IgG Leakage May Contribute to Neuronal Dysfunction in Drug-Refractory Epilepsies With Blood-Brain Barrier Disruption

Zuzanna Michalak, PhD, Aurore Lebrun, PhD, Mathieu Di Miceli, MSc, Marie-Claude Rousset, Arielle Crespel, MD, PhD, Philippe Coubes, MD, PhD, David C. Henshall, PhD, Mireille Lerner-Natoli, PhD, and Valérie Rigau, MD, PhD

Abstract

Focal epilepsies are often associated with blood-brain barrier disruption. In 4 entorhinal cortex tissue samples and 13 hippocampal samples from patients with pharmacoresistant temporal lobe epilepsy, we observed immunoglobulin G (IgG) leakage in the parenchyma and IgG-positive neurons that had evidence of neurodegeneration, such as shrinkage and eosinophilia. These findings were not present in samples from 12 nonepileptic control subjects. To complement these findings, we used a rat in vivo model that mimics the development of limbic epilepsy with blood-brain barrier disruption. During epileptogenesis, IgG leakage and neuronal IgG uptake increased concomitantly with the occurrence of seizures. Immunoglobulin G accumulation in neurons was selective, particularly for interneurons and pyramidal neurons. Immunohistochemistry and electron microscopy showed that IgG uptake in the rat neurons was associated with eosinophilia, shrinkage, and ultrastructural degenerative changes. Moreover, IgG-positive neurons lost their NeuN immunohistochemical staining. Together, these observations suggest that IgG leakage is related to neuronal impairment and may be a pathogenic mechanism in epileptogenesis and chronic epilepsy.

Key Words: Blood-brain barrier, Immunoglobulin G, NeuN, Neurodegeneration, Pilocarpine model, Temporal lobe epilepsy.

INTRODUCTION

Recent studies have identified a role for vascular impairment in a wide range of neurologic conditions. For example, perturbations in blood-brain barrier (BBB) function are described in Alzheimer disease, Parkinson disease, multiple sclerosis, head trauma, brain tumors, stroke, and epilepsy (1). Various clinical and experimental studies support the hypothesis that BBB dysfunction contributes directly to epileptogenesis in focal epilepsies and relevant animal models (2–7). The extravasation of leukocytes, serum proteins, and ions disrupts homeostasis, generating edema, and inflammatory/immune responses. These changes have been shown to modify the neuronal environment, leading to seizures (8–11). In turn, seizures induce angiogenic processes that disrupt the BBB, thereby maintaining epileptogenesis (6, 12, 13). Among the blood components that trigger seizures, albumin down-regulates the transcription of $K_{\text{IR}}$ channels and glutamate transporters in astrocytes. These changes increase neuronal activity and trigger epileptiform events (14–16).

We previously reported accumulation of immunoglobulin G (IgG) in neurons of hippocampi surgically removed from patients with intractable temporal lobe epilepsy (TLE) and in a rodent model of limbic epilepsy (13). Here, we focused on the pathogenicity of neuronal IgG accumulation in human epileptic tissues. We evaluated i) the link between BBB disruption and IgG leakage, ii) the percentage of IgG-positive neurons and their localization, and iii) the histopathologic profile of IgG-positive neurons. In parallel, we used the lithium-pilocarpine model, which induces limbic epilepsy in rats, to decipher the kinetics of IgG accumulation and its potential effects on neuron function and survival.

MATERIALS AND METHODS

Subjects and Clinical Data

This study was performed in accordance with the French Ethical Committee and received the approval of the Comité National Informatique et Libertés. All patients (or their families) were informed of additional studies performed on surgical tissue and provided a written consent. Tissue was obtained and used in a manner compliant with the Declaration of Helsinki. All patients had intractable partial complex...
seizures, and the epileptic foci were localized to the temporal lobe on the basis of neurologic examination, long-term EEG-video monitoring, morphologic magnetic resonance imaging (MRI), and \textsuperscript{99m}Tc-HMPAO-single-photon emission computed tomography. The MRI and T2-weighted images were used to detect hippocampal sclerosis. Hippocampal sclerosis and hippocampal atrophy were designated on the basis of MRI-T2 data and histologic observations (Table 1). Surgery consisted of anterior temporal lobectomy with amygdalo-hippocampectomy performed by the same neurosurgeon for all patients. Histology provided data on tissue remodeling, particularly gliosis, and neuronal loss according to the Blümcke classification (17). Four entorhinal cortex samples and 13 hippocampal surgical samples from patients with TLE were used. Clinical data and diagnosis are detailed in Table 1.

Nonepileptic specimens were obtained from 3 sources. Hippocampus (n = 3) was from autopsied adult patients from the tumor collection at the Montpellier hospital (Centre des Collections Biologiques Hospitalières de Montpellier, Collection tumorothèque, FINES 340780477, Montpellier, France). These patients had no history of epileptic seizures and no pathologic findings in the brain. The postmortem intervals ranged from 7 to 48 hours. Hippocampal samples (n = 5) from autopsied adult patients were also obtained from National Brain Tumor Reference Center, University of Bonn Medical Center, Bonn, Germany. Control entorhinal cortex samples (n = 3) were obtained from the Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD. These were from individuals who died from causes unrelated to neurologic disease.

The TLE and nonepileptic specimens (entorhinal cortex and hippocampus) were either rapidly frozen in liquid nitrogen or fixed by immersion in 10% buffered formalin and processed into paraffin for histologic evaluation and immunohistochemistry.

**Rat Model of Limbic Epilepsy**

All animal procedures were conducted in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC), and approved by the French Ministry of Agriculture (Authorization No. 34178, ML-N). Male Sprague-Dawley rats weighing 150 to 175 g (n = 25) were purchased from Janvier (Le Genest-St-Isle, France). All animals were maintained under standard laboratory conditions on

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<th>TABLE 1. Clinical Data</th>
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<td>Hanging</td>
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<td>P29</td>
<td>Cardiac infarct</td>
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AVM, arteriovenous malformation; Delay FPS-S, delay between first partial seizure and surgery; DNET, dysembryoplastic neuroepithelial tumor; F, female; HA, hippocampal atrophy; HS, hippocampal sclerosis; L, left; M, male; R right; TLE, temporal lobe epilepsy.
a 12-hour light/dark cycle (lights on at 6:00 AM). Lithium chloride (3 mEq kg; Sigma-Aldrich, St. Louis, MO) was administered intraperitoneally (i.p.) 18 to 20 hours before the injection of pilocarpine hydrochloride (30 mg/kg, i.p.; Sigma-Aldrich, St. Quentin Fallavier, France). Rats received 1 mg/kg methylscopolamine bromide i.p. (Sigma-Aldrich) 30 minutes before the convulsant to reduce the peripheral side effects of pilocarpine. Control animals also received lithium chloride, methylscopolamine (Sigma-Aldrich), and an equivalent volume of saline instead of pilocarpine. Approximately 30 minutes after pilocarpine injection, status epilepticus (SE) started, with long-lasting severe seizures. At 2 hours after SE onset, the severity of convulsions was reduced with 2 mg/kg diazepam i.p. (Roche Diagnostics, Meylan, France). Rats were killed at various time points after the onset of SE. Four rats died in the first week after SE. The numbers of animals used were as follows: control (n = 6); acute period, between 1 and 12 hours after SE (n = 5); ‘silent’ period, from 2 to 14 days after SE (n = 5); and chronic period, between 21 and 60 days (n = 5).

**Tissue Processing, Histology, Immunohistochemistry, and Immunoelectron Microscopy**

After fixation in formalin (Sigma-Aldrich), human samples were embedded in paraffin (4 μm thick) and processed routinely for histologic evaluation and immunohistochemistry. For particular protocols, they were processed without aldehyde fixation to detect tight junction (TJ) proteins; frozen tissue was cut on a cryostat in 15-μm coronal sections.

**Histology and Immunohistochemistry**

Neuronal damage was evaluated using hematoxylin and eosin (H&E; Vector Laboratories, Burlingame, CA) to detect cell body shrinkage or eosinophilic cytoplasm, which are hallmarks of neurodegeneration (18). Primary antibodies used for immunohistochemistry are listed in Table 2. The BBB integrity was investigated by double fluorescence immunohistochemistry using an antibody against zonula occludens 1 (ZO-1), an essential protein of TJs and specific anti-human IgG to detect serum leakage. The presence of plasma cells was assessed using an anti-syndecan antibody (CD138). Secondary antibodies were conjugated with either Alexa-488 or Alexa-555 (Molecular Probes, Eugene, OR) or with peroxidase (DAKO, Glostrup, Denmark) and counterstained with hematoxylin.

**Rat Brains**

Rats were killed at various time points after the onset of SE by decapitation after i.p. administration of 4 mg/kg diazepam. The brains were rapidly removed from the skull on ice, and hemispheres were separated and were either fixed in 4% paraformaldehyde (Sigma-Aldrich) in 0.1 mol/L PBS for immunohistochemical studies or directly frozen in liquid N₂. The tissue samples were cut with a vibratome into 25-μm-thick coronal sections that were carefully rinsed in PBS and subsequently treated for immunolabeling. For the rat brains, neuronal damage was evaluated on fixed sections using eosin and anti-IgG (both from Vector Laboratories).

**Immunohistochemistry**

Rat neurons were identified with an anti-NeuN antibody. The integrity of the BBB was investigated by double fluorescence staining with an antibody against rat endothelial cell antigen (RECA-1) and a specific rat IgG to detect serum leakage. After rinsing in PBS, sections were incubated for 4 hours at room temperature with the corresponding secondary antibodies conjugated with anti-rabbit Alexa-488 or anti-mouse Cy3 (Jackson ImmunoResearch, West Grove, PA). The primary and secondary antibodies were diluted in PBS containing 2% bovine serum albumin and 0.1% Triton X-100 (Sigma-Aldrich). Primary antibodies are listed in Table 2.

**Immunoelectron Microscopy**

Twelve rats were killed at various time points after the onset of SE: acute period (control, n = 2; SE, n = 2), silent (pre-epileptic) period (control, n = 2; SE, n = 2), and chronic period (control, n = 2; SE, n = 2). Under deep anesthesia with sodium pentobarbital (50 mg/kg; CEVA, Paris, France), rats were perfused through the ascending aorta with PBS (pH 7.4, 20 mL per animal), followed by 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 mol/L PBS (pH 7.4, 100 mL per animal). Brains were quickly dissected and postfixed by immersion in 4% paraformaldehyde (24–48 hours). They were cut on a vibratome into 50-μm coronal sections. Sections were treated with 1% H₂O₂ for 15 minutes for quenching endogenous

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**TABLE 2. Primary Antibodies and Vascular Markers**

<table>
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<tr>
<th>Primary Antibodies</th>
<th>Source</th>
<th>Isotype</th>
<th>Clone or Reference</th>
<th>Dilution</th>
<th>Supplier</th>
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<td>CD45RA Mouse mAb</td>
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<td>NB600-535</td>
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<td>-</td>
<td>N9528</td>
<td>1/1000</td>
<td>Sigma-Aldrich, France</td>
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<td>Other markers Human IgG Goat Alexa-555 coupled pAb</td>
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<td>A-21433</td>
<td>1/1000</td>
<td>Molecular Probes, France</td>
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+ technique used; IHC, immunohistochemistry; mAb, monoclonal antibody; pAb, polyclonal antibody; WB, Western blot.
peroxidase. After rinsing in PBS, sections were incubated in primary rabbit polyclonal antibodies against rat IgG (Vector Laboratories) diluted in PBS containing 2% bovine serum albumin and 0.1% saponin (48 hours, 4°C). After rinsing in PBS, sections were then incubated (12 hours, 4°C) with a peroxidase-labeled secondary goat anti-rabbit IgG (Jackson ImmunoResearch) and with 3,3′-diaminobenzidine. The specificity of the primary antibody was previously assessed by omitting the primary antibody and applying the secondary antibody alone. Sections were then rinsed in 0.1 mol/L cacodylate buffer, pH 7.3, postfixed in 1% osmium tetroxide in the same buffer, dehydrated in graded concentrations of ethanol, and embedded in Araldite. Punches of 1.5 μm in diameter were cut through the CA3 area of the hippocampus and mounted on Araldite blocks and cut in ultrathin sections (all other products for electron microscopy [EM] were purchased from Sigma-Aldrich).

**Western Blotting**

Rat brain hemispheres were frozen immediately after dissection and stored at -20°C. They were mechanically dissociated and homogenized in lysis buffer containing Tris (20 mmol/L, pH 7.4), EDTA (2 mmol/L), NaCl (100 mmol/L), Triton X-100 (1%), glycerol (10%), phosphatase inhibitors (10 mmol/L sodium fluoride, 1 mmol/L sodium pyrophosphate, and 2 mmol/L sodium orthovanadate), and protease inhibitor mixture (Roche Diagnostics). Samples underwent centrifugation at 37,000 rpm at 4°C for 30 minutes, and the supernatant was collected. Protein concentration was determined using a bicinchoninic acid protein assay (Sigma-Aldrich, St. Quentin Fallavier, France). Samples of 20 μg of protein boiled in Laemmli buffer were loaded onto a 4% to 12% gradient of acrylamide gel, separated electrophoretically, and transferred to polyvinylidifluoride membranes (Hybond-C-extra, Amersham Biosciences, UK). Membranes were incubated for 1 hour in Tris-buffered saline with Tween 5% (TBST) Euromedex, Souffelweyersheim, France) containing 5% skimmed milk, then overnight at 4°C with the following antibodies diluted in TBST in milk: rabbit polyclonal antibodies against rat IgG, mouse monoclonal antibody against rat CD45RA (a marker of lymphocytes), or mouse monoclonal antibody against β-actin. After washes in TBST, membranes were incubated for 2 hours at room temperature with peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibodies (1:2000 dilution) (Jackson ImmunoResearch). All other products for Western blotting were purchased from Sigma-Aldrich. After 3 washes, bands were visualized using chemiluminescence (Western Lightening; Perkin Elmer, Courtaboeuf, France). Densitometry was performed using ImageJ software (National Institutes of Health, Bethesda, MD). Data were statistically compared using the Mann-Whitney nonparametric test (* p ≤ 0.05).

**Microscopy**

Human sample sections double labeled for ZO-1 and IgG were observed using a wide-field microscope (Zeiss Axio Imager Z1, Göttingen, Germany) equipped with 40× and 100× objectives. Images were scanned at 1,388 × 1,040 pixel resolution. Sections labeled for IgG and counterstained with hematoxylin and H&E were observed using a Leica DM4000 microscope (Leica Lasertechnik, Heidelberg, Germany) equipped with 5× and 40× objectives and a DCF310 FX camera. Bright-field pictures were taken at 1,392 × 1,040 pixel resolution. For cell counting, digital images were captured using the whole slide scanner (NanoZoomer; Hamamatsu Photonics KK, Hamamatsu, Japan) under 20× magnification with a 0.50-μm/pixel resolution.

Rat sample sections double labeled for NeuN and IgG were imaged using a confocal microscope (Zeiss 510 Meta) equipped with 25× objective (multi-immersion, numeric opening 0.8). We used an argon laser (excitation 488, emission 505–530 nm) for Alexa-488 and a helium laser (excitation, 543 nm; emission, 585–615 nm) for Cy3. Images were collected sequentially to avoid cross-contamination between fluorochromes. Series of 10 optical sections were projected onto a single image plane and scanned at 1,024 × 1,024 pixel resolution. Rat sections double labeled for RECA-1 and IgG were observed using a Zeiss Axio Imager Z1 equipped with Apotome Cam function and with 5× objective. A series of 5 optical sections were projected onto a single image plane and scanned at 1,388 × 1,040 pixel resolution.

To detect eosin in IgG-positive cells, we combined bright-field images of IgG staining with images of eosin fluorescence emission and then superposed the images, as described (13). For immunoelectron microscopy, sections were examined with a Hitachi H 7100 electron microscope (Hitachi, Krefeld, Germany).

**Data Analysis and Statistics**

For human tissue, numbers of IgG-stained neurons and numbers of eosin-stained neurons were related to the total number of neurons in the hippocampus stained with hematoxylin and characterized based on their shape and position in the hippocampal layers. For each human sample, 25 images were taken of the pyramidal layer and 15 of the granular layer. The IgG-positive neurons were counted relative to the total number of neurons in each layer. The groups were then compared using a nonparametric unpaired t test. To evaluate the presence of ZO-1 on vessels, 15 photos were taken for each sample, and the length of ZO-1 staining was calculated relative to the total length of vessels. The groups were compared by a nonparametric unpaired t test.

In rat sections, neuron counting was based on NeuN staining and its shape and position in the hippocampal layers. We looked for NeuN and IgG staining and detected eosinophilic cytoplasmic inclusions by immunofluorescence. The number of IgG-stained neurons was determined relative to the total number of hippocampal neurons evaluated at each time point after SE. Ratios were compared using one-way analysis of variance and Tukey post hoc test.

**RESULTS**

**BBB Disruption and IgG Leakage in Human TLE**

In the entorhinal cortex of autopsied nonepileptic patients, the BBB integrity was assessed by the presence of ZO-1 staining at regular intervals corresponding to interendothelial junctions and a lack of IgG extravasation (Fig. 1A).
In contrast, the BBB was markedly altered in the entorhinal cortex of patients with TLE. Staining for ZO-1 was mainly absent from the TJs and was mislocalized in spots in endothelial cells, whereas IgGs were present as a diffuse stain in the parenchyma (Fig. 1B). Semiquantitative analysis of ZO-1 staining confirmed the significant loss of ZO-1 staining of the TJs along endothelial cells in the epileptic tissue samples (Fig. 1C).

**IgG Parenchymal Leakage With Selective Accumulation in Neurons in Human TLE**

In the entorhinal cortex and hippocampus of epileptic patients, there were large IgG halos found around microvessels (Fig. 2A) and a selective accumulation of IgGs in the cytoplasm of neurons to variable extents. Some dentate granule cells were faintly stained (Fig. 2B, D), whereas pyramidal neurons (PNs) of the hippocampus were strongly labeled (Fig. 2C, E). The scarce interneurons that were present were also IgG positive. Semiquantitative analysis confirmed that IgGs accumulate in most neurons, in particular, in PNs (Fig. 2F). Less commonly, there was IgG accumulation in astrocytes (Fig. 3C). To be certain that the IgGs observed in brain parenchyma leak from the disrupted BBB, we verified that IgGs were not carried by syndecan-positive cells extravasated in brain tissue (19–21) (Figure, Supplemental Digital Content 1, part A, http://links.lww.com/NEN/A364).

**Morphologic Characteristics of IgG-Positive Neurons in Human TLE**

Most of the IgG-positive PNs displayed shrunken perikarya, corkscrew-shaped dendrites (Fig. 3A–D), and variable degrees of disappearance of nuclear structures (Fig. 3E–J). In H&E-stained sections, PNs in TLE tissue were shrunken and darkly stained, with an indistinct nucleus (Fig. 4A, B). Some granule cells were also strongly shrunken, and there was often vacuolation of the adjacent neuropil (Fig. 4C, D). Semi-quantitative analysis determined that the percentage of eosinophilic neurons was significantly higher in epileptic tissue.
FIGURE 2. Leakage of IgG into the parenchyma and accumulation in neurons in human temporal lobe epilepsy (TLE). (A) The IgGs form halos around microvessels in the hippocampus (anti-human IgG antibody, hematoxylin counterstaining). (B–E) The accumulation of IgGs in some granule cells (GCs) of the dentate gyrus (B). Higher magnification (D) shows IgG accumulation in the cytoplasm of GCs (arrow) near microvessels (arrowhead). (C) The accumulation of IgG in pyramidal neurons (PNs) of the CA1 area. Higher magnification (E) shows IgGs in the cytoplasm, the nucleus, and a corkscrew-shaped dendrite of a PN (oval arrow) in the vicinity of microvessels (arrowhead). (F) Percentage of IgG-positive neurons (PNs and GCs). Scale bars = (A) 500 μm; (B, C) 50 μm; (E, F), 25 μm. *** p ≤ 0.0001. For each group, mean and SEM were compared by unpaired t test (mean was significantly different at p < 0.05, n = 13).
versus control tissue as follows: PNs, 43.5% TLE versus 15.5% nonepileptic; granule cells: 21.7% TLE versus 7.2% nonepileptic (Fig. 4E).

Rat Model of Limbic Epilepsy

To extend these human findings, we examined the same processes in tissue obtained shortly after SE in rats triggered by pilocarpine and in tissue sampled during the pre-epileptic and chronic epileptic phases. Leakage of IgG into the parenchyma and IgG accumulation in neurons were analyzed in the control, the acute period (continuous severe seizures for hours), the silent period (before spontaneous seizures but during tissue remodeling), and in the chronic period (recurrent spontaneous seizures).
FIGURE 4. Neuronal damage in human temporal lobe epilepsy (TLE). (A, B) The CA1 area of the hippocampus in nonepileptic (NE) (A) and TLE (B) tissues. Note that numerous pyramidal neurons (PNs) in TLE tissues are shrunken and hyperchromatic and display basophilic perikarya and prominent corkscrew-shaped dendrites (arrows). (C, D) Dentate gyrus in NE (C) and TLE (D). In TLE tissues, the granule cells (GCs) are shrunken and the neuropil is vacuolated. (E) Percentages of eosinophilic neurons (PNs and GCs) counted in H&E-stained sections. Scale bar = 50 μm. *** p < 0.0001. For each group, mean and SEM were compared by unpaired t test (p < 0.05, n = 8).
We detected neither endothelial damage nor IgG leakage in capillaries of control animals (Fig. 5A). During the acute period after SE in rats, large halos of IgGs were obvious around dilated microvessels, assessed by the endothelial marker RECA-1 (Fig. 5A). The leakage was very faint during the silent period and increased during the chronic period.

**FIGURE 5.** Leakage of IgGs and B cell accumulation during epileptogenesis in rats. (A) Double immunostaining of IgG (green) and vessels (RECA-1, red) in the hippocampus. Scale bar = 200 μm. (B) The IgG expression accumulated in brain parenchyma measured by Western blot. Data are expressed as a ratio of optical density (OD) of the IgG band to that of the actin band. (C) Expression of B lymphocyte assessed by Western blot. Data are expressed as a ratio of OD of the CD45RA band to that of the actin band. Control, n = 6; acute, n = 5; silent, n = 5; chronic, n = 5. For both Western blots, data are compared using the Mann-Whitney nonparametric test (\( p \leq 0.05 \)).

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FIGURE 6. Kinetics of neuronal accumulation of IgGs during epileptogenesis in rats. (A-D) Double immunostaining of IgG (green) and NeuN (red) in CA3 area of the hippocampus at each period. Arrows indicate IgG-positive neurons. Arrowheads indicate IgG-positive and NeuN-negative neurons. Asterisk shows IgG accumulation in brain parenchyma. Scale bar = 80 μm. (E) Ratio of IgG-stained neurons reported to the total number of hippocampal neurons, at each period of epileptogenesis (control, n = 4; each period, n = 5 rats). Data were statistically compared by one-way analysis of variance and Tukey post hoc test. ** p < 0.001; * p ≤ 0.05.
albeit not to a significant extent (Fig. 5A, B). The measurement of the level of IgG protein by Western blotting reveals the significant increase of IgG accumulation in the parenchyma during the acute period (Fig. 5B), concomitantly with a peak of CD45RA expression, indicating an acute infiltration of lymphocytes in the parenchyma (Fig. 5C).

**IgG Accumulation in Neurons During Epileptogenesis**

In control conditions, Western blotting determined that IgGs were absent from brain parenchyma (Figure, Supplemental Digital Content 1, part B, http://links.lww.com/NEN/A364), whereas immunostaining showed very few IgG-positive neurons in brain tissue (Fig. 6A). In the acute period after SE, many PNs accumulated IgGs in the cytoplasm (Fig. 6B). During the silent and chronic periods, there was an increase of PNs positive for IgGs (Fig. 6C, D). Quantification showed that the ratio between IgG-positive neurons and total neurons increased from 0.01 ± 0.01 in controls to 0.47 ± 0.06 in the silent period (Fig. 6E), indicating a significant increase in the number of IgG-positive neurons during this period. In the chronic epileptic phase, the increased ratio was less prominent because of a reduction of the total number of neurons caused by cell loss (Fig. 6D, E). Notably, many IgG-positive PNs lost their NeuN immunoreactivity (arrowheads in Fig. 6C). Hippocampal CA2 PNs were not damaged in this model but were still IgG positive in the chronic epileptic period, similar to observations of human CA2 PNs in TLE patients. In contrast, interneurons accumulated IgGs during the acute phase, but these neurons were not apparent at later time points (Figure, Supplemental Digital Content 2, http://links.lww.com/NEN/A365 and Figure, Supplemental Digital Content 3, http://links.lww.com/NEN/A366).

**Morphologic Characteristics of IgG-Positive Neurons During Epileptogenesis**

We examined IgG-stained cells using EM to visualize their ultrastructure. In control rats, neurons displayed intact subcellular structures and no IgG staining (Fig. 7A). During the acute period after SE, there was punctiform staining of IgGs in dendrites (Fig. 7B). Subsequently, in the silent and chronic periods, we observed diffuse IgG staining in the cytoplasm of shrunken neurons; astrocytic processes ensheathed these neurons (Fig. 7C, D). Notably, nuclear membrane, organelles, and postsynaptic densities were hardly distinguishable in these neurons (Fig. 7C, D, F). In rats killed 60 days after SE, IgG-positive shrunken neurons with corkscrew-shaped dendrites were strongly eosinophilic in the hippocampus and entorhinal cortex (Fig. 7G–I).

**DISCUSSION**

Disruption of the BBB is now considered to be a causal factor inictogenesis and epileptogenesis, whereas, in turn, epileptic activity participates in BBB alteration, inducing serum leakage ([1, 10-13]). The findings in this study support a link between BBB disruption and an abnormal accumulation of IgGs in neurons. A putative mechanism of IgG entry into brain parenchyma is the extravasation of B cells (19–21), but we did not observe plasma cells in epileptic tissue. Protein leakage is caused by the impaired functioning of BBB systems, that is, carrier-mediated transport, transcytosis, or paracellular pathway. In TLE and models of limbic epilepsy, we previously described changes in expression and localization of the TJ protein ZO-1, which controls TJ assembly. Downregulation of ZO-1 promotes BBB alterations and blood component leakage ([12, 13]). Moreover, other serum proteins leak into the parenchyma and accumulate selectively in neural cells; albumin in astrocytes and complement proteins in neurons modify their functions ([13, 22]).

Various studies have suggested that extravasation and accumulation of nondegraded serum proteins in the CNS participate in the pathogenesis of brain proteinopathies via passive accumulation and aggregation that perturb neuronal function and survival ([23]). Such neuronal uptake of serum proteins has been described in cases of BBB breakdown and neurodegenerative diseases ([24-27]).

Another hypothesis incriminates an autoimmune cause, as has been proposed for amyotrophic lateral sclerosis, Parkinson disease, and Alzheimer disease, based on assessment identification in patient sera of specific antibodies that recognize neuronal targets ([28-30]). Evidence supporting an autoimmune mechanism for pharmacoresistant epilepsies includes i) the efficacy of immunomodulatory therapies to reduce seizure occurrence and ii) the identification in patient sera of many autoantibodies that modify the function of neurotransmitter receptors and ion channels, such as n-methyl-d-aspartic acid (NMDA), amma-aminobutyric acid (GABA), and amino-3-hydroxy-5-methylisoxazol-4-propionate (AMPA) receptors, and the voltage-gated potassium channels (VGKC) comprising lacrimal gland antigen (LG1), contactin-associated protein-like 2 (CASPR2), and contactin-2. However, these targets are not exclusive to epilepsy because these autoantibodies have also been found in various encephalopathies ([30-33]). Stein et al ([27]) suggested that autoantibodies bind specifically to dying neurons. In human TLE tissue, IgGs were obvious in numerous PNs and only in few granule cells, which are less affected. However, we did not find any IgG-positive interneurons, although this assessment was hampered by severe depletion of this cell population in our material ([34]). In the rat model, we observed that IgG accumulation was selective; during the acute period after SE, IgGs seemed to enter interneurons before their subsequent disappearance. During the silent and chronic periods, IgGs were obvious in PNs, which displayed a marked evidence of degeneration and loss of NeuN staining. At the ultrastructural level, IgGs were obvious in dendrites of neurons in the acute period and in the cytoplasm and nucleus in later phases. Taken together, these observations favor the hypothesis of accumulation of IgGs in “healthy neurons” that progressively induces their deterioration.

We also confirmed that IgG uptake correlated with several features of neurodegeneration and/or dysfunction. In TLE, as well in the chronic period in the experimental model, shrunken neurons were eosinophilic, with corkscrew-shaped dendrites. These morphologic changes have been described in various brain diseases, particularly in the so-called hypoxic neurons or dark neurons ([18, 35, 36]). Similar patterns are also observed in other experimental models of epileptogenesis, showing that neurons die by a necrosis/apoptosis continuum ([37-41]). It is noteworthy that PNs of the CA2
sector survive in human TLE, as well as in experimental epi-
leptogenesis, thereby arguing that this region is resistant to
neurotoxicity (42, 43).

In conclusion, we describe an immune pathologic
process related to vascular permeability that may contribute to
epileptogenesis. The present pathologic observations suggest
that IgG leakage may promote neuronal dysfunction and
degeneration. Whether IgG accumulation results from auto-
immune binding or from protein diffusion mediated by
endocytosis, transporter, or receptor is not certain.

FIGURE 7. Effects of IgG accumulation on neurons during epileptogenesis in rats. (A, B) Accumulation of IgGs in neurons. Immunoelectron microscopy shows that, in control rats, neurons show intact subcellular structures and no IgG staining (A). In the acute period, there is punctiform staining of IgGs only in dendrites (B). (C, D) In the silent and chronic periods, there is diffuse IgG staining in the cytoplasm of shrunken neurons and astrocytic processes sheath IgG-positive neurons. Neurons are pseudocolored red, astrocytes blue, and dendrites green. Scale bar = 2 μm. (E, F) Subcellular damage. Immunoelectron microscopy shows intact organelles in neurons of control rats (E). In the silent period, nucleolus, nuclear membrane, and postsynaptic densities are hardly distinguishable (F). Scale bar = 1 μm. (G-I) Evidence of neurodegeneration. The IgG-positive shrunken neurons with corkscrew-shaped dendrites (G), eosinophilic neurons (H), and overlap (I) show that IgG-positive neurons are eosinophilic. Scale bar = 25 μm.
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