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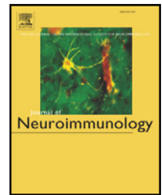

A diagnostic approach for identifying anti-neuronal antibodies in children with suspected autoimmune encephalitis

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A B S T R A C T

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.

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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.

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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/mm^3), and (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 that 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 h. Subsequently, the membranes were serially incubated with the following reagents at the indicated dilutions: patient sera (1:200) for 2 h at 37 °C, biotin-conjugated goat anti-human IgG (1:400, Jackson Immunoresearch, West Grove, PA, USA) for 1 h at 37 °C, and streptavidin-conjugated horseradish peroxidase (HRP) (1:400, Jackson Immunoresearch) for 30 min at 37 °C. Membranes were visualized using 3,3′-diaminobenzidine (DAB) (Vector Lab, Burlingame, CA, USA).

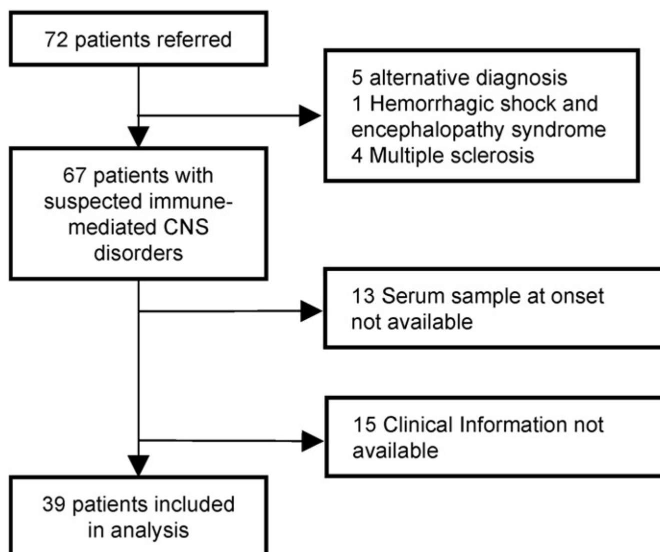

Fig. 1. Study flow chart. Among the 72 patients who were referred, five patients were excluded from the study because they were made alternative diagnoses; four were multiple sclerosis and one was hemorrhagic shock and encephalopathy syndrome. Patients whose serum samples at onset were not available (n = 13) and whose clinical information was not available (n = 15) were also excluded. Thus, 39 patients were finally enrolled in this study.

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 min at 4 °C, treated with 0.06% H_2O_2 for 20 min, and blocked with Protein Block (Dako, Glostrup, Denmark) for 1 h. Subsequently, they were serially incubated with patient serum (1:500) and/or CSF (1:9) for 2 h at 37 °C, biotinylated goat anti-human IgG (1:2000, Jackson Immunoresearch) for 1 h at 37 °C, and streptavidin-conjugated HRP (1:1000, Jackson Immunoresearch) for 30 min 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 min at 37 °C. Digests were then centrifuged at 1000 rpm for 4 min. 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 precoated 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% CO_2 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 h at room temperature (RT), incubated with patient sera (1:100) and/or CSF (1:9) for 1 h at RT, and with Alexa Fluor488® anti-human IgG (1:2000, Jackson Immunoresearch) for 30 min 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 manufacturer'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.

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