

# Tissue Immunofluorescence Confirmation of CNS Autoantibodies Identified by Immunoblot or Cell-Based Assay

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## Abstract

While many antigenic targets of autoimmune CNS disease have been identified, antibodies to each are detected only rarely. Therefore, reference laboratories often screen serum for CNS autoantibodies by immunofluorescence assay (IFA), using whole brain tissue, before reflexing positive samples to more specific assays for confirmatory testing. These include immunoblot assays (IBAs) and cell-based immunofluorescence assays (CBAs), which are commonly used to identify autoantibodies to intracellular and membrane-embedded antigens, respectively. However, it remains to be determined how sensitive tissue IFA screening is for the initial detection of CNS autoantibodies, compared to these alternative techniques. This study compared the sensitivity of brain tissue IFAs, IBAs, and CBAs, for the detection of CNS autoantibodies. All comprehensive paraneoplastic neurological syndrome panel results released from our laboratory over a two-year period (2017-2019) were reviewed. This panel was chosen for analyses because of its design; the comprehensive PNS panel does not incorporate a reflex testing algorithm and brain tissue IFAs, IBAs, and CBAs, are run in parallel and independently on all serum samples. The tissue IFA detected 47 of 56 (83.9%) autoantibodies identified by IBA but only 5 of 14 (35.7%) autoantibodies identified by CBA. Results of these retrospective analyses suggest that tissue IFAs of serum are more than twice as likely to confirm the presence of IBA identified autoantibodies to intracellular proteins than CBA identified autoantibodies to membrane embedded protein targets. These findings have important implications for the selection of methods to initially screen serum samples for CNS autoantibodies in clinical practice.

**Keywords:** Paraneoplastic Syndrome; CNS Autoantibody Detection; Tissue Immunofluorescence Assay; Immunoblot Assay; Cell based Assay

**List of Abbreviations:** PNS: Paraneoplastic Syndrome; IFA - Immunofluorescence assay; CBA - Cell based assay; IBA - Immunoblot assay

## Introduction

Over the last couple of decades, autoimmune diseases of the CNS have become a major focus of investigation within the field of neurology. Armed with an expanding arsenal of commercially available diagnostic tests, the medical community has come to appreciate that neuronal autoantibodies may directly cause or serve as biomarkers for a far wider range of CNS diseases than previously realized [1,2]. Neuronal autoantibody production is often associated with malignancies in non-neuronal tissues but not always. Current thinking is that these malignant cells have the potential to both express and break tolerance to neuronal antigens, thereby eliciting an autoimmune reaction within neuronal tissues, including the brain, optic nerves, spinal cord, peripheral nerves, neuromuscular junction, and muscle [3]. This likely explains why most CNS specific autoantibodies are more readily detected in serum than in cerebral spinal fluid.

Interestingly, most malignancy associated CNS autoantibodies target intracellular proteins. These autoantibodies are probably not directly pathogenic, but instead serve as surrogate markers of underlying T-cell mediated CNS pathology [3]. Neuronal cell surface proteins associated with neurotransmitter receptors and ion channels represent a separate class of antigenic targets whose association with cancer is much weaker. Moreover, these autoantibodies are thought to be directly pathogenic and contribute to neurological symptoms and disease [1-3]. These views are supported by several observations. For example, CNS diseases associated with intracellular autoantibodies tend to be more responsive to therapies that broadly target cellular immunity, such as high dose corticosteroids, while clinical responses are often partial rather than complete [1, 2]. In contrast, diseases associated with autoantibodies to neuronal cell surface proteins are more responsive to plasmapheresis and intravenous immunoglobulin therapy, and neurological recovery is often rapid, complete, and sustained [1, 2].

While many intracellular and membrane targets of CNS disease have been identified, autoantibodies to each are detected only rarely [4, 5], and there are likely additional antigenic targets that have yet to be identified [4, 5]. Therefore, reference laboratories often use immunofluorescence assays (IFAs) that include cerebellum, hippocampus, and potentially other tissues for the initial detection of CNS autoantibodies. Confirmatory testing is then done with assays designed to identify autoantibodies to individual target proteins. Of the techniques available, immunoblot assays (IBAs) are considered preferable for detecting autoantibodies to most intracellular targets, while detection of autoantibodies to membrane embedded proteins is generally better achieved with cell-based assays (CBAs) that utilize HEK293 cells transfected to express the protein of interest in its native conformation [4, 5]. Tissue IFAs have historically been considered very sensitive for the detection of autoantibodies. However, little is known about the sensitivity of tissue IFAs relative to IBAs and CBAs for the initial detection of autoantibodies to intracellular and membrane embedded proteins, respectively. To address this issue, we conducted retrospective analyses of tissue IFA, IBA, and CBA results from patient serum samples sent to our laboratory for concurrent CNS autoantibody testing by all three methods.

## Methods

### Study Design

To determine the frequency with which IBA and CBA identified CNS autoantibodies are also detected by tissue IFA, we reviewed almost 2300 consecutive comprehensive paraneoplastic neurological syndrome (PNS) panel results released from our laboratory over a two-year period (2017-2019). Results from this panel were selected because of the comprehensive PNS panel's design; a reflex testing algorithm is not used and tissue IFA, IBAs, and CBAs were run in parallel on each serum sample. Therefore, each tissue IFA result was obtained by lab technicians unbiased by prior knowledge of the IBA and CBA results. Only IBA and CBA identified autoantibodies and their corresponding tissue IFA results were considered in these analyses.

### Laboratory Techniques

Immunoblot assays were conducted with the Euroimmun (Lubeck Germany) Euroline PNS 12 antigen profile (DL 1111-1601-7 G), which includes individual immunoblots for Hu, Yo, Ri, CV2/collapsin response mediator protein 5 (CRMP5), Ma2, anti-gli-

al nuclear antibody (AGNA)/SOX1, Zic4, glutamic acid decarboxylase 65 (GAD65), amphiphysin, delta/notch-like epidermal growth factor-related receptor (DNER)/Tr, titin, and recoverin. Assays were conducted with the Euroblot One system, following manufacturer recommendations. Results were reported in signal intensity (SI) units and those  $\geq 11$  SI units were considered positive. Cell-based assays were conducted with the Euroimmun encephalitis mosaic panel 1 (FA 112d-1), which includes HEK293 cells transfected with  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor 1 and 2 (AMPA1, AMPA2), N-methyl-D-aspartate receptor (NMDAR1), contactin-associated protein 2 (CASPR2), gamma-aminobutyric acid B receptor (GABA<sub>B</sub>R) and leucine-rich glioma-inactivated protein 1 (LGI-1) according to the manufacturer’s instructions. Each CBA was initially run at a 1:10 dilution with serial 1:2 dilutions until endpoint. Slides were interpreted with the EuroPattern imaging system. Tissue IFAs were conducted with custom-designed Euroimmun slides that contain 9 tissues, including rat hippocampus, primate cerebellum, basal ganglia, sural nerve, intestine (plexus myentericus), kidney, pancreas, testis, and Hep2 cells. Sera were processed according to manufacturer guidelines and images were interpreted with the Euro Pattern imaging system at a 1:40 dilution. Although titin and recoverin antigens are included on the immunoblot strips used for these studies, tissues appropriate for autoantibody detection were not included on the tissue IFA slide, and therefore, these analytes were not included in the analyses.

### Statistics

The non-parametric Wilcoxon rank sum test was used to determine if the signal strength of IBA and CBA detected (SI and titer, respectively) CNS autoantibodies influenced the likelihood that they would also be detected by tissue IFA. A chi-square test was used to compare the relative sensitivity of the tissue IFA for the detection of IBA and CBA identified CNS autoantibodies. *P-values*  $\leq 0.05$  were considered statistically significant. Statistical analyses were conducted using R version 3.6.3, R Foundation for Statistical Computing (<https://www.R-project.org>).

### Results

Fifty-four serum samples with positive IBA results were identified that met study criteria. Two of the samples had autoantibodies to two target CNS proteins, while the rest had a single IBA detected CNS autoantibody. Of these 56 IBA identified CNS autoantibodies, 47 (83.9%) were also positive by tissue IFA (Table 1). The mean IBA signal intensities (SIs) of tissue IFA positive and negative samples were 36.7 SI (S.D.  $\pm$  37.1) and 22.3 (S.D.  $\pm$  6.4), respectively (*P* = 0.6). Although this difference was not statistically significant, it is noteworthy that 78% (33/42) of IBA detected CNS autoantibodies with a relatively weak signal (SI = 11-31) were confirmed by tissue IFA, while 100% (14/14) of IBA detected CNS autoantibodies with a stronger signal (SI = 32–175) were confirmed.

Analyte	Detected By Tissue IFA		Not Detected By Tissue IFA		Assay Agreement
	N	Sample SI (Mean)	N	Sample SI (Mean)	
Hu	3	18, 48, 114 (60)	0		3/3 (100%)
Yo	8	12,14,16,30,33,44,90,96 (41.9)	1	31 (31)	8/9 (88.9%)
Ri	0		0		0/0 (NA)
CV2/CRMP5	1	17 (17)	2	14, 25 (19.5)	1/3 (33.3%)
Ma2	3	12, 12, 50 (24.7)	0		3/3 (100%)
AGNA/SOX1	14	12,13,14,14,14,15,17,20,20,21,25,25,30,30 (19.3)	2	12,20 (16)	14/16 (87.5%)
Zic4	3	11,12,16 (13)	3	17,22,27 (22)	3/6 (50%)
GAD65	9	14,16,17,21,28,32,111,128,175 (60.2)	0		9/9 (100%)
Amphiphysin	5	12,13,26,60,97 (41.6)	1	15 (15)	5/6 (83.3%)
Tr/DNER	1	58 (58)	0		1/1 (100%)
<b>Total</b>	<b>47</b>	<b>(36.7; S.D. = <math>\pm</math> 37.1)</b>	<b>9</b>	<b>(20.3; S.D. = <math>\pm</math> 6.4) *</b>	<b>47/56 (83.9%)</b>

\*P-Value = 0.6 (not significant)

**Table 1:** Tissue IFA detection of IBA identified CNS autoantibodies

Analyte	Detected By Tissue IFA		Not Detected By Tissue IFA		Assay
	N	Titer of each sample (Mean)	N	Titer of each sample (Mean)	Agreement
AMPA1	0		0		0/0 (NA)
AMPA2	0		0		0/0 (NA)
NMDAR	1	1:160 ( <b>1:160</b> )	2	1:10, 1:80 ( <b>1:45</b> )	1/3 (33.3%)
CASPR 2	2	1:160, 1:160 ( <b>1:160</b> )	4	1:10, 1:20, 1:40, 1:80 ( <b>1:38</b> )	2/6 (33.3%)
GABA <sub>B</sub> R	1	1:80 ( <b>1:80</b> )	1	1:20 ( <b>1:20</b> )	1/2 (50%)
LGI-1	1	1:20( <b>1:20</b> )	2	1:10, 1:20 ( <b>1:15</b> )	1/3 (33.3%)
<b>Total</b>	<b>5</b>	<b>(1:116; S.D. = ± 63.9)</b>	<b>9</b>	<b>(1:32.2; S.D. = ± 28.6)*</b>	<b>5/14 (35.7%)</b>

\*P-Value = 0.02 (significant)

**Table 2:** Tissue IFA detection of CBA identified CNS autoantibodies

Fourteen serum samples with CBA detected CNS autoantibodies were identified that met study criteria. Each of them was positive for a single autoantibody and none had an additional IBA detected CNS autoantibody. Of these 14 autoantibodies, only 5 were also detected by tissue IFA (35.7%). The mean CBA titer of tissue IFA confirmed CNS autoantibodies was 1:116 (S.D.  $\pm$  1:63.9) compared to 1:32.2 (S.D.  $\pm$  1:28.6;  $P = 0.02$ ) for tissue IFA unconfirmed CNS autoantibodies. However, the tissue IFA still only detected 57.1% (4/7) of CBA identified CNS autoantibodies with a titer  $\geq$  1:40 and 14.2% (1/7) of autoantibodies with low titers (1:10 and 1:20). Finally, the tissue IFA was significantly less sensitive in detecting CNS autoantibodies identified by CBA than those identified by IBA (35.7% vs 84%, respectively;  $P = 0.001$ ).

## Discussion

Many laboratories currently use tissue IFA screening to detect a wide range of CNS autoantibodies in serum. Subsequently, IBAs and CBAs are used to confirm the tissue IFA result and identify the precise antigen specificity of the autoantibody. However, one limitation of this approach is that clinically relevant CNS autoantibodies are missed if not initially detected by tissue IFA. To address this concern, patient results released from our laboratory were reviewed to determine how often IFA results confirmed the presence of CNS autoantibodies identified by IBA or CBA. The tissue IFA was found to detect 83.9% of the CNS autoantibodies identified by IBA but only 35.7% of those identified by CBA. In consideration of these findings, the specific antigenic targets of IBAs used for these studies were all intracellular proteins, while CBAs were used exclusively for the detection of autoantibodies to cell surface receptors and other membrane embedded proteins. These considerations strongly suggest that the tissue IFA is more sensitive for the detection of autoantibodies to intracellular proteins than to membrane embedded proteins.

This retrospective study has two main limitations worthy of further comment. First, despite reviewing results for almost 2300 consecutive serum samples, some antigen specific autoantibodies identifiable by IBA and CBA were identified only rarely or not at all (Table 1 and 2). Therefore, it is possible that tissue IFA detection rates for select IBA and CBA identified autoantibodies might be significantly different from the overall IBA and CBA detection rates presented in these tables. Second, while patient serum samples sent to our laboratory for CNS autoantibody testing, patient diagnoses were not available. Therefore, findings reported herein do not directly address the diagnostic accuracy of tissue, IFA, IBA, or CBA results, only the likelihood that a CNS autoantibody identified by IBA or CBA will also be detected by tissue IFA if used to initially screen for these CNS autoantibodies. However, a major strength of these investigations is that serum samples used for these inter-assay comparison studies were not preselected because of prior CNS autoantibody detection by any of the three techniques being compared.

While not available for studies described herein, other investigators have used clinically characterized serum samples to compare the performance of the tissue IFA in detecting CNS autoantibodies initially identified by IBA, and their clinical significance [6]. When the clinical histories of 58 patients with an IBA detected and tissue IFA confirmed CNS autoantibody were reviewed, all had a neurological syndrome consistent with the autoantibody identified. Moreover, 50 of the 58 patients also had a malignancy

known to be associated with the identified CNS autoantibody. In contrast, IBA detected autoantibodies with a low signal intensity were often not confirmed by tissue IFA, were far less likely to be the cause of the patient's neurological symptoms and were rarely associated with malignancy [6]. Considered in conjunction with our own results, the findings of this study suggest that clinically relevant CNS autoantibodies to intracellular targets are unlikely to be missed by tissue IFA screening of serum, especially if present at higher concentrations, while those that are missed, are far less likely to be of clinical consequence.

Consistent with our findings, published investigations have concluded that CBAs offer significantly better sensitivity than tissue IFAs for the detection of serum autoantibodies to select membrane embedded proteins, including Aquaporin-4, myelin oligodendrocyte glycoprotein, and NMDAR [7-11]. Moreover, unlike IBA detected autoantibodies of low signal intensity, which often are not clinically relevant [6], a vast majority of CNS autoantibodies detected by CBA have clinical relevance [7-11]. Considered in this context, our findings suggest that potentially pathogenic CNS autoantibodies with membrane protein specificities, which are readily detected by CBA, are often missed by tissue IFA.

In summary, retrospective analyses presented in this paper support the continued use of the tissue IFA as a sensitive screening test for the initial detection of CNS autoantibodies to intracellular target proteins. Moreover, the tissue IFA has clinical utility for the detection of CNS autoantibodies to currently unidentified target proteins that may be of clinical relevance. However, based on findings presented in this paper, we strongly recommend that CBAs be included in the initial evaluation of all patients suspected of having autoimmune mediated CNS diseases.

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## **Declaration of Conflicting Interests**

All authors are paid employees of Quest Diagnostics except for ML, who has no identified conflict of interests to declare

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