

RESEARCH ARTICLE

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Comparison of Commercial Fixed and In-House Tissue-Based and Live Cell-Based Assays for the Detection of Common Neuronal Surface Autoantibodies

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ABSTRACT

Introduction: Autoimmune encephalitis (AIE) is diagnosed through detection of neuronal-surface antibodies (Abs) in serum and/or cerebrospinal fluid (CSF). Commercial biochip-based cell-based assay (CBA) using fixed cells and in-house CBAs using live cells may give incongruent results. We aimed to shed further light on features of AIE patients with discordant CBA results to establish factors related with diagnosis and treatment-response.

Methods: A total of 45 patients found positive for N-methyl-D-aspartate receptor (NMDAR), leucine rich glioma inactivated 1 (LG11), contactin-associated protein-like 2 (CASPR2)-Abs on routine screening done with fixed-CBA, were further tested using in-house live-CBA and tissue-based assay (TBA). Serum neurofilament light chain (NFL) levels were measured and response to immunotherapy was assessed in a follow-up period of 36.9±7.8 months.

Results: Thirty patients who were positive on both fixed and live-CBA/TBA, showed trends towards displaying limbic symptoms and EEG/MRI features

suggestive of AIE. By contrast, most of the 15 patients with only fixed-CBA positivity failed to fulfill AIE criteria. Positivity on live CBA/TBA did not predict response to immunotherapy or coexisting cancers. Some patients in the fixed-CBA positive category exhibited features highly suggestive of autoimmune encephalopathy and elevated NFL levels. Lower disease severity, presence of viral prodromal findings, absence of autonomic symptoms and facial dyskinesia were associated with treatment response.

Conclusion: Live CBA/TBA demonstrated higher diagnostic accuracy than fixed cell-based assays and substantially reduced the likelihood of false-positive antibody detection. Positive live-CBA/TBA results strongly coincide with definite AIE criteria. However, live CBA and NFL results fail to predict response to immunotherapy and identify coexisting cancers. This is putatively because some patients in the live-CBA/TBA negative category likely have immunotherapy-responsive neuroinflammatory disorders.

Keywords: Autoimmune encephalitis, autoimmunity, cell-based assay, n-methyl-D-aspartate receptor, neurofilament light chain.

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INTRODUCTION

Autoimmune encephalitis (AIE) encompasses a group of inflammatory neurological disorders typified with rapid progression and serum/cerebrospinal fluid (CSF) antibodies (Abs) targeting neuronal surface antigens (1). The diagnosis of AIE relies heavily upon determination of these Abs. However, currently used commercial test kits may potentially give false positive and false negative results and Ab positivity may also be detected in cases that do not meet AIE diagnostic criteria (2–5). This may potentially lead to difficulties and errors in diagnosis and treatment processes. Many diseases can resemble AIE and false positive Ab results and subsequent administration of immunotherapy in these

Highlights

- Live CBA/TBA positivity aligns with definite autoimmune encephalitis criteria.
- Fixed CBA shows a high false-positivity rate for common neuronal surface antibodies.
- Live CBA and serum NFL levels do not predict immunotherapy response or cancer risk.

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so-called “AIE mimics” may lead to serious adverse events or delays in the diagnosis. On the other hand, as high as 56% of AIE mimics with no identifiable underlying cause may give favorable response to immunotherapy (2). These observations prompt the improvement of Ab-detection methods and increased understanding of factors related to treatment-response, cancer co-occurrence in AIE and AIE mimics.

The gold standard in diagnosis of AIE is detection of neuronal surface Abs in CSF of patients by live cell-based assay (CBA) utilizing live human embryonal kidney 293T (HEK293T) cells expressing the target neuronal surface autoantigen of interest (6). Nevertheless, due to the unavailability of these live CBAs in most clinical centers around the world, the diagnosis of AIE is mostly carried out by commercially available biochip-based assays utilizing fixed HEK293T cells. Only a small number of studies have so far compared the utility of fixed versus live CBAs for N-methyl-D-aspartate receptor (NMDAR), leucine rich glioma inactivated 1 (LG11), contactin-associated protein-like 2 (CASPR2) and gamma-amino-butyric acid B receptor (GABABR)-Abs. Although majority of the patient samples appear to yield concordant results by both methods, incongruent results may be obtained in a substantial proportion of patients (3–5). Also, both CBAs appear to have considerable flaws in immunotherapy and cancer screening decisions.

Therefore, we aimed to establish clinical and laboratory criteria that can effectively guide diagnosis and treatment in AIE cases and to test the validity of existing criteria predicting anti-neuronal Ab positivity in neurological diseases. Commercially available fixed and in-house live CBAs designed for the three most common neuronal surface Abs (NMDAR, LG11 and CASPR2-Abs) were compared in terms of Ab detection rate, association with clinical and demographic characteristics, response to immunotherapy and compliance with AIE diagnosis (6).

METHODS

Enrollment of Patients

Serum and CSF samples of patients sent to our laboratory between 2017–2025 for the differential diagnosis of AIE were screened by a standard commercial fixed CBA (Autoimmune Encephalitis Mosaic; FA 112 d-1010-6; Euroimmun, Luebeck, Germany) for NMDAR, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA1 and 2), LG11, CASPR2, GABABR and dipeptidyl aminopeptidase-like protein 6 (DPPX)-Abs and by ELISA for glutamic acid decarboxylase (GAD)-Ab (EA 1022-9601-2; Euroimmun, Luebeck, Germany). As per the manufacturer's recommendation, CSF was tested undiluted, and sera were incubated at a 1:10 dilution. Ab positivity score was established for each sample on a scale between 0 and 4 (0, no binding; 1, weak; 2, moderate; 3, strong; 4, very strong binding) by two independent experts blinded to the clinical features of the patients, and discrepancies were resolved by consensus. A total of 208 patients were found positive for NMDAR, LG11 and CASPR2-Abs and none of these patients were positive for more than one Ab. Forty-five of these patients with paired serum/CSF samples available in our archives and sufficiently detailed clinical history and follow-up data were enrolled for our study and stratified into definite, probable and possible AIE, as per previously proposed diagnostic guidelines (6). All included patients had a subacute onset of less than 3 months, the average ($\pm$ standard deviation) follow-up period was 36.9 ± 7.8 months (range: 14–192 months) and potential alternative causes had been excluded after meticulous differential diagnostic procedures in all patients.

In addition to demographic features, clinical symptoms and supportive laboratory findings, maximum modified Rankin scale (mRS) score during the follow-up, response to immunosuppressive treatment, presence of treatment resistant seizures and coexisting diseases were also noted

(Table 1). Thirty-eight patients gave consent for immunotherapy and had detailed follow-up data for assessment of response to first-line immunotherapy (i.e., iv pulse methylprednisolone and iv immunoglobulin or plasma exchange). Response to immunotherapy was defined as either complete functional recovery (final mRS=0) or a clinically meaningful neurological improvement corresponding to a reduction of at least 3 points from the maximum mRS score during follow-up. Resistance to seizure treatment was defined as failure to suppress clinical seizure activity despite the use of two or more anti-epileptic drugs. The study was approved by the Institutional Review Board and all the participants provided their written informed consent. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki.

In-House Live CBA

All samples were collected during the active stage of AIE before commencement of any immunotherapy and stored at -80°C until use. Live CBA was performed on HEK293T cells expressing human NMDAR-EGFP, LG11-EGFP, and CASPR2-EGFP. Transfected cells were incubated 1 hour at room temperature with diluted serum (for NMDAR 1:20; LG11 1:20; CASPR2 1:100) and CSF (1:2 dilution ratio for each antigen) samples in Dulbecco's Modified Eagle Medium (DMEM) containing N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) and 5% bovine serum albumin (BSA). Following incubation, the cells were fixed with 4% PFA in PBS for 10 minutes, were washed and incubated with Alexa Fluor 568 goat anti-human IgG secondary Ab (1:750, A-21090, Thermo Fisher Scientific, Waltham, MA, USA) at room temperature for 45 minutes. After washes, 4',6-diamidino-2-phenylindole (DAPI) was applied for nuclear staining. Fluorescent microscopy (CTR6000, Leica, Wetzlar, Germany) was used to assess the presence of specific IgG binding to antigen-expressing HEK293T cells.

In-House Tissue-Based Assay (TBA)

Immunohistochemical staining for TBA was performed on 4% paraformaldehyde (PFA)-fixed rat brain sections (10- μm -thick) for anti-neuronal Ab screening in case samples. After consecutive 0.3% hydrogen peroxide and 5% normal goat serum (NGS) blockage, sections were incubated with patient samples (for serum 1:200; CSF 1:2) overnight at $+4^{\circ}\text{C}$. The next day, after washing steps, secondary biotinylated goat anti-human immunoglobulin G (IgG) (1:750, BA-3000, Vector Laboratories, Newark, CA, USA) was applied for 1 hour at room temperature. Following the avidin-biotin complex and chromogenic detection with 3,3'-diaminobenzidine (DAB) (ZH0306, Vector Laboratories, Newark, CA, USA), the sections were counterstained with hematoxylin and mounted. The staining patterns of each neuronal surface Ab, particularly in the hippocampus region, were evaluated under the microscope (CTR6000, Leica, Wetzlar, Germany).

Serum NFL Levels

Neurofilament light chain levels were measured by the Siemens Healthineers Atellica Analyzer (Siemens Healthcare Diagnostics, Tarrytown, NY, USA), as per the manufacturer's instructions. Serum NFL levels higher than 13.9 pg/ml were accepted as elevated, as recommended by the manufacturer. Neurofilament light chain measurement results of this assay have been shown to significantly correlate with other assays used for serum NFL measurements (7). For all assays, testing personnel were blinded to clinical diagnosis.

Statistical Analysis

Two-group comparisons were carried out using Mann-Whitney U (mRS score), Student's t-test (age, serum NFL levels, CSF cell counts) and chi-square (all remaining categorical variables), as required. $p < 0.05$ were considered as statistically significant.

Table 1. Comparison of the clinical features of patients with antibodies detected by both live and fixed cell-based assays (CBA) versus only fixed CBA

	Live+fixed CBA positive cases (n=30)	Only fixed CBA positive cases (n=15)	p value
Age	51.9±18.9	49.7±18.7	0.359
Sex	17 men	9 men	0.831
Anti-neuronal antibody positive in CSF by fixed CBA	15	7	0.833
Antibody positivity score	1/2–19 3/4–11	1/2–13 3/4–2	0.103
Type of anti-neuronal antibody	CASPR2-4 LGI1-9 NMDAR-17	CASPR2-4 LGI1-1 NMDAR-10	0.163
Diagnosis due to autoimmune encephalitis criteria	21 - definite 9 - possible 0 - not encephalitis	0 - definite 4 - possible 11 - not encephalitis	<0.001
Viral prodromal findings	3	3	0.352
Short-term memory loss	23	4	0.001
Altered mental status	15	1	0.004
Catatonia	1	1	0.609
Psychiatric symptoms	22	3	0.001
Autonomic symptoms	5	1	0.352
Movement disorders	8	2	0.311
Facial dyskinesia	3	0	0.205
Epileptic seizures	19	3	0.006
Myoclonic seizures	5	0	0.093
Generalized tonic-clonic seizures	15	3	0.053
Focal seizures	11	0	0.007
Status epilepticus	5	2	0.771
Treatment-resistant seizures	6	0	0.063
Maximum mRS score	2.6±1.8	2.1±1.1	0.206
Positive response to immunotherapy*	18/26	5/12	0.106
Coexisting disease	4 cancer 3 HSV encephalitis [#] 2 autoimmune disease 21 none	2 cancer 0 HSV encephalitis 1 autoimmune disease 12 none	0.651
Abnormal EEG findings**	23	3	<0.001
Periodic wave activity on EEG	2	1	>0.999
Abnormal response to photic stimulation on EEG	6	1	0.245
Diffuse slow wave activity on EEG	18	3	0.011
Diffuse sharp/spike wave activity on EEG	4	0	0.138
Diffuse slow or sharp/spike wave activity on EEG	20	3	0.003
Bilateral temporal lobe lesions on MRI	24	1	<0.001
CSF pleocytosis (>5 mm ³)	14	3	0.082
CSF cell count (cells/mm ³)	24.9±16.4	162.3±206.3	0.184
Serum neurofilament light chain (pg/ml)	41.1±27.9	111.0±91.4	0.020

Parametric variables are denoted in terms of mean ± standard deviation.

* Immunotherapy was administered to 38 cases.

** Presence of one or more of periodic wave activity, abnormal response to photic stimulation, diffuse slow wave, diffuse sharp/spike wave activity.

[#]These three patients were diagnosed as post-HSV autoimmune encephalitis.

Bold characters signify statistically significant p values.

CSF: cerebrospinal fluid; mRS: modified Rankin scale; HSV: herpes simplex virus.

RESULTS

Results of Screening for Neuronal Surface and Synapse Abs

Using fixed CBA, NMDAR, LGI1 and CASPR2-Abs were identified in 27, 10 and 8 patients, respectively. None of the 45 patients were positive for AMPAR1/2, GABABR, DPPX, and GAD-Abs.

The in-house live CBA confirmed NMDAR, LGI1 and CASPR2-Ab positivity in 30 (67%) patients. Among these 30 patients, NMDAR-Ab was found in 17 (7 serum and CSF, 5 only CSF, 5 only serum), LGI1-Ab was found in 9 (1 serum and CSF, 1 only CSF, 7 only serum) and

CASPR2-Ab was found in 4 (1 serum and CSF, 3 only serum) patients by fixed CBA. The live CBA found NMDAR-Ab in the CSF of additional 4 patients with only NMDAR-Ab seropositivity on fixed CBA (11 serum and CSF, 5 only CSF, 1 only serum by live CBA) and CASPR2-Ab in the CSF of additional 2 patients with only CASPR2-Ab seropositivity on fixed CBA (3 serum and CSF, 1 only serum by live CBA). The in-house TBA showed previously described (8,9) characteristic staining patterns in the respective NMDAR, LGI1 and CASPR2-Ab positive patients, determined by live CBA. There was no discordance between live CBA and TBA results.

In remaining 15 (33%) fixed CBA positive patients with NMDAR (4 only serum, 6 serum and CSF), LGI1 (1 only serum) and CASPR2 (3 only serum, 1 serum and CSF)-Abs (Table 2), immune reactivity could not be confirmed by live CBA. Likewise, the in-house TBA did not yield any appreciable staining congruent with known or as yet uncharacterized IgG-reactivity patterns. There was no reactivity with glial cells on frozen rat brain sections, as well. Overall, failure to confirm fixed CBA results by live CBA/TBA studies were 37%, 10% and 50% for NMDAR, LGI1, and CASPR2-Abs, respectively.

Comparison of Fixed Versus Live CBA Positive Patients

There were no significant differences between patients with only fixed CBA (n=15) and fixed/live CBA (n=30) Ab positive patients in terms of age, sex, Ab positivity scores, Ab positivity in CSF samples and Ab types. Nevertheless,

patients exhibiting positive results with live CBA showed trends towards displaying higher Ab positivity scores with fixed CBA. Significantly higher number of patients with positive live CBA results fulfilled the criteria for definite AIE (n=21) and all live CBA positive patients fulfilled either the definite or possible (n=9) AIE criteria. By contrast, only 4 of fixed CBA positive patients fulfilled possible AIE criteria and the remaining 11 patients did not fulfill definite, probable or possible AIE criteria (Table 2).

Live CBA positive patients showed significantly higher ratios of short-term memory loss, altered mental status, psychiatric symptoms, epileptic seizures, focal seizures, and abnormal EEG and MRI findings suggestive of AIE than patients with only fixed CBA positive Ab results. There were no significant differences by means of maximum mRS scores and response

Table 2. Demographic and clinical features of cases with anti-neuronal antibodies found positive with fixed cell-based assay (CBA) but not detected with live CBA or tissue-based assay

Case number	Age/Sex	Serum Ab/score*	CSF Ab/score*	VP	Limbic symptoms	Other symptoms	MRI	CSF	EEG	Coexisting disease	Response to IT
1	36/M†	NMDAR+++	(-)	Absent	None	Loss of vision	BL occipital lesions	Normal	Normal	None	Responsive
2	42/M†	NMDAR+++	(-)	Present	None	Ataxia	Cerebellar, brainstem lesions	Normal	Normal	None	Responsive
3	52/F†	CASPR2+	CASPR2+	Absent	None	Ataxia	Normal	Normal	Normal	Breast Ca	Responsive
4	28/F†	NMDAR+	NMDAR++	Present	None	Loss of vision	Meningeal contrast enhancement	400 cells/mm ³	Diffuse slow waves	Ovarian teratoma	Responsive
5	62/M†	NMDAR+	(-)	Present	None	Ataxia	Cerebellar, brainstem lesions	57 cells/mm ³	Normal	None	Responsive
6	61/M†	CASPR2+	(-)	Absent	None	Parkinsonism	Normal	Normal	Normal	None	No response
7	58/F†	LGI1+	(-)	Absent	ST memory loss	None	Normal	Normal	Diffuse slow waves	SLE	No response
8	28/F†	NMDAR+	NMDAR+	Absent	ST memory loss, psychosis	Catatonia	Normal	Normal	Normal	None	No response
9	50/F†	NMDAR+	(-)	Absent	None	Ataxia	Normal	Normal	Normal	None	No treatment
10	28/M†	NMDAR+	NMDAR+	Absent	None	Ataxia	Normal	Normal	Normal	None	No treatment
11	80/F†	NMDAR+	NMDAR+	Absent	None	Dystonia	Normal	Normal	Normal	None	No treatment
12	48/M††	CASPR2+	(-)	Absent	Psychosis	None	BL medial temporal lesions	Normal	Normal	None	No response
13	69/M††	NMDAR++	NMDAR++	Absent	ST memory loss, coma, first seizure, GTCS-SE	Autonomic symptoms	Normal	30 cells/mm ³	Diffuse slow waves, right temporal periodic waves, abnormal response to photic stimulation	None	No response
14	24/M††	CASPR2+	(-)	Absent	ST memory loss, first seizure, GTCS-SE	None	Normal	Normal	Normal	None	No response
15	80/M††	NMDAR+	NMDAR+	Absent	Psychosis, first seizure, GTCS	None	Normal	Normal	Normal	None	No response

* Antibody binding scores with fixed CBA (-, no binding; +, weak; ++, moderate; +++, strong binding)

† Cases with clinical features fulfilling neither definite nor possible autoimmune encephalitis criteria.

†† Cases with clinical features fulfilling possible but not definite autoimmune encephalitis criteria.

M: male; F: female; Ab: antibody; CSF: cerebrospinal fluid; Ca: cancer; SLE: systemic lupus erythematosus; IT: immunotherapy; ST: short term; BL: bilateral; GTCS: generalized tonic clonic seizures; SE: status epilepticus; VP: viral prodromal findings.

to immunotherapy between groups. Nevertheless, NMDAR-Ab positive patients (3.0 ± 1.0) exhibited significantly higher mRS scores than LGI1/CASPR2-Ab positive patients (1.8 ± 1.5) ($p=0.033$).

Although coexisting disorder profiles were comparable in both groups, post-herpes simplex virus (HSV) encephalitis was found only in the live CBA positive group ($n=3$). Likewise, although not statistically significant, certain clinical features such as facial dyskinesia, myoclonic seizures, treatment-resistant seizures and diffuse sharp/spike wave activity on EEG were exclusively found in the live CBA positive group. While CSF pleocytosis ratios and CSF cell counts were comparable among live and fixed CBA positive groups, patients with positive live CBA results showed significantly lower serum NFL levels (Table 1). Twenty-eight of 30 live CBA positive and 12 of 15 fixed CBA positive patients showed elevated (>13.9 pg/ml) serum NFL levels ($p=0.180$). Serum NFL levels did not correlate with parametric variables including mRS scores.

Characteristics of Only Fixed CBA Positive Patients

To better characterize the patients with incongruent CBA results, we cast a spotlight on clinical features of patients, whose samples were found positive on fixed CBA but negative on live CBA (Table 2). Patients, who did not fulfill definite or possible AIE criteria, mostly lacked limbic symptoms and supportive laboratory features suggestive of AIE (Case 1–6, 9–11). Nevertheless, some patients of this group displayed limbic symptoms (Case 7, 8, 12–15), bilateral medial temporal lesions (Case 12), CSF pleocytosis (Case 4, 5, 13), abnormal diffuse EEG findings (Case 4, 7, 13), underlying cancers and response to immunotherapy (Case 1–5), suggestive of autoimmune encephalopathy. Findings of Case 4 (NMDAR-Ab in serum and CSF, meningeal contrast enhancement, CSF pleocytosis, diffuse slow waves, ovarian teratoma and response to immunotherapy) were particularly suggestive of paraneoplastic autoimmune meningoencephalitis. Although these findings were congruent with glial fibrillary acidic protein astrocytopathy, which can be accompanied

Table 3. Comparison of the clinical features of fixed cell-based assay (CBA) positive patients with and without good response to immunotherapy*.

	Responsive (n=23)	Non-responsive (n=15)	p value
Age	51.8±13.4	50.3±22.6	0.410
Sex	14 men	8 men	0.646
Anti-neuronal antibody positive in CSF by fixed CBA	10	8	0.552
Antibody positivity score	1/2–16 3/4–7	1/2–11 3/4–4	0.802
Type of anti-neuronal antibody	CASPR2-3 LGI1-7 NMDAR-13	CASPR2-3 LGI1-3 NMDAR-9	0.015
Diagnosis due to autoimmune encephalitis criteria	12 - definite 6 - possible 5 - not encephalitis	5 - definite 7 - possible 3 - not encephalitis	0.395
Viral prodromal findings	6	0	0.031
Short-term memory loss	13	11	0.294
Altered mental status	8	7	0.464
Catatonia	1	1	0.477
Psychiatric symptoms	14	8	0.646
Autonomic symptoms	1	4	0.014
Movement disorders	4	4	0.493
Facial dyskinesia	0	1	0.209
Epileptic seizures	11	8	0.740
Myoclonic seizures	3	2	0.979
Generalized tonic-clonic seizures	8	7	0.464
Focal seizures	5	5	0.428
Status epilepticus	2	4	0.137
Treatment-resistant seizures	3	3	0.502
Maximum mRS score	1.8±0.9	3.6±2.0	0.010
Coexisting disease	4 cancer 1 HSV encephalitis* 2 autoimmune disease 16 none	1 cancer 1 HSV encephalitis* 1 autoimmune disease 12 none	0.785
Abnormal EEG findings**	14	9	0.804
Periodic wave activity on EEG	1	2	0.315
Abnormal response to photic stimulation on EEG	3	3	0.565
Diffuse slow wave activity on EEG	10	8	0.552
Diffuse sharp/spike wave activity on EEG	1	1	0.754
Diffuse slow or sharp/spike wave activity on EEG	11	8	0.740
Bilateral temporal lobe lesions on MRI	16	9	0.543
CSF pleocytosis ($>5 \text{ mm}^3$)	10	6	0.832
CSF cell count (cells/mm ³)	62.7±119.5	32.5±19.6	0.226
Serum neurofilament light chain (pg/ml)	63.8±64.0	73.8±80.4	0.376

Parametric variables are denoted in terms of mean ± standard deviation.

* Immunotherapy was administered to 38 of 45 fixed CBA positive cases.

** Presence of one or more of periodic wave activity, abnormal response to photic stimulation, diffuse slow wave, diffuse sharp/spike wave activity.

These two patients were diagnosed as post-HSV autoimmune encephalitis.

Bold characters signify statistically significant p values.

CSF: cerebrospinal fluid; mRS: modified Rankin scale; HSV: herpes simplex virus.

Table 4. Comparison of the clinical features of fixed and live cell-based assay (CBA) positive patients with and without good response to immunotherapy*

	Responsive (n=18)	Non-responsive (n=8)	p value
Age	54.0±12.9	48.4±25.4	0.284
Sex	11 men	3 men	0.285
Anti-neuronal antibody positive in CSF by fixed CBA	8	5	0.395
Antibody positivity score	1/2–13 3/4–5	1/2–4 3/4–4	0.272
Type of anti-neuronal antibody	CASPR2-2 LGI1-7 NMDAR-9	CASPR2-0 LGI1-2 NMDAR-6	0.407
Diagnosis due to autoimmune encephalitis criteria	12 - definite 6 - possible	5 - definite 3 - possible	0.837
Viral prodromal findings	3	0	0.219
Short-term memory loss	13	7	0.393
Altered mental status	7	6	0.089
Catatonia	1	0	0.496
Psychiatric symptoms	14	5	0.954
Autonomic symptoms	1	3	0.037
Movement disorders	4	2	0.876
Facial dyskinesia	0	2	0.027
Epileptic seizures	11	5	0.946
Myoclonic seizures	2	2	0.365
Generalized tonic-clonic seizures	8	4	0.793
Focal seizures	5	5	0.093
Epileptic status	2	2	0.365
Treatment-resistant seizures	3	3	0.245
Maximum mRS score	1.6±0.7	4.3±2.0	0.005
Coexisting disease	2 cancer 1 HSV encephalitis* 2 autoimmune disease 13 none	1 cancer 1 HSV encephalitis* 0 autoimmune disease 6 none	0.741
Abnormal EEG findings**	13	7	0.393
Periodic wave activity on EEG	1	1	0.539
Abnormal response to photic stimulation on EEG	3	2	0.619
Diffuse slow wave activity on EEG	9	7	0.069
Diffuse sharp/spike wave activity on EEG	2	1	0.918
Diffuse slow or sharp/spike wave activity on EEG	10	7	0.114
Bilateral temporal lobe lesions on MRI	14	7	0.561
CSF pleocytosis (>5 mm ³)	8	5	0.395
CSF cell count (cells/mm ³)	21.3±12.0	33.0±21.9	0.159
Serum neurofilament light chain (pg/ml)	37.8±24.3	39.4±21.2	0.447

Parametric variables are denoted in terms of mean ± standard deviation

* Immunotherapy was administered to 26 of 30 live CBA positive cases.

** Presence of one or more of periodic wave activity, abnormal response to photic stimulation, diffuse slow wave, diffuse sharp/spike wave activity

These two patients were diagnosed as post-HSV autoimmune encephalitis.

Bold characters signify statistically significant p values.

CSF: cerebrospinal fluid; mRS: modified Rankin scale score; HSV: herpes simplex virus.

with ovarian teratoma (10), serum and CSF of the patient did not yield astrocytic staining pattern on TBA. Likewise, Case 13, also with serum/CSF NMDAR-Ab, fulfilled the criteria for probable anti-NMDAR encephalitis (6) but failed to display any specific IgG reactivity on NMDAR-expressing HEK293T cells or frozen rat brain sections.

Factors Associated with Positive Response to Immunotherapy

When the entire cohort of patients having received first-line immunotherapy (n=38) were analyzed, patients with higher maximum mRS score, with viral prodromal findings and without autonomic symptoms were found out to be more likely to respond to immunotherapy. Also, non-treatment-responsive group had a slightly higher NMDAR-Ab prevalence probably due to the fact that the maximum mRS score of NMDAR encephalitis

patients (3.0±1.0) were significantly higher than LGI1/CASPR2 encephalitis (1.8±1.5, p=0.033) patients (Table 3). Similar factors were associated with treatment-responsiveness, when patients with neuronal Abs detected by both fixed and live-CBA were analyzed (n=26). Patients with higher maximum mRS score, without facial dyskinesia and without autonomic symptoms were more likely to respond to immunotherapy (Table 4). Demographic features, Ab positivity scores, coexisting disorders, limbic symptoms, supportive laboratory findings, and NFL levels were not associated with response to immunotherapy (Table 3, 4).

DISCUSSION

Our findings demonstrate that live cell-based and tissue-based assays provide higher diagnostic reliability than fixed cell-based assays for

the detection of neuronal surface antibodies. Patients with antibodies confirmed by live CBA/TBA frequently fulfilled criteria for definite autoimmune encephalitis and displayed limbic symptoms and MRI/EEG/CSF features consistent with central nervous system inflammation (6). In contrast, a substantial proportion of patients with antibody positivity detected only by fixed CBA did not meet AIE diagnostic criteria, supporting previous observations that fixed CBA may yield clinically irrelevant or false-positive results. Our findings therefore confirm, in a novel ethno-geographic setting, that integration of live CBA/TBA results with established diagnostic criteria can improve the distinction between true AIE and AIE mimics (3–5). By contrast, patients with Abs identified by live and fixed CBAs displayed comparable treatment response and cancer rates, indicating that the main clinical value of live CBA lies primarily in improving diagnostic accuracy rather than predicting treatment response or cancer risk.

In fixed CBA, the fixative may alter the conformation of the target antigen thus decreasing the likelihood of patient IgG binding and overall sensitivity of the assay. Preservation of the native conformation of antigens in live CBA and TBA results in higher sensitivity. Therefore, it is possible that this superior aspect of live CBA/TBA enabled identification of CSF Abs that were not detectable by fixed CBA. Moreover, in alignment with previous studies (3,5), our results demonstrated that false positivity of fixed CBA may be as high as 33% for the most common three neuronal surface Abs to NMDAR, LGI1 and CASPR2. This emphasizes the fact that during diagnostic procedures Ab results should be assessed cautiously and clinicians should always be seeking for an association between the clinical syndrome and identified Ab type.

In five live CBA/TBA negative patients not meeting definite AIE criteria, fixed CBA showed Abs in the CSF, suggesting that detection of Abs in the CSF does not necessarily eliminate false positivity. Although not statistically significant, live CBA/TBA positive patients showed trends towards displaying higher Ab-binding scores on fixed CBA. Therefore, we recommend that in patients showing weak-moderate binding score by fixed CBA and not satisfying the AIE criteria, Ab results should be confirmed by live CBA.

As for the accompanying disorders, the history of HSV encephalitis was the only etiological feature that was exclusively found in live CBA/TBA positive patients and thus could be used as an auxiliary supportive feature for AIE diagnosis. Coexisting autoimmune disorders and cancer were equally prevalent among live CBA/TBA positive and negative patients. Notably, in case 7 having the history of systemic lupus erythematosus, none of the investigated Abs could be identified by live CBA/TBA, in alignment with our previous report (11).

Among supportive laboratory findings, diffuse slow waves on EEG and bilateral temporal lesions on MRI stood out as factors closely associated with live CBA/TBA positivity. Furthermore, investigation of a wide array of EEG abnormalities such as periodic activity and response to photic stimulation in addition to slow and sharp/spike wave activity increased the sensitivity of EEG as a tool for diagnosing AIE. Notably, patients with live CBA/TBA positivity showed trends towards exhibiting increased prevalence of focal, myoclonic, generalized-tonic clonic and treatment-resistant seizures, implying that these features can be a part of the AIE diagnostic criteria. Similar to a previous publication (2), CSF pleocytosis is found in both live and fixed CBA positive patients and thus inflammatory CSF features have lower supportive influence in the diagnosis of AIE.

Notably, live CBA/TBA positive and negative patients showed comparable response to immunotherapy, suggesting that patients with Abs identified by fixed CBA only may also show response to immunosuppressive treatment. Closer inspection of fixed CBA positive patients in Table 2 revealed that a significant proportion of the patients exhibited features

suggestive of AIE such as underlying cancer, viral prodromal findings, bilateral temporal lobe lesions, CSF pleocytosis, limbic symptoms and favorable response to immunotherapy and Case 13 even met the criteria for probable anti-NMDAR encephalitis. Similar live CBA negative patients with probable autoimmune-like features have been reported and in a recent publication, these individuals were categorized as patients with probable neuroinflammatory disorder (2).

It is well known that NFL levels are increased in AIE, as was the case in our AIE cohort (12). Interestingly, NFL levels were even higher in the fixed CBA positive group than those of the live CBA positive patients fulfilling AIE criteria. Also, patients with and without treatment response showed comparable NFL levels. These findings reduce the utility of NFL as a diagnostic and prognostic biomarker in AIE. The lack of correlation between disease activity and NFL levels has already been reported (12). Moreover, increased NFL levels along with elevated CSF cell counts and failure to identify an underlying etiological factor in a respectable follow-up period in the fixed CBA positive group suggests that at least some of the patients of this group might be undergoing a neuroinflammation-induced neurodegeneration process, as discussed above. Absence of an appreciable IgG reactivity with neurons and glial cells of rat brain sections suggests that cellular (rather than humoral) immunity might be involved in these patients.

Finally, it is tempting to speculate that serum/CSF Abs identified by only fixed CBA might be reacting with intracellular linear epitopes released and exposed to the immune system through inflammation-induced neuronal destruction and might actually be an indication of the ongoing neuroinflammation. This assumption can be verified through comparison of clinical, laboratory, treatment response and Ab-detection features of individuals with neurological symptoms that are negative on both fixed and live CBA/TBA and absence of such a control group was a limitation of this study. Another limitation is the potential selection bias resulting from the inclusion of only a subset of initially identified antibody-positive patients. Although 208 positive cases were detected during the screening period, the present analysis focused on 45 patients with available paired serum/CSF samples and sufficiently detailed clinical follow-up data. While this approach allowed a more reliable clinicoradiological correlation, it may limit the generalizability of the findings. In addition, our data were retrospectively collected, and the sample size was limited for less common antibodies such as LGI1 and CASPR2.

Overall, our results confirm the previously established overlap between live CBA results and definite AIE criteria, verify previously proposed AIE-associated clinical and laboratory features and offer new factors that might potentially be helpful in the differential diagnosis and treatment decisions of AIE. Our findings also emphasize the presence of non-AIE patients with probable neuroinflammatory disorder and bring forward the need for innovation of clinical criteria and novel biomarkers for immunotherapy responsiveness.

Ethics Committee Approval: The study protocol was approved by the Istanbul University Istanbul Medical Faculty Clinical Research Ethics Committee (date:25/02/2022, number: 2022/338).

Informed Consent: Written informed consent was obtained from all patients.

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Data Availability Statement: Anonymized data will be shared at request by qualified investigators after the execution of appropriate materials transfer agreements.

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