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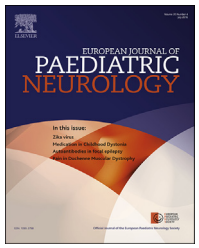

Original article

Autoantibodies to neuronal antigens in children with focal epilepsy and no *prima facie* signs of encephalitis

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ABSTRACT

Objective: There is increasing awareness of neuronal autoantibodies and their impact on the pathogenesis of epilepsy. We investigated children with focal epilepsy in order to provide an estimate of autoantibody frequency within a pediatric population without *prima facie* evidence of encephalitis using a broad panel of autoantibodies. This was done to assess the specificity of antibodies and to see whether antibodies might be of modifying influence on the course of focal epilepsies.

Method: We searched for autoantibodies in 124 patients with focal epilepsy (1–18 years; mean 10; 6 years). Sera were tested using a broad panel of surface and intracellular antigens.

Results: We found autoantibodies in 5/124 patients (4%): high-positive GAD65 antibodies ($n = 1$), low-positive GAD65 antibodies ($N = 1$), VGKC complex antibodies not reactive with LGI1 or CASPR2 ($n = 3$). We did not find any distinctive features distinguishing antibody positive patients from those without antibodies.

Conclusions: The antibodies found in this cohort are probably neither disease-specific nor pathogenic. This has been suggested before for these antigenic targets. Moreover, they do not seem to modify disease severity in the antibody-positive epilepsy patients.

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1. Introduction

There is an increasing interest in immunopathogenic mechanisms in the epilepsies. During the last decade, specific autoantibodies in patients with autoimmune encephalitides have been identified. About 80% of these patients with pathogenic effects of the adaptive immune system have seizures.[1] "Autoimmune epilepsies", i.e., autoimmune encephalitides with seizures as the only or the predominant symptom, have been diagnosed not only in adults but also in pediatric patients. In series selected by the clinical suspicion of autoimmune encephalitis, up to 44% of children had antineural antibodies,[2] One may hypothesize that not only patients with typical encephalitic features but also other epilepsy cases may have an autoantibody-related etiology. Recent data linking autoimmunity in general to a higher risk of epilepsy may point into this direction: Ong et al. demonstrated a significantly heightened risk of epilepsy in patients with other autoimmune diseases with an odds ratio of 3.8 in a large population based study with more than 2.5 million patients. In children, this odds ratio was even as high as 5.2.[3] Studies in distinct autoimmune conditions point to the same direction: The prevalence of epilepsy is eight times higher in patients with systemic lupus erythematosus (SLE) than in the general population, multiple sclerosis and epilepsy may occur together more commonly than by chance.[4,5]

The specificity of antibodies has been estimated by the plausible association with specific encephalitic syndromes. Here, those clinical constellations were deliberately excluded. So, first of all, this cohort is a kind of "negative control" cohort for antibody specificity. That means: any antibodies found in this cohort may be considered as being of doubtful specificity.

In addition, we considered the possibility that antibodies in this cohort even though not being qualitatively specific (syndrome-specific), they might still be related "quantitatively" to the severity of the epilepsies, i.e., might be associated with a more severe, pharmacoresistant phenotype. Children with structural-metabolic epilepsies more often have persisting seizures despite adequately chosen drugs than e.g. patients with typical benign epilepsy with centrotemporal spikes (BECTS). Yet again, some patients with presumed problematic constellation will become seizure free without any problems whereas others without any obvious findings might run a devastating course. A comparison of patients with well controlled epilepsies with a cohort of difficult to treat patients might add information on a possible modifying role of autoimmune mechanisms on the course of epilepsies in children.

2. Methods

2.1. Participants

The study population consisted of patients ≥1 and ≤18 years with focal epilepsy. Two different groups were recruited depending on the course of epilepsy of last six months irrespective of autoantibodies which were analyzed *en bloc* at the end of the study. The patients were classified before the antibody analysis was done in terms of epilepsy type and treatability. We did not intend to include all patients with epilepsy at the participating centers but rather to create two distinctive groups: well controlled epilepsies compared to a cohort of difficult to treat epilepsies. In order to avoid any overlap the first group consisted of patients without severe problems concerning seizure control ("easy to treat group" – ett, group 1). Inclusion criteria were a maximum of one seizure during the last six months, a present combination therapy of at most two drugs and not more than three different drugs for long term treatment in their treatment history. Additional emergency treatment with diazepam, lorazepam, etc. in the past was accepted. The other group consisted of patients with difficult to treat epilepsies. Criteria were persisting seizures – at least two seizures during the last six months despite adequately chosen drugs – and treatment with at least three different drugs in the past ("difficult to treat group" – dtt, group 2). Patients not completely fulfilling the criteria of respective groups were not included in the study. This also accounted for children in whom either the patients themselves or their parents were not willing to participate.

In Germany pediatric patients with an uncomplicated course are mainly treated in specialized medical practices. Depending on regional needs also some ambulatory services of hospitals are allowed to treat patients on an outpatient base. For patients with a more complicated course epilepsy centers serve as tertiary referral centers with special emphasis on difficult to treat epilepsies. As we intended to include patients from both ends of the spectrum, different sites were asked to participate. No financial compensation was paid for the inclusion of a patient and the documentation for study purpose and work-up. Thus, we anticipated that due to workload most centers might be able to recruit just a limited series of patients. To keep a possible bias as small as possible we tried to include patients at least as a consecutive series in different centers.: Center for Child and Adolescent Medicine, HELIOS Hospital Wuppertal, Epilepsy Center Kork, Epilepsy Center Vogtareuth, Neuropediatric Department of the University Kiel, the Neuropediatric Medical Office Hirschaid and the Center of Developmental Neurology Frankfurt included the patients and collected blood samples. Patients' history including detailed information on the course of the epilepsy was derived from patients' records and completed by recent history given either by the patients themselves or their parents. Seizures and epilepsies were classified according to the new organization of the epilepsies by the participating centers as provided by the International League against Epilepsy (ILAE).[6]

2.2. Laboratory methods

After obtaining informed consent of patients and their parents the additional blood samples for study purpose were taken on the different study sites when a routine blood test was ordered. Cerebrospinal fluid testing was not part of the study. Blood samples were centrifuged at 5.000 U/min and serum was stored at study sites at a temperature of −20 °C or

less. All specimens were pseudonymized. At the end of the study blood samples were analyzed *en bloc* at the laboratory of the Epilepsy Center Bethel (CGB) for IgG autoantibodies. Cell based assays (Euroimmun, Lübeck, Germany) were used to find IgG antibodies against glutamic acid decarboxylase 65 (GAD65), the N-methyl-D-aspartate receptor (NMDAR), gamma-aminobutyric-acid-B-receptor ($GABA_BR$), 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid receptor subunits 1/2 (AMPA1/2-R), glycin-receptor, leucine-rich, glioma inactivated 1 (LGI1), contactin-associated protein-like 2 (CASPR2), metabotropic glutamate receptor subunit 5 (mGluR5), and dipeptidyl-peptidase-like protein (DPPX). The secondary system consisted of a goat-anti-human IgG (heavy and light chain [H + L]) antibody conjugated with DyLight 594 produced by Jackson ImmunoResearch, West Grove, PA, USA, Code No. 109-515-088 at a dilution of 1:100. IgG positivities were confirmed by use of a second test round with goat-anti-human IgG-Fcγ fragment, Jackson 109-515-008 DyLight 594 at a dilution of 1:100. Positive samples were endpoint titrated on the transfected cells to multiples of 1:2 with the H + L antibody. Evaluation was done by two experienced investigators; in cases of disagreement, the mean of the two values was noted. High-titer GAD65 antibodies were defined as in Saiz et al. as ≥1:500.[7] All other titers were considered low-positive. Immunoblots (Ravo, Freiburg, Germany) were used to detect the following antibodies: SOX-1, Delta/Notch-like Epidermal Growth Factor-Related Receptor (DNET, formerly known as Tr), amphiphysin, collapsing response mediator-proteine 5 (CV2.1/CRMP5), Ma1, Ma2, Hu, Ri, Yo, Zic4. The diagnosis of these antibodies required a band on the immunoblot and corresponding staining patterns on indirect immunofluorescence study on a mouse brain sections. Voltage-gated potassium channel complex (VGKC) IgG antibodies were diagnosed by radioimmunoprecipitation with dendrotoxin (RSR Cardiff, UK) by the Krone Laboratory (DM) in accordance with the manufacturer's recommendations. Values >100 pmol/l were taken as positive according to the index publication on this assay in patients with limbic encephalitis.[8] Readers of all tests were blind to the clinical and demographic information of the patients.

2.3. *Ethics, sample size*

All procedures of this study were consistent with the Declaration of Helsinki. The study was approved by the institutional ethics committee of Witten/Herdecke University as well as by the respective ethic committees of the participating study sites. Written informed consent was obtained from all children if appropriate for age and all parents. A sample size of 120 was intended as the existing studies with a similar scope had sample seizes in the range of 50–120.

3. Results

With end of May 2014, recruiting was terminated with different time periods of recruiting and included patients due to organizational issues and time constraints. Most patients were included in Wuppertal (**n** = 62; April 2011 to December 2013) and Hirschaid (*n* = 22; April 2013 to August 2013). The other participating centers mainly included patients of the "difficult to treat group" (group 2): Kork (*n* = 12; April 2012 to June 2012), Vogtareuth (*n* = 7; July 2011 to August 2011, June 2012), Kiel (*n* = 16; March 2014 to May 2014), Frankfurt (*n* = 5; November 2012 to December 2012). For characteristics of the 124 participants, see Table 1. There were no statistical significant differences concerning age and sex. Seizures in the group with difficult to treat epilepsies (group 2) started significantly earlier than in the "easy to treat group" (group 1) leading to a longer disease duration. There were no statistical significant differences ($p = 0.106$; chi^2) between both groups with respect to etiology of epilepsy according to the ILAE.[6]

3.1. *Antibodies*

Five patients had serum antibodies (Table 2): high-positive GAD65 antibodies (*n* = 1, 1:64,000), low-positive GAD65 antibodies (*n* = 1, 1:100), VGKC complex antibodies not reactive with LGI1 or CASPR2 (*n* = 3: 223 pmol/l, 147 pmol/l, 142 pmol/l).

We did not find any distinctive features distinguishing antibody positive patients from those without antibodies. This also holds true for a comparison of the two groups: Three patients were "easy to treat" (group 1: the two with GAD antibodies and one with VGKC complex antibodies) and two were "difficult to treat" (group 2: VGKC complex antibodies). Even on re-evaluation after breaking the blind, they were not felt as having specific features or being comparable to the known encephalitides that have been associated with GAD65 or VGKC complex antibodies. None of the patients in the antibody positive group were treated with immunotherapy.

4. Discussion

We found serum autoantibodies in 5/124 (4%) of these unselected children with focal epilepsy. Interestingly, no antibodies that are as specific as those against LGI1 or CASPR2 or antibodies against the NMDAR were detected. The antibodies that were detected have been considered as being of doubtful relevance in the literature. GAD65 antibodies – especially those with low titers – can be related to purely non-neurological autoimmunity, especially to Diabetes mellitus type I.[9] High-titer antibodies have been found in patients with neurological syndromes like limbic encephalitis, cerebellar ataxia or stiff-man syndrome; the specificity and pathogenicity of the GAD65 antibodies themselves, however, has be questioned.[10] Intrathecal synthesis of GAD65 antibodies might be an additional argument for their pathogenicity, but cerebrospinal fluid (CSF) was not available in this study.[7] Similarly, serum antibodies to the VGKC complex that do not bind to the defined and specific antigens LGI1 or CASPR2 are of doubtful pathogenic significance. One reason is that the antigen could be located on the intracellular side of the cell membrane.[11,12]

Table 1 – Characterization of the 124 patients.

	Male/female	Median age (mean, SD)	Median disease duration (mean, SD, range)	Positive family history	Epilepsy classification	History of status epilepticus
All patients	62/62	11; 0 yrs (10; 6 ± 4; 11 yrs)	4; 3 yrs (5; 6 ± 4; 3 yrs. 0; 7–17; 3 yrs)	30/124	Structural/metabolic: 79/124 Unknown: 45/124	22/124
Easy to treat group (group 1)	21/29	11; 11 yrs (11; 3 ± 4; 9 yrs)	3; 4 yrs (4; 5 ± 3; 9 yrs. 0; 7–16; 3 yrs)	17/50	Structural/metabolic: 31/50 Unknown: 19/50	2/50
Difficult to treat group (group 2)	41/33	11; 0 yrs (10; 0 ± 4; 11 yrs)	5; 4 yrs (6; 3 ± 4; 6 yrs. 0; 8–17; 3 yrs)	13/74	Structural/metabolic: 48/74 Unknown: 26/74	20/74

SD = standard deviation.

In the present series, only patients with duration of their epilepsy of at least six months were included, ruling out patients with an acute encephalitic presentation. Even a second, unblinded look to the antibody-positive cases did not reveal encephalitic features. This suggests that the antibodies found here were of uncertain significance. There were no highly specific antibodies in this cohort. This means that definite antibody mediated adaptive autoimmunity seems to be very rare in the pediatric population with focal epilepsies. Our series might not be representative for all pediatric epilepsies as we excluded patients with generalized epilepsies as well as some patients with focal epilepsies not matching our inclusion criteria. However, both groups represent a wide spectrum of childhood epilepsies including both, patients with benign development of their epilepsy as well as children running a devastating course.

Suleiman et al. prospectively studied 114 children for serum autoantibodies.[13] The main difference to our study was that they included only children with new onset epilepsy. They found a 10% rate of children with autoantibodies ($n = 11$). The proportion in a control group without seizures was 5% (i.e., close to our figure of 4%), the difference not being significant. The authors found serum IgG against the following antigen: VGKC complex, but neither LGI1 nor CASPR2 ($n = 4$), CASPR2 ($n = 3$), NMDAR ($n = 2$, CSF not tested, therefore of questionable significance),[12] or double-positive ($n = 2$) with VGKC-complex antibodies and NMDAR antibodies (CSF not tested). The three positive control patients (non-epilepsy hospital patients) had antibodies against the VGKC complex, but neither LGI1 nor CASPR2 ($n = 2$) or against the NMDAR (CSF not tested).

Bektas et al. examined 80 patients with epilepsies that responded well to steroids or immunomodulatory agents and an acute or subacute (<12 weeks) onset but undetermined underlying cause.[14] The group found antineural antibodies in 23 patients (33%): against GAD65 ($n = 7$, no titers given); the VGKC complex ($n = 13$, none reactive with LGI1 or CASRP2); onconeural antibodies (Ma2, $n = 3$; Yo, $n = 3$, demonstrated by immunoblot only, therefore of uncertain specificity).[15]

A third study retrospectively reviewed clinical data from 48 patients with probable autoimmune encephalitis according to the clinical presentation; 83% of them had seizures.[2] Among these patients, serum antibodies were detected in 21 cases (44%): to the NMDAR ($n = 13$; one of two available CSF samples was negative for NMDAR antibodies), to the VGKC complex but neither LGI1 nor CASPR2 ($n = 7$; two of them also had GAD65 antibodies) or to the glycine receptor ($n = 1$, positivity in serum and CSF).

Taken together, a plausible gradient in positive antibody results emerges: In series selected by clinical suspicion of encephalitis, the rate of positives is 33% or even 44%[2,14]; in new-onset epilepsies, the overall antibody rate is 10%; and in an explicitly non-encephalitic group (present series), 4% have antibodies.

The specificity and potential pathogenicity of these antibodies to GAD and the VGKC complex (but not LGI1 or CASPR2) are, however, doubtful. Their presence is also unrelated to the individual course of epilepsy. A recent study on the relevance of VGKC complex antibodies (mostly not

Table 2 – Antibody positive patients.

No.	Sex	Age (yrs; mo)	Disease duration (mo)	Antibody diagnosis	Seizures	MRI	Family history	Epilepsy classification	Status epilepticus	Group	Additional problems
1	f	17; 4	7	GAD65 abs 1:64,000	Sz free	Vermis hypoplasia; Hydrocephalus e vacuo; temporal arachnoidal cysts	Negative	Structural	No	ett	Learning-disabled, Hashimoto thyroiditis
2	m	13; 9	12	GAD65 abs 1:100	Sz free	Normal	Negative	Unknown	No	ett	None
3	m	2; 5	13	VGKC complex abs 147 pmol/l	5–10 Series of myoclonias with loss of consciousness per day	Normal	Positive	Probably genetic[a]	Yes	dtt	Physically handicapped
4	f	6; 6	63	VGKC complex abs 223 pmol/l	Sz free	Shunted Hydrocephalus	Negative	Structural	No	ett	Mentally handicapped
5	f	5; 6	54	VGKC complex abs 142 pmol/l	Several startle szs per day and 2 GTCS/ day	Periventricular leukomalacia with cystic defects	Negative	Structural	No	dtt	None

Abbreviations: abs = antibodies; f = female; m = male; GAD = glutamic acid decarboxylase; GTCS = generalized tonic-clonic seizures; mo = months; yrs = years; Sz(s) = seizure(s); ett = easy to treat (group 1); dtt = difficult to treat (group 2); VGKC = voltage gated potassium channel.

[a] Four different mutations with uncertain significance (SCN1A, CLCN2, CACNA1A und SPTAN1), as the father (without seizures) also has the SCN1A-mutation and the mother (also without seizures) carries the other three mutations.

directed to LGI1 or CASPR2) offered another explanation: These antibodies may be directed to diverse antigens within the VGKC complex, partly intracellular and partly extracellular. They are (especially if present at high concentrations) associated with peripheral or central inflammatory conditions in a broad sense, sometimes with other, more specific antineural antibodies. Therefore, VGKC complex antibodies were no longer interpreted as being specific for distinct neurological syndromes.[16] This suggests a new arrangement of antineural antibodies: antibodies with tight syndromic associations and pathogenic effects (like antibodies to the NMDAR or LGI1) might need to be distinguished from antibodies that in some cases (like biomarkers) merely suggest an "inflammatory condition", partly accompanying other, potentially more specific antibodies.[17,18] An example for such a non-specific association could be patient 1 in Table 2: This 17-year old female had epilepsy because of hydrocephalus and – probably unrelated to the seizure disorder – GAD antibodies in the presence of Hashimoto thyroiditis.

4.1. *Limitations*

As we compared two different groups of patients with epilepsy, a control group is lacking which is a limitation of our study. One further limitation is missing data on patients and family history of autoimmunity and the circumstance of presentation of the first seizures when comparing antibody "positive" and negative group. This might play a role of more importance in patients with an encephalitic course. In the present series, only patients with duration of their epilepsy of at least six months were included, excluding patients with an acute encephalitic presentation. Furthermore, due to workload of participating centers we tried to keep the documentation as short as possible and did not collect these specific data. As our study was designed before publication of the definition of drug resistant epilepsy (DRE) we did not include this concept with respect to creation of our two groups.[19] The concept of DRE provides a good platform for future comparisons of different groups and should be considered in further studies.

What is already known on this topic

- Autoimmune encephalitides with seizures as the only or the predominant symptom, have been diagnosed not only in adults but also in pediatric patients.
- Some encephalitic syndromes are associated with specific antibodies.
- Also patients besides those with encephalitic features may have an autoimmune-related etiology as patients with autoimmune diseases carry a higher risk of epilepsy.

What this paper adds

- The low rate of autoantibodies in children with focal epilepsy without *prima facie* evidence for encephalitis supports previous results in unselected patients and is similar to those of control groups.
- We did not find any distinctive features distinguishing antibody positive patients from those without antibodies.
- These antibodies are not syndrome specific, probably not pathogenic and may in some cases be biomarkers for inflammatory processes in a broad sense.

5. Conclusion

Assessment of children with epilepsy and the pathogenic role of antibodies require diagnostics at the first presentation of epilepsy with systematic evaluation of CSF and plasma at the time if patients present with a typical encephalitic presentation. In these cases antibodies might be etiological. In patients without encephalitic features, antibodies are probably not predictive regarding the course of epilepsy over time. Relations between the immune system and epilepsies exist but the mechanisms are rather due to other causations but direct influence of autoantibodies.

Disclosure of conflicts of interest

Peter Borusiak has formerly received support from, has served as a speaker for and taken part in investigations of Novartis (Nürnberg, Germany), Janssen Cilag (Neuss, Germany), UCB (Monheim, Germany) and Desitin (Hamburg, Germany) He has taken part in an investigation of Dibropharm (Baden-Baden, Germany).

Ulrich Bettendorf has formerly received support from, has served as a speaker for and taken part in investigations of Novartis (Nürnberg, Germany), UCB (Monheim, Germany) and Desitin (Hamburg, Germany).

Gert Wiegand obtained honoraria for speaking engagements from Desitin (Hamburg, Germany) and Novartis (Nürnberg, Germany). He gave scientific advice for PTC Therapeutics (Frankfurt, Germany).

Dieter Münstermann undertook industry-funded travel with support of Abbott (Wiesbaden, Germany), DiaSorin (Dietzenbach, Germany), Roche (Mannheim, Germany), Mikrogen (München, Germany) and Beckman Coulter (Krefeld, Germany). He obtained honoraria for speaking engagements from Beckmann Coulter and Roche and received research support from Hologic (Wiesbaden, Germany). He runs a commercial laboratory, which performed the detection of VGKC-antibodies described in this study. External senders are charged for antibody diagnostics.

Christian G. Bien gave scientific advice to Eisai (Frankfurt, Germany) and UCB (Monheim, Germany), undertook industry-funded travel with support of Eisai (Frankfurt, Germany), UCB (Monheim, Germany), Desitin (Hamburg, Germany), and Grifols (Frankfurt, Germany), obtained honoraria for speaking engagements from Eisai (Frankfurt, Germany), UCB (Monheim, Germany), Desitin (Hamburg, Germany), diamed (Köln, Germany), Fresenius Medical Care (Bad Homburg, Germany),

and received research support from Astellas Pharma (München, Germany), Octapharma (Langenfeld, Germany), diamed (Köln, Germany) and Fresenius Medical Care (Bad Homburg, Germany). His employer (Krankenhaus Mara, Bielefeld, Germany) runs a laboratory for the detection of auto-antibodies including those described in this study; external senders are charged for antibody diagnostics.

The remaining authors (Thomas Bast, Gerhard Kluger, Heike Philippi) have no conflicts of interest.

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