Original Paper

A diagnostic approach for identifying anti-neuronal antibodies in children with suspected autoimmune encephalitis

Eri Nakahara a, b, Hiroshi Sakuma a, Junko Kimura-Kuroda a, Toshiaki Shimizu b, Akihisa Okumura b, c, Masaharu Hayashi a

a Department of Brain Development and Neural Regeneration, Tokyo Metropolitan Institute of Medical Science, 2-1-6 Kamikitazawa, Setagaya-ku, Tokyo, Japan

b Department of Pediatrics, Juntendo University Faculty of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo, Japan

c Department of Pediatrics, Aichi Medical University Faculty of Medicine, 1-1 yazakokarimata, Nagakute, Aichi, Japan

Correspondence to: Hiroshi Sakuma, Department of Brain Development and Neural Regeneration, Tokyo Metropolitan Institute of Medical Science, 2-1-6, Kamikitazawa,
Setagaya-ku, Tokyo, 156-8506, Japan. Email: sakuma-hs@igakuen.or.jp

Telephone and fax number: +81-3-5316-3100, +81-3-5316-3150

Word count for the body of manuscript: 2,932 words
Abstract

We assessed the validity of immunoblotting, immunohistochemistry (IHC), and immunocytochemistry (ICC) to detect anti-neuronal antibodies in an attempt to establish a diagnostic approach for pediatric autoimmune encephalitis. Both IHC and ICC had higher sensitivity than immunoblotting and could differentiate between antibodies directed towards intracellular and cell surface antigens. There was a significant correlation between the IHC and ICC results. When patients were divided into encephalitis and non-encephalitis groups, there was no difference in the positivity rate and staining pattern of IHC and ICC between them. In conclusion, IHC and ICC are useful methods to screen for anti-neuronal antibodies. A combination of IHC, ICC, and specific cell-based assays is expected to be an efficient approach for the diagnosis of autoantibody-mediated encephalitis.

Keywords: Autoimmune encephalitis; Anti-neuronal antibody; Immunoblot; Immunohistochemistry; Immunocytochemistry; Cell-based assay
1. Introduction

Autoantibodies to neuronal or glial components are important causes of autoimmune diseases affecting the central nervous system (CNS). For example, anti-aquaporin-4 antibodies are associated with neuromyelitis optica, a demyelinating disease of the CNS (Takahashi et al., 2007). Over the last decade, autoantibodies targeting extracellular synaptic receptor epitopes and components of trans-synaptic protein complexes have been identified in several forms of autoimmune encephalitis (Dalmau and Rosenfeld, 2008). These autoantibodies include those directed at N-methyl-D-aspartate receptors (NMDAR) (Dalmau et al., 2007), voltage-gated potassium channel complexes (Irani et al., 2010), gamma-aminobutyric acid (GABA)-A and GABA-B receptors (Lancaster et al., 2010; Petit-Pedrol et al., 2014), and dopamine D2 receptors (Dale et al., 2012).

Autoimmune encephalitis caused by anti-neuronal antibodies is commonly observed in the pediatric population. For example, 40% of NMDAR encephalitis cases are pediatric (Florance et al., 2009). The differential diagnosis for pediatric autoimmune encephalitis is diverse, including infectious, para-infectious, metabolic, traumatic, genetic, malignant, and toxic etiologies (Hacohen et al., 2013). Therefore, testing for
anti-neuronal antibodies is important in pediatric clinical practice. For efficient
diagnosis of these diseases, the development of a comprehensive anti-neuronal antibody
survey is required. In the present study, we assessed the advantages and disadvantages
of four methods to detect anti-neuronal antibodies in patients with suspected pediatric
immune-mediated CNS disorders.

2. Materials and Methods

2.1. Patients

The subjects in this study were pediatric patients (≤18 years of age at the time of
symptom onset) with suspected immune-mediated CNS disorders whose sera and/or
cerebrospinal fluid (CSF) were sent to the Tokyo Metropolitan Institute of Medical
Science for immunological analyses. Immune-mediated CNS disorders were suspected
when patients fulfilled all of the following criteria: (1) acute/subacute onset of CNS
symptoms (seizure, psychiatric symptoms, behavioral abnormalities, movement
disorders, ataxia, etc.), (2) evidence of an immune response (triggered by infection or
vaccination, or a good response to immunomodulatory treatments), and (3) exclusion of
other neurological disorders (structural, tumor, metabolic, genetic, degenerative, or primary viral encephalitis). Cases were excluded if there was insufficient clinical information.

Patients were categorized into encephalitis and non-encephalitis groups. The encephalitis group was defined as having at least one of the following features: (1) the presence of a well-defined clinical syndrome, such as NMDAR encephalitis or limbic encephalitis, (2) CSF pleocytosis (>5 white cells/mm³), (3) CNS imaging abnormality suggestive of inflammatory or autoimmune encephalitis, including increased signal in the mesiotemporal lobe or basal ganglia (Zuliani et al., 2012).

Patients (n = 39) suspected of having autoimmune CNS disorders were enrolled in the study from January 2013 to March 2014 (Fig. 1). Clinical data, laboratory results, and electrophysiological and neuroimaging testing data from the onset of the disease were compiled using a questionnaire. Specimens were obtained between 1 day and 6 months after onset. Thirty-five samples were taken before immunotherapy. CSF samples were available from 33 patients. All samples were tested for autoimmune antibodies using four different methods, as described below, at the Tokyo Metropolitan
Institute of Medical Science. Patient samples were frozen at -80°C until use.

2.2. Anti-neuronal antibody assays

2.2.1. Test optimization

Fixation and incubation conditions for immunoblotting, immunohistochemistry (IHC), and immunocytochemistry (ICC) were optimized using two control sera obtained from healthy adults and four sera with known positivity for autoantibodies (anti-NMDAR antibodies [n = 2] and anti-nuclear antibodies [n = 2]). Ice-cold acetone was selected for fixation because both paraformaldehyde and methanol abolish typical staining of neuronal dendrites with anti-NMDAR antibodies. Regarding immunostaining, we used starting points suggested by a previous report (Graus et al., 2008) and determined the optimal antibody dilution yielding the best signal-noise ratio. Preliminary studies demonstrated eight additional control sera obtained from healthy adults were all negative by immunoblot, IHC, and ICC.

2.2.2. Immunoblotting
Immunoblotting was performed using a pre-made polyvinylidene fluoride (PVDF) membrane onto which denatured proteins from whole human brain tissue lysates were electrotransferred (IMGENEX, San-Diego, CA, USA). Membranes were immersed in 100% methanol and blocked with phosphate-buffered saline containing 0.1% Tween 20, 5% normal goat serum, and 5% non-fat dry milk for 1 hour. Subsequently, the membranes were serially incubated with the following reagents at the indicated dilutions: patient sera (1:200) for 2 hours at 37°C, biotin-conjugated goat anti-human IgG (1:400, Jackson Immunoresearch, West Grove, PA, USA) for 1 hour at 37°C, and streptavidin-conjugated horseradish peroxidase (HRP) (1:400, Jackson Immunoresearch) for 30 minutes at 37°C. Membranes were visualized using 3, 3′-diaminobenzidine (DAB) (Vector Lab, Burlingame, CA, USA).

2.2.3. IHC of frozen rat brain sections

Indirect IHC was performed using 10-µm frozen rat brain tissue sections, including hippocampi. Sections were fixed with ice-cold acetone for 5 minutes at 4°C, treated with 0.06% H₂O₂ for 20 minutes, and blocked with Protein Block (Dako, Glostrup,
Denmark) for 1 hour. Subsequently, they were serially incubated with patient serum (1:500) and/or CSF (1:9) for 2 hours at 37°C, biotinylated goat anti-human IgG (1:2000, Jackson Immunoresearch) for 1 hour at 37°C, and streptavidin-conjugated HRP (1:1000, Jackson Immunoresearch) for 30 minutes at 37°C. Sections were visualized using DAB (Vector Lab).

2.2.4. ICC of primary rat neuronal cultures

Primary neuronal cultures were prepared as previously described (Kimura-Kuroda et al., 1994) with a slight modification. Briefly, cerebral cortices were dissected from 18-day-old embryonic Sprague Dawley rats (Clea Japan, Inc., Tokyo, Japan). After dissection, the cerebral cortices were treated with 2 µg/ml papain (Worthington, Lakewood, NJ), 0.01% DNase (Roche, Mannheim, Germany), 2 mg/ml bovine albumin (Invitrogen, Carlsbad, CA, USA), 2 mg/ml DL-cysteine hydrochloride (Wako, Osaka, Japan), and 50 mg/ml glucose in phosphate-buffered saline for 25 minutes at 37°C. Digests were then centrifuged at 1,000 rpm for 4 minutes. The pelleted cells were mechanically dissociated by repeated passages through pipettes. The cells were then
triturated in neurobasal medium supplemented with 2% B27 medium (Life Technologies, Carlsbad, CA, USA) and 1% fetal bovine serum. The cells were then plated at a density of 200,000 cells in 0.2 ml/well on 8-well chamber slides pre-coated with 100 µg/ml poly-L-lysine (Sigma-Aldrich, St. Louis, MO, USA) and 10 µg/ml laminin (BD Bioscience, Franklin Lakes, NJ, USA) then kept at 37°C in a humidified, 5% CO₂ incubator. Half of the culture medium was replaced with fresh neurobasal and B27 medium without serum every 3–4 days for 2 weeks. Cells were then fixed in acetone and kept frozen at -30°C until use. They were serially blocked with protein block for 2 hours at room temperature (RT), incubated with patient sera (1:100) and/or CSF (1:9) for 1 hour at RT, and with Alexa Fluor488® anti-human IgG (1:2000, Jackson Immunoresearch) for 30 minutes at RT. Nuclei were visualized using 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Invitrogen). Confocal laser images were captured using a FV1000 fluorescence microscope (Olympus, Tokyo, Japan).

2. 2.5. Cell-based assay
Autoantibodies to specific neuronal antigens, including NMDAR, 
α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) 1, 
AMPAR2, leucine-rich glioma inactivated 1 protein (LGI1), contactin-associated 2 protein (CASPR2), and GABA-B receptor, were tested by indirect immunofluorescence according to the manufacture’s protocol (Autoimmune Encephalitis Mosaic 1 Kit, Euroimmun, Lübeck, Germany).

2.2.6. Criteria for immunoblotting, IHC, and ICC results
All membranes/slides were evaluated independently by two blinded investigators. The staining evaluations were standardized regarding staining pattern definitions, as shown in Table 1.

2.3. Ethics statement
This study was approved by the Institutional Review Board of the Tokyo Metropolitan Institute of Medical Science (12-34). All patients and/or their family members provided written informed consent.
2.4. Statistics

Concordance between results of the four methods was evaluated by the kappa coefficient for qualitative variables using the Excel statistics 2012 software (SSRI, Tokyo, Japan). Correlations between results of the two different categories were assessed by Fisher’s exact probability tests for qualitative variables using the StatView-5.0 software (HULINKS, Tokyo, Japan). Statistical significance was accepted at a value of p less than 0.05.

3. Results

3.1 Clinical and demographic features (Suppl. Table 1)

We identified 22 patients for the encephalitis group (11 boys; median age, 8.95 years; age range, 8 months–14 years) and 17 for the non-encephalitis group (12 boys; median age, 9.33 years; age range, 6 months–16 years). There were no differences in age or sex between the groups. Patients of the encephalitis group more commonly had involuntary
movements (p = 0.021, Fisher’s exact probability test). There was no significant
difference in the frequency of other symptoms between the groups (data not shown).

3.2. Immunoblot (Fig. 2, Suppl. Table 2)

The membrane incubated with secondary antibody only, without any patient sample,
(Fig. 1, Lane 1) showed bands at 25, 50, 70, and 120 kDa. The 25- and 50-kDa bands
presumably indicated the IgG light and heavy chain, respectively. Sera from 15/39
patients showed extra bands of various molecular sizes. Sera from nine patients
showed multiple bands. It was difficult to judge whether similar size bands in different
patients represented antibodies to the same antigen.

3.3. IHC of frozen rat brain sections (Fig. 3, Table 3, Suppl. Table 2)

IHC results were positive for 22/39 samples (56.4%). CSF IHC results were positive for
7/31 samples (22.5%). Control sera showed faint background staining (Fig. 2A, 2B).

IHC could differentiate antibodies directed against the neuronal nuclei, cytoplasm, and
neuropil. Autoantibodies to nuclei were observed most frequently (serum: 14/39, 35.8%,
CSF: 5/31, 16.1%) (Fig. 3G, 3H). In most cases, neuronal as well as glial and endothelial nuclei were stained, suggesting that these autoantibodies were not specific to neurons. Autoantibodies to the cytoplasm (serum: 4/39, 33.3%, CSF: 0/31, 0.0%) (Fig. 3E, 3F) reacted most strongly to hippocampal CA3 region neurons. Autoantibodies to the neuropil (serum: 6/39, 15.3%, CSF: 3/31, 9.6%) selectively stained the molecular layer of the dentate gyri, where neuronal dendrites are densely localized (Fig. 3C, 3D).

3.4. ICC in primary rat neuronal culture (Fig 4, Table 3, Suppl. Table 2)

Serum ICC results were positive for 25/39 samples (64.1%). CSF ICC resulted in 8/31 (25.8%) positive cases. Control sera did not stain neurons (Fig. 4A, 4B). Similar to IHC, ICC also distinguished autoantibodies towards dendrites (serum: 5/39, 12.8%, CSF: 3/31, 9.6%) (Fig. 4C, 4D, Supplemental figure), nuclei (serum: 9/39, 23.0%, CSF: 3/31, 9.6%) (Fig. 4G, 4H), and cytoplasm (serum: 13/39, 33.3%, CSF: 4/31, 12.9%) (Fig. 4E, 4F). Multicolor immunostaining demonstrated the co-localization of reactivity between NMDARE patient’s sera and MAP2, indicating specific neuronal staining
3.5. Cell-based assay

Both sera and CSF cell-based assays identified only one case positive for anti-NMDAR antibodies. Both serum and CSF from this patient reacted with the neuropil by IHC and with dendrites by ICC. Other known anti-neuronal antibodies were negative in all tested cases.

3.6. Clinical-laboratory correlation (Table 2)

We compared the profile of anti-neuronal antibodies between the encephalitis and non-encephalitis groups. There were no significant differences in the positivity rate and staining patterns of immunoblotting, IHC, ICC, and cell-based assays. However, most cases with positive neuropil/dendrite staining belonged to the encephalitis group (4/6 cases by IHC and 5/5 cases by ICC).

3.7. Concordance between the results of different methods (Table 3, 4)
Finally, we studied the concordance rate between the three methods described above.
Results for IHC were significantly concordant with those of ICC (serum: concordance rate = 0.2207, p = 0.0051, CSF: concordance rate = 0.3261, p = 0.0035). There was no significant concordance between the results of immunoblotting and those of IHC/ICC.

4. Discussion

Autoantibodies to neuronal antigens are divided into two categories based on antigen location: those to intracellular antigens and those to cell surface antigens (Tuzun and Dalmau, 2007). It has been speculated that antibodies to cell surface antigens are potentially pathogenic, whereas those to intracellular antigens do not directly cause cell injury (Vincent et al., 2011). Anti-NMDAR antibodies reduce the surface expression of NMDA receptors via receptor internalization (Hughes et al., 2010). In contrast, it is assumed that encephalitis associated with autoantibodies to intracellular antigens is mainly mediated by cytotoxic T cells, rather than humoral immunity (Bien et al., 2012). Therefore, it is clinically important to determine whether autoantibodies are directed
against cell surface or intracellular antigens.

We used four analysis methods to detect anti-neuronal antibodies: Western blot of human brain tissue, IHC on rat hippocampal sections, ICC on rat primary neuronal cultures, and a cell-based assay. Western blots were used to identify basal ganglia antibodies (Dale et al., 2001) and are used clinically for detecting classical paraneoplastic autoantibodies, including anti-Hu (Graus et al., 2001). In the present study, the overall positive rate of immunoblotting was lower than that of IHC and ICC, presumably due to the lower sensitivity of immunoblotting. Immunoblotting is advantageous in predicting the molecular size of target antigens. However, this method is not suited for detecting antibodies to cell surface antigens because these molecules may lose their antigenicity after protein denaturation. Additionally, since whole brain lysates were used as antigens, non-specific binding of serum proteins led to a poor signal-to-noise ratio and hindered the identification of true-positive bands. Specific bands may be visualized using neuronal extracts that are immunoprecipitated as the antigen with patient sera (Ohkawa et al., 2013).

IHC and ICC showed relatively higher positive rates, and there was a
significant concordance between the IHC and ICC results. A major advantage of IHC and ICC is their superiority in distinguishing antibodies to intracellular antigens from those to cell surface antigens. More than half of these antibodies targeted cellular nuclei. Although their pathogenic roles remain unknown, these antibodies may represent low titer anti-nuclear antibodies because they react with the nuclei of all cells, not just neuronal cells. It was unclear whether antibodies showing a cytoplasmic pattern truly target neuronal antigens because primary neuronal culture contained non-neuronal cells, including astrocytes. In contrast, autoantibodies showing a neuropil/dendritic pattern were likely to be clinically significant.

ICC on rat neuronal cultures clearly helped visualize dendrites when tested with anti-NMDAR antibody-positive serum. This method is particularly powerful for identifying antibodies to cell surface antigens enriched in synaptic regions, and is potentially beneficial in detecting antibodies against synaptic antigens. ICC may specify antibodies that react with region-specific antigens in the brain. These methods were suitable for autoantibodies screening, and a combination of ICC and IHC is expected to reduce false negative results. Autoantibodies that bind exclusively to human antigens
might be excluded by this method, but most have been successfully detected due to
cross-reactivity between rats and humans. We were unable to determine the sensitivity
and specificity of IHC and ICC because there were very few samples positive for
known anti-neuronal antibodies. It is unclear whether these methods can detect
anti-neuronal autoantibodies in samples from children because their serum IgG levels
are lower than that of adults. However, our data proved that we could obtain stable
measurements of anti-neuronal antibodies in a pediatric cohort.

Various techniques have been used to detect autoantibodies, including IHC,
western blot, radioimmunoassay, enzyme-linked immunosorbent assay (ELISA), and
cell-based assays (Vincent and Bien, 2011). Immunoblotting and ELISA are
conventional methods for detecting autoantibodies (Takahashi et al., 2003). Cell-based
assays using mammalian cells transfected with autoantigens have been established as
the standard technique for identifying antibodies to cell-surface antigens. Cell-based
assay kits consisting of a panel of neuronal antigens are commercially available and
easy to use in a hospital-based laboratory. To date, however, commercial kits to detect
several autoantibodies, including anti-glycine receptor and anti-GABA-A receptor
antibodies, are not available. Additionally, the number of newly identified autoantibodies associated with autoimmune CNS diseases is growing. To overcome this problem, a screening method for anti-neuronal antibodies using IHC and ICC has been proposed (Hoftberger et al., 2012). There is another recommendation that antibodies to neuronal surface antigens should be screened using cell-based assays first, and IHC and ICC used for negative cases to detect unknown antigens (Lancaster et al., 2011). An advantage of IHC and ICC is their potential to discover novel autoantibodies. In the present cohort, five patients each were positive for anti-neuronal antibodies to cell surface antigens by IHC and ICC. However, we did not identify known cell surface antibodies, except the anti-NMDAR antibody, using a cell-based assay, suggesting the existence of unknown autoantibodies. Collectively, the current gold standard for anti-neuronal antibody assays is to use cell-based assays as well as ICC and/or IHC in parallel.

In the present cohort, there was no significant difference in the results of immunoblot, IHC, ICC, and cell-based assays between the encephalitis and non-encephalitis groups, probably due to the low positivity rate. Many cases in the
encephalitis group were negative for anti-neuronal antibodies, implying diversity in the
pathogenesis of suspected autoimmune encephalitis. Antibodies showing a
neuropil/dendritic pattern, however, were rarely found in the non-encephalitis group.
This result is consistent with the observation that antibodies to synaptic surface antigens
are relevant to autoimmune encephalitis.

5. Conclusion

The present study demonstrated that IHC on rat hippocampal sections and ICC on
primary rat neuron cultures were suitable to detect anti-neuronal antibodies in sera
obtained from a pediatric population. These tests differentiated antibodies directed
against cellular nuclei, cytoplasm, and neuronal dendrites, which seem to differ in their
pathogenicity. Each technique has advantages and disadvantages and a combination of
IHC, ICC, and specific cell-based assays provides an efficient diagnostic tool for
autoantibody detection in pediatric autoimmune encephalitis.
Acknowledgments

H.S. was supported by a research grant from The Mother and Child Health Foundation (24-4).

References


investigations and outcomes in patients with or without antibodies to known central nervous system autoantigens. J Neurol Neurosurg Psychiatry. 84, 748-755.


Takahashi T, Fujihara K, Nakashima I, Misu T, Miyazawa I, Nakamura M, Watanabe S,


Table 1. Definitions of staining patterns for immunohistochemistry and immunocytochemistry

<table>
<thead>
<tr>
<th>Staining Pattern</th>
<th>Immunohistochemistry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear pattern</td>
<td>Diffuse, granular, spotty, or peripheral staining of cellular nuclei</td>
</tr>
<tr>
<td>Cytoplasmic pattern</td>
<td>Diffuse or granular staining of cytoplasm with absent nuclear staining</td>
</tr>
<tr>
<td>Neuropil pattern</td>
<td>Selective staining of the molecular layer of the dentate gyri with preservation of granular cell layer</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Staining Pattern</th>
<th>Immunocytochemistry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear pattern</td>
<td>Diffuse, granular, spotty, or peripheral staining of cellular nuclei that merges with DAPI</td>
</tr>
<tr>
<td>Cytoplasmic pattern</td>
<td>Diffuse or granular staining of cytoplasm with absent nuclear staining</td>
</tr>
<tr>
<td>Dendritic pattern</td>
<td>Granular staining with dendritic appearance</td>
</tr>
</tbody>
</table>

DAPI = 4',6-diamidino-2-phenylindole

Table 2. Positivity rate of anti-neuronal antibody tests

<table>
<thead>
<tr>
<th></th>
<th>Encephalitis group</th>
<th>Non-encephalitis group</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>n = 22</td>
<td>n = 17</td>
<td></td>
</tr>
<tr>
<td>Immunoblot</td>
<td>11/22 (50.0%)</td>
<td>4/17 (23.5%)</td>
<td>0.1116</td>
</tr>
<tr>
<td>IHC</td>
<td>14/22 (63.6%)</td>
<td>8/17 (47.0%)</td>
<td>0.3454</td>
</tr>
<tr>
<td>neuropil</td>
<td>4/22 (18.1%)</td>
<td>1/17 (5.8%)</td>
<td>0.3634</td>
</tr>
<tr>
<td>cytoplasm</td>
<td>2/22 (9.0%)</td>
<td>2/17 (11.7%)</td>
<td>&gt;0.9999</td>
</tr>
<tr>
<td>nucleus</td>
<td>9/22 (40.9%)</td>
<td>6/17 (35.2%)</td>
<td>0.7526</td>
</tr>
<tr>
<td>ICC</td>
<td>14/22 (63.6%)</td>
<td>10/17 (58.8%)</td>
<td>&gt;0.9999</td>
</tr>
<tr>
<td>dendrite</td>
<td>5/22 (22.7%)</td>
<td>0/17 (0.0%)</td>
<td>0.0565</td>
</tr>
<tr>
<td>cytoplasm</td>
<td>7/22 (31.8%)</td>
<td>6/17 (35.2%)</td>
<td>&gt;0.9999</td>
</tr>
</tbody>
</table>
### Cell-based assay

<table>
<thead>
<tr>
<th></th>
<th>n = 22</th>
<th>n = 17</th>
</tr>
</thead>
<tbody>
<tr>
<td>nucleus</td>
<td>9/22 (40.9%)</td>
<td>5/17 (29.4%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>n = 21</th>
<th>n = 13</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IHC</td>
<td>7/21 (33.3%)</td>
<td>1/13 (7.6%)</td>
</tr>
<tr>
<td>neuropil</td>
<td>3/21 (14.2%)</td>
<td>0/13 (0.0%)</td>
</tr>
<tr>
<td>cytoplasm</td>
<td>0/21 (0.0%)</td>
<td>0/13 (0.0%)</td>
</tr>
<tr>
<td>nucleus</td>
<td>4/21 (19.0%)</td>
<td>1/13 (7.6%)</td>
</tr>
<tr>
<td>ICC</td>
<td>4/21 (19.0%)</td>
<td>3/13 (23.0%)</td>
</tr>
<tr>
<td>dendrite</td>
<td>2/21 (9.5%)</td>
<td>2/13 (15.3%)</td>
</tr>
<tr>
<td>cytoplasm</td>
<td>2/21 (9.5%)</td>
<td>2/13 (15.3%)</td>
</tr>
<tr>
<td>nucleus</td>
<td>1/21 (4.7%)</td>
<td>1/13 (7.6%)</td>
</tr>
</tbody>
</table>

\[\text{ICC} = \text{immunocytochemistry}, \quad \text{IHC} = \text{immunohistochemistry}, \quad \text{p-values were calculated by Fisher’s exact probability tests.}\]

### Table 3. Concordance between serum IHC and ICC results

<table>
<thead>
<tr>
<th></th>
<th>neuro</th>
<th>cyto</th>
<th>nuc</th>
<th>neuro + cyto</th>
<th>neuro + nuc</th>
<th>cyto + nuc</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICC</td>
<td>dend</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>cyto</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>nuc</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>dend + cyto</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>dend + nuc</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>cyto + nuc</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N</td>
<td>3</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>9</td>
</tr>
</tbody>
</table>
Kappa coefficient = 0.2207, p = 0.0051
IHC = immunohistochemistry, ICC = immunocytochemistry, neuro = neuropil, cyto = cytoplasm, nuc = nucleus, dend = dendrite, N = negative

Table 4. Concordance between CSF IHC and ICC results

<table>
<thead>
<tr>
<th></th>
<th>CSF</th>
<th>IHC</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>neuro</td>
<td>cyto</td>
<td>nuc</td>
<td>neuro + cyto</td>
<td>neuro + nuc</td>
<td>cyto + nuc</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>ICC</td>
<td>dend</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>cyto</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>nuc</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>dend + cyto</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>dend + nuc</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cyto + nuc</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>19</td>
</tr>
</tbody>
</table>

Kappa coefficient = 0.3261, p = 0.0035
IHC = immunohistochemistry, ICC = immunocytochemistry, neuro = neuropil, cyto = cytoplasm, nuc = nucleus, dend = dendrite, N = negative
FIGURE LEGENDS

Figure 1. Immunoblotting

Membranes on which denatured proteins from whole human brain lysates were electrotransferred were immunostained with either secondary anti-human IgG without the primary antibody (Lane 1) as control staining, or sera from patients (Lanes 2–5). Arrows indicate positive extra bands. Lanes 2, 3, and 4 had no specific band. Lane 5 had three extra bands at 22, 24, and 50 kDa.

Figure 2. Immunohistochemistry of frozen rat brain sections

Frozen sections of rat hippocampus immunostained with patient sera. A, B: negative staining. C, D: positive staining of the neuropil, sparing neuronal cell bodies. Note that the region of the molecular layer adjacent to the granular cells of the dentate gyrus is selectively stained. E, F: positive staining of the cell bodies. G, H: positive staining of the nuclei. Scale bar = 500 μm (A, B, C, E) or 50 μm (D, F).
Figure 3. Immunocytochemistry of primary rat neuronal cultures

72 patients referred

67 patients with suspected immune-mediated CNS disorders

5 alternative diagnosis
1 Hemorrhagic shock and encephalopathy syndrome
4 Multiple sclerosis

13 Serum sample at onset not available

15 Clinical Information not available

39 patients included in analysis
Figure 2
Click here to download high resolution image