves diagnostic accuracy and the identification of patients with a relapsing disease. Usual methods to detect autoantibodies are usually divided into 3 categories: tissue-based assays, protein-based assays and cell-based assays (CBA). Tissue-based assays are commonly used for initial identification of autoantibodies based on staining patterns and co-localization. Once the antigen is known, autoantibodies can be detected using other antigen-specific methods based on recombinant proteins and CBA using transfected cells expressing the protein in their cell membranes. Compared to traditional methods using recombinant proteins such as ELISA and western blot, the CBA have advantage of detecting conformational sensitive antibodies using natively folded proteins in the cell membrane. This article reviews the utility of CBA into the clinical practice.

1. Autoantibodies as disease biomarkers

The acquired inflammatory central nervous system (CNS) diseases encompass a heterogeneous group that includes multiple sclerosis (MS), neuromyelitis optica spectrum disorders (NMOSD), acute disseminated encephalomyelitis (ADEM) and autoimmune encephalitis, among others (Hu and Lucchinetti, 2009). Sometimes, the initial clinical presentation may overlap (e.g. isolated optic neuritis, myelitis or solitary brain lesions), making precise diagnosis very difficult without surrogate markers.

In the recent years, autoantibodies have emerged as important tools as biomarkers for the diagnosis of an increasing number of diseases. In 2004, antibodies against aquaporin-4 (AQP4) water channels expressed in the end-foot processes of astrocytes were found in patients with NMOSD (Lennon et al., 2004, 2005). In 2007, antibodies against N-methyl-D-aspartate receptor (NMDA) were described in paraneoplastic encephalitis associated with ovarian teratoma (Dalmau et al., 2007, 2008), following an increasing number of autoimmune encephalitis associated with antibodies targeting neuronal surface antigens (Dalmau and Graus, 2018). More recently, antibody against myelin oligodendrocyte glycoprotein (MOG) have been associated with transverse myelitis (Ramanathan et al., 2014), optic neuritis (ON) (Chalmoukou et al., 2015; Nakajima et al., 2015), ADEM (O'Connor, 2007; Di Pauli and Berger, 2018), cortical encephalitis (Ogawa et al., 2017) and antibodies to aquaporin-4 (AQP4-Ab) seronegative NMOSD (Sato et al., 2014).

Identification of these autoantibodies as biomarkers can not only help us better understand the underlying mechanism of each disorder, but also improve diagnostic accuracy, stratify prognosis (including the risk of a relapsing disease) and even help developing new treatments targeting specific molecules. In this review, we will describe the main techniques for the detection of autoantibodies, specially the clinical application of cell-based assays in neurological diseases.

2. Identification of antibody in CNS inflammatory disorders

Over 40 different methods have been described to identify specific autoantibodies. Some of these tests are developed using in-house

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research protocols, others are already commercially available. In common, all of them require the binding of patient's antibody to animal tissue slices, recombinant proteins (Lennon et al., 2004) or transfected cells expressing the protein of interest in the membrane surface (Takahashi et al., 2006, 2007; Waters et al., 2008). These assays can be divided into three categories: tissue-based assays, protein-based assays and cell-based assays (CBA) (Wolburg et al., 2011).

2.1. Tissue-based assays

Tissue-based immunohistochemistry (TBI) assays use animal tissue sections (e.g. brain slices from mouse, rat or monkeys) with a few micrometers of thickness. These slices are chemically fixed and pre-treated to reduce unspecific binding. After these slices have been exposed to diluted patient serum samples, they are labelled with a secondary anti-human antibody conjugated to fluorophores or non-fluorescent peroxidase-conjugated polymer (conventional immunohistochemistry) (Jarius and Wildemann, 2013). The immunofluorescence techniques have advantage of allowing co-localization of different antibodies, allowing the confirmation that the human antibody is binding at the same antigen from a well-characterized monoclonal antibody produced against it (e.g. aquaporin-4, NMDA receptor Glu1 subunit).

TBI assays are commonly used for initial identification of autoantibodies based on staining patterns for unknown CNS antigens. The potential antigen can be identified by the mass-spectroscopy or co-localization using a monoclonal antibody using immunofluorescence with confocal laser microscopy. However, TBI results interpretation is subjective and requires an experienced observer to discriminate specific and unspecific binding patterns. In addition, sensitivity can be very low, and TBI protocols are time- and resource-consuming (Fazio et al., 2009; Chan et al., 2010).

2.2. Protein-based assays

2.2.1. Enzyme-Linked Immunosorbent Assay

Compared to other tests, ELISA is a simple method widely commercially available in routine clinical laboratories, as it does not require cutting-edge research laboratory resources. ELISA usually uses coated wells with recombinant proteins or linear peptides produced in transfected bacteria (e.g. *E. coli*) or baculovirus-infected insect cells (Engvall and Perlmann, 1972; Possee, 1997; Manole et al., 2018).

ELISA-based antibody assays usually have a low variability between laboratories and allows high-output testing of a large number of samples, since it can be fully-automated. However, it cannot quantify samples that exceed the reference range of its standard curve (Jarius et al., 2012).

The test has a sensitivity usually is higher than IHC, as the antigen concentration many times higher than it is expressed on normal tissue (Jarius et al., 2012). However, ELISA-based antibody assays may not discriminate unspecific binding patterns and have higher rate false-positive results. Moreover, ELISA is not suitable for the detection of conformational sensitive antibodies, which requires expression of human protein in the cellular membrane of eukaryotic cells. In the study by Pittock et al. (2014), it was seen that in a cohort of 1040 MS cases, 7 patients demonstrated AQP4-Ab positivity by CBA and ELISA assays. The frequency of a false-positive result for both assays was 0.5% (ELISA) and 0.1% (CBA) demonstrating the superior specificity of CBA (Pittock et al., 2014). Moreover, Di Pauli et al. (2011) found discordant results when comparing samples tested using CBA and ELISA for MOG-Ab. Unlike ELISA did not correlate with the disease groups (Di Pauli et al., 2011).

2.2.2. Western blot

Western Blot (WB) uses proteins extracted from a protein lysate from cells expressing the targeted protein. After the exposition to the patient sample, proteins are separated by gel electrophoresis based on their weight and electrical properties (Towbin et al., 1979; Jensen, 2012). The proteins are then transferred to a nitrocellulose or polyvinylidene fluoride (PVDF) membrane, blocked to reduce non-specific binding of proteins, incubated with a peroxidase-labeled antibody, and protein line blots can be visualized by chemiluminescence (Manole et al., 2018).

WB is useful to confirm the antibody binding to single or multiple proteins that may be present in a single sample. However, since WB requires protein denaturation, it is not suitable for conformational sensitive antibodies. Moreover, WB assays may have nonspecific protein binding, leading to a lower specificity compared to assays using natively folded membrane expressed proteins (Bass et al., 2017) .

2.2.3. Fluorescence immunoprecipitation assay

Fluorescence immunoprecipitation assay (FIPA) uses recombinant proteins based on the protein of interest tagged with a fluorescent protein produced in eukaryotic cells. The antigen-antibody complex is purified using protein-A or -G beads binding to immunoglobulins. The advantage of protein-based assays such as FIPA is to reduce the substrate complexity, eliminating non-target proteins that may provide unspecific binding seen on the TBI. FIPA may provide quantitative antibody titer results, as the fluorescence signal is proportional to the amount of antibodies binding to the antigen (Kim et al., 2012; Jarius and Wildemann, 2013). Waters et al. (2012) compared ELISA, flow cytometry, CBA, tissue-based indirect immunofluorescence and FIPA assays. Results showed that FIPA and tissue-based indirect immunofluorescence had the lowest sensitivities for detecting AQP4-Ab (48%−53%). Due to the low sensitivity, FIPA is not usually recommended for use in clinical practice (Waters et al., 2012). This may be attributable to changes in the protein conformation after the protein processing or due to the protein tagging. For some antigens, the loss of specific membrane arrays may also interfere in the sensitivity. FIPA is also an time- and resource-consuming technique, requiring multiple step protocols (Waters and Vincent, 2008; Jarius and Wildemann, 2013). However, FIPA demonstrates high reproducibility and it may be useful for monitoring circulating antibody levels as a surrogate marker of antibody production and disease activity.

2.2.4. Radioimmunoassay

Radioimmunoassay (RIPA) expresses a recombinant protein in eukaryotic cells and it is labeled with the radioactive 35S-methionine portion, incubated with the patient sample and subsequently with Protein A (Patrono and Peskar, 1987). The radioactivity quantification of the antigen-antibody bound is done throughout a scintillation conter, and indicated the amount of autoantibodies present in the patient serum. For AQP4-Ab, RIPA has a relatively low sensitivity, but provides high specificity compared to CBA using flow cytometry (Paul et al., 2007). The lower sensitivity may be partly explained by the protein production using a reticulocyte lysate-free cell-based in vitro transcription/translation system to express AQP4, which may have affected protein conformation (Fazio et al., 2009). Despite the clinical use for the detection of AChR antibodies, the low sensitivity, cost and time required for execution limited the use in the clinical practice for CNS antigens.

2.3. Cell-based assays

CBA uses eukaryotic cell lines (such as HEK293 and CHO) transfected with plasmids encoding the human sequence of the protein of interest. Mock-transfected cells are usually used as controls. Transfected cells used in CBA express high levels of natively folded protein in the cell membrane, being useful for the validation of new protein targets for autoantibodies (Ricken et al., 2018). Therefore, CBA usually have a higher sensitivity compared to TBI and protein-based assays (Kang et al., 2012; Höftberger et al., 2013; Jarius and Wildemann, 2013; Fryer et al., 2014) .

The pattern of the antigen-antibody binding can be verified using indirect immunofluorescence or by multiparametric flow-cytometry, but some antigens may not be suitable for both methodologies. Indirect immunofluorescence requires attention on binding patterns and a highly trained reader. Antibodies titers are usually provided in semi-quantitative serial dilutions using indirect immunofluorescence. Flow-cytometry requires a multi-channel flow-cytometer equipment and experienced user on cell gating and analysis protocol. Antibodies titers using flow-cytometry are usually calculated in two main formats - mean fluorescence intensity (MFI) between transfected and non-transfected cells or a ratio between the patient's sample MFI and a negative sample MFI (Chan et al., 2010; Kang et al., 2012; Amatoury et al., 2013; Ramberger et al., 2015; Gastaldi et al., 2018; Ricken et al., 2018).

Pre-fixed cells containing transfected cells may be produced on a commercial scale and stored for a relatively long period of time on low temperatures (Kang et al., 2012), thus, it can be performed in routine clinical laboratories using indirect immunofluorescence protocols. However, there may have some loss of sensitivity and specificity compared to the live CBA performed in research laboratories (Fryer et al., 2014; Waters et al., 2016). Commercially available kits are usually expensive, and their performance may depend on the experience with the interpretation of indirect immunofluorescence assays (Waters et al., 2016). Nevertheless, they do not require specialized environment for the transfection and maintenance of eukaryotic cell culture lines for live-CBA inaccessible for most research laboratories, especially in poor-resource or developing countries (Jarius and Wildemann, 2010).

3. Clinical applications of CBA in CNS inflammatory disorders

The main current clinical applications of CBA in CNS inflammatory disorders are summarized in the Table 1.

3.1. NMOSD

After the discovery of AQP4-Ab in 2004 in NMOSD patients using TBI assay (Lennon et al., 2004), an increasing number of AQP4-Ab positive patients were described having limited forms such as longitudinally extensive myelitis (LETM) or recurrent and/or bilateral ON, area postrema attacks with persistent (>48 h) hiccups, nausea and vomiting and those patients with associated diencephalic, brainstem and cerebral lesions or coexisting autoimmune systemic diseases. According to the international consensus diagnostic criteria for NMOSD published on 2015, the diagnosis of NMOSD for AQP4-Ab positive cases is possible if there is a suggestive attack with involvement 1 of 6 core locations (optic nerve, spinal cord, area postrema of the dorsal medulla, brainstem, diencephalon or cerebrum). In seronegative patients, two or more core locations must be affected, with at least one of the attacks in the optic nerve, spinal cord or the area postrema, and additional magnetic resonance imaging (MRI) criteria should be fulfilled (Wingerchuk et al., 2015).

The 2015 NMOSD diagnostic criteria recommends that AQP4-Ab should be tested with CBA. Waters et al. (2012) performed a multicenter study that compared the AQP4-Ab assays, concluding that the most sensitive and specific assays were those using CBA protocols (73–77%) (Waters et al., 2012). In general, CBA using AQP4 M23-isoform results in slightly higher sensitivity compared to assays using AQP4 M1-isoform due to the formation of AQP4 orthogonal array of particles. In addition, CBA using live transfected cells usually have lower background and higher sensitivity compared to pre-fixed cells.

3.2. MOG-associated disease

MOG-associated disease has recently emerged as a new subgroup of inflammatory CNS demyelinating disorder distinct from MS. Although antibodies to MOG were firstly described in patients with clinical isolated syndromes converting to definitive MS using a WB assay with recombinant MOG peptide (Reindl et al., 2013), this finding was not confirmed in other studies indicating limited clinical utility of this methodology (Kuhle et al., 2007). Further several studies demonstrated antibody negativity in different cohorts of adult MS patients (Tanaka and Tanaka, 2014; Waters et al., 2015; Jurynczyk et al., 2017; Wynford-Thomas et al., 2019).

The clinical spectrum of antibodies to myelin oligodendrocyte glycoprotein (MOG-Ab) positive patients clarified when conformational sensitive antibodies against MOG were evaluated using CBA. The first studies reported the association of MOG-Ab with ADEM in pediatric patients (O'Connor et al., 2007; Brilot et al., 2009; Mclaughlin et al., 2009). Moreover, patients with persistent MOG-Ab have a higher rate of relapsing disease among those that remained seropositive after an ADEM-like episode (Pröbstel et al., 2011).

Further studies related MOG-Ab seropositivity in NMOSD patients who were negative to AQP4-Ab (Reindl et al., 2013; Sato et al., 2014). Sato et al. (2014) described the clinical features of NMOSD MOG-Ab positive in comparison to AQP4-Ab positive patients (Sato et al., 2014). NMOSD MOG-Ab patients have no gender predominance, with high frequency of ON and with better functional recovery after an attack compared to AQP4-Ab positive patients. When myelitis was present it commonly involved lower spinal segments compared to AQP4-Ab patients. Other studies correlated MOG-Ab to recurrent and/or bilateral isolated ON (Ramanathan et al., 2014; Chalmoukou et al., 2015; Nakajima et al., 2015), isolated LETM (Cobo-Calvo et al., 2016), ON following an ADEM episode (Baumann et al., 2016) and encephalitis (Ogawa et al., 2017). Taken together, the identification of MOG-IgG using CBA allowed the clinical characterization of the patients encompassed under the term MONEM (MOG-IgG-associated ON, encephalitis - including ADEM-like lesions and cortical encephalitis, and myelitis) (dos Passos et al., 2018). As the clinical spectrum of MOG-associated disease expands, the clinical phenotypes associated with

Table 1
Clinical application of autoantibodies in patients using CBA.

Disease	Antigen	Diagnostic application	Prognostic application
NMOSD	AQP4	Yes	High-risk of relapsing disease; Antibody titers might be used in selected individual cases for disease monitoring
MONEM	MOG	Yes	Seroconvertion to negative status during the follow-up may indicate a lower risk of relapsing disease
Autoimmune encephalitis*	NMDA receptor LGI1 Caspr2 AM A receptor GABAb receptor GABAa receptor DPPX Dopamine-2 receptor IgLON5 P-Q-type VGCC mGLUR1 mGLUR5 Glycine receptor	Yes	Unknown

NMOSD: Neuromyelitis optica spectrum disorders, MONEM: MOG-associated optic neuritis, encephalitis and myelitis, AQP4: Aquaporin-4, MOG: Myelin oligodendrocyte glycoprotein, NMDA: N-methyl-D-aspartate, LGI1: Leucine-rich glioma inactivated 1, CASPR2: Contactin-associated protein-like 2, AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, GABA: Gamma-aminobutyric acid, DPPX: Dipeptidyl-peptidase-like protein-6, VGCC: voltage-gated calcium channel, mGLUR: Metabotropic glutamate receptor.

* This list refers only to cell-surface autoantigens with detected autoantibodies.

MOG-Ab are not limited to ADEM and aquaporin-4-IgG-negative NMOSD (dos Passos et al., 2018).

Even though significant advances have been accomplished to guarantee high specificity of detection of MOG-Ab using CBA, there is still some issues that need to be improved. Waters et al. (2015) evaluated the specificity of the secondary autoantibodies used in CBA assays and they found that use of anti-human IgG1 secondary antibodies was more specific than IgG that recognizes heavy and light chains. This was partially explained by cross-reactivity of IgG light chains secondary antibody with IgM. The use of secondary IgG1 anti-human antibody increased the assay accuracy and differentiated MOG-Ab positive from MS patients (sensitivity 24%, 95% confidence interval [CI] 9%–45%; specificity 100%, 95% CI 88%–100%) (Waters et al., 2015). Therefore, it is recommended to select secondary antibodies against human IgG without cross-reactivity to other immunoglobulin subclasses.

Although these findings still need to be confirmed by larger studies, apparently MONEM represents a new phenotype of CNS demyelinating disorder with MOG-Ab detected by CBA. For persistent MOG-Ab seropositive patients, MOG-Ab titers might be a prognostic tool that could be used to guide therapeutic decision-making. The follow-up of the titers of this autoantibody may help to identify the cases that remain seropositive and potentially might have a higher recurrence rate and could benefit from long-term immunosuppression (Oliveira et al., 2018).

3.3. Autoimmune encephalitis

Autoimmune encephalitis is a group of recently described diseases with autoantibodies directed to cell-surface neuronal antigens that are pathogenic by altering the function of neuronal receptors (Dalmau et al., 2017). Following the antibodies against N-methyl-D-aspartate positive cases, this emerging group of diseases has been characterized from the observation of groups of patients with similar phenotypes and patterns of IHC staining that allowed the identification of the specific antigens by mass-spectroscopy (Dalmau et al., 2017). Then, these antigens were validated in CBA with transfected cells.

The antibodies directed to these cell-surface antigens can only be detected if they are in their native conformation preserving protein folding (Graus et al., 2016). This is accomplished by CBA, IHC of fresh animal brain sections or immunocytochemistry of live neurons (Lai et al., 2009). CBA has been used in commercial kits of prefixed transfected cells in several centers. Even though this method has been proven to be specific, it may lack sensitivity to some antibodies (McCracken et al., 2017). Since, most patients with autoimmune encephalitis have antibodies relevant to their clinical phenotype in cerebrospinal fluid (CSF), testing both serum and cerebrospinal fluid is recommended. If the antibodies were detected only in serum or if the clinical phenotype is not compatible with the identified antibody using CBA, the results should be confirmed by brain IHC or culture of neurons. (Graus et al., 2016).

Overall, the detection of autoantibody in autoimmune encephalitis using CBA allows the definition of the diagnosis, helps to make differential diagnosis in atypical cases (Graus et al., 2016) and in research, and helps to define the spectrum of phenotypes associated with each autoantibody. Nevertheless, its prognosis value is still under investigation.

CBA has several advantages in the evaluation of autoimmune encephalitis. It is more available than other methods that preserve the native conformation of the antigens making possible to test a higher number of suspected cases. Moreover, some auto-Ab, such as glycine receptor antibody, are better detected using specific CBA than IHC (McCracken et al., 2017). However, it is important to evaluate the results of the CBA (as any other diagnostic method) according to the clinical hypothesis to define its interpretation and the necessity of further analysis.

4. Conclusions

Autoantibodies have revolutionized the diagnosis of inflammatory CNS disorders. NMOSD diagnostic criteria have incorporated the presence of AQP4-Ab as a major criterion, and MOG-associated disease have been described as a new potential disease entity. Moreover, an increased number of surface neuronal antigens have been described in autoimmune encephalitis. All these autoantibodies have high diagnostic application in the clinical practice with the development of CBA detecting conformational sensitive antibodies. Nevertheless, the prognostic value of most of them is still unclear and requires further research.

CRediT authorship contribution statement

Rachel Dias Molina: Writing - original draft, Writing - review & editing. **Lucas Piccoli Conzatti:** Writing - original draft, Writing - review & editing. **Ana Paula Bornes da Silva:** Writing - original draft, Writing - review & editing. **Leise Daniele Sckenal Goi:** Writing - original draft, Writing - review & editing. **Bruna Klein da Costa:** Writing - original draft, Writing - review & editing. **Denise Cantarelli Machado:** Writing - original draft, Writing - review & editing. **Douglas Kazutoshi Sato:** Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

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