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MORPHOLOGICAL STUDIES OF GABA-ERGIC NEURONS IN FOCAL EPILEPSY IN PRIMATES

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INTRODUCTION

Experimental models of focal epilepsy have been examined for specific cellular and biochemical changes to obtain an understanding of certain types of human epilepsy(1-3). The alumina gel model in monkeys is an excellent model of post-traumatic epilepsy in humans(4). Also, this model best approximates the histology of human foci in that they are both characterized by neuronal loss, reactive gliosis, and dendritic abnormalities(4,5). Seizures usually begin 2-3 months following the alumina gel implants into the hand region of the sensori-motor cortex. Epileptic monkeys with alumina gel implants display spike and wave activity on electroencephalograms (EEG's) and electrocorticograms (ECoG's) which arises from the site adjacent to the alumina granuloma and a surrounding area of cortex (6). Excision of these two regions abolishes epileptic activity (7). In addition, extracellular recordings from cells in the focus display abnormal excitatory post-synaptic potentials (6).

A growing body of evidence indicates that neurons containing the neurotransmitter gamma-aminobutyric acid (GABA) are preferentially lost in the epileptic foci of monkeys treated with alumina gel. Binding studies of these monkeys have indicated a decrease in the number of GABA receptors and biochemical studies have demonstrated a loss of tissue GAD activity in the focus (1). Consistent with these findings were the initial immunocytochemical data that demonstrated a 58-62% loss of GABAergic axon terminals in the epileptic focus of chronically seizing monkeys that had not reached status epilepticus (8). Subsequent studies have indicated that greater than 80% of the axon terminals of GABAergic chandelier and basket cells have degenerated at epileptic foci (9,10). More recent immunocytochemical studies have shown that the loss of these terminals at chronic epileptic foci was due to a loss of GABAergic somata (11). However, it was not shown whether a significant loss of GABAergic somata precedes the onset of seizure activity because only one pre-seizing monkey was used in that study (4). This question needs to be resolved because recent findings have shown a loss of GABAergic terminals prior to the onset of clinical seizures (12). Therefore, the present study of alumina gel treated monkeys was undertaken using a larger number of pre-seizing monkeys to determine whether a significant loss of GABAergic somata occurs prior to the onset of seizure activity. Additional monkeys with chronic epilepsy were also studied.

MATERIALS AND METHODS

Ten adolescent monkeys (Macaca mulatta) were used in this study. Under general anesthesia, nine of the monkeys underwent a left frontoparietal craniotomy. The precentral (Brodman's area 4) motor cortex for the hand-face area was identified by electrocortical stimulation. Seven monkeys were injected with 0.2 cc. of aluminum hydroxide (alumina gel) into the left pre- and post-central gyri using the Ward modification of the Kopeloff technique (13). Two monkeys were used as surgical controls in which a subpial resection was made of a 5 x 5 mm. area down to the white matter. This area is approximately equivalent to the area of a mature alumina granuloma. One monkey received neither an injection of alumina gel nor a surgical lesion and served as a control animal.

Three monkeys were sacrificed two to four weeks post-injection time which was prior to the development of seizure activity. These animals are referred to as pre-seizing monkeys, and although they did not display seizures, they did demonstrate a sharp wave adjacent to the granuloma on the electrocorticogram (ECoG). The acute seizing monkey was sacrificed six weeks following the injection. This animal displayed two to three seizures per day for four days. The three chronic monkeys displayed a minimum of one seizure per week and were sacrificed 3-6 months following the injection. These chronically seizing animals showed an ECoG consistent with spike and wave discharges in the area immediately adjacent to the developing granuloma.

After the ECoG, each monkey was anesthetized with sodium phenobarbital and perfused transcardially with fixative. A tissue block from the left precentral gyrus was immediately removed and included an area adjacent to the alumina gel injection and an area 1-2 cm superior to the injection site. A block of tissue was also taken from the right precentral gyrus to be used as control tissue. These blocks were placed in a 10% formalin solution for further fixation. The tissue was then placed in a cryoprotectant solution of 30% sucrose in phosphate buffered saline (PBS) for 24 hrs. Sections of 40 micron thickness were cut on a freezing microtome and placed in 0.1M PBS for light microscopic immunocytochemistry. Free floating sections were processed for the immunocytochemical localization of glutamic acid decarboxylase (GAD), the synthesizing enzyme for GABA, using an anti-GAD serum developed by Oertel et al.(14). Sections from the right and left hemispheres were incubated at the same time.

Sections of tissue were examined under a light microscope equipped with a 40X objective lens. All sections were analyzed for the content of GAD-positive cells. The GAD-positive cells were identified as cells with dense, brownish reaction product within their perikaryal cytoplasm. The alumina gel treated animals were examined and counted for GAD-positive cells at four sites. The area adjacent to the alumina granuloma was referred to as the focus, whereas an area one centimeter from the focus was called the parafocus. Two other sites were examined for these monkeys, and they were located in the contralateral hemisphere. The two surgical control animals were examined at a proximal

site adjacent to the lesion, at a distal site about 1 cm away from the lesion, and at two other sites on the contralateral side. The normal monkey was counted for the number of GAD-positive cells at two sites 1 cm apart in both right and left motor cortices.

RESULTS

All layers of the normal and non-epileptic motor cortex contained GAD-positive cells that were, in general, homogeneously distributed throughout all layers. The GAD-positive cells contained a brown reaction product in their perikaryal cytoplasm and rarely were their dendrites stained. Many of these neurons displayed eccentrically placed nuclei. The shapes of the cell bodies were clearly non-pyramidal (Fig. 1). Thus, the GAD-positive somata were round oval, teardrop-shaped, or fusiform shaped.

GAD-positive somata that were found in the focus and parafocus sites were similar in shape and size to those in the normal and non-epileptic cortical sites (Figs. 1A and B) Also, they were distributed throughout the cortical layers. However, reduced numbers of GAD-positive somata occurred in these regions of chronically seizing monkeys as compared to the two sites examined in the contralateral cortex (Figs. 1A and B). The pre-seizing monkeys displayed reduced numbers of GABAergic neurons in the focus. The parafocus in these monkeys appeared similar to the contralateral non-epileptic cortex. In addition to these observations for GAD-positive somata, the focus in both pre-seizing and chronically seizing monkeys displayed a reduction in the number of GAD-positive puncta as compared to the contralateral cortex (Figs. 1A and B). These puncta are the light microscopic correlates of axon terminals.

The pre-seizing monkeys displayed significant differences in the number of GABAergic somata compared between the focus and both contralateral sites and between the focus and parafocus ($p<0.05$, Fisher probable least significant difference test). The quantitative data demonstrated that the loss of GABAergic neurons at the focus for the pre-seizing monkeys ranged from 23%-44% as compared to the control sites (Fig. 5). There was no significant loss of GAD-positive cells at the parafocus of pre-seizing monkeys. The chronically-seizing monkeys also displayed a significant loss of GAD-positive somata ($p<0.05$) between the focus vs. parafocus and between the focus vs. both contralateral sites. In addition, the parafocus showed a significant loss of neurons as compared to both contralateral sites in the chronically-seizing monkey ($p<0.05$, Fisher probable least significant difference test). The data indicated a loss of GABAergic neurons at the focus and parafocus ranging from 42%-61% and 15%-26%, respectively. The acute monkey had no differences between any of the sites that were statistically significant because only one subject was used. However, the focus of this monkey displayed a 20% loss of GABAergic neurons as compared to the contralateral sites. The normal and surgical control animals did not show any significant loss of GAD-positive neurons among their sites.

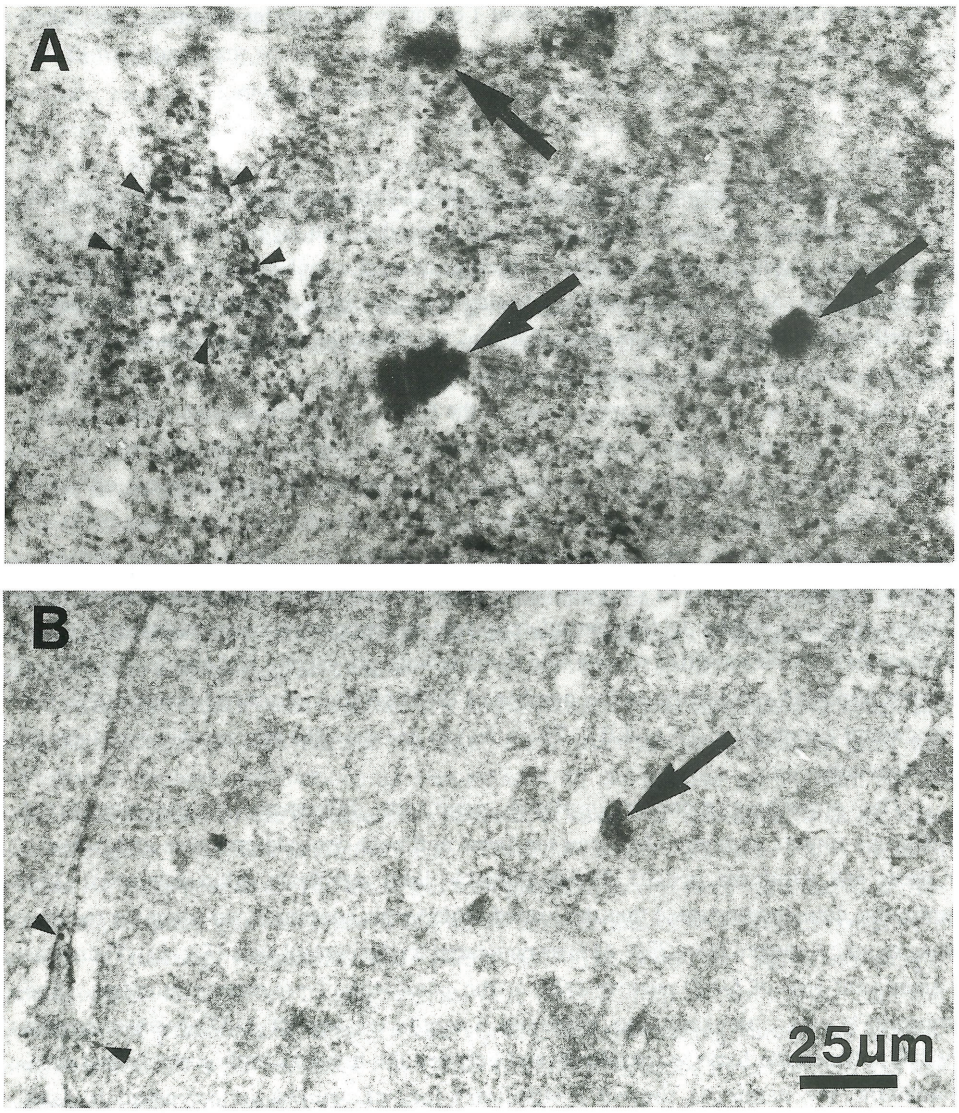

Fig. 1 Light photomicrographs of GAD-immunoreacted sections from a chronically seizing, alumina gel treated monkey. **A** shows layer V in the contralateral non-epileptic site that contains a number of densely stained GAD-positive cells (arrows) and numerous GAD-positive puncta (arrowheads). Some of the puncta are concentrated around a pyramidal cell. **B** shows the distribution of GAD-positive cells (arrow) in layer V at the focus adjacent to the alumina gel granuloma. The number of GAD-positive cells (arrows) and puncta (arrowheads) is less than that found in **A**.

DISCUSSION

The major finding of this study is the demonstration of a significant and selective loss of GABAergic somata at the focus site in the pre-seizing alumina gel treated animals. The magnitude of the loss of GABAergic neurons was 23-47% as compared to the contralateral, non-epileptic cortex. Since no significant loss of total neurons occurred in the pre-seizing focus (15), the loss of GABAergic neurons at focal sites of alumina gel application in pre-seizing monkeys is selective for this neuronal type. In addition, these data suggest that GABAergic neuronal loss precedes the onset of clinical seizures, a finding that is consistent with the results of Houser et al. (12), who showed a significant loss of GABAergic terminals in pre-seizing monkeys. Since this latter study showed degenerating terminals at the electron microscopic level, it is likely that the reduction in the number of GAD-immunoreactive somata reflects a degeneration of these neurons and not simply a loss of immunocytochemically detectable levels of GAD in cortical GABAergic neurons as observed in the kindling model of epilepsy (16). The loss of GABAergic neurons in pre-seizing monkeys is probably a result of the alumina gel implants because the surgical control animals did not show a significant loss of GABAergic neurons. Therefore, the alumina gel may somehow affect the cortical vascular supply to cause ischemia which may be the cause of the selective destruction of GABAergic neurons as previously proposed (10).

Another important finding of this study was that the chronically seizing monkeys displayed a significant loss of GABAergic neurons at both the focal and parafocal sites. A previous study showed a significant loss of GAD-positive cells at the focus (11). However, the parafocal region in these same chronic monkeys did not show a significant loss. This was probably due to the fact that those chronic animals were sacrificed 2 to 2 1/2 months after the alumina gel implant. In the present study, the chronic animals were sacrificed 3 to 6 1/2 months after the implant. These data add support to an old adage, that seizures beget more seizures. In other words, the longer the time interval between implantation of the alumina gel and the time of examination of brain tissue, the more seizures will have occurred and the more severe will be the degeneration of GABAergic neurons and terminals. Thus, the results of the present study show that alumina gel causes a significant loss of GABAergic neurons prior to seizures at the focus and that as seizures progress, more GABAergic neurons are lost at both the focus and parafocus.

The relevance of these experimental findings in monkeys to the understanding of human epilepsy has recently been revealed with an analysis of a young girl with intractable epilepsy who had a tumor removed form her left temporal lobe (17). The temporal lobe including the hippocampal formation and associated parahippocampal structures was immersed into fixative for light- and electron microscopic preparations. Light microscopy revealed a ganglioglioma in the entorhinal cortex where pyramidal cell bodies were apposed by tumor cells. The dentate gyrus displayed a decrease in the number of granule cells, whereas the CA1 region (Sommer's sector) was devoid of virtually all pyramidal cells.

Cortical interneurons labeled with immunocytochemical methods were decreased in the entorhinal cortex and dentate gyrus (17). The basket plexus of axon terminals around pyramidal cells was labeled in CA3 but not in CA1 where the pyramidal cells were lost. Electron microscopic preparations of the entorhinal cortex showed a normal appearance of pyramidal cells and interneurons in tissue distal from the tumor. However, pyramidal cells within the tumor were apposed by numerous layers of reactive astrocytic processes and lacked symmetric axosomatic synapses. In contrast, asymmetric (excitatory) axodendritic synapses were found in the adjacent neuropil. These findings suggest that the projection neurons in this area have lost their inhibitory feedback control and may cause the loss of hippocampal neurons by their high physiologic activity.

These studies show that a loss of GABAergic neurons in the cerebral cortex may provide the basis for epileptic activity. This conclusion is well founded in our monkey studies. Further work is required to demonstrate this conclusion in specific types of human epilepsy.

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