Clinical commentary

Epileptic Disord 2016; 18 (2): 204-10

Neuronal autoantibodies in patients with Rasmussen's encephalitis

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Received January 14, 2016; Accepted April 07, 2016

ABSTRACT – *Aim*. Rasmussen's encephalitis (RE) is a rare disease with unknown pathophysiology. To disclose whether anti-neuronal autoimmunity participates in the aetiology of RE, various neuronal autoantibodies (NAAbs) were investigated in sera of patients with RE and controls.
Methods. The study included five patients who fulfilled the RE diagnostic criteria (clinical, EEG, and MRI findings) as the patient group, and 50 multiple sclerosis patients and 50 healthy subjects as the control groups. Sera were evaluated for various NAAbs by radioimmunoassay or cell-based assays. All sera were also screened for uncharacterized antibodies to neuronal cell surface or synapse antigens by indirect immunofluorescence using hippocampal cell cultures.
Results. The mean age at onset of seizures was 8.3±3.4 years (range: 4-13.5) and mean follow-up time was 11.2±5.4 years (range: 5-19). All patients had unihemispheric atrophy of the cerebral cortex and *epilepsia partialis continua*. Two of the patients had moderate cognitive impairment, while the others were severely affected, as shown by neuropsychological testing. NAAb positivity was not detected in any of the patients.
Conclusion. Immune aetiology is thought to have a role in RE, but the responsible players have not yet been elucidated. Our extensive antibody screening in a small number of patients does not support the presence of antigen-specific anti-neuronal autoimmunity in RE pathophysiology.

Key words: anti-neuronal antibodies, autoimmunity, neuronal autoantibodies, Rasmussen's encephalitis, Rasmussen syndrome

Rasmussen's encephalitis (RE) is a rare chronic progressive disease characterized by drug-resistant focal epilepsy, hemiparesis, cognitive decline, and unihemispheric atrophy of the cerebral cortex (Bien *et al*., 2005). Approximately half of the patients have epilepsia partialis continua (EPC). The disease affects mostly children and young adults and its diagnosis can be confirmed by the consensus criteria that include clinical, electroencephalographic, and neuroimaging findings (Bien *et al*., 2005).

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doi:10.1684/epd.2016.0829

As RE is hypothesized to have an immune-mediated aetiology, some studies have been focused on autoantibodies. The first neuronal autoantibody (NAAb) identified in RE was anti-GluR3, implicating the presence of antibody-mediated mechanisms in RE pathophysiology (Rogers *et al*., 1994; Wiendl *et al*., 2001). Other studies also found antibodies against some other neuronal antigens, such as α-7 nicotinic acetylcholine receptor, GluR2-GluR3 subunit complex of the AMPAR, and mammalian uncoordinated (Munc)-18-1 (Yang *et al*., 2000; Watson *et al*., 2005; Alvarez-Baron *et al*., 2008; Nibber *et al*., 2016). However, all these antibodies could only be demonstrated in a small group of patients, and these results were not replicated by other authors (Watson *et al*., 2004).

In recent years, various newly discovered NAAbs were reported to be associated with drug-resistant epilepsy syndromes (Ekizoglu *et al*., 2014). Uncovering the possible role of specific NAAbs in the pathophysiology of RE is very important in terms of disease management and treatment options. The aim of this study was to investigate the presence of NAAbs, and thus specific antibody-mediated anti-neuronal autoimmunity in RE.

Methods

Five patients (three females and two males), diagnosed with RE in two epilepsy centres in Istanbul during a one-year period according to the suggested criteria (Bien *et al*., 2005), were included in this study. The study was approved by the ethics committee and written informed consent was obtained from all patients. The patients were evaluated by a neurologist in terms of age at onset, follow-up time, seizure types, neurological examination, EEG, and MRI findings. All patients underwent neuropsychological testing by an experienced neuropsychologist.

Blood samples were obtained in order to evaluate antibodies against voltage-gated potassium channel (VGKC)-complex antigens, contactin-associated protein-like 2 (CASPR2), leucine-rich glioma inactivated 1 (LGI1), glutamic acid decarboxylase (GAD), N-methyl-D-aspartate receptor (NMDAR), glycine receptor (GlyR), alpha-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid receptor (AMPAR), and type A and B gamma aminobutyric acid receptors ($GABA_AR$ and $GABA_BR$). For all antibody assays, the sera from autoimmune limbic encephalitis patients with well-characterized antibodies (5-8 patients for each antibody), 50 relapsing remitting multiple sclerosis (MS) patients, and 50 healthy individuals were used as controls. Sera were stored at -80°C until analysis. GlyR and $GABA_AR$ antibody assays were performed by one of the authors (EE) at the University of Oxford, UK, and the remaining assays were performed at the University of Istanbul, Turkey. Ion channel antibodies were analysed using a commercial kit (Euroimmun, Luebeck, Germany) involving human embryonic kidney 293 (HEK293) cells transfected with plasmids containing the genes for the NR1/NR2 subunits of NMDAR, GluR1/GluR2 subunits of AMPAR, CASPR2, LGI1, and $GABA_BR$. Antibodies against the α1 subunit of GlyR and α1/γ2/β2 subunits of $GABA_AR$ were similarly analysed using HEK293 cells transfected with plasmids containing the respective genes. Transfected cells were incubated with patients' sera (1:20) and the appropriate Alexa Fluor secondary antibody, as described earlier (Carvajal-González *et al*., 2014; Pettingill *et al*., 2015). HEK293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum (TCS Cellworks Ltd, Buckingham, UK) and 100 units/ml each of penicillin G and streptomycin at 37°C in a 5% CO_2 atmosphere. Cells were grown on 13-mm glass coverslips placed in six-well cell culture plates for microscopy. Using polyethylenimine, cells were transiently co-transfected with the plasmids mentioned above. Forty-eight hours after transfection, the coverslips were transferred to individual wells in 24-well culture plates and incubated at room temperature for 45 minutes with patient serum (1:20); these were diluted in Dulbecco's modified Eagle's medium buffered with HEPES, with added 1% bovine serum albumin to block non-specific binding. The cells were subsequently washed three times in Dulbecco's modified Eagle's medium/HEPES buffer and fixed with 3% formaldehyde in phosphate buffered saline at room temperature for 15 minutes, followed by further washing. They were then labelled for 45 minutes at room temperature with anti-human IgGAlexa Fluor 568-conjugated secondary antibody (Invitrogen-Molecular Probes, Paisley, UK) at 1:750 in 1% bovine serum albumin/Dulbecco's modified Eagle's medium/HEPES buffer. All coverslips were subsequently washed three times in phosphate buffered saline and mounted on slides in fluorescent mounting medium (DakoCytomation, Cambridge, UK) with DAPI. The binding was scored visually on a range from 0 (negative) to 4 (very strong), as previously described (Irani *et al*., 2010a, 2010b; Zandi *et al*., 2015). Only scores greater than 1 were considered positive due to the low level of non-specific signal. For detection of antibodies against uncharacterized VGKC-complex antigens, a radioimmunoassay (RIA) kit was utilized (normal value: <50 pM; RSR, Cardiff, UK). Patients' sera were added to detergent-solubilized VGKC complexes extracted from rabbit brain tissue and ^{125}I-labeled α-dendrotoxin was added to form a complex with the antibodies. After incubation at 2-8°C overnight, the resulting antigen-antibody complexes were immunoprecipitated by the addition of anti-human IgG. After a second incubation of 1.5 hours,

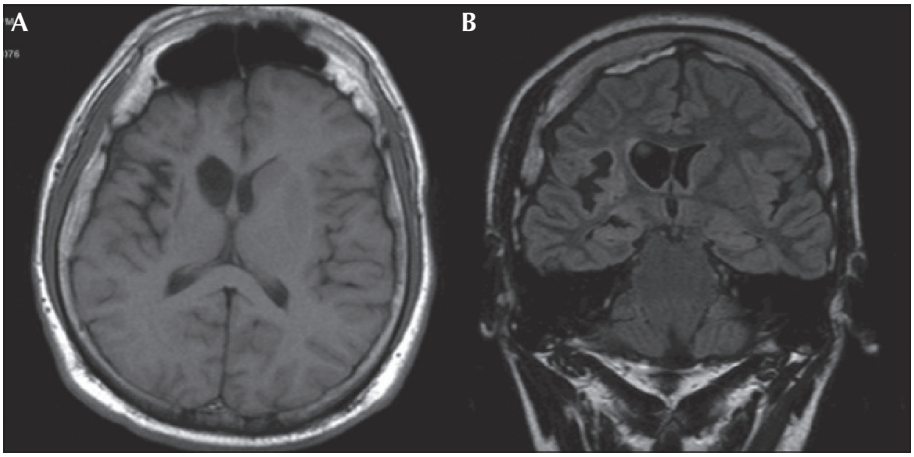

Figure 1. Axial T1-weighted (A) and coronal FLAIR (B) MR images of Patient 2, demonstrating typical atrophy on the right hemisphere.

assay buffer was added and the samples were centrifuged. Unbound ^{125}I-labelled α-dendrotoxin-VGKC complex was removed from the tubes by aspiration of the supernatant. The level of radioactivity remaining in the tube, which is proportional to the antibody level in the test sample, was measured with a gamma counter and expressed as pM. GAD antibodies were measured quantitatively using an ELISA kit, as per the manufacturer's recommendations (normal value: <10 U/ml; Euroimmun, Luebeck, Germany). Sera were incubated with GAD coated onto a microplate and biotin-labelled GAD, consecutively. To detect the bound biotin, a third incubation was performed using enzyme-labelled avidin, promoting a colour reaction, the intensity of which is proportional to GAD antibody levels. The obtained optical density levels were converted to U/ml values using serial standard concentrations.

In addition, antibodies against uncharacterized neuronal surface and synapse antigens were investigated, as described previously (Irani *et al*., 2010b). Briefly, sera (1:250) were incubated for one hour at room temperature with neurons cultured from newborn (P1) rat hippocampus. After extensive washing, neurons were fixed with 3% formaldehyde for 15 minutes and incubated with Alexa Fluor 488-conjugated anti-human IgG (Invitrogen, UK) at 1:1,000 for 45 minutes. Subsequently, the cells were permeabilized with 0.3% Triton X-100 in PBS for 15 minutes and were incubated for one hour at room temperature with mouse monoclonal antibody against microtubule-associated protein 2 (MAP-2: 1:1,000; Sigma-Aldrich, UK), a marker of axonal and dendritic processes. The cells were then immunolabelled with Alexa Fluor 548-conjugated anti-mouse IgG (Invitrogen) at 1:1,000 dilution for 45 minutes. Cells were mounted and images were photographed under a Zeiss fluorescence microscope with a digital camera using the Zeiss Axiovision software.

Results

The mean age at onset of RE was 8.3±3.4 years (range: 4-13.5) and mean follow-up period was 11.2±5.4 years (range: 5-19). All patients had unihemispheric atrophy of the cerebral cortex and EPC. MR images of one illustrative case are shown in *figure 1*. While two of the patients had moderate cognitive impairment, other patients were severely affected, as shown by neuropsychological testing. Demographic features and clinical, EEG, and neuroimaging findings of all patients are presented in *table 1* (Tekturk *et al*., 2016).

None of the RE patients, MS patients, or healthy controls showed positivity for any of the well-characterized antibodies. Likewise, none of these participants' sera displayed antibodies to uncharacterized neuronal cell surface/synapse antigens investigated by immunofluorescence performed using hippocampal neuronal cultures. In contrast, all sera from control limbic encephalitis patients showed intense reactivity to cultured neuronal cells and HEK293 cells expressing NR1/NR2 heteromers of NMDAR, as shown in *figure 2*.

Discussion

RE has long been considered as the prototypic inflammatory epilepsy syndrome, and is presumed to have an immune basis with unknown mechanisms (Bien *et al*., 2013; Varadkar *et al*., 2014). T-cell/antibody-mediated mechanisms and microglia-induced toxicity are speculated to regulate potential immunopathological mechanisms that might have a role in central nervous system degeneration.

In this study, we aimed to delineate antibody-mediated mechanisms as a potential source of neuronal death in RE. Since antibodies against cell surface and synapse

Table 1. Clinical, neuroimaging, and EEG findings of patients diagnosed with Rasmussen encephalitis.

Patient	Gender	Age at onset (years)	Follow-up duration (years)	Seizure types	Neurological examination + cognitive impairment	MRI	EEG
1	F	9	5	Left focal + EPC	Left pyramidal signs + moderate cognitive impairment	Right hemisphere atrophy	Right centro-temporal and frontal spike and wave discharges
2	M	13.5	10	Left focal + EPC + generalized and right focal	Left pyramidal signs + moderate cognitive impairment	Right hemisphere atrophy	Generalized slowing and secondary generalized epileptiform discharges prominent in left frontocentral region
3	F	4	8	Left focal + EPC	Moderate motor impairment + severe cognitive impairment	Right rolandic, parietal and occipital atrophy	Right fronto-centro-temporal epileptic discharges
4	F	7	14	Left focal + EPC + secondary generalization	Left pyramidal signs + severe cognitive impairment	Right hemisphere atrophy prominent in frontal lobe	Epileptic activity over right fronto-central region, later diffusing over left hemisphere
5	M	8	19	Right focal + EPC	Right pyramidal signs + severe cognitive impairment	Left hemisphere atrophy	Slowing of the background activity and multifocal epileptic activity over the left hemisphere

EPC: epilepsia partialis continua. The patients included in this study were already diagnosed and treated as patients with Rasmussen encephalitis (Tekturk *et al*., 2016).

antigens are generally believed to have a pathogenic significance (Vincent *et al*., 2010), we screened sera of RE patients with a large panel of antibodies against neuronal ion channels and synapses. However, extensive antibody screening for well-characterized and, as yet, uncharacterized antibodies against neuronal cell surface antigens failed to identify any specific anti-neuronal autoimmunity in RE patients.

The first NAAb against GluR3 was shown in animal models and in a small number of RE patients, implicating the possible role of these antibodies in RE pathogenesis (Rogers *et al*., 1994). However, in subsequent studies, GluR3 NAAb positivity was found in a minority of RE cohorts (Wiendl *et al*., 2001) and only a few RE patients benefited from plasmapheresis or IVIg treatments (Granata *et al*., 2003). In other studies, antibodies against alpha-7 nicotinic acetylcholine receptor or Munc-18-1 were detected in the sera of some RE patients, however, lack of these antibodies in subsequent studies suggested that these antibodies are not specific or important players in RE (Yang *et al*., 2000; Alvarez-Baron *et al*., 2008).

Although GluR3 antibodies initially evoked some enthusiasm, their presence and pathogenic role in RE have not really been reliably confirmed (Watson *et al*., 2004). Many previous studies have used ELISA to detect the antibodies against short linear peptides of GluR3 (Wiendl *et al*., 2001; Levite and Ganor, 2008), but these

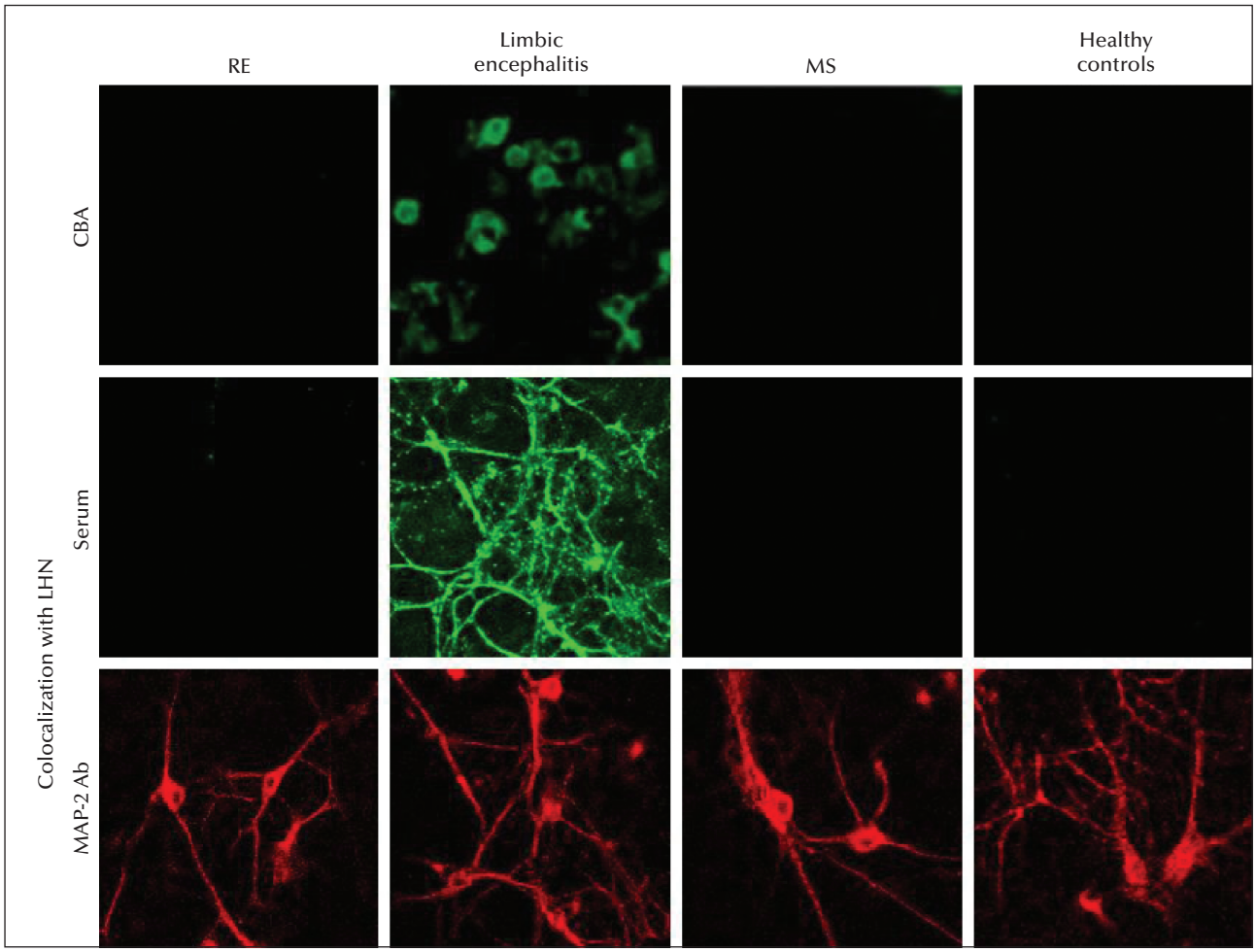

Figure 2. Antibody assays performed using sera from patients with Rasmussen encephalitis (RE), autoimmune limbic encephalitis, multiple sclerosis (MS), and healthy controls. The cell-based assay (CBA) shows that serum antibodies from an autoimmune limbic encephalitis patient reacted (green) with HEK 293 cells expressing NR1/NR2 heteromers of the N-methyl-D-aspartate receptor (NMDAR). In contrast, serum IgGs of RE and MS patients and healthy controls did not show any immunoreactivity (upper row). Likewise, cultured hippocampal neurons incubated with serum from an NMDAR-antibody positive limbic encephalitis patient demonstrated intense immunolabelling (green) of neuronal membrane/synapse antigens, whereas serum antibodies from other patients and healthy controls did not show any reactivity to cultured hippocampal neurons (middle row). Immunoreactivity of serum IgG from a limbic encephalitis patient significantly co-localized with microtubule-associated protein-2 (MAP-2) antibody (Ab) (red) (lower row). Original magnification in upper panels (400x) and middle and lower panels (800x).

results have not been confirmed by demonstrating binding to native GluR3 expressed in intact mammalian cells (Watson *et al*., 2004).

Therefore, instead of investigating non-specific antibodies directed against linear epitopes of GluR3 peptides, we investigated antibodies against GluR1/GluR2 subunits of the AMPA receptor, which have been detected frequently in autoimmune encephalitis patients and have been found to be specific to certain forms of limbic encephalitis (Peng *et al*., 2015). In a report by Nibber *et al*. (2016), at the University of Oxford, published shortly after this manuscript was submitted for publication, antibodies against GluR2/GluR3 were identified for the first time using HEK cells co-transfected with these AMPAR subunits. Since these antibodies putatively recognize conformational epitopes expressed by the GluR2/GluR3 complex, they are completely different to previously described GluR3 antibodies. In this very recent study, GlyR antibodies were not studied. Overall, our findings therefore largely support the data of Nibber *et al*. and together reveal a comprehensive panel of anti-neuronal antibodies in Rasmussen encephalitis.

Clinical descriptions and reports of positive responses to immunotherapy in patients with LGI1, AMPAR,

$GABA_BR$, and NMDAR antibody-mediated limbic encephalitis presenting with seizures (Vincent *et al*., 2010) revived the idea of an antibody-mediated basis to RE. The fact that these antibodies can cause a wide range of clinical presentations and mimic the early stages of RE was shown in a case report of anti-NMDAR antibody-mediated encephalopathy (Greiner *et al*., 2011). Recently, VGKC-complex antibodies, unreactive to LGI1 or CASPR2, were reported to be positive in a child with RE (Spitz *et al*., 2014). However, the small number of antibody-positive RE cases in the entire medical literature, as well as our results, suggest that these ion channel antibodies form a response to a pathological inflammation process rather than being the main cause. Also, the lack of desired success with plasmapheresis treatment in RE cases has indicated that the role of NAAb in the pathology of RE is highly questionable. On the other hand, immunosuppressive therapy has been shown to slow the expected rate of progression in RE, particularly the hemispheric atrophy on MRI (Bien *et al*., 2013), and this finding suggests an immune basis of the disease.

In conclusion, the nature of RE immunopathogenesis is still unclear. Our study suggests that antigen-specific neuronal autoimmunity is probably not the main cause of RE pathogenesis. Therefore, investigation of antibodies against other cell types (*e.g*. glial or endothelial cells) and delineation of T-cell mediated and microglia-induced disease mechanisms in large RE cohorts is of great importance for appropriate management of this disease and development of new therapeutic strategies. □

Acknowledgements and disclosures.

The authors thank the participants for taking part in the present study. We would like to particularly thank Sian Peach (Nuffield Department of Clinical Neurosciences, University of Oxford, UK) for her help and contribution to GlyR and $GABA_AR$ antibody assays.

This study was supported by the Turkish Scientific and Technical Research Council; no. 214S170.

None of the authors have any conflict of interest to disclose.

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TEST YOURSELF

(1) Are the major players in the presumed immune aetiology of Rasmussen's encephalitis known?

(2) Do patients with Rasmussen's encephalitis have antibodies against VGKC-complex, CASPR2, LGI1, GAD, NMDAR, GlyR, AMPAR, $GABA_AR$, and $GABA_BR$ antigens?

(3) Does the sera from patients with Rasmussen's encephalitis contain some uncharacterized antibodies against neuronal cell surface or synapse antigens when investigated by indirect immunofluorescence using hippocampal cell cultures?

Note: Reading the manuscript provides an answer to all questions. Correct answers may be accessed on the website, www.epilepticdisorders.com, under the section "The EpiCentre".