TRANSMISSIBLE MINK ENCEPHALOPATHY IN CARNIVORES: CLINICAL, LIGHT AND ELECTRON MICROSCOPIC STUDIES IN RACCONS, SKUNKS AND FERRETS *

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Abstract: Four raccoons and one of two skunks inoculated with brain suspensions containing the transmissible mink encephalopathy (TME) agent developed a neurologic disease characterized by alterations of behavior, by incoordination and by slowing of motor activity. Histologic examination of the brains revealed a spongiform polioencephalopathy as is characteristic of the disease in mink. Fourteen ferrets inoculated with TME brain suspensions remained asymptomatic until sacrifice 2 years post-inoculation. A spongiform degeneration of gray matter was present in all ferret brains. However, the lesions and their topographical distribution were distinctly different from those seen in the brains of all other species susceptible to TME infection.

INTRODUCTION

Transmissible mink encephalopathy (TME) is a rare sporadic disease of ranch mink first known to occur in Wisconsin in 1947, with additional outbreaks in Wisconsin in 1961 and 1963.[1] In 1963, TME also was recognized in ranch mink in Idaho[2] and Ontario.[3] One additional outbreak was reported in 1967 from East Germany.[4] Epizootiologic evidence suggested that the disease in Wisconsin mink had occurred by introduction of the agent in the animals' diet.[1] Morbidity in these outbreaks was usually high and almost entirely limited to adults. TME is a progressive, uniformly fatal disease of mink restricted in its pathology to the central nervous system (CNS). The experimentally induced disease lasts from a few days to 6 weeks and follows a long incubation period, 7-10 months after oral

inoculation and 5-6 months after parenteral inoculation.[2,4,5,6] TME has never been reported in wild mink.

The purpose of this study was to further define the experimental host-range of the TME agent, specifically for carnivores other than mink, and to describe the clinical and pathologic features of the experimentally induced disease.

MATERIALS AND METHODS

Wild raccoons (Procyon lotor) and striped skunks (Mephitis mephitis) of unknown age were trapped in Dane and Iowa counties, Wisconsin. The origin of one of the experimental raccoons was not traced. Randomly selected domesticated, pregnant albino ferrets (Mustela putorius fero) were obtained from the Fur Animal Farm, Genetics Department, University

of Wisconsin. Each raccoon and skunk was inoculated with a suspension of either infected mink brain containing the 4th passage TME agent (Wisconsin isolate) or with normal mink brain. The intracerebral (I.C.) route of inoculation was used for skunks and raccoons with the exception of one raccoon which was inoculated only *per os* (P.O.). Details of inoculation techniques have been described elsewhere.[7] The 15 fetuses of two ferret dams were inoculated with TME mink brain suspensions approximately 1 week prior to parturition. After surgical exposure of the uterus each fetus was held firmly and an attempt was made to introduce 0.1 ml of inoculum through the uterine wall into the body below the head. This inoculation procedure was designated as the fetal trunk (F.T.) route. It has to be assumed that the dams received concomitant inoculation by inadvertent spilling of inoculum into the uterus.

Brain and general body tissues were collected for light microscopy from sernylan[4]-anesthetized or from dead animals. For electron microscopy, three cerebral cortical biopsies were taken prior to death from each of five raccoons (Table 1: #770, #324, #246, #399, #400). The specimens were minced and fixed in 3% phosphate buffered osmium tetroxide. Adjacent pieces of cortex were fixed in formalin-ammonium-bromide according to the Cajal technique. Cortical tissue from some animals was frozen for bioassay in mink. After this, the remainder of the brain was removed and fixed in 10% formalin. Spinal cords were removed from all animals. A complete necropsy examination was performed and all major organs were sampled for histology.

RESULTS

Raccoons

Three racoons (#324, #770, #772) inoculated I.C. with 0.3 ml of a 10% TME mink brain suspension developed a similar, rapidly progressive disease characterized by alterations in behavior, weakness, incoordination especially of the hind limbs, and generalized slowing of motor function. The incubation periods were 190, 174 and 167 days respectively, and the clinical course ranged from 7 to 17 days. Two raccoons (#324, #770) were killed during the terminal stage of the disease while raccoon #772 was killed at an earlier stage.

Approximately 1-2 weeks prior to the onset of incoordination, two raccoons (#324, #772) which were usually shy, became friendly and playful, grasping the human hand when offered. The animals stayed out of their nest boxes during the day, even in the presence of man. A striking behavioral change was noted in the third raccoon (#770). This animal had previously remained calm in the nest box while the caretaker opened and cleaned the cage. Five weeks prior to the onset of incoordination, she became aggressive and would attack. About 1 week prior to death, she lost the aggressiveness and appeared clinically similar to the other two sick raccoons.

Incoordination and weakness were first detected when the animals had difficulty climbing into their nest boxes located 50 cm above the cage floor. They were able to pull themselves up with their forelegs but were unable to hoist the hindlegs into the opening. These attempts were repeated several times per day. Only a few days after this, the animals made no further attempts to reach the boxes and remained on the cage floor day and night. When placed on the floor of the room, they would follow the caretaker up and down the aisle, like pets, and could be led back to their cage. Movements were slow and incoordinated. When objects were placed in their path, the raccoons did not circumvent them but climbed over them in an awkward stumbling fashion. At the time of euthanasia, two animals were unable to walk. None of the animals appeared to be blind, and none showed tremors or seizures. All three raccoons continued to

[4] Phencyclidine hydrochloride 20 mg/ml, Bioceutic Labs., Inc.

TABLE 1. Susceptibility of Carnivores to Transmissible Mink Encephalopathy (TME).

Species	Animal #	Inoculum [1]	Inoculation [2]	Incubation Period	Microscopic Findings	Brain Assay [5]
Raccoon	772	TME	I.C.	167 days	TME	166, 155 days
	770	TME	I.C.	174 days	TME	148, 152 days
	324	TME	I.C.	190 days	TME	N.D.
	246	TME	P.O.	306 days	TME	N.D.
	399	NMB	P.O.	Asympt. 425 days	Normal	N.D.
	400	NMB	I.C.	Asympt. 425 days	Normal	N.D.
Skunk	769	TME	I.C.	133 days	TME	130, 141 days
	776	TME	I.C.	Asympt. 395 days	TME	N.D.
Ferret						
Dams	1	TME	I.U.	82 days[3]	Normal	N.D.
	2	TME	I.U.	Asympt. 700 days	TME[4]	N.D.
Offspring	3	TME	F.T.	59 days[3]	Normal	N.D.
	4	TME	F.T.	89 days[3]	Normal	N.D.
	5-17	TME	F.T.	Asympt. 700 days	TME[4]	N.D.

[1] Either TME infected mink brain (TME) or normal mink brain (NMB).

[2] Inoculation by either one of the following routes: intracerebral (I.C.), per os (P.O.), intrauterine (I.U.) or into fetal trunk (F.T.).

[3] One of two dams and 2 of 15 kits died of intercurrent disease 60-90 days post-inoculation.

[4] Spongiform polioencephalopathy, but not typical of TME.

[5] Assay for presence of TME agent by I.C. inoculation of mink is reported as the incubation period, or, if not done, as N.D.

eat and drink, and remained bright and alert. Somnolence, which is characteristic of TME in mink, hamsters and monkeys, was not observed. Brain suspensions from raccoons #770 and #772 were inoculated I.C. into two mink each and produced typical TME after incubation periods of 148-166 days (Table 1).

A fourth raccoon (#246) had been inoculated P.O. with 3.0 ml of a 1% suspension of TME mink brain. After an incubation period of 306 days, the animal became incoordinated and weak and had difficulty getting into and out of his nest box. Behavioral changes had started 2 weeks earlier. Although his previous shyness disappeared, a pet-like friendliness never developed. Just prior to sacrifice when the alert animal was placed on the floor of the room, he was unable to walk or stand and showed a slight trembling of the head and fore limbs.

In all four raccoons, light microscopic lesions were limited to the brain (Fig. 1)

and were typical for TME as described previously in other species.[2,3,5,6,7,8] Vacuolization of gray matter, neuronal degeneration, and hyperplasia and hypertrophy of astrocytes were bilaterally symmetrical, diffuse, and widespread, involving the cerebral cortex, the non-cortical telencephalon, the diencephalon and mesencephalon, and to a lesser degree the brainstem. Lesions were absent in the cerebellum and spinal cord. Only in the amygdaloid nuclei did the encephalopathy have a patchy rather than diffuse character. No distinction could be made in the extent of the overall encephalopathy as present in the P.O. inoculated animal versus two of the I.C. inoculated ones. The third I.C. inoculated raccoon (#324), however, showed an even more severe and extensive involvement of the cerebral cortex. This difference was noticeable in the frontal and occipital portions of the brain. By the Cajal method, a moderate to marked cortical astrocytosis was demonstrated in all animals (Fig. 2 A&B).
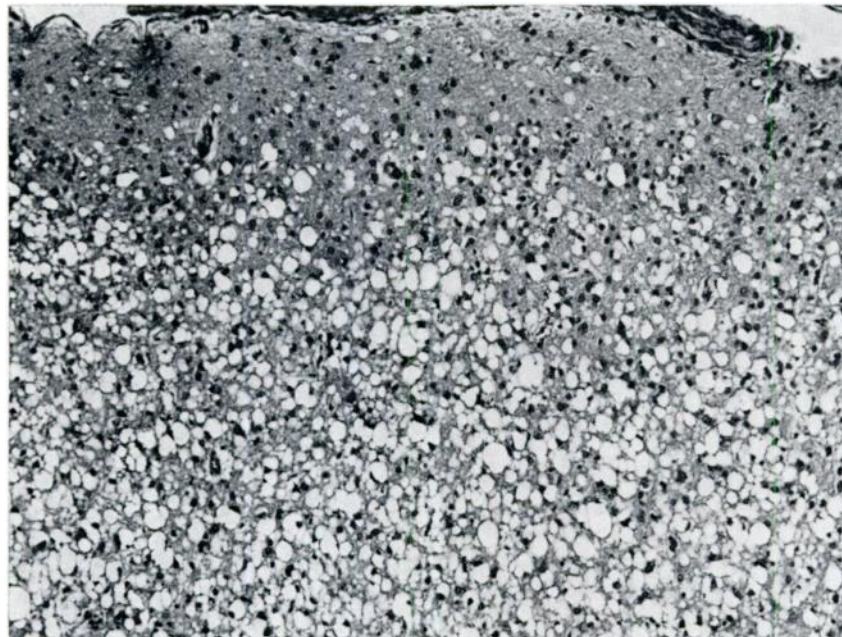

FIGURE 1. Diffuse spongiform polioencephalopathy of the cerebral cortex of TME affected raccoon. H&E x 100.
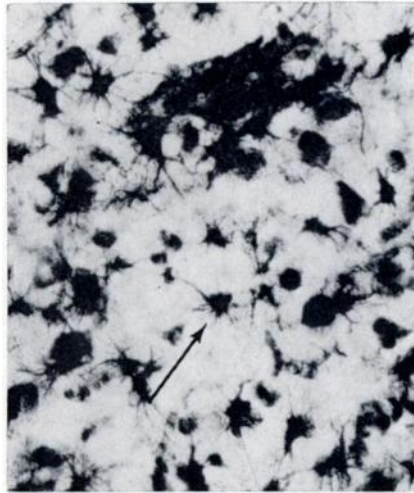
FIGURE 2A. Hypertrophied astrocytes (arrow) with numerous processes in vacuolated cerebral cortex of TME affected raccoon. Frozen section, Cajal's gold chloride sublimate x 375.
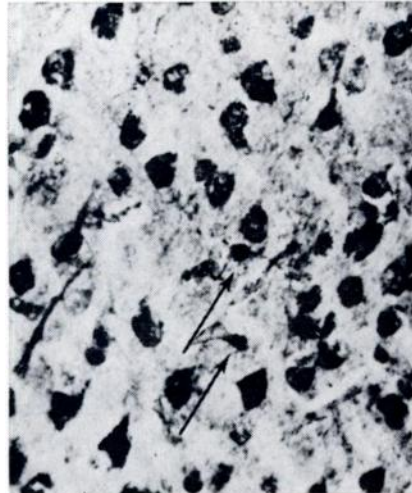
FIGURE 2B. Cerebral cortex of control raccoon with astrocytes (arrows) of average size and number surrounded by neurons. Frozen section Cajal's gold chloride sublimate x 375.

Ultrastructural studies of three TME infected raccoons (#770, #324, #246) revealed, as the chief abnormality, well-defined electron lucent vacuolar areas within the neuropil (Fig. 3). These were not related to blood vessels. They measured up to 13 micra in width (study based on 174 electron micrographs). Most of the vacuoles contained innumerable kinked filaments approximately 100 nm long and less than 5 nm wide (Fig. 4). In addition, aggregates of curled or whorled or vesicular (60-200 nm) membrane profiles were seen along the periphery of vacuoles (Figs. 3 and 4). Some of these could be traced in their origin to the enclosing membrane of the vacuole. This membrane showed focal dehiscences by which connections to the extracellular compartment had been established. The derivation of many of these vacuoles remained uncertain. However, along the periphery of some, synaptic junctions were found (Fig. 5), indicating participation of neuronal dendrites in the formation of lesions. The second most frequently encountered abnormality in the neuropil consisted of focal dendritic alterations. Dendrites showed swelling up to more than 4 micra and, especially at their synaptic junctions, rarification of organelles, presence of vesicles and disruptions or invaginations of their surface membrane (Fig. 6). Presynaptic axonal abnormalities were found but rarely and consisted of accumulation of vesicles from 250-300 nm in width. Myelinated axons on occasion showed swelling of the axoplasm (Fig. 7), rarification of organelles, and presence of 1-2 micra vacuoles. The perikarya of a few neurons contained small electron-lucent areas with or without enveloping membranes. Focal cytoplasmic degradation also was noticed. Astrocytes showed no distinct changes other than hypertrophy.

Two control raccoons, one inoculated I.C. (#400), the other P.O. (#399) with normal mink brain were asymptomatic when killed 14 months post-inoculation. Their tissues were unremarkable in light microscopic and ultrastructural studies (45 electron micrographs).

Skunks

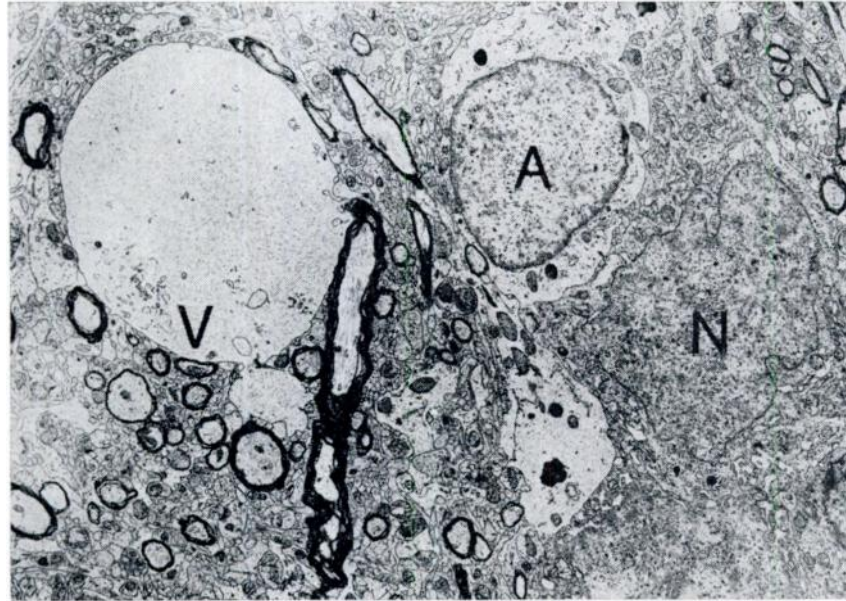One (#769) of two skunks, inoculated I.C. with 0.1 ml of a 10% suspension of

FIGURE 3. Vacuole (V) separated by myelinated axons and other cell processes from astrocyte (A) and nerve cell (N). x 3600. In Figs. 3-7, sections contrasted with uranyl acetate and lead citrate.
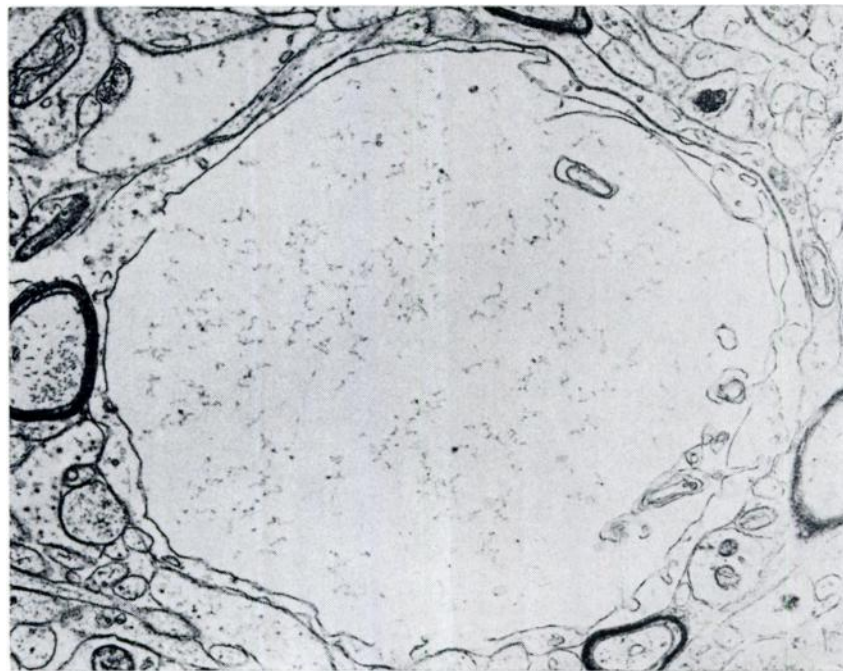
FIGURE 4. Vacuole within a greatly expanded cell process of the neuropil. x 16000.
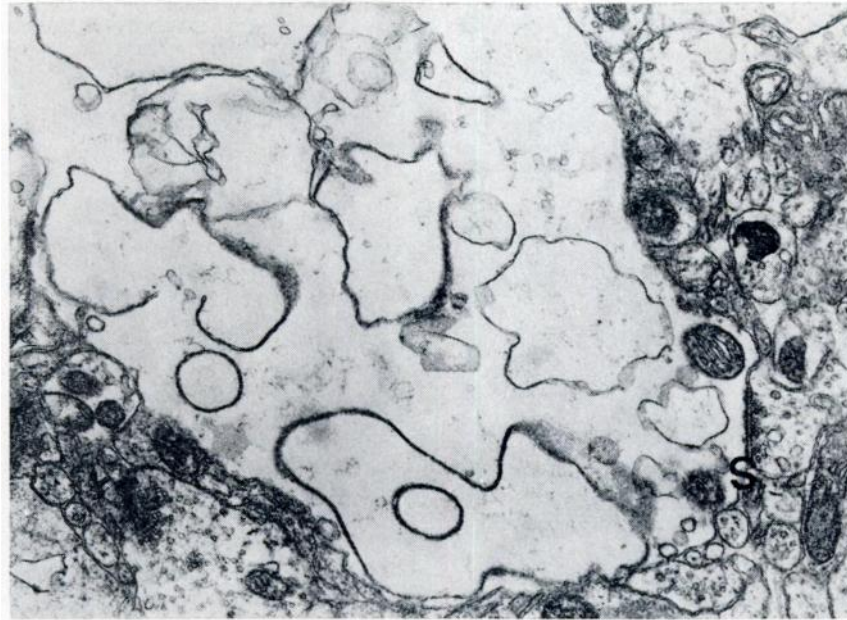
FIGURE 5. Vacuole of more complex content than in Figs. 3 & 4, possibly derived from several degenerated cell processes. The post-synaptic portion of the synapse (S) has been incorporated into the lesion. x 18800.

FIGURE 6. Axo-dendritic synapse (S) with membrane alterations and cytoplasmic "clearing" of post-synaptic portion. Normal synapse at top right of photograph. x 16000.

FIGURE 7. Abnormal myelinated axon and unaltered axon (bottom). x 5700.

TME mink brain, developed a personality change 4½ months post-inoculation. She became very shy and refused to move forward to eat if the caretaker remained nearby. During the next few weeks, she developed progressive incoordination, general body tremor and apathy. One month later she appeared terminally ill and was euthanized.
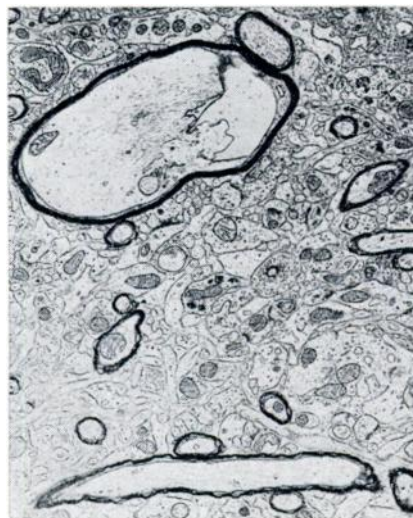
Microscopic examination of the brain revealed a typical spongiform polioencephalopathy as seen in TME affected mink. The sponginess was severe in the non-cortical telencephalon and in the diencephalon. The cerebral cortical lesions were less severe and had a limited topographical distribution. No astrocytosis was demonstrated in a small sampling of Cajal-stained sections. Small perivascular mononuclear infiltrates in the leptomeninges and diencephalon were thought to be related to the presence of a round worm found under the dura during removal of the calvarium. Inoculation of brain suspensions from this skunk I.C. into two mink produced typical TME after incubation periods of 130 and 141 days (Table 1).

The second skunk (#776) remained asymptomatic and was found dead 13 months post-inoculation. Microscopic examination of the brain revealed spongiform encephalopathy of a less severe degree and a more limited distribution than in the first skunk. No vacuolization was found in the cerebral cortex, but shrunken neurons and neuronophagia were common in some cortical areas. An area of vacuolization was present in the most ventro-medial portion of each caudate nucleus and there were patchy lesions throughout the thalami. In the hypothalmus, spongiform lesions of a slight degree occurred quite diffusely. In the mesencephalon, lesions involved the substantia nigra and the central tegmental field. There were no inflammatory infiltrates. The cause of death was unclear, but was not considered to have been the TME encephalopathy.

Ferrets

One dam (#1) and two offspring (#3, #4) died of intercurrent disease 60-90 days post-inoculation. Their brains were free of gross and microscopic lesions. The other dam and a total of 13 offspring of albino and dark variety remained asymptomatic until they were killed 2 years post-inoculation.

Microscopic examination of these brains revealed focal areas of vacuolization in the middle zone of the cerebral cortex (Fig. 8). The lesions were present in both hemispheres and were most prominent along their lateral aspects. Vacuoles in small numbers tended to be clustered in groups with an occasional proximity to blood vessels (Fig. 9). The vacuoles tended to be multilocular. When oblong, they were oriented toward the cortical surface. Neuronal degeneration was not prominent. Hypertrophied astrocytes were present in some vacuolated areas. Clusters of vacuoles were seen in both thalami. The lateral cuneate nuclei seen in the non-standardized sections of four ferret brains appeared diffusely vacuolated.

DISCUSSION

The range of animal hosts susceptible to experimental infection with TME agent is wide.[7,9,10,11,12] The short incubation periods in the first passage of TME in skunks and raccoons, which is similar to that of mink, suggests that they are particularly susceptible. In contrast, when the high titer TME agent was inoculated I.C. into species such as primates[11] or hamsters[10] relatively long incubation periods resulted (11 to 23 months, and 18 months respectively). Since oral infection appears to be important in outbreaks of TME in ranch mink, the susceptibility of one raccoon (#246) to infection by the oral route in this study suggests that natural infection may occur following consumption of infected tissues. Inoculation by the oral route greatly increased the length at the incubation period in both mink[8] and raccoon. Other routes of inoculation may extend the incubation period beyond the life span of the species such as in short lived rodents, or beyond a reasonable period of exprimental observation, as probably occurred in the ferrets in this study. One carnivore,

FIGURE 8. Focal areas of vacuolization in the middle zone of the cerebral cortex of an asymptomatic TME -inoculated ferret. H&E x 60.
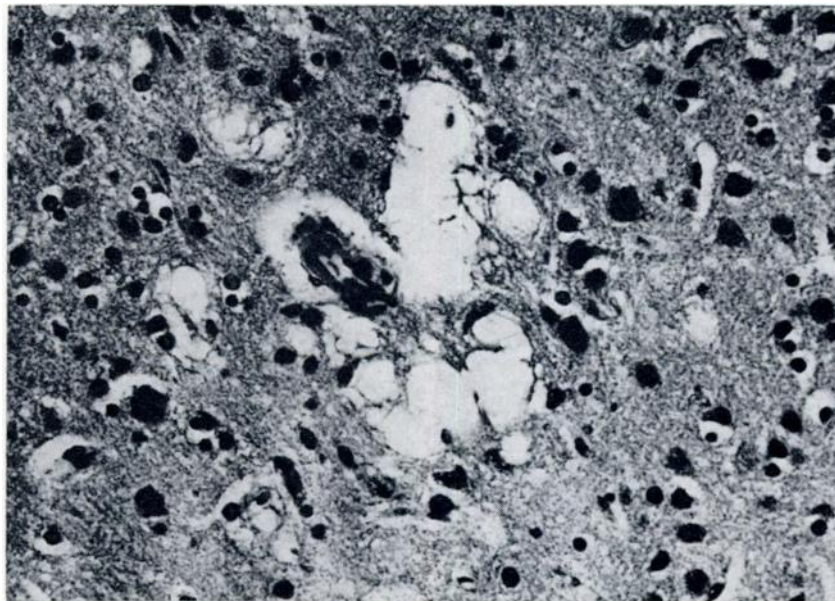

FIGURE 9. Group of focally confluent vacuoles in close proximity to a blood vessel in the cerebral cortex of an asymptomatic TME-inoculated ferret. H&E x 400.

the cat, has failed to develop a disease following subcutaneous (S.C.) inoculation with TME agent and remains asymptomatic more than 5 years post-inoculation.[10]

Personality change in the raccoons and one skunk was a more readily recognizable aspect of the CNS disease than in mink, where it occurred chiefly in the prodromal period.[6] Various distinct personality changes occur in TME affected primates.[11,13]

Microscopic lesions and their topographical distribution in the clinically affected raccoons and skunks were remarkably similar to those observed in mink. The presence of lesions in one skunk that had been asymptomatic after a much longer observation period would indicate a later development or a slower progression of the disease. In contrast to mink, however, in which earliest vacuolization involves the cerebral cortex,[7] it would appear that the earliest vacuolization in the skunk involved the deep subcortical gray matter.

Ultrastructural analysis of the TME infected raccoon brains revealed the same basic features as noticed previously in other species.[11,13,14,15] The vacuoles seen by light microscopy do not simply represent focal dilatation of the extracellular compartment but can be traced in their origin to neural elements and in particular to dendritic cell processes. Factors involved in the formation of the "empty" lesions seem to be swelling of the dendritic cytoplasm, at times in neighboring dendrites, loss of constituent organelles, breaks in the surface membranes and secondary incorporation of some extracellular space. Whether the swelling of the dendrites was an exclusively hydropic phenomenon and whether the chief content of the vacuoles was water are questions not yet completely answered by the techniques employed. The elusiveness of the TME agent to ultrastructural identification is a vexing problem. It can be assumed that the agent while replicating in the tissues instigates various biochemical reactions which lead to dysfunction of individual neurons, and, if the injury is extensive enough, to clinical symptoms.

A suspension of electric transmission at the synapses is probably a more frequent event than the loss of the current-producing nerve cell itself.

The reaction of ferrets to inoculation with TME agent deserves special attention. Marsh et al.[10] observed a clinically vague disease and numerous large vacuoles in the frontal and parietal cortex in albino ferrets sacrificed 15 months after S.C. or I.C. inoculation with TME agent. Dark ferrets remained asymptomatic for three years and their brains were normal at necropsy. Four albino ferrets which developed clinical disease after 14-15 months, recovered within 4-6 weeks and remained asymptomatic through the 28th month of the experiment.[10] Recovery from clinical disease has never been observed in TME affected mink or primates. In the present study, all ferrets, albino or dark, remained healthy 2 years after inoculation, but all had brain lesions as described previously.[10] The topography of the lesions was different from that seen in mink and was more limited in the cerebral cortex, where it, however, involved precisely that zone most prominently affected in mink.[7] The overall degree of disruption of the gray matter was comparable to that seen in mink and monkeys prior to the onset of disease.[7] Perhaps these animals would have developed symptoms at an even later date.

It would appear that the age of the ferrets and the route of inoculation chosen in this study did not affect the unusual variation of spongiform encephalopathy since similar lesions had been produced after S.C. and I.C. inoculation of adult albino ferrets. The previously assumed resistance of dark ferrets to infection, however, may have been overcome by the mode of inoculation used in this study. The disease in ferrets warrants further investigation. Electron microscopic studies were not performed on this material, and the ferret brains were not assayed for the presence of TME agent by I.C. inoculation of mink. In addition, the effect of normal mink brain suspension inoculated by the same mode was not determined.

The possibility that the spongiform lesions in ferrets were based on an entirely different etiology has to be considered. However, to the best of our knowledge no such cases have been reported in the literature. The diet was carefully controlled and — at necropsy — no organic disease outside the CNS was found and there were no parasites. The lesions do not resemble those seen in experimental allergic encephalomyelitis.[16] In fact, no hypersensitivity type lesions have been found to date in the CNS of the various species employed in TME experiments when inoculated for control purpose by any one of several routes (exclusive of F.T.) with either homologous or heterologous brain suspensions.

It should be noted that the three ferrets dying from inter-current infection 60-90 days after exposure to TME agent were free of spongiform lesions. This could indicate that the lesions found at 700 days had developed long after inoculation, which is consistent with a "slow" virus infection, such as TME.

The ease with which disease is produced in mink, raccoons and skunks suggests that it might occur naturally in these wild animal species if their diet included TME infected tissues. Abnormal behavior of wild skunks and raccoons is usually regarded as a sign of rabies. In many countries, however, the brains of rabies suspects are not examined unless a human has been bitten. Investigators of wildlife diseases should be alerted to the possibility that abnormal behavior and neurologic deficits may be caused by TME.

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