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Natural and experimental oral infection of nonhuman primates by bovine spongiform encephalopathy agents

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ABSTRACT **Experimental lemurs either were infected orally with the agent of bovine spongiform encephalopathy (BSE) or were maintained as uninfected control animals. Immunohistochemical examination for proteinase-resistant protein (prion protein or PrP) was performed on tissues from two infected but still asymptomatic lemurs, killed 5 months after infection, and from three uninfected control lemurs. Control tissues showed no staining, whereas PrP was detected in the infected animals in tonsil, gastrointestinal tract and associated lymphatic tissues, and spleen. In addition, PrP was detected in ventral and dorsal roots of the cervical spinal cord, and within the spinal cord PrP could be traced in nerve tracts as far as the cerebral cortex. Similar patterns of PrP immunoreactivity were seen in two symptomatic and 18 apparently healthy lemurs in three different French primate centers, all of which had been fed diets supplemented with a beef protein product manufactured by a British company that has since ceased to include beef in its veterinary nutritional products. This study of BSE-infected lemurs early in their incubation period extends previous pathogenesis studies of the distribution of infectivity and PrP in natural and experimental scrapie. The similarity of neuropathology and PrP immunostaining patterns in experimentally infected animals to those observed in both symptomatic and asymptomatic animals in primate centers suggests that BSE contamination of zoo animals may have been more widespread than is generally appreciated.**

In previous papers (1, 2), we reported that a rhesus monkey and two lemurs housed in the Zoological Park in Montpellier, France, died of neurological illnesses associated with spongiform encephalopathy and the presence of proteinase-resistant protein (prion protein, or PrP). In this paper, we bolster the presumption that the zoo animals had been infected with the agent of bovine spongiform encephalopathy (BSE) with epidemiological and experimental observations describing spongiform encephalopathy and PrP in an additional 20 lemurs that had been exposed to beef protein dietary supplements in three different primate facilities (Montpellier, Besançon, and Strasbourg, France), and show that the distribution of PrP in the tissues of these lemurs was similar to that seen in two experimental lemurs fed with BSE-infected brain tissue.

MATERIALS AND METHODS

Epidemiological Study. A detailed study was undertaken of 61 primates belonging to 11 species housed in the Montpellier Zoological Park to evaluate the possible role of diet on the longevity of the animals. The animals live in very large cages spread out in a natural garrigue (Mediterranean forest). Depending on animal size, no more than three simians or five lemurians live in any one cage. A questionnaire also was mailed to other zoos and primate breeding facilities in France, asking for information about neurological or unexplained primate deaths and dietary practices. In the course of this inquiry, we were informed that a number of apparently healthy lemurs in the Besançon zoo and the Strasbourg breeding facility were going to be euthanized because of a new French regulation concerning hybrid primates, and so we obtained an additional group of 18 animals (six from Besançon and 12 from Strasbourg).

These 79 animals were all large-sized, long-lived monkeys and lemurs (over 1,000 g in body weight and more than 20 years longevity), who were fed a daily diet of vegetables and fruits supplemented by 20–40 g/kg of commercial food products containing animal-derived proteins (Singe 107, MP, or Marex). According to the manufacturers, this food contained various items, including gross protein (19.2–25.4%), fats (5.7–7.5%), corn, soya, carob bean, alfalfa, mineral, yeasts, vitamins A, C, D3, and E, and cracklings (the so-called "fifth quarter of beef" suitable for human consumption).

Experimental Study. This study involved a group of five lemurs belonging to the small-sized and short-lived species *Microcebus murinus* (around 100 g in body weight, 8–10 years longevity). These animals, from a colony housed at the Center for Laboratory Animals of the Montpellier University of Science, were 1-year-old adults and had never been fed commercial food containing meat. Three lemurs (control animals nos. 538, 593, and 655) were allowed to remain in the colony. Two lemurs (nos. 654 and 656) were reared in a locale protected under French law, one animal (no. 654) having been fed a single 0.5-g dose of a BSE-infected cattle brain (obtained from Centre National d'Etudes Veterinaires et Alimentaires, Lyon, France), and the other (no. 656) having been fed two 0.5-g doses, spaced 2 months apart, of the same cattle brain. The brain fragments were mixed with apple compote and given to the animals before their customary daily diet.

Immunohistology. Animals were anaesthetized by an i.p. injection of pentobarbital (0.5 ml/kg). The various organs were dissected, and samples were fixed by immersion in paraformaldehyde (4% in 0.1 M phosphate buffer, pH 7.4) and Carnoy's liquid. After routine histological protocols, 6-μm microscopic sections of different parts of the gastrointestinal tract, spleen, tonsil, thymus, spinal cord, and brain were prepared for PrP immunohistological study as follows: sections were immersed in 85% formic acid for 45 min, washed in distilled water, immersed in 5% hydrogen peroxide for 10 min,

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Abbreviations: PrP, proteinase-resistant protein, or prion protein; BSE, bovine spongiform encephalopathy; GFAP, glial fibrillary acidic protein.

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immersed in distilled water, and autoclaved for 10 min at 121°C.

The sections then were rinsed in Tris-buffered saline (TBS) before overnight incubation at 4°C with either of two mouse monoclonal primary antibodies: anti-$PrP_{106–126}$ (dilution 1:2) or anti-PrP 3F4 (dilutions 1:200, 1:500, or 1:1,000). Sections then were incubated for 1 h with a secondary anti-mouse IgG antibody coupled to peroxidase (Boehringer Mannheim). Color was developed with 0.2% diaminobenzidine (Sigma) in TBS containing 0.02% hydrogen peroxide and counterstained with Harris' hematoxylin. Histological sections of brain, spleen, and gastrointestinal tract from several different *Eulemur* spp. were independently studied in the laboratory of P. Belli, using the laboratory's own rabbit polyclonal antibody RS1 and revealed by the kit Duet (Dako) according to the protocol of Tagliavini *et al.* (3).

Selected brain and spinal cord sections also were treated with the polyclonal antibody 961S28T (4) (1:200 dilution for 5 days), which stains abnormal neuronal Tau proteins, and the polyclonal glial fibrillary acidic protein antibody (GFAP) (Dako, 1:100 dilution overnight), which stains reactive astrocytes. The protocol was identical to that used for anti-PrP antibodies, except for the omission of formic acid and autoclaving pretreatment. Quantitative studies were performed on brain sections chosen with reference to the microcebe brain atlas (5); the distribution of cortical neurons containing abnormal aggregated Tau proteins was mapped with an image analysis computer (Biocom Histo 200, Paris).

Because no anti-PrP antibody is capable of distinguishing between the normal and pathological isoforms of PrP in fixed tissue, and because discrimination by proteinase K partial digestion also is rendered ineffective by fixation, it is essential that a number of methodological criteria be met for a proper interpretation of immunostaining results. These criteria include: unequivocal staining having a characteristic morphological appearance, with little or no background noise; and the absence of such staining in parallel sections treated with (*i*) preimmune serum from the animal in which the primary antibody was raised, (*ii*) immune serum preabsorbed with its corresponding PrP antigen, (*iii*) secondary antibody without previous incubation with the anti-PrP antibody, and (*iv*) at least one other antibody unrelated to PrP. In addition, staining must not occur in identically prepared sections from tissues of healthy control animals, and the results should be duplicated by an independent laboratory using the same or different immunohistochemical techniques and antibodies. Our study meets all of these criteria, and we therefore have accepted positive staining results as representing the presence of the pathological isoform of PrP.

RESULTS

Epidemiological Study. Among the primates in the Montpellier zoo, 26 deaths were recorded between 1989–1998, of which 23 occurred between 1989 and 1993 (Table 1). The date of arrival of each primate at the zoo was always known, but the date and the locality of its birth were often unknown (many animals came from other zoological parks). Although detailed clinical information rarely was recorded in the zoo registers, clinical signs were observed before death in 14 animals, of which 12 were characterized as having had serious neurological abnormalities.

In view of the multiple geographic origins of the animals dying at the Montpellier zoo, it is not possible to state that infection in all animals occurred in this locale. However, three animals dying from spongiform encephalopathy must certainly have been infected in Montpellier: two lemurs (nos. 481 and 586) came directly from Madagascar to Montpellier in 1974 and 1979, well before the era of BSE, and one animal (no. 474) was born and raised in the Montpellier zoo.

We received nine responses (representing only about a 10% response rate) from our mailed questionnaire to other primate holding facilities: one respondent zoo had no primates, and of the eight respondent zoos with primates, seven denied any suspicious or neurological deaths, and one (Lille) noted three deaths in January 1996 in primates after neurological illnesses similar to those seen in the Montpellier primates.

All of the primates in Lille, Strasbourg, Besançon, and Montpellier, as well as animals in the seven zoos that reported no neurological deaths, had diets that included nutritional supplements containing meat meal, sold under the names Singe 107, MP, or Marex. The supplements are produced by two different companies (one of which is based in the United Kingdom), which distribute them through a French company to zoos and animal breeding facilities. It is highly likely that British beef was included in the source of meat powder, especially as the British manufacturer announced that as of June 1996 it ceased to use beef meal in its nutritional supplements.

Immunohistological Studies. We studied two lemurs (microcebes) that were experimentally fed with BSE-infected brain tissue and three unexposed control lemurs. After the killing of one of the BSE-fed lemurs (no. 654) by its cage mates, we sacrificed one of the two remaining BSE-fed animals (no. 656) to have optimally preserved tissues for examination from at least one animal during the incubation phase of disease (5 months postinfection). Other animals are being held under observation until such time as they may show signs of neurological disease.

We also studied two additional symptomatic lemurs in the Montpellier zoo (nos. 456 and 586), and 18 asymptomatic lemurs (nos. 700–717) in captivity in either Besançon or Strasbourg. All of these animals were 6–16 years of age (except for two animals 25 years of age), with body weights of 1,500–1,800 g. The presence and distribution of PrP immunoreactivity described in the following paragraphs was similar in the captive lemurs and in the two microcebes that had been experimentally infected with BSE (Tables 2 and 3). Uninfected control animals showed no PrP immunoreactivity.

In the tonsils, PrP was seen in the peripheral epithelium, lymphoid nodules, and in scattered cells inside the glands. In the esophagus, PrP was present in the stratified epithelial cells, but not in the mucigen-secreting esophageal glands. Immunoreactive lymphocytes were scattered throughout the connective tissue of the lamina propria and infiltrating the muscularis mucosae and the submucosa. An abrupt transition between the esophagus and the stomach was conspicuous by a different PrP distribution starting at the cardia: the gastric columnar epithelium bordering the lumen and the gastric pits were PrP-negative but the gastric glands were positive. The underlying lymphoreticular tissue in the lamina propria also was labeled (Fig. 1 *E* and *F*).

In the small intestine, including the duodenum, finely particulate PrP was spread throughout the cytoplasm of the epithelial cells (except in goblet cells), located close to the lumen as well in the villi. The PrP was located within the striated border cells, the glandular cells located at the base of the villi, and the specialized M cells associated with lymphocytes infiltrating the epithelium (Fig. 1 *G* and *J*). The lamina propria and the submucosa contained labeled lymphocytes as did the wall of the lymph and blood vessels. In these areas, PrP-labeled cellular elements also were observed at the periphery of both lymphoid structures associated with the intestine: the elongated Peyer's patches (Fig. 1*H*) and the spherical lymph nodes. In the colon, PrP immunoreactivity was noted in the columnar epithelial cells near the lumen but not in the crypts. The tunica muscularis of the different regions of the gastrointestinal tract never exhibited immunoreactivity. The spleen showed an obvious staining of numerous cells located

Table 1. Epidemiological summary of primates housed in the Montpellier Zoological Park during the period 1989–1998

	Status as of December 1998		Description of dead primates						
Primate species	Alive	Dead	Origin	Date of arrival	Date of death	Age at death, yr	Clinical signs	Spongiform encephalopathy	No. of animal
Lemurians									
Eulemur fulvus mayottensis	5	6	Madagascar	1974	1992	>18	Neurological signs + blindness	Yes	481
			Madagascar	1974	1990	>16			
			Montpellier	1984	1991	7			
			Montpellier	1983	1989	6	Neurological signs (cerebral hemorrhage)		
			Montpellier	1988	1992	4	Neurological signs	Yes	474
			Montpellier	1995	1996	1	Neurological signs + blindness	No	584
Eulemur fulvus albifrons	4	4	Paris	1988	1992	>4	Neurological signs	Yes	456
			Paris	1988	1990	>2			
			Paris	1988	1992	>4			
			Paris	1988	1990	>2			
Eulemur mongoz	0	3	Mulhouse	1989	1991	"Old"	Neurological signs		
			Mulhouse	1989	1990	"Old"	Neurological signs		
			Madagascar	1979	1992	>13	Neurological signs	Yes	586
Eulemur macaco	3	1	Montpellier	1981	1996	15	Neurological signs		
Lemur catta	3	1	Montpellier	1976	1994	18	Motor deficits		
Varecia variegata variegata	4	2	Mulhouse	1985	1990	7	Neurological signs		
			?	1993	1994	1			
Varecia varietata rubra	4	0							
Simians									
Macaca mulatta	0	3	United Kingdom	1986	1992	10	Neurological signs	Yes	455
			United Kingdom	1986	1993	11	Neurological signs		
			United Kingdom	1988	1991	>3	Pneumonia		
Macaca sylvanus	5	1	?	1985	1993	7			
Macaca fuscata	3	0							
Saimiri sciureus	4	5	Fréjus	1987	1990	>3	Neurological signs		
			Fréjus	1989	1990	>3			
			Fréjus	1989	1990	>3			
			Fréjus	1989	1990	>3			
			Fréjus	1989	1989	>3			

All animals died of undetermined causes unless otherwise indicated. Blank spaces in the last two columns indicate that no autopsy examinations were performed on these animals.

in the red pulp (Fig. 1*I*) and, in lower number, at the periphery of the white pulp.

In the central nervous system of large-size lemurs in the preclinical stage of disease, we observed PrP particles in both dorsal and ventral roots of the spinal cord in the cervical region and scattered along vacuolated fibers in the spinal cord (Fig. 1*A*). PrP was also visible as dust-like particles in layer IV of the cerebral cortex near PrP-labeled fibers originating from the corpus callosum (Fig. 1*B*). Moreover, clearly degenerative central nervous system processes were seen in both the zoo eulemurs and the experimental microcebes. This degeneration was manifested by three abnormalities, which were never detected in the brains of control animals.

First, numerous aggregated Tau-containing neurons were present throughout the cerebrum, particularly in the cerebral cortex, the brain stem, the superior colliculus, and the thalamus (Fig. 1*D*). As the evolution of Tau proteins in the cortical pyramidal neurones is well studied in microcebes (6, 7), we were able to compare their number to those in the experimental microcebe with optimally preserved tissue (the condition of the tissue from the lemur killed by his cage mates was not good enough for quantitative study). The BSE-infected lemur had more than 10 times as many degenerating neurones as aged normal lemurs (8–13 years), and nearly 300 times as many as young lemurs of comparable age (1–2 years). In particular, degeneration of the pyramidal cortical neurones in healthy young adult microcebes begins in the occipital cortex, and aggregated Tau-containing neurones are never observed in the parietal and frontal cortices, whereas, on average, 280

Table 2. PrP immunostaining in non-nervous system tissues of spontaneous cases of spongiform encephalopathy in eulemurs and in microcebes fed with BSE-infected brain tissue (nos. 654 and 656)

Species and animal no.	Tonsil	Digestive tract						Spleen
		Esophagus		Stomach		Intestine		
		Epithelium	Lymphoreticular cells	Epithelium	Lymphoreticular cells	Epithelium	Peyer's patches	
Eulemurs								
456								
586								
700		0	+	++	+	0	0	0
701		0	+	+	+	0	0	(+)
702				+	+	++		(+)
703				0	(+)	++		(+)
704				0	(+)	++	++	+
705				0	(+)	(+)	(+)	+
706	+			(+)	(+)	(+)	(+)	
707	+			0	(+)	++	(+)	+
708	+			0	(+)	++		+
709	+			(+)	+	+	++	
710	(+)							(+)
711	+							
712								(+)
713	++							+
714	++							+
715	+							+
716		0	+	+	+	+	+	
717								
Microcebes								
654	+	+	+	+	+	+		
656		(+)	0	+	++	++	++	+

Immunostaining symbols: 0, absent; (+), trace positive; +, moderate positive; ++, strong positive; +++, widespread strong positive.

and 269 abnormal neurones were found in these areas of the BSE-infected lemur.

Second, innumerable small vacuoles were present in the cortical parenchyma (Fig. 1*C*), often in close contact with the hyperphosphorylated Tau-containing neurones. In the brains and spinal cords of all animals, a majority of large nerve tract fibres exhibited vacuolation, and in some large bundle tracts, such as the reticular formation and corpus callosum, it was

Table 3. PrP, Tau, and GFAP immunopositivity, and micro-vacuolation in nervous system tissues of spontaneous cases of spongiform encephalopathy in eulemurs, and in microcebes fed with BSE-infected brain tissue (nos. 654 and 656)

Species and animal no.	Brain						Spinal cord	
	PrP				Vacuoles			
	Neurons	Fiber tracts	Tau	GFAP	Parenchyma	Fiber tracts	PrP	GFAP
Eulemurs								
456	+	+			+++	+++		
586	+	+			+	+		
700	0	0	+	+	++	(+)	+	++
701	0	++	+	+	+++	+++	+	++
702	(+)	++	+	+++	+++	++		
703	(+)	++	+	+	+++	+	+	++
704	0	0	+	+	0	(+)		+
705	0	+	+	++	+++	++		
706	0	+	+	+++	+++	++	+	
707	(+)	++	++	++	++	++		
708	(+)	+	++	++	(+)	(+)		
709	(+)	++	+++	+	+	+		
710	0	+	++	(+)	+++	++		
711	0	+	++	++	+	+		
712	(+)	+	+++	+++	+++	++		++
713	(+)	+	+++	+++	+++	+		
714	+	+	+++	+	++	+		
715	0	+	++	+	+++	++		
716	0	+	+++	+	++	++		
717	0	+	+++	(+)	+++	+		
Microcebes								
654								
656	0	+	+++	++	++	+++	+	++

Immunostaining symbols: 0, absent; (+), trace positive; +, moderate positive; ++, strong positive; +++, widespread positive.

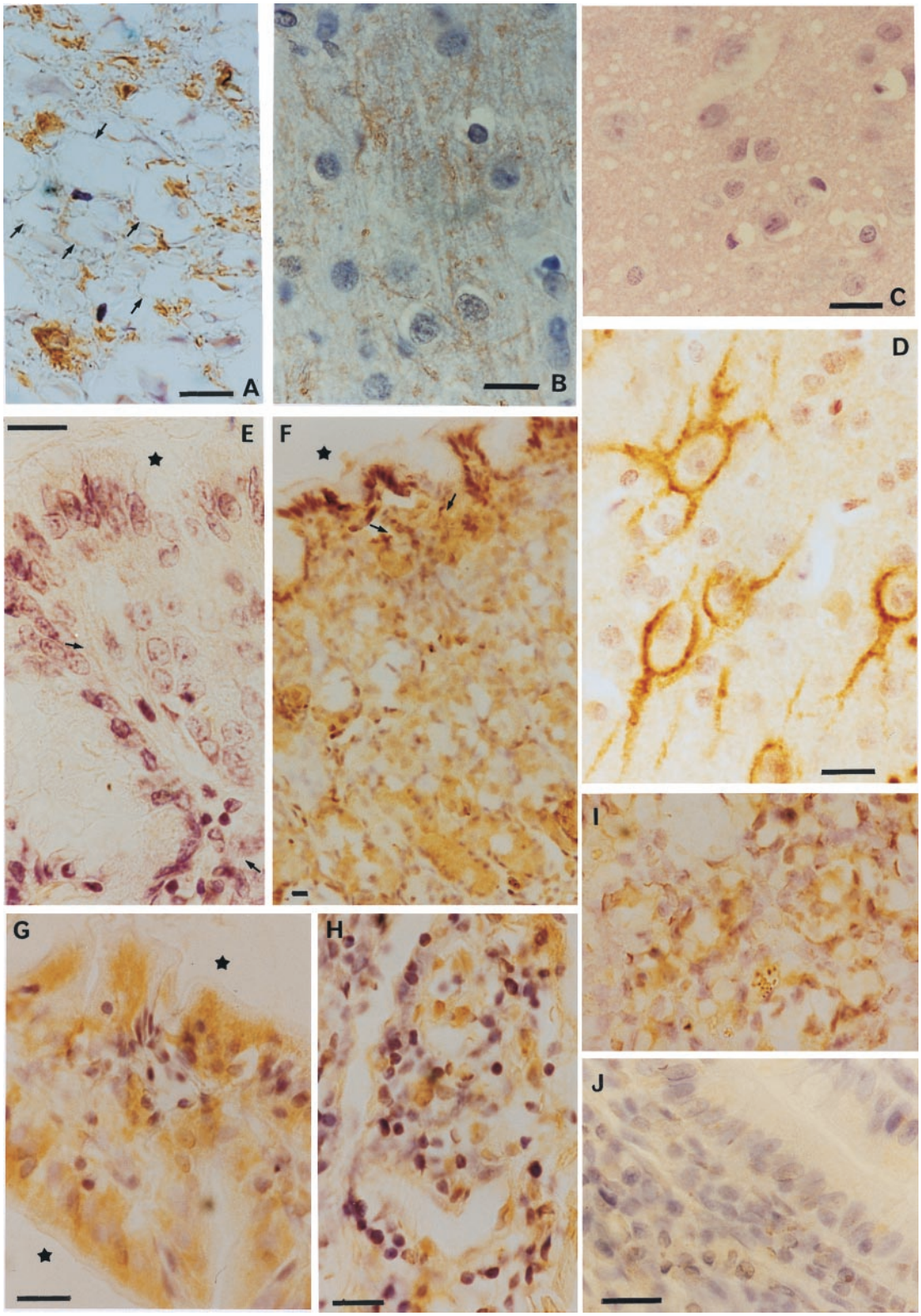

FIG. 1. (*A*) Zoo lemur no. 703. PrP deposits in large vacuolated fibers of the ventral funiculus of the cervical spinal cord. Arrows point to fiber membranes. Anti-PrP 3F4, 1:200. (*B*) Zoo lemur no. 712. Nerve fibers showing PrP immunoreactivity (brown) in layer IV of the cerebral cortex. Anti-PrP 3F4, 1:200. (*C*) Experimental BSE-infected microcebe no. 656. Microvacuolation in the neuropil of the parietal cortex (layer V). Hematoxylin and eosin. (*D*) Experimental BSE-infected microcebe no. 656. Abnormal Tau proteins inside pyramidal neurons of the parietal cortex layer III. Anti-tau 961S28T, 1:200. (*E*) Experimental control microcebe no. 593. High magnification of the stomach wall: no PrP immunoreactivity is detected in the epithelium, secretory glands, or various lymphoreticular tissue elements (arrows). Star indicates luminal surface. Anti-PrP 3F4, 1:200. (*F*) Experimental BSE-infected microcebe no. 656. PrP distribution in the stomach wall. Arrows point to reticulolymphatic elements; star indicates luminal surface. Anti-PrP 3F4, 1:500. (*G*) Experimental BSE-infected microcebe no. 656. PrP localization in an intestinal villus. Note the interrupted epithelium at the level of M cells containing a lymphocyte, and the immunoreactivity of lymphoid reticular structures. Stars indicate luminal surfaces. Anti-$PrP_{106–126}$, 1:2. (*H*) Experimental BSE-infected microcebe no. 656. Peyer's patch with PrP immunoreactive lymphoid structures. Anti-$PrP_{106–126}$, 1:2. (*I*) Experimental BSE-infected microcebe no. 656. PrP labeling in splenic red pulp. Anti-PrP 3F4, 1:500. (*J*) Experimental BSE-infected microcebe no. 656. Small intestine. Anti-PrP 3F4, 1:200, pre-adsorbed with PrP antigen.

possible to distinguish between discrete vacuolated and non-vacuolated tracts.

Third, astrocytic gliosis was evident in the large increase of reactive astrocytes showing GFAP immunoreactivity, particularly well developed in the white matter of the brain, in layers I, V, and VI of the cortex, and in proximity to blood vessels. Blood vessesls in the pia matter also were surrounded by reactive astrocytes. In the spinal cord, GFAP-labeled astrocytes were very numerous in the white matter but also scattered in the central gray matter. Aggregated Tau proteins were seen in fibers of the spinal cord tracts and in the axoplasm of myelinated fibers in peripheral nerves near the spinal cord.

DISCUSSION

Pathogenesis has been a continuing subject of importance in the study of transmissible spongiform encephalopathies, having been first addressed systematically by Hadlow *et al.* (8–10) in a landmark set of experiments in which the sequential appearance of infectivity in different organs was determined in both naturally and experimentally acquired disease, continued by Kimberlin and Walker (11, 12) in a series of experiments on orally infected mice, and most recently extended by Beekes *et al.* (13, 14) to include parallel studies of PrP in tissues after oral infection and by Klein *et al.* (15) with particular attention to the role of B cells in neuroinvasion. All of these studies were undertaken by using scrapie as the model of infection, but preliminary investigations also have been reported on BSE in naturally and experimentally infected cattle (16).

From the ensemble of these studies it has become clear that, after oral infection, infectivity and pathologic PrP first appear in the digestive tract and its contained or proximate lymphoid tissues (tonsils, lymph nodes, Peyer's patches, and spleen), before moving, presumably through autonomic nervous system fibers, to the spinal cord and up to the brain. Natural and experimental BSE in bovines is notable in the comparatively limited distribution of infectivity outside the central nervous system, having been demonstrated only in the trigeminal and dorsal root ganglia, distal ileum, and (possibly) bone marrow and retina.

The present study, which extends our earlier investigations of two lemurs and one monkey dying with spongiform encephalopathy in the Montpellier zoo (1, 2), contributes two additional pieces of information about oral infection by transmissible spongiform encephalopathy agents. First, the immunohistochemical results of our experimental study of BSE-fed lemurs has precisely defined the distribution and localization of PrP within a variety of tissues early in the incubation period of disease. PrP (and by implication, the infectious agent) evidently is taken up by epithelial cells lining the lumen of the digestive tract (including those of the tonsil), initiating a reaction of the M cells and lymphocytes within the tissues of the digestive tract and in their lymphatic drainage system (including lymph nodes and spleen). Our observations also show that even before PrP can be detected in the central nervous system in the pattern typical of terminal illness, it can be traced along nerve pathways from ventral and dorsal root ganglia through the spinal cord into the brain cortex. These results are consistent with the observed distribution and progression of infectivity and PrP during the evolution of scrapie, as measured by infectivity assays (12) and Western blots of extracted PrP (14).

Second, the similar neuropathology and distribution of PrP in orally infected experimental lemurs and spontaneously affected zoo lemurs, together with the epidemiological observations confirming the occurrence of spongiform encephalopathy in animals fed a diet supplemented with meat protein that until 1996 had very likely included rendered British beef, leave little room for doubt that cases of spongiform encephalopathy in French primates resulted from infection by BSE-contaminated meat, just as in felines and ungulates in zoos elsewhere. Our unexpected finding that the same patterns of PrP distribution and brain degeneration were present in asymptomatic lemurs from two other French primate facilities suggests that BSE-contaminated diets may have been far more widespread than appreciated and mandates continued surveillance of primates in European zoos and breeding facilities.

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