Review Article

How to improve the sensitivity and specificity of cell-based assays in detecting autoantibodies in neuroimmune diseases

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Abstract: Autoantibodies are associated with neuroimmune diseases that affect the central and peripheral nervous systems. There are many methods for detecting autoantibodies, among which cell-based assay (CBA) is a relatively novel and important technology that is widely used. CBAs, as novel indirect immunofluorescence assays with known antigen epitopes, have revolutionized the identification of autoantibodies compared with the traditional immunoassays, such as the radioimmunoprecipitation and enzyme-linked immunosorbent assays, as well as the tissue-based assays (TBAs). However, the results of the same sample might exhibit obvious differences between different laboratories, or among repeated testing in the same laboratory, which influence the sensitivity and specificity in the diagnostic performance for a specific neuroimmune disease. In this paper, we review the establishment of CBA technology, and discuss potential interfering factors in CBA methods on its sensitivity and specificity for the autoantibodies associated with neuroimmune diseases.

Keywords: Neuroimmunology; antibody; cell-based assay (CBA); sensitivity; specificity

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Introduction

Pathogenetic autoantibodies tend to recognize cell surface antigens in their native conformation. Cell surface antigens account for most of the antibodies associated with neuroimmune diseases. Therefore, methods that detect antibodies against the antigens remaining in their native three-dimensional conformation might be more accurate than the denatured or linear configuration. Cell-based assays (CBA) detect antibodies against target antigens of three-dimensional conformations (1). The expert consensus and treatment guidelines for anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis (2) and anti-aquaporin-4 (AQP4)-positive neuromyelitis optica spectrum disease (NMOSD) (3) recommend CBAs as a preferred method. In this article, we seek to discuss the rationale for improving the sensitivity and specificity of CBAs and their diagnostic performance in neuroimmune diseases.

The principle of CBAs includes the constructing a plasmid DNA containing the genes of interest, transfecting the correct plasmid to a living eukaryotic cell, and then inducing protein expression. Any antibodies against the protein present in the serum or cerebrospinal fluid may be recognized using fluorescein-labeled anti-human IgG. Membrane proteins account for ~30% of all genomically expressed proteins. They are often expressed at low levels (4). Most synaptic proteins manifest a transmembrane structure with three-dimensional spatial conformation. Assembling the interested gene segment into a plasmid allows for translation of the target proteins in their native three-dimensional

conformations. Plasmid transfection allows protein overexpression proteins on the surface of the eukaryotic cells for autoantibody recognition. The eukaryotic expression system can simulate the natural state and make the target protein present a three-dimensional structure, which is easily recognized by autoantibodies *in vitro*.

Certain autoantibodies against membrane proteins, such as anti-NMDAR and anti-AQP4, are commercially available (5,6). Their manipulation and analysis are very convenient. However, for newly discovered autoantibodies, most institutions conduct in-house CBA tests (7-11) with various details in the testing system and with variation in the expression of the antigenic epitopes in repeated testing. Therefore, there are differences in the sensitivity, specificity, and accuracy among and even within the same institution. For this reason, detection schemes for autoantibodies should be designed systematically. The sensitivity and specificity of CBA procedures must be established in-house and then adapted to clinical performance. This article will elaborate on several aspects.

Establishment of a CBA detection system

Establishing a complete CBA detection system involves selection of the target protein isotypes, modification of gene structures for more stable and native protein structure, vector construction, cell transfection, fluorescein-labeled anti-human IgG selection, and data interpretation (12). Each of these steps has a varying degree of influence on the detection system and uniquely influences test accuracy.

Protein isotypes

Selection of the target protein isotype associated with neuroimmune diseases is the core of the CBA system. If newly reported or previously unreported target proteins with multiple gene transcripts are to be identified, the transcript containing the full length of the amino acid sequence should be selected first, but this is not absolute. Other homologous transcripts should be studied similarly. The optimal target protein isoform transcript is screened out according to the sensitivity and specificity of antigen-antibody reactions for cloning and plasmid construction.

The transcripts which have the best binding to the pathogenic antibody are selected from plasmid construction databases. For instance, there are eight transcripts in the database for anti-glial fibrillary acidic protein (GFAP) astrocyte disease. The GFAP IgG binds to GFAP-α to produce an antigen-antibody complex. Only a small proportion of the IgG binds to both GFAP-ε and GFAP-α. It has been found that the sensitivity of CBA employing GFAP-α is 100% in 102 cerebrospinal fluid (CSF) samples. GFAP-IgG positive patients, which is significantly higher than those employing GFAP-ε (81%) and κ (54%). Serum and/or CSF IgG of 22/451 (5%) patients bound to human GFAP, of which 22/22 bound to GFAPα, 14/22 to both GFAP-α and GFAP-δ and none to the GFAP-δ isoform only (13,14). However, in 19 Chinese patients who underwent CSF testing, we found fourteen cases that were GFAP-α-IgG positive, and five cases who were only GFAP-ε-IgG positive (15). Thus, the target protein is selected from transcript 1 of GFAP-α.

AQP4 has two main isoforms with different N-terminal lengths, the shorter AQP4-M23 and longer AQP4-M1. Approximately 90% of NMO patients and more than half of NMOSD patients are positive for AQP4 autoantibodies, and their binding antigen is AQP4-expressing M23 (16). It has been found that optimizing the CBA method by co-transfection of AQP4-M23 and EGFP in HEK293 was more sensitive than that of AQP4-M1. It is possible that AQP4-M23 can form orthogonal arrays of particles (OAPs) independently. Compared with the binding ability of AQP4 tetramer, anti-AQP4-Ab has higher binding ability to OAPs. There are potential conformational sites recognized by AQP4-Ab in OAPs (17,18), whereas M1 itself forms little or no OAPs, and thus AQP4-M23 is higher in specificity and sensitivity than M1. Test sensitivity was found 97.5% in an AQP4-M23 based assay, 27.5% in an AQP4-M1 based assay, and 0% in an AQP4 $M1^{M23I}$ (a AQP4-M1 mutant)I based assay, indicating the AQP4-M23 expressing vector is the best candidate for high sensitivity tests.

Vector construction

The choice of eukaryotic expression vector greatly influences the CBA ability to detect autoantibodies in neuroimmune diseases. Eukaryotic expression vectors contain various promoter elements, multiple cloning sites (MCS), antibiotic resistance genes and tags, etc. Tags can be used to locate and purify proteins. Several N- or C-terminus vectors have different labels, such as green fluorescent protein (GFP), red fluorescent protein (mCherry), etc. Adding a reporter gene makes it easier to calculate the transfection efficiency and interpret and analyze the results. Common examples include the pCMV-Tag2B-expressing HMGCR (19), pCMV-Myc-HMGCR plasmid

expressing the C-terminus of HMGCR (20), pIRES2-Ds red2 vector plasmid expressing full-length MOG (21), and expression vector pcDNA3.1/Hygro(-) (22) expressing the NMDAR subunit. All of the expression vectors contain the characteristics of the above eukaryotic expression vectors.

The pCMV6-AC-GFP (Origene) eukaryotic expression vector has recently been studied and applied in our laboratory. Compared with other plasmid, the C-terminus has a TurboGFP tag driven by the CMV promoter of the strong expression of GFP. And its MCS includes seven restriction sites which provide more chances to clone foreign gene. When a foreign gene is inserted and fused to GFP, the efficiency of the transfected cells is assessed from the fluorescence. Strong expression of GFP is more helpful to observe the transfection effect, which may influence the detection sensitivity. At the same time, the fluorescence intensity of GFP can indicate the expression level of foreign protein because of the fusion expression. Then the double colors of green-red fluorescent CBA detection system that eliminates nonspecific fluorescent signal interference and enhances detection specificity can be established on this basis. Pisani *et al.* (23) found that if GFP is placed at the N-terminal, it affects sensitivity. Therefore, selecting the optimal elements (position, expression of GFP tag) to construct the expression vector is important.

Cell transfection

Cell transfection approaches are divided into transient and stable depending on the experimental objectives. The cell lines used in transient transfection need to be transfected in each batch which would decrease antigen reproductivity and testing repeatability. Nevertheless, transient transfection has several advantages, including being a simple procedure, high expression efficiency, good safety, short period, and low cost. Therefore, the technique is convenient for clinical investigation on the role of autoantibodies in the diseases. However, because of the proportionality in the binding process between the antigen and antibody, low-titer antibodies may be undetected by transient transfection methods, leading to false negatives and decreased sensitivity. As a rule, the efficiency of transient transfection must be ≥60% to achieve the required sensitivity.

Stable transfection can be established on the basis of transient transfection. Stable transfection requires antibiotic resistance gene screening to obtain stable cell lines, which will continue to express the target protein as the cells proliferation. With the cell proliferation, plasmids are randomly distributed to the daughter cells and diluted with every division, until they eventually disappear. Stable transfection is suitable for long-term pharmacological studies, genetic regulatory mechanisms, large-scale protein synthesis, as well as in laboratory diagnosis of specific diseases. It requires a long process period which increases both input costs and uncertainty. The target proteins express in each cell in a stable transfection status. In addition, stable transfection can improve the sensitivity in detecting autoantibodies using the CBA method.

Transfection efficiency directly influences the detection results. Factors affecting transfection efficiency include technique, reagents, cell types, cell status, DNA quality, and vector selection.

The most common plasmid transfection methods used in CBAs are liposomes and viral infection. The most effective transfection method of viral infection involves the use of adeno-associated virus vectors and lentiviral vectors. However, they have disadvantages, such as long experiment periods, complex procedures, and potential safety issues. Lipofection reagents have high transfection efficiency, low mortality, simplicity, and low cytotoxicity (24). For example, transfection mediated with liposomes is used to detect anti-AQP4, anti-NF155, anti-NF186, anti-MOG, anti-GFAP, and anti-NMDAR antibodies (25-29). Some researchers have also adopted cationic polymer gene transfection reagents, such as polyethyleneimine (PEI). Detection of anti-MuSK antibodies using this method has also been established (30). Compared with liposomes, PEI has the advantages of low cost, no pollution, simple use and high transfection efficiency *in vivo*. Liposomes are widely used in cell transfection. Different liposomes (turbo 8.0, Lipofectamine^{TM}2000, Lipofectamine^{TM}3000) from different manufacturers have different transfection efficiency, influencing sensitivity and specificity to some extent. Transfection efficiency is also associated with transfected cell types and status. HEK293T (31), HEK293A (32), TE671 (33), cos-7, and CHO (34) are used as tools for expressing the target antigens of autoantibodies in neuroimmune diseases. HEK293 is the most common cell line widely used to detect autoantibodies in neuroimmune diseases (30,32). Transient transfection of HEK293T cells is a convenient way of overexpressing proteins. HEK293 cells adhere and grow well within a few generations. However, after several generations, the cells gather easily, and their adhesion performance is weak. These deficiencies may influence subsequent experiments and immunofluorescence detection. To solve these problem, newly purchased cell strains should be frozen in large

quantities to avoid serial passage and cell slides can be pretreated with polylysine or laminin to enhance adhesion. Poor cell status also reduces transfection efficiency. Generally, the preferred number of transfected cells and confluence are <50 generations (logarithmic growth phase, during S or G2/M phase) and 50–60%, respectively. Cell density also has some effect on the transfection efficiency, which should depend on the instructions for the transfection reagent. To obtain the best transfection efficiency (≥60%), transfection should be conducted under the optimal physiological state of cells. If the cell density is less than 60%, it will affect the cell state and the transfection efficiency, thus reduce the sensitivity.

Cell transfection efficiency is also dependent on plasmid quality. Commonly used transfection techniques are all based on the principle of charge attraction. Low plasmid concentrations and poor plasmid purity greatly reduce transfection efficiency. According to the manuscript of transfection agent in Invigentech, the target plasmid concentration and purity should be up to 2 $\mu g \cdot \mu L^{-1}$ and 1.8–2.0, respectively.

Western blot verification

Western blotting (WB) is critical to ensure the specificity of the CBA detection system. The lysates of untransfected cells, cells transfected with empty plasmid-transfected, and plasmids expressing the target gene should be prepared. After SDS-PAGE, the target protein is transferred to a polyvinylidene difluoride membrane. The fusion-expressing protein band is labeled with an anti-tag antibody. The position of the band is noted to determine whether the target protein is expressed and the molecular weight is correct. A monoclonal antibody is then used to label a target protein and verify its biological activity. Examples include the construction of a CBA detection system against AQP4 (35) and NMDAR (31) antibodies.

Only when the molecular weight of the genetically engineered protein is correct and the protein expressed has specific immunoreactivity should it be used in the next detection step.

Selection of fluorescently labeled anti-human antibodies

CBA technology using cells as the carrier of antigens is equivalent to solid phase which carry the antigens in the determination of antibodies. Various Ig subclasses or subtypes can be detected using the corresponding fluorescently labeled anti-human antibodies (secondary antibodies). This process is essential for detecting and identifying pathogenic autoantibodies in neuroimmune diseases.

The use of other Ig subclasses in addition to IgG will add diagnostic sensitivity. Researchers (36,37) have used an established in-house CBA with HEK293 cells transfected with a full-length MOG fused with EGFP at the C-terminal. The serum samples of 120 patients with MOG-IgG and 114 patients without detecting MOG-IgG were assessed to determine the frequency and relevance of IgM and IgA antibodies against MOG. IgM or IgA MOG antibodies were identified in 19% of patients with MOG-IgG and in 1.7% without MOG-IgG. Hence, missed diagnoses would ensue following MOG-IgG-only detection. To summarize, identifying different Ig subclasses can improve the positive rate.

However, secondary antibodies could also affect the sensitivity and specificity of CBAs. One study on CBAs (30) found that the serum from 18% of seronegative myasthenia gravis, 11% of healthy controls, and 19% of NMDAR encephalitis patients showed positive binding to MuSK-transfected cells using anti-human IgG (H+L) antibody, indicating unspecific binding. When anti-human IgG (H+L) was replaced with IgG Fc(γ)-specific secondary antibody, MuSK-Abs were detected in 68/69 (99%) of definite MuSK-MG patients, in 0/35 of healthy control individuals, in 0/16 of patients with NMDAR-Ab, and 14/169 (8%) of seronegative MG patients, providing both higher sensitivity and specificity. This is because the anti-human IgG (H+L) binds not only to IgG heavy chains but also cross-reacts with different Igs via the light chains that are not Ig class specific. Similar problems exist in MOG antibodies detection, which may be due to the cross-reactivity in the extracellular immunoglobulin-like domains and/or glycosylation state of MOG proteins (38). Therefore, it is suggested that caution be used regarding anti-IgG (H+L) for detecting IgG binding to similar antigens.

Data interpretation

Test equipment performance greatly impacts CBA sensitivity, specificity, and accuracy. The most commonly used equipment comprises ordinary fluorescence and laser scanning confocal fluorescence microscopes. Both are used for the microscopic observation of living cells in CBAs.

In the case of an elevated autoantibody concentration, live cells display high-intensity fluorescent signals.

Therefore, the results may be readily evaluated using a common fluorescence microscope. However, if the autoantibody concentration is low, the live cells produce weak fluorescent signals. In this situation, more than one trained laboratory technician should observe and analyze the same fluorescent film (39). The results may also be interpreted by laser scanning confocal fluorescence microscopy (LSCM) (40).

CBA results are commonly reported as antibody-positive cell counts or fluorescence intensity. Strong fluorescent signals generally indicate a high affinity of autoantibodies to target antigens, except when the transient transfection efficiency is very low (<20%). In the in-house testing, the fluorescent signals can be quantified at a given dilution. In the positive cell counting method, five different microscope fields are selected at high magnification (200×), and the positive cells are counted. Data are reported as means ± standard deviation (SD). The fluorescence intensity quantification method determines the number of fluorescence-reactive cells and the fluorescence intensity in five different high-power fields using laser scanning confocal fluorescence microscopy. The results are relatively easy for fluorescence quantification. Commercially available kits usually report CBA results by the antibody titer (the largest dilution in which the tested fluorescent signals is above a pre-specified detecting level).

The aforementioned CBA data interpretation methods have their unique advantages and characteristics. The first two reporting models are subjective, whereas the third is relatively objective. When the findings are ambiguous, laboratories may resort to other test methods, such as ELISA or WB, for validation or confirmation. The standard for determining a positive cell is the average fluorescence intensity of the negative serum ± 2 SD. If the number of positive cells is greater than ten per visual field (200×) or the titer is greater than 1:10, it can be determined as positive. In this way, a relatively quantitative interpretation of the CBA data is established to mitigate the impacts of human error and bias. Moreover, laser scanning confocal microscopy has superior fluorescent signal detection sensitivity as it can scan cells in multiple layers and capture fluorescence at various depths on the cell surface.

Comparison of IF-CBA and FACS

The most commonly used CBAs comprise IF-CBA (indirect immunofluorescence) and flow cytometry (FACS). Both IF-CBA and FACS use living plasmid-transfected cells and fluorescent signals to detect autoantibodies. De Vidi *et al.* (41) used IF-CBA, FACS, and IIF techniques to detect the AQP4 antibody and compared the sensitivity among the three methods. They carried out serial dilution of an AQP4 antibody-positive serum and compared the ability of the three methods to detect low antibody concentration. The results indicated that IF-CBA and FACS could detect the antibody at dilution 1/51,200, whereas staining by the tissue-based assay (TBA) disappeared at 1/3,200, indicating that the IF-CBA and FACS have a higher sensitivity. In addition, IF-CBA can be semi-quantitative by antibody titers, while FACS can be quantitative by mean fluorescence intensity (MFI). Therefore, FACS is presumed as more sensitive. However, Ramberger *et al.* (42) compared data generated by commercially available IF-CBA and FACS and found that the latter showed lower sensitivity and higher variability. Thus, IF-CBA is a more reliable detection method. In MG studies, researchers consider that CBA is highly sensitive and has unique clinical value in diagnosing seronegative myasthenia gravis using RIPA (43,44).

Summary on the sensitivity and specificity of CBA

Compared with other testing techniques, CBAs have obvious advantages, including the use of native target antigens, high signal, reconstruction of a visible three-dimensional image, and target antigen localization. Therefore, it has been adopted as a new clinical laboratory detection method. It is particularly advantageous for detecting autoantibodies against membrane antigens. However, obtaining accurate quantitative data with CBA technology may be difficult.

Although CBAs have been widely used in the laboratory diagnosis of neuroimmune diseases, there are currently no definite criteria for evaluating their sensitivity and specificity. In general, CBA sensitivity and specificity can be improved mainly through the following aspects: (I) choosing the optimal expression plasmid, (II) the number of cells expressing target antigen protein is not less than 60%, (III) exploring the best sample dilutions, and (IV) using Fc (γ) as the fluorescent secondary antibody.

Tyramide signal amplification (TSA) technology is recently used to enhance the immunohistochemical detection of proteins, nucleic acids, or other molecules *in situ*, which was proved extremely high sensitivity and detect very low level of target antigens. In the CBA-TSA, HRP-labeled secondary antibody and fluorescently labeled tyramine

are added successively. Tyramine can be activated by HRP and deposited *in situ* to achieve signal amplification (45). Therefore, weakly positive antibodies can be detected using TSA. Its applications in CBAs may significantly improve the sensitivity or discovery of novel antibodies in neuroimmune diseases.

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Footnote

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