Temporal Profile of Clinical Signs and Histopathologic Changes in an F-344 Rat Model of Kainic Acid–induced Mesial Temporal Lobe Epilepsy

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ABSTRACT

Since there is limited information in the literature, the purpose of this study was to investigate clinical signs, morphology, and temporal progression of lesions from Days 3 to 168 in a kainic acid (KA)-induced Fischer-344 (F-344) rat model of mesial temporal lobe epilepsy (MTLE). Following a single KA subcutaneous dose of 9 mg/kg to young adult male rats, 95% survived, 93% exhibited status epilepticus, and 80% eventually developed spontaneous motor seizures. Histopathology included hematoxylin and eosin (H&E), autofluorescence, Fluoro-Jade B, Timm’s, ED-1/CD68, GFAP, doublecortin, and Ki-67. Neuronal degeneration occurred on Day 3 in the hippocampal CA1, CA3, and dentate hilar regions; amygdaloid and thalamic nuclei; and frontoparietotemporal, entorhinal and piriform cortices. Degeneration severity peaked on Day 6 and decreased progressively until Day 168. Aberrant mossy fiber (MF) sprouting was present in the inner molecular layer of dentate gyrus on Days 6–168. Microgliosis and astrogliosis peaked on Day 28 and generally colocalized with the distribution of neuronal degeneration. Important correlates to human MTLE included induction of spontaneous seizures, more severe neuronal damage of CA1 than CA3 (in contrast to other animal models but similar to humans), hilar neuronal loss, activated microgliosis and astrogliosis, aberrant MF sprouting, and dentate granule cell neurogenesis. Aberrant MF sprouting prior to spontaneous motor seizures and reduced seizure frequency with a decrease in aberrant MF sprouting support the hypothesis that MF sprouts are necessary for spontaneous seizure generation and maintenance.

Keywords: mesial temporal lobe epilepsy (MTLE); Fischer-344 rat; animal model; histopathology; lesions; neurodegeneration; microgliosis; astrogliosis; mossy fiber sprouting; neurogenesis.

INTRODUCTION

Mesial temporal lobe epilepsy (MTLE) is one of the most common types of temporal lobe epilepsy (TLE) in human beings and is characterized by seizure generation from the mesial temporal lobe (Falconer et al. 1964; Margerison and Corsellis 1966). It is hypothesized that MTLE is initiated by injuries to the brain such as those caused by febrile convulsions, status epilepticus, encephalitis, or trauma, which are followed by years of latency during which additional changes lead to spontaneous motor seizures (Engel 1993).

Hippocampal sclerosis (HS) is a common finding in approximately 70% of patients with TLE. Patients with HS have neuronal degeneration, especially of CA1, with pronounced microgliosis and astrogliosis (Babb and Brown 1986) and aberrant mossy fiber (MF) sprouting in the inner molecular layer of the dentate gyrus.

Although the clinical signs and pathologic findings have been described for human MTLE, the relationships of those findings to the pathogenesis of the disease are unknown.

Rodent models have been developed to study the pathogenesis of MTLE (Buckmaster 2004; Coulter et al. 2002; Sharma et al. 2007; White 2002). Examples include chemoconvulsant- and electrical stimulation-induced post-status epilepticus, kindling, tetanus toxin, hyperthermia, post-traumatic epilepsy, and perinatal hypoxia/ischemia models. Features of the chemiconvulsant and electrical stimulation-induced post-status epilepticus (SE) rodent models, such as HS and MF sprouting (Chakravarty et al. 1997; Okazaki et al. 1995; Tauck and Nadler 1985), are similar to those reported in human MTLE (Babb and Brown 1986; de Lanerolle et al. 1989; de Lanerolle and Lee 2005; Franck et al. 1995).

Kainic acid (KA) [2-carboxy-4-(1-methylethenyl)-3-pyrrolidinacetic acid] is a cyclic analog of L-glutamate and an agonist of ionotropic, non-NMDA glutamate AMPA and KA receptors. KA is commonly administered systemically or intracerebrally to cause sustained neuronal depolarization and hyperexcitability, especially in the hippocampus (Cossart et al. 1998; Frerking et al. 1998; McGeer and McGeer 1982; Olney et al. 1974).

Strain-based variations in susceptibility to epileptogenic treatments have been reported in mice. Hippocampal neuronal...
degeneration and aberrant MF sprouting have been observed in 129/SvEms mice and not in imprinting control region (ICR) mice following KA administration (Cantallops and Routtenberg 2000; McNamara et al. 1996; Schauwecker and Steward 1997). We were interested in developing a rodent model that closely resembled human MTLE. Although the characteristic lesions have been reported in KA-induced Sprague-Dawley (SD) rat models of TLE (Buckmaster and Dudek 1997; Cronin and Dudek 1988), less is known about the long-term temporal profile of histopathologic lesions in the smaller Fischer 344 (F-344) rat (Rao et al. 2006; Zhang et al. 2002). Additionally, a detailed and long-term description using special staining methods—such as Fluoro Jade B (FJB), Timm’s stain, ED-1/CD68, GFAP, doublecortin (DCX), and Ki-67—characterizing the progression of neuronal degeneration, the associated cell types, aberrant MF sprouting, and neurogenesis has not been reported in F-344 rats.

In previous studies KA has been given at doses of 8 to 12 mg/kg via the subcutaneous (s.c.), intraperitoneal (i.p.), or intravenous (i.v.) routes (Ben-Ari et al. 1981; Lothman and Collins 1981; Milgram et al. 1991; Sloviter 1992; Tauck and Nadler 1985; Zucker et al. 1981). KA has been used in more than 10 mg/kg results in a 50% mortality rate (Brines et al. 1995; Sperk 1994).

Early mortality following KA administration is associated with SE. SE has been blocked by administration of thionembutal (a barbiturate) ninety minutes following SE onset (Covolan and Mello 2000) and by diazepam after several hours of SE onset (Ben-Ari et al. 1980) following KA administration in rats. In one study, 36% rats died following attenuation of SE by administration of 5 mg/kg of diazepam after three hours of KA administration in fifty-six rats (Dernovsek and Sket 1998). Diazepam is a benzodiazepine that potentiates the inhibitory effect of gamma-aminobutyric acid (GABA) leading to anticonvulsant activity (Koob et al. 1987). Dose selection for the current study was based, in part, on these studies and on an in-house pilot study.

The purpose of the current study was to characterize the temporal morphological changes as they correlated to clinical observations in an F-344 rat model of MTLE to gain insight into the pathogenesis of human MTLE. Specific objectives included characterization of neuronal degeneration, astrogliosis, activated microgliosis, aberrant MF sprouting, and dentate granule cell neurogenesis at Days 3, 6, 14, 28, 84, and 168 following a single s.c. dose of KA in F-344 rats. Secondary objectives were to compare the results obtained with the F-344 rat to those reported for other rodent models of MTLE and to study any improvement in survival following KA administration.

**Materials and Methods**

In this study, F-344 rats were administered KA to cause SE and ensuing spontaneous motor seizures. Following euthanasia, brains were processed for routine and special histopathologic staining and immunohistochemical labeling to investigate the temporal profile of morphologic alterations.

**Pilot Study: Dose and Temporal Profile Selection**

To select an appropriate dose of KA, an in-house dose-ranging study was conducted, where five different groups of six rats each were administered one s.c. dose of 7 to 9 mg/kg. Since no SE was observed below 8.5 mg/kg, we selected 9 mg/kg, where five of six rats developed SE. Based on our understanding that the multiple low doses likely maintained moderate systemic levels of KA, we selected the s.c. route, as a slow absorption from the injection site would lead to maintenance of moderate systemic KA levels for a longer period of time (Wilkinson 2001). Based on lack of any neurodegeneration observed in H&E- and FJB-stained sections immediately following SE on the day of KA administration (Day 0), and previous experience with KA in rats (unpublished data), Day 3 was selected as the first point to study morphologic changes, especially those resulting from SE. Days 6 and 14 were included to investigate morphologic changes during epileptogenesis and early epilepsy, whereas Days 28, 84, and 168 were selected to study progressive changes during established epilepsy.

**Animals**

One hundred seven- to eight-week-old male F-344 rats weighing 200 to 300 g were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, IN, USA) and housed individually in stainless steel cages for one week prior to the treatment. Eighty rats were used for the treatment group, and twenty rats were used for the control group. The rats were allowed free access to a normal laboratory diet (Certified Rodent Diet 5002, Pellet, supplied by PMI Nutrition International Inc., St. Louis, MO, USA) and chlorinated potable water. All rats were acclimated for at least one week to the housing facilities and diet before being used in the study. The controls in the animal room were set to maintain a temperature of 20°C to 24°C and 30% to 70% relative humidity. Rats were maintained on a twelve-hour light/dark cycle. The Animal Care and Use Committee of Lilly Research Laboratories approved all study protocols.

**Treatment**

Rats were randomly assigned to treatment and control groups and sequentially numbered. The twenty control rats were administered normal saline via the s.c. route, and eighty rats were administered a single s.c. dose of 9 mg/kg KA (Sigma Chemical, St. Louis, MO, USA).

To diminish mortality, SE was blocked by 10 mg/kg i.p. diazepam (Hospira Inc, Lake Forest, IL, USA) approximately one hour and forty-five minutes following initiation of status epilepticus. The time of diazepam administration was based on our experience of very high mortality after one hour and forty-five minutes of SE (unpublished data). Five to 10 mL of normal saline were also administered s.c. to dehydrated rats until they were able to drink water on their own. The s.c. fluid was generally given on Day 0 of the study.
Behavior

Control and treated rats were evaluated continuously for clinical signs progressing to SE for four hours post-KA administration. On subsequent days, all surviving rats were examined cageside for spontaneous motor seizures by two well-trained technicians and a trained pathologist in a nonblinded fashion for four approximately equally spaced thirty-minute periods between 7:00 AM and 5:00 PM on weekdays until study termination. SE and post-latency spontaneous motor seizures were recorded (Table 1) on a scale of stages 1 through 5 (Racine 1972).

Assignment of Rats to Treatment Groups

In addition to the control rats, only those treated rats with status epilepticus, characterized by stage 4 to 5 limbic seizures occurring usually within forty-five minutes, were included in the primary evaluation of the study. Post-KA administration scheduled necropsy intervals included Days 3, 6, 14, 28, 84, and 168. For each time point (day), three control and six treated rats meeting SE selection criteria were randomly selected for tissue collection. Selection criteria for the treated rats included rats with SE for Days 3 and 6 and rats with spontaneous motor seizures from Day 14 to study termination. Rats that experienced SE but did not develop spontaneous motor seizures were also terminated on Day 168 (last day of the study) and evaluated histopathologically.

Euthanasia, Tissue Collection, and Tissue Processing

Immediately after rats were humanely euthanized by carbon dioxide asphyxiation, brains from control and treated rats were collected and fixed for routine and special histopathology. With the exception of samples for the Timm’s stain, fresh right hemibrains were placed in Timm’s fixative at 4°C for two days. Three coronal slices of brain selected to include dorsal and ventral hippocampus were microtomed at 5 µm in steps between Bregma -2.8 and -4.8 (Paxinos and Watson 2005), processed, and mounted on glass slides. Timm’s fixative was freshly prepared by adding 0.37% Na,S and 4% glutaraldehyde in PBS (pH 7.5). Fresh hemibrains were immersed in fixative and maintained at 4°C for forty-eight hours. Fixed hemibrains were trimmed and processed routinely for paraffin embedding.

Special Histologic Stains and Procedures

In addition to routine H&E staining, FJB staining and autofluorescence were used to investigate neurodegeneration, whereas Timm’s histochemistry was conducted to study development of aberrant MF sprouting in the inner molecular layer of the dentate gyrus. The glial markers ED-1/CD68 and GFAP and neurogenesis/cell proliferation markers doublecortin (DCX) and Ki-67 were used to investigate gliosis and dentate neurogenesis, respectively.

FJB staining was conducted as described by Schmued and Hopkins (2000). Both the FJB- and H&E-stained sections were viewed under a fluorescence microscope with an FITC filter (approximately 450–490 nm) to demonstrate, respectively, fluorescence and autofluorescence (Jordan et al. 2007) of degenerating neurons and neuronal processes.

For Timm’s staining, mounted sections were immersed in a “physical developer” in darkness at 21°C. The physical developer was prepared as follows: 30 mL aqueous solution of 7.65 g citric acid and 7.05 g of sodium citrate were added to 180 mL gum Arabic (500 g/L water); 5.0 g hydroquinone in 90 mL distilled water and 1.5 mL of 15% silver nitrate solution were added to the previous solution just before use (in the dark). The sections were periodically examined under the microscope to judge adequate staining during the developing stage (Sloviter 1982).

Immunohistochemistry

Antigen retrieval was performed by immersing the rehydrated brain sections in Dako Target Retrieval solution (Dako Corp., Carpinteria, CA, USA), heating them to 95°C for ten minutes using MicroMED T/T Mega Laboratory Microwave Systems (Milestone, Sorisole, Italy), and then cooling the sections at room temperature for twenty minutes. For ED-1/CD68, sections were pretreated with trypsin (Invitrogen Corp., Carlsbad, CA, USA) that was diluted according to the manufacturer’s specifications and incubated at 37°C for ten minutes. Staining was performed on the Dako Autostainer Plus (S38-0259-01) using the antibodies listed in Table 2. Endogenous peroxidase activity was blocked by incubation with Power Block (Biogenex, San Ramon, CA, USA). Immunolabeling for ED-1/CD68 and GFAP was performed by incubating with the

<table>
<thead>
<tr>
<th>Stage</th>
<th>Clinical signs</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Immobility, eye closure, ear twitching, twitching of vibrissae, sniffing, facial clonus</td>
</tr>
<tr>
<td>2</td>
<td>Head nodding associated with more severe facial clonus</td>
</tr>
<tr>
<td>3</td>
<td>Clonus of one forelimb</td>
</tr>
<tr>
<td>4</td>
<td>Bilateral clonus accompanied with rearing</td>
</tr>
<tr>
<td>4.5</td>
<td>Generalized clonic seizures without rearing and falling</td>
</tr>
<tr>
<td>5</td>
<td>Rearing and falling accompanied by generalized clonic seizures</td>
</tr>
</tbody>
</table>

Source: Racine 1972.
corresponding primary antibodies in Dako Antibody Diluent (Dako) at room temperature for thirty minutes followed by incubation with biotinylated secondary antibody in 1.5% horse or goat serum diluent, respectively. To rule out any nonspecific labeling, sections were incubated with Dako Wash Buffer (Dako) without addition of primary antibody followed by incubation with avidin-biotin complex (Vector). Peroxidase was developed with diaminobenzidine (Dako). Labeled slides were counterstained with hematoxylin.

For DCX and Ki-67, fluorescent immunolabeling was performed in the dark by incubating with primary antibody for sixty minutes followed by incubation for forty-five minutes at room temperature with secondary antibody in 1.5% rabbit and 1.5% donkey serum diluent, respectively. Negative control sections were incubated with Dako Wash Buffer without addition of either of the primary antibodies. Sections were then rinsed with wash buffer and mounted with ProLong Gold antifade reagent with DAPI (Molecular Probes, Eugene, OR, USA).

**Lesion Score Criteria**

Severity grading for morphologic changes in slices of brain was performed in a blinded fashion by a trained pathologist in a semiquantitative manner so that all areas evaluated were considered to be either normal or to fall within five levels of severity, as outlined in Table 3. The observations were peer reviewed by an experienced neuropathologist. Numbers of degenerated neurons were estimated in four equivalent 40X fields in individual affected areas in a single section of brain per animal (Table 4). Only shrunken or condensed neurons with hypereosinophilic cytoplasm and condensed or karyorrhectic nuclei were considered as degenerated or necrotic neurons. The neurons from the same region fluoresced under the FITC filter in H&E- and FJB-stained sections. Scoring for ED-1/CD68- and GFAP-positive cells was based on estimating four equivalent 40X fields in CA1, CA3, and the dentate hilus. Additional areas were examined qualitatively but not reported as data. The scoring for aberrant MF sprouting, outlined in Table 3, was based on a semiquantitative scale published previously (Holmes et al. 1999). Timm’s scoring was performed on equivalent portions of suprapyramidal and infrapyramidal blades of dentate gyrus on a single brain section between Bregma -3.00 and -3.48 (Paxinos and Watson 2005).

For dentate SGZ neurogenesis or proliferation, DCX- or Ki-67-positive cells were counted in the subgranular zone of the ventral and dorsal blades of the dentate gyrus, as that is the location where neurogenesis has usually been reported in the dentate gyrus of adult rats. For this purpose, scoring was performed on approximately 7-mm-long segments of SGZ in suprapyramidal and infrapyramidal blades of dentate gyrus from a single section of brain between Bregma -3.00 and -3.48 (Paxinos and Watson 2005).

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**Table 2.** Primary and secondary antibodies used for immunohistochemistry.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Used to detect</th>
<th>References</th>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>ED-1/CD68</td>
<td>Activated microglial cells</td>
<td>(Bonde et al. 2006)</td>
<td>Monoclonal mouse anti-rat ED-1/CD68</td>
<td>Horse anti-mouse biotinylated IgG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Serotec), 1 µg/mL</td>
<td>(Vector)</td>
</tr>
<tr>
<td>GFAP</td>
<td>Astrocytes</td>
<td>(Bendotti et al. 2000)</td>
<td>Polyclonal rabbit anti-GFAP antibody</td>
<td>Goat anti-rabbit biotinylated IgG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Dako), 2 µg/mL</td>
<td>(Vector)</td>
</tr>
<tr>
<td>Ki-67</td>
<td>Proliferating cells</td>
<td>(Tanapat et al. 1999)</td>
<td>Monoclonal mouse anti-rat Ki-67</td>
<td>Rabbit anti-mouse Alexa Fluor</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>antibody (Dako), 23.75 µg/mL</td>
<td>594 IgG (Molecular Probes)</td>
</tr>
<tr>
<td>DCX</td>
<td>Postmitotic immature neurons</td>
<td>(Couillard-Despres et al. 2005; Francis et al. 1999)</td>
<td>Goat anti-doublecortin antibody</td>
<td>Donkey anti-goat Alexa Fluor</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Santa Cruz Biotechnical), 4 µg/mL</td>
<td>488 IgG (Molecular Probes)</td>
</tr>
</tbody>
</table>

**Table 3.** Scoring criteria for neuronal degeneration, microgliosis, astrogliosis, and aberrant MF sprouting.

<table>
<thead>
<tr>
<th>Severity grades</th>
<th>Number of degenerated neurons</th>
<th>Number of ED-1/CD68 and GFAP positive glial cells</th>
<th>Aberrant MF sprouting (granule distribution in inner molecular layer of dentate gyrus) (Holmes et al. 1999)</th>
<th>Dentate subgranular zone (SGZ) neurogenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>0–30</td>
</tr>
<tr>
<td>1</td>
<td>&lt;10</td>
<td>Scattered</td>
<td>Occasional</td>
<td>31–60</td>
</tr>
<tr>
<td>2</td>
<td>10–20</td>
<td>&lt;20</td>
<td>Numerous (patchy)</td>
<td>61–90</td>
</tr>
<tr>
<td>3</td>
<td>20–50</td>
<td>20–50</td>
<td>Nearly continuous</td>
<td>91–120</td>
</tr>
<tr>
<td>4</td>
<td>50–80</td>
<td>50–80</td>
<td>Continuous highly concentrated band</td>
<td>121–150</td>
</tr>
<tr>
<td>5</td>
<td>&gt;80</td>
<td>&gt;80</td>
<td>—</td>
<td>&gt;150</td>
</tr>
</tbody>
</table>

*Grading scale: normal = 0, minimal = 1, slight = 2, moderate = 3, marked = 4, severe = 5.*
Medians were calculated for the ordinal scores of all lesions. These scores were tested using the Mann-Whitney U test, a nonparametric hypothesis test (Mann and Whitney 1947). Data were considered significantly different at a minimum confidence level of $p \leq .05$.

**RESULTS**

**Behavioral Observations**

Following a single KA treatment of 9 mg/kg, seventy-six of the eighty rats survived the treatment (given supportive fluid replacement as needed), and seventy of the survivors exhibited SE within forty-five to sixty minutes post-KA administration. Important clinical signs included changes in physical activity, hypersalivation, stereotypic grooming, wet dog shakes (a type of motor automatism characterized by shaking the body like wet dog), varying intensities of continuous tremors, and continuous clonus for two and one half to three hours after KA administration until cessation by diazepam. Stages 3 to 5 spontaneous motor seizures developed in fifty-six of the seventy rats that had earlier experienced SE following a variable latency period ranging from one to eight weeks. Fourteen of fifty-six epileptic rats (25%) had spontaneous motor seizures on Day 7.

In general, the incidence of seizure frequency progressively decreased until the termination of the study (Figure 1A). The incidence of seizure frequency per rat was 1.03 ($n = 30$) by the first seven days, almost doubled ($n = 24$) from Day 7 to Day 14, and reduced slightly ($n = 24$) between Days 21 and 28. Seizures were noted infrequently near the Days 84 and 168 termination times.

**Histopathology**

The most important histopathological changes were time dependent and included neuronal degeneration and regeneration, microgliosis, astrogliosis, and neurogenesis. Figure 1B shows the temporal progression of various histopathological parameters in the hippocampus. Neurodegeneration was apparent on Day 3, peaked on Day 6, and decreased after Day 28.

Astrogliosis developed more slowly, peaked on Day 28, and persisted until study termination. The distribution and severity patterns of neurodegeneration and activated microglial infiltration were similar through Day 28. However, activated microgliosis decreased minimally, and the neurodegeneration decreased sharply on Days 84 and 168. The onset of the regenerative process, characterized by aberrant MF sprouting within the inner molecular layer of dentate gyrus, was first seen on Day 6 (just before the onset of observed spontaneous motor seizures), peaked on Day 14, and decreased in prominence thereafter. No important changes were found using this battery of stains in the brains of the five rats that experienced SE on Day 0 but failed to develop spontaneous motor seizures and were killed on Day 168.

**Neuronal Degeneration**

A time-dependent induction of neurodegeneration, occasionally accompanied by mineralization, was evident in the hippocampus (CA1, CA3, and dentate hilus), thalamus, and
V ol. 36, No. 7, 2008  HISTOPATHOLOGY OF MTLE IN F-344 RAT MODEL 937
cerebral cortex (Table 4). Degenerating neurons had pyknotic
nuclei with indiscernible nucleoli and condensed hyper-e-
osophilic neuroplasm. Degenerating neurons in multiple
brain areas examined between Days 3 and 168 had bright green
fluorescence of the neuronal bodies, axons, and dendrites by
autofluorescence or FJB staining.
Slight to severe neuronal degeneration was present in CA1
on Day 3 in six of six rats (Figures 2A, 2B, and 2D), and con-
sequent loss of neurons resulted in a discernible reduction in
the thickness of CA1 at Days 84 and 168 in four of twelve rats.
Neuronal degeneration was less severe in CA3 than in CA1.
Mineralization was found as early as Day 28 (Figure 2 E) in
damaged thalamic nuclei and in the thalamus of four of twelve
rats on Days 84 and 168. Complete focal loss of neurons with
subsequent mineralization occasionally occurred in CA1 on
Day 168 (Figure 2F).

Aberrant Mossy Fiber Sprouting

Aberrant MF sprouting, characterized by Timm’s-positive
blackish-brown granules in the inner molecular layer of the
dentate gyrus, appeared to be correlated with the total cumula-
tive numbers of seizures exhibited by rats at corresponding
time points. Minimal to slight aberrant MF sprouting, charac-
terized by occasional blackish-brown granular deposits, was
evident in two of the six treated rats on Day 6. Slight to mod-
erate aberrant MF sprouting, characterized by patchy to nearly
continuous blackish-brown punctuate staining, was observed in
all treated rats on Days 14 and 28 (Figure 3B). Consistent with
the decreased incidence of spontaneous seizures near the end of
the study, the intensity of Timm’s staining decreased to mini-
mal to slight in four of six rats by Day 84 and was minimal in
two of six rats by Day 168.

Activated Microglial Cells

The distribution and severity patterns of activated microglial
cell infiltration, as indicated by ED-1/CD68 immunolabeling,
were similar to those of neurodegeneration described above.
However, the peak severity of the glial reaction was delayed
relative to the degeneration (Figure 1B).
Both control and treated rats had low numbers of ED-1/
CD68-positive cells at the periphery of blood vessels throughout
all the sections evaluated. This perivascular staining was consid-
ered to represent normal background labeling. In treated rats, the
ED-1/CD68-positive microglial cells were low in number on
Day 3 and gradually increased to moderate numbers on Day 28,
but decreased to minimum on Days 84 and 168. Throughout all
time points, these activated microglial cells were discernible in
all the areas with neuronal degeneration and/or loss, namely CA1,
CA3, dentate gyrus, dentate hilus, lateral septal, various thalamic and amygdaloid nuclei, and frontoparietotemporal, entorhinal, and piriform cortices (Figures 4B and 4D). The numbers of ED-1/CD68-positive activated microglial cells generally appeared to be correlated with the size of the damaged area except in the dentate gyrus and dentate hilus, where large numbers of activated microglial cells were identified even though the neuronal degeneration was minimal to slight in those areas. Activated microglial cells were also associated with the mineralized thalamic nuclei and CA1 during later time points.

Astrogliosis

Although they became apparent later than the onset of neurodegeneration and activated microglial cell infiltration, the distribution and severity patterns of astrogliosis were similar to those of neurodegeneration and microgliosis. Astrogliosis was characterized by increased size of astrocytes and increased thickness of astrocytic processes. This phenomenon was especially discernible in areas of neuronal degeneration with mineralization such as CA1, dentate hilus, and in multiple thalamic nuclei (Figures 5B and 5D).

Dentate Subgranular Zone (SGZ) Neurogenesis

In this study, DCX-expressing neurons were found clustered in the SGZ of control and treated rats. The SGZ is an area that is known to contain neuronal precursors, which proliferate and migrate into the dentate granule cell layer in adult rats (Cameron et al. 1993; Kuhn et al. 1996). The DCX-labeled processes of these neurons coursed through the entire dentate granule cell layer (Figure 6). On Day 6, four of the six treated rats had minimal increases in DCX-labeled immature neurons in the SGZ of the dentate gyrus. Five of the six treated rats on Day 14 had minimal to slight increases in DCX-labeled neurons in the SGZ. Occasionally, DCX-labeled neurons expressed Ki-67 (Figure 6). However, no differences in Ki-67 labeling were observed between control and treated rats.

DISCUSSION

The current study evaluated a temporal profile of clinical signs and histopathologic changes and refined an appropriate KA dose for producing a high incidence of spontaneous motor seizures in rats. We more thoroughly characterized the pathologic findings through special stains and immunohistochemical labeling. Three of the novel findings in this study were related to KA dose, correlation of hippocampal subanatomic site severity with human MTLE, and improved correlation of MF sprouting with spontaneous seizure incidence. Along with timely diazepam administration and supportive care when needed, the use of a lower single s.c. dose of KA (9 mg/kg), in comparison to those previously used in SD or F-344 rats (Balchen et al. 1993; Rao et al. 2006; Riljak et al. 2005), led to lower mortality and successful production of spontaneous motor seizures in a larger proportion of rats. The neurodegeneration of CA1 and CA3 in this study was of similar severity and distribution to what is reported in human patients, whereas previous rat studies had more damage to CA3 than CA1 (Balchen et al. 1993; Rao et al. 2006; Riljak et al. 2005). In the current study, aberrant MF sprouting occurred before the onset of spontaneous motor seizures and, in contrast to previous reports (Hellier et al. 1998; Rao et al. 2006), the frequency of seizures decreased with decreases in aberrant MF sprouting. The latter observation provides strong evidence for a role of aberrant MF sprouting in the generation and maintenance of spontaneous seizures.

In studies where KA was given by the s.c., i.p., or i.v. route at 12 to 14 mg/kg, mortality was high (Buckmaster 2004; Cronin and Dudek 1988; Schwob et al. 1980). In the current study, 95% of the rats survived the single s.c. KA dose of 9 mg/kg, and spontaneous motor seizures were first observed on Day 7 in 26% of the survivors. It was likely that a combination of a low dose of KA and the sufficiently slow and constant rate
FIGURE 4.—Activated microgliosis. ED-1/CD68 and GFAP-labeled microglial cells and astrocytes, respectively. Activated microglial cells in an area of neuronal degeneration in CA1 on Day 3 (B) and in the dorsolateral (D) thalamic nucleus on Day 28. A and C are from nontreated control rats.

FIGURE 5.—Astrogliosis. In contrast to pale GFAP labeling of small astrocytes with thin processes within the dentate hilus of a control rat on Day 14 (A), GFAP labeling of the hilus of a KA-treated rat on Day 14 exhibits darkly staining plump astrocytes with thickened processes (B). Similar activated astrocytes are also present in the degenerated dorso-lateral thalamic nucleus on Day 28 (D). C is from a nontreated control.
of absorption following the s.c. route of administration helped maintain moderate levels of KA within the brain (Wilkinson 2001). Diazepam administration and supportive fluid therapy likely reduced early neuronal degeneration, contributing to the low mortality without blocking the subsequent development of spontaneous motor seizures. The protocol used in the present study had the advantage of being less labor intensive in comparison to the ones with repeated low doses (Hellier et al. 1998; Riljak et al. 2005), as the latter typically required four to ten doses of KA for a total of 20–50 mg/kg of KA.

Morphological changes progressed from hippocampal neuronal degeneration and/or loss at Day 3 to regeneration in the form of aberrant MF sprouting in the inner molecular layer of the dentate gyrus on Day 6, prior to observation of the first spontaneous motor seizures on Day 7. Minimal to moderate microgliosis detected by ED-1/CD68 labeling on Day 3 was followed by widespread astrogliosis detected by GFAP labeling within and around the foci of neuronal degeneration by Day 6. The morphological changes were comparable to those reported for both SD rats given KA and to those reported in MTLE patients. The nature and progression of these changes are consistent with a leading hypothesis (Buckmaster et al. 2002; Scharfman et al. 2003) that spontaneous motor seizures occur as a result of recurrent excitatory circuitry formed by aberrant MF sprouts between the granule cells.

Previously, repeated low doses (Riljak et al. 2005) or a single high dose (Balchen et al. 1993) of KA caused greater damage to CA3 than CA1, which probably resulted in death of CA3 neurons before excitatory input through the Schaffer collateral pathway could damage the CA1 pyramidal neurons. Although rat strain differences cannot be completely discounted, the greater damage observed in CA1 in our study most likely was the result of sufficient excitatory input to the CA1 from surviving CA3 neurons. The hippocampal changes in our model closely mimic the morphological changes reported in human MTLE, where patients exhibited relative resistance of CA3 and more susceptibility of CA1 pyramidal neurons (Babb and Brown 1986; Lieb et al. 1989). The significance of greater CA1 damage as observed in human patients and rats in our model is undetermined and needs further evaluation.

In the current study, the autofluorescent nature of degenerating neurons proved to be a useful feature for finding individual or clusters of degenerating neurons. In general, the intensity of neuronal degeneration peaked in the hippocampus, FPT, piriform and entorhinal cortices, and amygdaloid nuclei on Day 6 and in the lateral septal and thalamic nuclei on Day 14 and decreased thereafter. This pattern of neuronal degeneration could be attributable to either a direct effect of KA treatment or to a secondary effect following the development of spontaneous motor seizures. This study was unable to illustrate which cause was more likely because of the sampling time points relative to disease progression. The role of seizures in inducing neuronal degeneration could be further defined by a study blocking spontaneous motor seizures. The distribution and severity of neuronal degeneration decreased remarkably on Days 84 and 168. This finding may be owing to neuronal loss following neurodegeneration. The pattern of neuronal degeneration most often observed with human MTLE (Babb and Brown 1986) was more closely reproduced with the current model of KA-induced epileptogenesis in F-344 rats than with previous models in SD rats, including both KA-induced and pilocarpine-induced models (Ben-Ari et al. 1980; Schwob et al. 1980; Turski et al. 1983). It would be interesting to use the current study protocol in SD rats to study any strain variation in the clinical and histopathological outcome.

The primary role of early neuronal degeneration, especially of CA3 pyramidal neurons, in epileptogenesis may be in aberrant MF sprouting as granular cell neurons of the dentate gyrus attempt to reestablish connections that were lost with injury to CA3 neurons. The results of the current study support previous evidence that aberrant MFs play a primary role in the pathogenesis of spontaneous motor seizure generation in
MTLE. Two of six treated rats had aberrant MF sprouting on Day 6 that preceded the first appearance of spontaneous motor seizures on Day 7. Although aberrant MF sprouting was not detected in the remaining four rats on Day 6, this finding was consistent with the small proportion of epileptic rats (fourteen of fifty-six) that developed spontaneous motor seizures on Day 7. Aberrant MF sprouts have been reported in MTLE patients (Babb et al. 1991; Buckmaster et al. 2002; Houser et al. 1990; Okazaki et al. 1999; Sutula et al. 1989), KA-induced models (Buckmaster and Dudek 1997; Buckmaster et al. 2002; Represa et al. 1993; Sundstrom et al. 1993; Sutula et al. 1998), and other animal models of MTLE (Houser and Esclapez 1996; Obenaus et al. 1993; Represa et al. 1993; White 2002). Aberrant MF sprouts form synapses with dendrites of granule cells, leading to recurrent excitatory circuitry (Buckmaster et al. 2002; Houser and Esclapez 1996; Lothman et al. 1992; Obenaus et al. 1993; Scharffman et al. 2003), which leads to spontaneous motor seizures.

In addition to the direct effects of KA, the role of additional processes in neurodegeneration and epileptogenesis must be considered. Activated microglia have been reported in the sclerotic hippocampus from temporal lobectomized MTLE patients (Beach et al. 1995). Microglial cells are the main antigen-presenting cells activated following a variety of insults in the central nervous system and may have a role in neurodegeneration (Chao and Hu 1994; Chao et al. 1992; Chao et al. 1995; Kitamura et al. 1998). In the current study, the ED-1/CD68 positive-activated microglial cells were detectable in all of the areas containing neuronal degeneration and/or loss. Microgliosis increased from Days 3 to 28, but was decreased slightly on Days 84 and 168. In contrast, Hattiangady et al. (2004) reported drastic reductions in microglial cell density following i.p. KA administration in three divided doses of 5 mg/kg from Day 16 to five months when evaluated at these two time points. Activated microglial cells may play a role in neurodegeneration (Wang et al. 2006) and epileptogenesis (Oprica et al. 2006; Vezzani et al. 1999; Vezzani et al. 2002). Further work is required to establish the role of activated microglial cells in neurodegeneration and/or epileptogenesis in our model. Our findings regarding astrogliosis are in partial agreement with those of Gray and Sundstrom (1998), which leads to recurrent excitatory circuitry (Buckmaster et al. 2002; Houser and Esclapez 1996; Lothman et al. 1992; Obenaus et al. 1993; Scharffman et al. 2003), which leads to spontaneous motor seizures.

In the present study, a small proportion of DCX-positive neurons expressing the Ki-67 were the mitotic neurons, whereas DCX-positive neurons without Ki-67 were the postmitotic immature neurons. We did not attempt double labeling with Ki-67 and the glial markers ED-1/CD68 or GFAP. However, based on single labeling with these glial markers, we can suggest that the cells with only Ki-67 labeling present in the dentate granule cell layer and the dentate hilus were most likely proliferating glial cells. The findings in the present study are in partial agreement with those of Gray and Sundstrom (1998), who observed neurogenesis by BrdU labeling in the dentate granule cell layer and the SGZ on Day 7 following intraventricular KA administration in rats. However, that study was terminated on Day 7. The exact molecular pathways supporting the neurogenesis have yet to be determined.

We did not find Ki-67 to be as sensitive as DCX to detect neurogenesis. This may be because Ki-67 was not expressed by mature cells in the G0 stage. A combination of both DCX and Ki-67, as was used in the present study, should provide a better picture of neurogenesis owing to spontaneous motor seizures. The sensitivity of both markers could likely be increased by examining serial sections of hippocampus and using stereology, rather than a subjective scoring technique as was used in the present study.

CONCLUSIONS AND FUTURE DIRECTIONS

The results of the current study support the value of s.c. administration of KA at 9 mg/kg in F-344 rats as a method for producing a promising new model of human MTLE. Important correlates with MTLE include: induction of spontaneous seizures; focus of the neuronal damage in CA1 prior to CA3 damage resembling human lesions as opposed to CA3 dominance observed in some rodent models; hilar neuronal loss; activated microgliosis; astrogliosis; and aberrant MF sprouting in the inner molecular layer of dentate gyrus. Development of aberrant MF sprouting prior to induction of spontaneous motor seizures and subsequent decreased prominence of aberrant MF sprouting concurrent with decreased spontaneous seizure incidence were important findings in the present study. Although not definitive, this apparent correlation strongly supports the hypothesis that MF sprouts are important in the pathogenesis of epileptogenesis in MTLE and its post-SE models.

Molecular biology techniques including gene profiling using microarrays, in situ hybridization, and immunohistochemistry are very promising tools to further explore the complex mechanisms of epileptogenesis in MTLE. Microarrays will undoubtedly generate large quantities of data, the majority of which may be correlative with no causal role in epileptogenesis. However, this exercise will be worthwhile, as arrays can be followed by in situ hybridization or immunohistochemistry to localize the selected genes or their products at the tissue level and individual mechanistic pathways can be dissected by using RNA silencing (siRNA) technique and/or knockout mice models.

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**References**


