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Intracellular and circulating neuronal antinuclear antibodies in human epilepsy☆

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ABSTRACT

There are overwhelming data supporting the inflammatory origin of some epilepsies (*e.g.*, Rasmussen's encephalitis and limbic encephalitis). Inflammatory epilepsies with an autoimmune component are characterized by autoantibodies against *membrane-bound*, *intracellular* or *secreted proteins* (*e.g.*, voltage gated potassium channels). Comparably, little is known regarding autoantibodies targeting *nuclear* antigen. We tested the hypothesis that in addition to known epilepsy-related autoantigens, the human brain tissue and serum from patients with epilepsy contain autoantibodies recognizing nuclear targets. We also determined the specific nuclear proteins acting as autoantigen in patients with epilepsy.

Brain tissue samples were obtained from patients undergoing brain resections to treat refractory seizures, from the brain with arteriovenous malformations or from *post-mortem* multiple sclerosis brain. Patients with epilepsy had no known history of autoimmune disease and were not diagnosed with autoimmune epilepsy. Tissue was processed for immunohistochemical staining. We also obtained subcellular fractions to extract intracellular IgGs. After separating nuclear antibody–antigen complexes, the purified autoantigen was analyzed by mass spectrometry. Western blots using autoantigen or total histones were probed to detect the presence of antinuclear antibodies in the serum of patients with epilepsy. Additionally, HEp-2 assays and antinuclear antibody ELISA were used to detect the staining pattern and specific presence of antinuclear antibodies in the serum of patients with epilepsy. Brain regions from patients with epilepsy characterized by blood–brain barrier disruption (visualized by extravasated albumin) contained extravasated IgGs. Intracellular antibodies were found in epilepsy ($n = 13/13$) but not in multiple sclerosis brain ($n = 4/4$). In the brain from patients with epilepsy, neurons displayed higher levels of nuclear IgGs compared to glia. IgG colocalized with extravasated albumin. All subcellular fractions from brain resections of patients with epilepsy contained extravasated IgGs ($n = 10/10$), but epileptogenic cortex, where seizures originated from, displayed the highest levels of chromatin-bound IgGs. In the nuclear IgG pool, anti-histone autoantibodies were identified by two independent immunodetection methods. HEp-2 assay and ELISA confirmed the presence of anti-histone ($n = 5/8$) and anti-chromatin antibodies in the serum from patients with epilepsy.

We developed a multi-step approach to unmask autoantigens in the brain and sera of patients with epilepsy. This approach revealed antigen-bound antinuclear antibodies in neurons and free antinuclear IgGs in the serum of patients with epilepsy. Conditions with blood–brain barrier disruption but not seizures, were characterized by extravasated but not chromatin-bound IgGs. Our results show that the pool of intracellular IgG in the brain of patients with epilepsy consists of nucleus-specific autoantibodies targeting chromatin and histones. Seizures may be the trigger of neuronal uptake of antinuclear antibodies.

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Introduction

Studies on inflammatory mechanisms in epilepsy have been burgeoning, with a 300% increase in published articles on PubMed from 1993 to 2003 compared to the previous decade. It is thus not surprising that new models of seizures have emerged. These models take into account the knowledge gained from clinical studies, and are based on mechanisms, receptors, and pathways that were formerly reserved for the immunologist (Galanopoulou, 2013; Marchi et al., 2007b, 2009; Vezzani et al., 2012). Evidence to support a role for inflammation and autoimmunity in epilepsy has come from indirect and direct sources. For example, the anticonvulsant activity of steroids in some epilepsies (indirect (Marchi et al., 2011)), together with the presence of inflammatory signs and markers in the serum or cerebrospinal fluid (CSF) of patients (direct (Nabbout, 2012)) have been interpreted as clues suggestive of a role for the immune response. In addition, well-established models of seizures which were developed to specifically target neurons have been re-examined to reveal an underlying inflammatory etiology. For example, research has shown that a putative muscarinic convulsant, pilocarpine, acts by immune activation and not as previously suspected by a CNS exclusive action on muscarinic receptors (Fabene et al., 2008; Marchi et al., 2007b, 2009; Uva et al., 2007). The role of inflammation in seizure disorders has therefore been recognized as an etiologic reality and as an important target for therapy (Marchi et al., 2009, 2010, 2012b; Vezzani et al., 2011).

There are three groups of "inflammation-related seizures" (IRS): 1) Seizures caused by the presence of a pathogen. These are perhaps the least studied cluster of IRS and include seizures due to meningitis, neurotropic pathogens, *etc.* In developing countries, pathogens are considered the highest risk factor for acute seizure and increase the risk of epilepsy by eleven fold (Bharucha et al., 2008). 2) A large family of IRS encompasses autoimmune epilepsy syndromes, where one of the etiological mechanisms is believed to be the presence of anti-neuronal autoantibodies typically targeting either ion channels, intracellular epitopes or neurotransmitter receptors (Graus and Dalmau, 2012; Lancaster and Dalmau, 2012; McKnight et al., 2005). 3) A number of seizure disorders lacking either of these features (pathogen or autoantibodies) can be classified as IRS based on a therapeutic response to immunomodulators (Granata et al., 2008, 2011), vascular changes consistent with an ongoing inflammatory process (*e.g.*, blood–brain barrier disruption; for a review see Janigro, 2012), or concomitant brain changes that mimic some, but not all, signs of inflammation (Granata et al., 2011; Nabbout, 2012; Nabbout et al., 2011).

As mentioned above, the third type of IRS may be linked to blood–brain barrier (BBB) disruption. The BBB is the gatekeeper of immune privilege in the CNS (Bechmann et al., 2007; Galea et al., 2007). The BBB maintains ionic homeostasis which, in turn, controls neuronal excitability (de Vries et al., 2012; Janigro, 2012; Marchi et al., 2012b; Seiffert et al., 2004). Thus, BBB disruption (BBBD) not only causes loss of immune privilege but may also directly result in seizures (Marchi et al., 2007a). A reporter of BBB failure, extravasated albumin levels in CSF, has also been used to detect focal BBBD by immunohistochemistry. Interestingly, after diffusion through the CNS extracellular space, albumin accumulates in neurons and glia (David et al., 2009a,b; Seiffert et al., 2004). Regions of focal BBBD can also be measured by detection of extravasated IgGs. Whether extravasated IgGs also enter into the brain cells it has not been fully elucidated (Michalak et al., 2012).

The presence of IgGs in the brain from patients with epilepsy, together with our understanding of the pathophysiology of multiple sclerosis (MS), has been used to propose autoimmunity as an etiologic factor in seizure disorders. Autoantibodies to the NMDA, $GABA_B$ and AMPA receptors, as well as the voltage-gated potassium channel and its components LGI1 and CASPR2 have been detected in the CSF or serum of patients with seizures (Lancaster and Dalmau, 2012). In addition, autoimmune diseases such as systemic lupus erythematosus (SLE) greatly influence seizure susceptibility (Adelow et al., 2012). Thus, seizure threshold can be lowered by direct action on CNS targets (*e.g.*, glutamate receptors), by exposure to endotoxin (Galic et al., 2008) or by autoimmune targeting of a specific antigen, such as nuclear components. A recent paper has shown that even in the absence of autoimmune disease, IgG can be found in the brain of mice after lithium/pilocarpine-induced seizures (Michalak et al., 2012). This is also consistent with previous work showing that BBBD, as seen in regions of seizure generation in the human brain, is characterized by large deposits of extravasated IgG (Michalak et al., 2012). However, to date, the significance or consequences of IgG extravasation into the CNS has not been fully elucidated.

The CNS of patients with epilepsy provides a unique environment where the coupling of seizure with inflammation, loss of immune privilege and cell death may provide a mechanism for the generation and uptake of autoantibodies against intracellular proteins. Therefore, we examined whether or not autoantibodies against intracellular proteins existed in the CNS and serum of patients with epilepsy where an autoimmune or infectious etiology was ruled out. By using a number of techniques and an approach based on comparison of different pathologies all characterized by BBBD, we isolated autoantigens from subcellular fractions of the brain from patients with epilepsy. MS was used as a comparative "neuro-autoimmune" disease, and brain resections derived from cerebrovascular malformations as a means to study BBBD independent of seizures. Our results demonstrate the presence of antinuclear antibodies in the brain and serum from patients with epilepsy, and the accumulation of autoantibodies in neuronal nuclei.

Materials and methods

The multimodal approach used for the experiments detailed in this section is depicted graphically in Fig. 1.

Patient selection

Brain tissue specimens were obtained from patients conforming to the guidelines of the Declaration of Helsinki. All patients signed an informed consent according to institutional review protocols at the Cleveland Clinic Foundation. Patient information and experimental use of patient samples are summarized in Table 1. All brain tissue samples were obtained from surgical resections with the exception of *post-mortem* MS brain. *Post-mortem* samples were a generous gift of Dr. Bruce Trapp's laboratory at the Cleveland Clinic Foundation Lerner Research Institute. Inclusion criteria were willingness to participate to the study and lack of positive diagnosis for an autoimmune disease. One patient was identified as RA *post-facto* and is considered a positive control (Fig. 6).

Detection and discovery

Immunohistochemical staining of brain tissue sections

The brain tissue was mounted using Tissue-Tek OCT compound (Sakura Finetek Europe B.V., The Netherlands) and sectioned at approximately 25 μm on a Leica CM3050 cryostat (Leica Microsystems Inc., Buffalo Grove, IL). Nine patients with epilepsy, 4 multiple sclerosis patients, and 3 arteriovenous malformation (AVM) patients were included in these experiments.

Immunofluorescent detection of IgG and albumin in neurons, glia and brain parenchyma

Free-floating sections were stained for IgG and albumin. Non-specific binding was minimized by incubation in a 3% goat serum blocking solution at room temperature for 1 h. Sections of the brain tissue were incubated with monoclonal mouse anti-human albumin antibody (1:1000; Sigma-Aldrich, St. Louis, MO). Fluorescently-labeled secondary antibodies used were as follows: Alexa Fluor 594 polyclonal donkey anti-mouse IgG (1:100; Jackson ImmunoResearch, West