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

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Short report

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Abstract

Background: While many immunologic targets of autoimmune CNS disease have been identified, autoantibodies to each, are detected only rarely. Therefore, serum is often screened by immunofluorescence assay (IFA) with whole brain tissue before reflexing positive samples to more specific assays for confirmation and identification. The purpose of this study was to compare the sensitivity of immunofluorescence positive staining of whole brain tissue to immunoblot assays (IBAs) and cell-based immunofluorescence assays (CBAs) for the initial detection of autoantibodies associated with paraneoplastic syndrome (PNS) and other autoimmune diseases of the CNS.

Methods: All comprehensive paraneoplastic neurological syndrome panel results released from our laboratory over a two-year period (2017-2019) were reviewed. This panel was specifically chosen for these retrospective analyses because of the comprehensive PNS panel's design; a reflex testing algorithm is not used and a tissue IFAs, IBAs, and CBAs, are run in parallel and independently on all serum samples.

Results: For autoantibodies to intracellular targets, tissue IFA results were positive for 47 of 56 (84%) samples that were positive by IBA. In contrast, for autoantibodies to membrane-imbedded antigenic targets, tissue IFA results were positive for only 5 of 14 (35.7%) samples that were positive by CBA.

Conclusions: 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 imbedded protein targets. Moreover, these findings have important implications for the use of tissue IFA screening for the initial detection of CNS autoantibodies.

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). These include demyelinating diseases, encephalopathy, dementia, epilepsy, and ataxia. Neuronal autoantibody production is often associated with malignancy but not always.

Current thinking is that malignant cells have the potential to both express and break tolerance to neuronal antigens, thereby eliciting an autoimmune reaction within non-malignant 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 all 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-glial 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 ≥ 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 α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor 1 and 2 (AMPA1, AMPAR2), N-methyl-D-aspartate receptor (NMDAR1), contactin-associated protein 2 (CASPR2), gamma-aminobutyric acid B receptor (GABABR) 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 monkey cerebellum and rat hippocampus. Sera were processed according to manufacturer guidelines and images were interpreted with the EuroPattern 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 ≤ 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 (84%) 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.

Table 1
Tissue IFA detection of IBA identified CNS autoantibodies

Analyte	Detected By Tissue IFA		Not Detected By Tissue IFA		Assay Agreement
	N	Sample SI (Mean $\pm$ S.D.)	N	Sample SI (Mean $\pm$ S.D.)	
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%)
Total	47	(36.7 $\pm$ 37.1)	9	(20.3 $\pm$ 6.4) *	47/56 (84%)
*P-Value = 0.6; None of the samples was positive for Ri.					

Table 2
Tissue IFA detection of CBA identified CNS autoantibodies

Detected By Tissue IFA			Not Detected By Tissue IFA		Assay Agreement
Analyte	N	Titer of each sample (Mean $\pm$ S.D.)	N	Titer of each sample (Mean $\pm$ S.D.)	
AMPAR1	0		0		1/3 (33.3%)
AMPAR2	0		0		0/0 (NA)
NMDAR	1	1:160 (1:160)	2	1:10, 1:80 (1:45)	1/3 (33.3%)
CASPR 2	2	1:160, 1:160 (1:160)	4	1:10, 1:20, 1:40, 1:80 (1:38)	2/6 (33.3%)
GABAbR	1	1:80 (1:80)	1	1:20 (1:20)	1/2 (50%)
LGI-1	1	1:20(1:20)	2	1:10, 1:20 (1:15)	1/3 (33.3%)
Total	5	(1:116 $\pm$ 63.9)	9	(1:32.2 $\pm$ 28.6) *	5/14 (36%)
*P-Value = 0.02; None of the samples were positive for AMPAR1 or AMPAR2					

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 (35.5% vs 84%, respectively; $P = 0.001$).

Conclusions

Many laboratories currently use tissue IFA screening as a predicate method 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 potential limitation of this approach is that clinically relevant CNS autoantibodies would be missed if not initially detected by tissue IFA. To address this concern, we conducted a retrospective study to determine how often tissue IFA results confirmed the presence of CNS autoantibodies identified by IBA or CBA. The tissue IFA was found to detect 84% of the CNS autoantibodies identified by IBA but only 34% of those identified by CBA. In

consideration of these findings, it is worth considering that 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.

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 SI 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, other 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 SI, 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, findings presented previously suggest that potentially pathogenic CNS autoantibodies with membrane protein specificities, which are readily detected by CBA, are often missed by tissue IFA.

In summary, these findings 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 retrospective analyses 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.

List Of Abbreviations

PNS - Paraneoplastic Syndrome

IFA - Immunofluorescence assay

CBA - Cell based assay

IBA – Immunoblot assay

Declarations

- Ethics approval and consent to participate: *Our internal IRB was consulted. These studies did not require their approval*
- Consent for publication: *Not applicable*
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- Authors' contributions: *RWA and LM collected data and prepared tables for this paper. LM, JGR, JHD HHT were largely responsible for generating the data presented in this paper. FXHS conducted all pertinent statistical analyses for the paper. ML helped craft the paper and provided critical editorial input. AAH oversaw data collection and statistical analyses. Moreover, AAH wrote a majority of the paper and is primarily responsible for its content*
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