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Immunopathology and Infectious Diseases

Spread of Classic BSE Prions from the Gut via the Peripheral Nervous System to the Brain

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An experimental oral bovine spongiform encephalopathy (BSE) challenge study was performed to elucidate the route of infectious prions from the gut to the central nervous system in preclinical and clinical infected animals. Tissue samples collected from the gut and the central and autonomic nervous system from animals sacrificed between 16 and 44 months post infection (mpi) were examined for the presence of the pathological prion protein (PrP^{Sc}) by IHC. Moreover, parts of these samples were also bioassayed using bovine cellular prion protein (PrP^{C}) overexpressing transgenic mice (Tgbov XV) that lack the species barrier for bovine prions. A distinct accumulation of PrP^{Sc} was observed in the distal ileum, confined to follicles and/or the enteric nervous system, in almost all animals. BSE prions were found in the sympathetic nervous system starting at 16 mpi, and in the parasympathetic nervous system from 20 mpi. A clear dissociation between prion infectivity and detectable PrP^{Sc} deposition became obvious. The earliest presence of infectivity in the brain stem was detected at 24 mpi, whereas PrP^{Sc} accumulation was first detected after 28 mpi. In summary, our results decipher the centripetal spread of BSE prions along the autonomic nervous system to the central nervous system, starting already halfway in the incubation time. ***(Am J Pathol 2012, 181:515–524; http://dx.doi.org/10.1016/j.ajpath.2012.05.001)***

Bovine spongiform encephalopathy (BSE) is a fatal neurodegenerative disease in cattle. Other transmissible spongiform encephalopathies (TSEs) include scrapie in sheep and goats, Creutzfeldt-Jakob disease in humans, and chronic wasting disease in cervids. The host-encoded cellular prion protein (PrP^{C}) is converted into its pathological, fibrillogenic, and partially proteinase K–resistant isoform, designated PrP^{Sc}.[1] A variant form of Creutzfeldt-Jakob disease has been linked to the consumption of BSE-contaminated food.[2–4]

Several studies have clarified the pathogenesis of classic scrapie, in particular the spread of the TSE agent in sheep after oral inoculation, showing a clear involvement of the peripheral nervous system and the lymphoreticular system.[5] However, only a few studies have been performed to address the BSE pathogenesis in cattle after an oral inoculation. Two consecutive challenge studies were performed at the Veterinary Laboratory Agency (Weybridge, UK), and another study was conducted at the Friedrich-Loeffler-Institute (Greifswald-Insel Riems, Germany), which form the basis of the hitherto reported investigation.

An earlier attack rate study using orally challenged cattle showed that the number of animals that develop a clinical disease is linked to the infectious dose.[6] A 100-g dose led to definite clinical signs 31 months post infection (mpi), and initial PrP^{Sc} detection was first observed after 30 mpi in the Veterinary Laboratory Agency studies,[7] as against PrP^{Sc} detection after 24 mpi using the same dose in the current analyzed pathogenesis experiment.[8]

After an oral challenge, the initial PrP^{Sc} accumulation occurs in bovines exclusively in the gut-associated lymphoid tissue. Because PrP^{Sc} is detectable as early as 4 mpi in the ileal Peyer's patches, this location is considered to be the primary site of entry in bovines.[9] By using a bovine transgenic mouse bioassay, BSE infectivity was also detected in lymphoid localization of the Peyer's patches in the jejunum at 8 mpi[9] and in the tonsil at 10 and 20 mpi, respectively.[10,11]

Previous studies on orally challenged bovine (100-g BSE brain stem homogenate) have shown PrP^{Sc} deposition in the celiac mesenteric ganglion complex (CMGC)

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in a preclinical animal sacrificed at 24 mpi.[8] Moreover, in this animal, a minor PrP^{Sc} deposition was present in the central nervous system (CNS; obex and spinal cord). Bioassay results from preclinical animals also detected infectivity in the brain stem at 27 mpi and in the sciatic nerve at 30 mpi.[10] In preclinical animals, PrP^{Sc} depositions were also revealed in the trigeminal ganglion and in the dorsal root ganglia from 32 mpi, whereas this disease marker was absent in the cranial cervical (GCC) and stellate ganglia for all examined animals.[7] However, all previous studies on asymptomatic or clinically affected cattle show an involvement of the autonomic nervous system (ANS) only after the CNS has been affected.

Massive PrP^{Sc} depositions were found regularly in the CNS of challenged bovine that developed clinical disease. Moreover, by using tissues from experimentally infected cattle from the first Veterinary Laboratory Agency experiment, PrP^{Sc} was demonstrated in the sciatic nerve[10] and peripheral ganglia nerves and in the adrenal glands of clinically affected cattle at 35 mpi and later.[12] Earlier reports documented the presence of infectivity in the retina, sciatic nerve, and optical and facial nerves of naturally infected cattle.[13] Outside the nervous system, infectivity was shown at low concentrations in the musculus semitendinosus, whereas most of the lymphatic tissues (with the exception of gut-associated lymphoid tissue, tonsil, and bone marrow) were free of infectivity.[13,14]

The aim of this study was to elucidate the pathogenesis of BSE in the natural host on oral infection. Sequentially dissected cattle in this study provided tissue samples over the entire incubation period. Because of the known main restriction of BSE prions to the central/peripheral nervous system, only representative samples from this pathway were challenged using the ultrasensitive mouse bioassay. We wanted to determine the spread of infectivity with incubation period and the pathway along peripheral nerves. Immunohistochemical (IHC) examinations were performed to reveal the cell types that are involved in the propagation of PrP^{Sc}. Our results shed light on the barely known pathogenesis in cattle in the early incubation period and provide knowledge about the ascension of BSE prion to the brain. Results have implications for diagnostic strategies and food safety measures.

Materials and Methods

Ethical Approval

The challenge experiments in cattle and mice described herein were approved by the competent authority of the Federal State of Mecklenburg–Western Pommerania, Germany, on the basis of national and European legislation (ie, the European Union Council Directive 86/609/EEC for the protection of animals used for experiments).

Animals

Within the German BSE pathogenesis study, 56 Simmental crossbred calves between the ages of 4 and 6 months were challenged orally with 100 g of a brain stem homogenate pooled from clinically infected BSE cattle. The infectivity load in the homogenate was $10^{6.1}$ ID_{50}/g tissues, as determined by end point titration in Tgbov XV mice.[8,13] Eighteen calves that were inoculated orally with a BSE-negative brain stem homogenate served as negative controls. Two to five BSE-challenged animals were selected randomly and euthanized every 4 months. More than 150 tissue and body fluid samples were taken during the necropsy from each animal under TSE-sterile conditions, using single-use instruments for each sample. Tissues that occur in bilateral symmetry were sampled from both sides. Therefore, the tissues collected from the left side were fixed in 4% neutral-buffered formalin, whereas right-sided samples were frozen for bioassay and biochemical analysis. Singular tissues were sampled twice in direct vicinity.

Table 1. List of Samples Collected During BSE Pathogenesis Study and Analyzed during This Survey

Body system	Body site
Central nervous system	Medulla oblongata (level at obex)
	Spinal cord, thoracal segment 7
	Spinal cord, lumbal segment 3
Somatic nervous system	Ganglion trigeminale*
Sympathetic nervous system	Splanchnic nerves
	GCC
	Ganglion stellatum
Parasympathetic nervous system	Vagus nerve (thoracal and cervical segment)
	Ganglion nodosum
Sympathetic and parasympathetic fraction	CMGC
	Ganglion mesenteriale caudale
Gut	Peyer's patches, ileum*

*Only IHC was performed.

For the study presented herein, an IHC examination was conducted on representative samples collected (Table 1) from two randomly selected animals sacrificed at relevant time points between 16 and 44 months after inoculation and from one control animal sacrificed 20 mpi. The same selected and predominant preclinical infected cattle between 16 and 36 mpi were studied in the mouse bioassay using Tgbov XV mice. Intestinal samples from control animals served as contamination controls for the bioassay experiments.[9] The samples examined in this study are listed in Table 2.

To complete the study performed with samples from cattle experimentally challenged with an extremely high dose, the corresponding samples collected during the necropsy of 6-year-old naturally infected field BSE cattle in the terminal stage of disease were also tested by IHC alone.

IHC Data

With some modifications, tissue samples were processed as previously described.[15] The tissues were fixed in 4% neutral-buffered formalin for at least 2 weeks. Before the dehydration procedure and embedding in paraffin, samples were incubated for 1 hour in 98% formic acid and rinsed in tap water for 40 minutes.

Sections (3 μm thick) were prepared and mounted on Superfrost plus slides (Menzel, Darmstadt, Germany). We used a

Table 2. Overview on the Brain Stem Results

Months p.i.	Animal no.	Clinical status*	Brain stem affection: Obex IHC†	Brain stem affection: Caudal medulla BA‡
16	28	0	–	0/12 (473 ± 104)
	46	0	–	0/13 (448 ± 97)
20	50	0	–	0/10 (476 ± 108)
	60	0	–	0/13 (464 ± 116)
24	24	0	–	**1/7** (433)
	58	0	–	0/9 (308 ± 36)
28	21	0	+ (1)	**8/12** (337 ± 37)
	52	0	–	0/8 (420 ± 119)
32	09	0–1	+ (4)	ND
	61	0–1	+ (1)	0/9 (387 ± 129)
36	11	0–1	+ (4)	ND
	49	3	+ (6)	ND
40	25	0–1	+ (1)	ND
	56	0	+ (2)	ND
44	22	3	+ (4)	ND
	38	2	+ (4)	ND

Positive results are boldfaced.
*0, none; 1, probable; 2, likely; and 3, definitive BSE clinical signs.
†Positive (+) or negative (−) for IHC. Numbers in parentheses indicate the grade of staining: 1 to 2, mild; 3 to 4, moderate; and 5 to 6, severe.
‡Number of positive/total examined mice (mean ± SEM incubation time).
BA, bioassay; ND, not done; PP, Peyer's Patches.

serial section procedure. According to this procedure, we examined five different levels per block with a plane distance of approximately 24 μm. This added up to a maximal distance between the first and the last section of one tissue of 150 to 200 μm. Two PrP-specific monoclonal antibodies (mAbs) were used for IHC staining: 12F10 (Cayman Chemical, Ann Arbor, MI) and 6C2 (Jan Langeveld, Central Institute for Animal Disease Control, Lelystad, the Netherlands). The pretreatment necessary for the applied IHC staining includes rehydration, incubation for 15 minutes (12F10) or 30 minutes (6C2) in 98% formic acid, rinsing in tap water, and inhibition of the endogenous peroxidase with 3% H_2O_2 (Merck, Darmstadt, Germany) in methanol for 30 minutes. Sections stained with mAb 12F10 were incubated for 15 minutes with proteinase K (4 μg/mL; Boehringer Mannheim, Mannheim, Germany) at 37°C. In contrast, the pretreatment protocol for IHC staining with mAb 6C2 includes an autoclaving step for 20 minutes at 121°C in citrate buffer. These primary antibodies were applied at a dilution of 1:500 (12F10) and 1:50 (6C2), diluted in goat serum and incubated for 2 hours at room temperature. Negative control sections were incubated with goat serum alone. As a secondary antibody system, we used the EnVision reagent (Dako, Glostrup, Denmark) containing a peroxidase-conjugated polymer backbone. The incubation time of the secondary antibody on the sections was 30 minutes at room temperature. The slides were finally developed in diaminobenzidine tetrahydrochloride (Fluka, Munich, Germany) and counterstained with Mayer's hematoxylin. All sections were examined by light microscopy.

Challenge of Transgenic Mice with BSE Samples

Transgenic mice overexpressing bovine PrPs (Tgbov XV) that are highly sensitive to a BSE infection[13] were used for the mouse bioassay. We inoculated 30 μL of 10% homogenates of the tissue samples in sterile 0.9% sodium chloride solution by the intracerebral route. Fifteen mice were challenged per group, and control mice were inoculated with 0.9% sodium chloride alone. All mice were supervised for the onset of clinical symptoms at least twice weekly. Mice showing typical signs of disease were sacrificed, and mouse brains were tested for the accumulation of PrP^{Sc} by phosphotungstic acid (PTA) immunoblotting.

PrP^{Sc} Detection in the Brains of Infected Mice

For the highly sensitive detection of PrP^{Sc} accumulation, a specific precipitation by PTA was performed, followed by using Western blot analysis. The PTA precipitation was performed according to the protocol previously established,[16,17] with some modifications.

For all samples, 10% (w/v) brain homogenates were prepared in 0.42 mmol/L sucrose solution containing 0.5% deoxycholic acid sodium salt and 0.5% Nonidet P-40 by using a Ribolyser (Hybaid, Heidelberg, Germany). Gross cellular debris was removed by centrifugation at 6000 rpm for 5 minutes at room temperature. A 200-μL aliquot of the supernatant was adjusted with proteinase K to a final concentration of 50 μg/mL proteinase K and incubated at 55°C for 1 hour. Digestion was terminated by addition of 4 μL of Pefabloc (Roche, Mannheim, Germany) and heating for 5 minutes at 95°C. Digested homogenates were mixed with PTA to a final concentration in the sample of 0.3% (w/v) PTA. Samples were incubated at 37°C for 60 minutes with constant agitation before centrifugation at 13,300 rpm for 30 minutes at room temperature. After careful removal of the supernatant, pellets were resuspended in sample buffer in varying volumes depending on the PrP^{Sc} concentration in the sample and heated for 5 minutes at 95°C. After a short centrifugation, samples were loaded on 16% Tris-polyacrylamide gels. Gels were transferred onto polyvinylidene fluoride membrane (Millipore, Billerica, MA) and blocked for 1 hour in 5% (w/v) nonfat milk powder in PBS containing 0.1% (v/v) Tween-20 (PBST). PrP^{Sc} bound to the membrane was detected using the mAb L42 (R-Biopharm, Darmstadt, Germany) at a concentration of 0.4 μg/mL, which was incubated on the membranes for 1 hour at room temperature. The membranes were then washed three times with PBST and then incubated in a 0.15 μg/mL concentration of alkaline phosphatase–conjugated anti-mouse Ig (Dianova, Hamburg, Germany) in PBST for 1 hour at room temperature. The membranes were finally washed three times with PBST, and the bound

Table 3. Results of Samples Collected from the CNS, the Gut, and the ANS of Cattle Sacrificed between 16 and 44 Months after Oral Challenge, Including One German Field BSE Case

			CNS						Gut	
			Obex	Caudal medulla	Spinal cord T7		Spinal cord L3		Distal ileum IHC	
Month p.i.	Animal no.	Clinical status*	IHC†	BA‡	IHC†	BA‡	IHC†	BA‡	PP§	ENS¶
No involvement of brain stem										
16	IT28	0	–	0/12 (473 ± 104)	–	**2/13** (529 ± 248)	–	0/13 (428 ± 133)	+ 1/76 (f/g)	+ pm
	IT46	0	–	0/13 (448 ± 97)	–	0/11 (437 ± 113)	–	0/14 (381 ± 62)	– 0/87	–
20	IT50	0	–	0/10 (476 ± 108)	–	0/12 (456 ± 108)	–	0/12 (389 ± 86)	+ 1/253 (f/g)	–
	IT60	0	–	0/13 (464 ± 116)	–	0/14 (488 ± 92)	–	0/13 (457 ± 66)	+ 1/184 (f/g)	–
24	IT58	0	–	0/9 (308 ± 36)	–	0/10 (364 ± 87)	–	0/12 (345 ± 67)	+ 1/82 (f/g)	–
28	IT52	0	–	0/8 (420 ± 119)	–	0/11 (381 ± 89)	–	0/10 (389 ± 107)	+ 1/332 (f)	–
Mild involvement of brain stem										
24	IT24	0	–	**1/13** 433	–	0/9 (475 ± 111)	–	0/10 (482 ± 108)	+ 15/272 (f/g)	+ pm
32	IT61	0–1	+ (1)	0/9 (387 ± 129)	–	**1/14** 292	–	**2/12** (508 ± 100)	+ 2/78 (f/g)	–
40	IT25	0–1	+ (1)	ND	+ (2)	ND	+ (2)	ND	– 0/8	+ pm/ps
Moderate/severe involvement of brain stem										
28	IT21	0	+ (1)	**8/13** (337 ± 37)	+ (1)	ND	+ (1)	ND	+ 1/101 (g)	–
32	IT09	0–1	+ (4)	ND	+ (6)	ND	+ (6)	ND	+ 2/12 (f)	+ pm/ps
36	IT11	0–1	+ (4)	ND	+ (6)	ND	+ (6)	ND	– 0/69	+ pm
	IT49	3	+ (6)	ND	+ (5)	ND	+ (5)	ND	+ 3/60 (f)	+ pm
40	IT56	0	+ (2–3)		+ (3)		+ (3)		+ 1/124 (f)	+ pm
44	IT22	3	+ (4)		+ (6)		+ (6)		– 0/41	+ pm/ps
	IT38	2	+ (4)		+ (3)		+ (3)		– 0/23	–
R09/06			+ (4)	ND	NS	NS	NS	NS	–	–

table continues

Every sample has two columns: the first column indicates the results obtained by IHC, and the second column shows the results from the mouse BA, which were detected by PTA-immunoblot of the mouse brains. Positive results are boldfaced.

*0, none; 1, probable; 2, likely; and 3, definitive BSE clinical signs.

†Positive (+) or negative (−) for IHC. The numbers in parentheses indicate the grade of staining: 1 to 2, mild; 3 to 4, moderate; and 5 to 6, severe.

‡Number positive by BA/total examined mice (means ± SEM incubation time).

§Number of positive follicles/total number examined (staining pattern: f, fine; g, granular).

¶ENS localization: pm, myenteric plexus; ps, submucosal plexus.

BA, bioassay; ND, not done; PP, Peyer's Patches.

antibodies were detected using the chemiluminescent substrate CDP Star (Tropix, Bedford, MA) and were visualized directly in an image analysis system (Versa Doc, Quantity One; Bio-Rad, Munich, Germany).

Results

In this study, the early pathogenesis of classic BSE was assessed using samples of the distal ileum and the peripheral (autonomic) nervous system and CNS of 16 experimentally challenged preclinical and clinically affected cattle. Samples from all bovines were investigated by IHC for the detection of PrP^{Sc} accumulations. Bioassays were conducted on samples from animals sacrificed between 16 and 36 mpi. The results of the brain stem are shown in Table 2, whereas all other results are summarized in Table 3. Detailed observations for particular regions are given later.

For each cow, clear signs of a successful challenge were obtained in at least one sample, although individual differences between cattle of the same time point existed,

Table 3. *Continued*

ANS																SNS
Mixed sympathetic and parasympathetic				Sympathetic nervous system						Parasympathetic nervous system						
Ganglion coeliacum		Ganglion mesenteriale caudale		Nervi splanchnici		Ganglion cervicale cranial		Ganglion stellatum		Vagus nerve (cervical)		Vagus nerve (thoracal)		Ganglion nodosum		Ganglion trigeminale
IHC†	BA‡	IHC†	BA‡	IHC†	BA‡	IHC†	BA‡	IHC†	BA‡	IHC†	BA‡	IHC†	BA‡	IHC†	BA‡	IHC†
–	0/11 (398 ± 77)	–	0/12 (340 ± 77)	–	**1/10** (377)	–	**5/13** (436 ± 77)	–	0/5 (522 ± 109)	–	0/9 (451 ± 103)	–	0/12 (439 ± 155)	–	0/11 (401 ± 109)	–
–	2/12 (409 ± 63)	–	0/14 (365 ± 89)	–	3/11 (598 ± 67)	–	1/12 (472)	–	0/9 (385 ± 124)	–	0/11 (497 ± 149)	–	0/9 (495 ± 98)	–	0/9 (526 ± 113)	–
–	**1/13** (495)	–	0/11 (308 ± 62)	–	0/10 (433 ± 140)	–	0/12 (345 ± 64)	–	0/6 (463 ± 132)	–	0/7 (376 ± 72)	–	0/9 (439 ± 132)	–	**5/13** (535 ± 79)	–
–	**7/15** (329 ± 39)	–	0/14 (370 ± 79)	–	0/12 (478 ± 126)	–	0/11 (364 ± 75)	–	0/6 (549 ± 98)	–	**1/9** (483)	–	0/12 (404 ± 130)	–	0/8 (437 ± 88)	–
–	0/5 (372 ± 121)	–	0/9 (335 ± 52)	–	0/8 (464 ± 155)	–	0/11 (376 ± 123)	–	0/4 (530 ± 66)	–	0/7 (419 ± 124)	–	0/7 (362 ± 134)	–	0/6 (407 ± 109)	–
–	3/12 (314 ± 25)	–	0/13 (391 ± 135)	–	0/3 (462 ± 121)	–	1/12 (624)	–	1/12 (427)	–	0/9 (394 ± 103)	–	0/7 (427 ± 98)	–	0/3 (371 ± 99)	–
–	0/12 (368 ± 70)	–	0/11 (412 ± 50)	–	**6/12** (499 ± 85)	–	**3/9** (411 ± 37)	–	**11/11** (402 ± 53)	–	0/8 (428 ± 127)	–	0/8 (478 ± 140)	–	0/7 (460 ± 127)	–
+ (1)	**9/13** (348 ± 60)	–	0/12 (328 ± 85)	–	0/6 (371 ± 125)	–	3/9 (388 ± 166)	–	0/8 (448 ± 102)	–	0/7 (361 ± 93)	–	0/7 (488 ± 45)	–	0/11 (430 ± 123)	–
+ (1)	ND	–	ND	–	ND	–	ND	–	ND	–	ND	–	ND	–	ND	+ (1)
–	0/10 (352 ± 70)	–	0/8 (37 ± 103)	–	**10/15** (523 ± 90)	–	**11/11** (333 ± 41)	–	**9/15** (459 ± 58)	–	**2/15** (498 ± 100)	–	**1/11** (561)	–	**4/13** (525 ± 57)	–
+ (1)	**12/15** (331 ± 25)	+ (1)	**15/15** (288 ± 26)	–	**12/12** (340 ± 37)	–	**13/15** (298 ± 28)	–	**11/11** (332 ± 23)	+ (1)	**10/12** (359 ± 59)	–	**13/15** (383 ± 70)	–	**10/14** (340 ± 55)	+ (1)
–	**11/11** (257 ± 39)	+	**10/11** (308 ± 35)	–	**14/14** (311 ± 59)	+ (1)	**12/14** (278 ± 39)	–	**12/12** (326 ± 34)	–	**8/8** (339 ± 51)	–	**14/14** (331 ± 43)	–	**9/10** (389 ± 89)	+ (1)
+ (1)	**7/9** (251 ± 17)	–	**6/14** (468 ± 41)	–	**14/14** (323 ± 40)	–	**12/13** (281 ± 37)	–	**8/8** (337 ± 28)	–	**12/13** (348 ± 29)	–	**9/10** (356 ± 41)	–	**9/13** (310 ± 36)	+ (1)
+ (1)		–		–		–		–		+ (1)		–		–		+ (1)
+ (1)		–		–		–		–		–		+ (1)		+ (1)		+ (1)
+ (1)		–		–		–		–		+ (1)		–		–		+ (1)
+ (1)	ND	NS	NS	NS	NS	+ (1)	NS	+ (1)	NS	+ (1)	ND	NS	NS	–	ND	+ (1)

leading to clear differences in the incubation periods. The first traces of infectivity (low transmission rates and long incubation periods in the recipient mice) were shown after 16 mpi in the ANS of both cows examined (IT28 and IT46) and after 24 mpi in the caudal parts of the medulla oblongata of one cow (IT24). Initial PrP^{Sc} accumulation in the ANS occurred at 32 mpi in both cows (IT09 and IT61), whereas the first slight deposits of the pathological prion protein in the CNS (Table 2) of a different bovine (IT21) were detected already after 28 mpi. Moreover, the sympathetic part of the ANS seemed more widely involved in the early pathogenesis than its parasympathetic counterpart. Strikingly, more preclinical bovines revealed infectivity in sympathetic structures at a higher degree than in the parasympathetic tissue samples.

Clinical Signs

A clear association between clinical signs and the detectable amounts of PrP^{Sc} was only demonstrated at

later stages of the disease. Low levels of infectivity or PrP^{Sc} accumulation in the CNS were present in cows, in which possibly BSE-associated symptoms appeared (IT61, 32 mpi; IT25, 40 mpi). Cattle displaying moderate deposits of PrP^{Sc} in the CNS either were clinically inconspicuous (IT56, 40 mpi) or exhibited highly suspicious (IT09, 32 mpi; IT11, 36 mpi) clinical signs. Two bovines displayed definitive clinical signs after incubation times of at least 36 mpi, showing moderate (IT22, 44 mpi) or severe (IT49, 36 mpi) PrP^{Sc} accumulations at the level of the obex.

Distal Ileum

Several different locations of the distal ileum were examined by IHC for the presence of PrP^{Sc} in the Peyer's patches and the enteric nervous system (ENS). In doing so, PrP^{Sc}-specific staining was determined in both structures of the distal ileum during the entire time course investigated in this study. In the ileal Peyer's patches (Figure 1A), the proportion of widely dispersed positive follicles was lower than 1%, whereas three cattle showed an increased level of labeled follicles (5.5%, IT24, 24 mpi; 16.7%, IT09, 32 mpi; 5%, IT49, 36 mpi).

Although immunostaining in the ENS, concerning individual plexuses, was first seen at 16 mpi, we only observed a more intense effect at later stages of the disease, showing a random distribution of PrP^{Sc}. Perineuronal immunolabeling and an association to satellite cells were seen in the plexus submucosus and in the plexus myentericus of the ENS (Figure 1, B and C). We did not observe any obvious association between PrP^{Sc} detection in the ENS and the presence of positive follicles in the Peyer's patches.

ANS Data

For only one cow analyzed in this study (IT58, 28 mpi), no pathological prion protein was detectable in the ANS. In cattle lacking CNS involvement, the earliest time point for the detection of infectivity in the sympathetic nervous system (GCC and splanchnic nerve) was at 16 mpi (IT28 and IT46), whereas the parasympathetic nervous system (vagus nerve and nodose ganglion) became involved after 20 mpi (IT50 and IT60). The predominantly affected structures from the sympathetic or parasympathetic system were the GCC and the vagal nerve, respectively. Starting at 16 mpi, PrP^{Sc} detection in the ANS was generally correlated with the presence of infectivity in the CMGC, which contains sympathetic and parasympathetic fiber (mixed) ganglion.

For one bovine (IT24, 24 mpi), moderate to high levels of infectivity in the examined samples of the sympathetic nervous system coincided with low infectivity levels in the caudal part of the medulla oblongata but were absent in the parasympathetic structures and the spinal cord. For the cow IT21 (28 mpi), the ANS was widely affected and distinct infectivity levels were seen in the sympathetic and parasympathetic structures.

The IHC-detectable PrP^{Sc} amounts in sympathetic, parasympathetic, or mixed structures were also observed in cows sacrificed after incubation periods longer than 32 mpi (Figure 2, A–G). Strikingly, these cows also revealed a moderate to severe accumulation of PrP^{Sc} in the CNS. The CMGC and the vagal nerve were the affected structures that consistently showed perineuronal

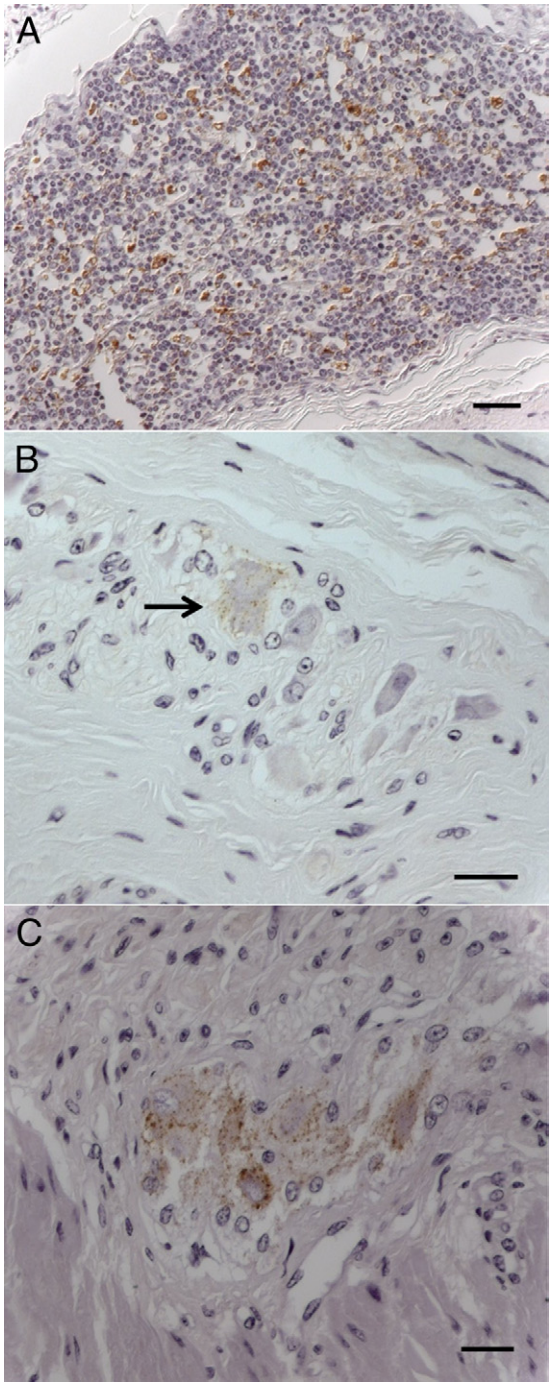

Figure 1. PrP^{Sc} detection in the gut. **A:** Lymphoid follicle of the Peyer's patches with moderate immunoreactivity in the cytoplasm of tingible body macrophages and a slight follicular dendritic cells–associated reaction pattern (IT60, 20 mpi). Neurons of the ENS are labeled in the submucosal plexus (IT22, 44 mpi) (**B**) and in the myenteric plexus (IT09, 32 mpi) (**C**) with a perineuronal, an intraneuronal, and an intraglial reaction pattern. **Arrow** denotes a labeled neutron. IHC: PrP mAb 12F10 is used. Scale bars: 50 μm (**A**); 20 μm (**B** and **C**).

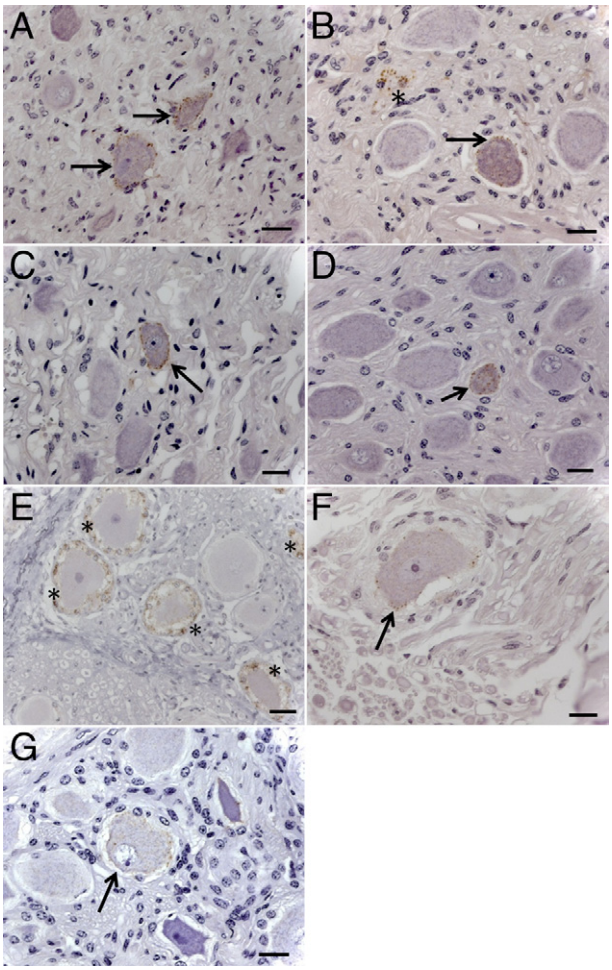

Figure 2. Distinct PrP^{Sc} immunolabeling in the ANS. Sympathetic and parasympathetic fibers contain celiac ganglion (IT09, 32 mpi) (**A**) and caudal mesenteric ganglion (IT09, 32 mpi) (**B**) with a clear perineuronal, but a sparse intraneuronal, reaction pattern, including a mild effect on the satellite cells in the latter. The sympathetic nervous system is represented by perineuronal-labeled stellate (R09/06) (**C**) and mainly intraneuronally labeled cervical cranial ganglion (IT11, 36 mpi) (**D**). **E:** Predominantly satellite cells are labeled for PrP^{Sc} in the trigeminal ganglion (IT49, 36 mpi). **F:** For parasympathetic tissues, perineuronal PrP^{Sc} depositions are shown in the nodose ganglion (IT22, 44 mpi). **G:** The vagal nerve displays perineuronal and sparse intraneuronal labeling at IT38, 44 mpi. **Arrows**, neurons; **asterisks**, satellite cells. IHC: PrP mAbs 6C2 (**E**) and 12F10 (**A–D**, **F**, and **G**) are used. Scale bars: 50 μm (**E**); 20 μm (**A–D**, **F**, and **G**).

and intraneuronal reaction patterns and only a minor intracellular staining of satellite cells.

CNS Data

Infectivity was first demonstrated in the CNS in the thoracic part of the spinal cord of one cow (IT28) at 16 mpi and in the brain stem of another cow sacrificed at 24 mpi (IT24) by transgenic mouse bioassay in Tgbov XV mice with low transmission rates and long incubation periods (the latter without any prior involvement of the spinal cord).

Mild accumulation of PrP^{Sc} in the CNS was also demonstrated in three cows at 28 mpi (IT21), 32 mpi (IT61), and 40 mpi (IT25). Although the IHC results of the animal IT21 correlated with higher transmission rates in the bioassay, either none or only small amounts of infectivity were detectable in the brain stem and spinal cord of animal IT61. No bioassay was performed for samples of the IT25 (40 mpi) because of the long incubation period being greater than 36 mpi.

When analyzing the obex region by IHC, we observed an intraneuronal and perineuronal reaction pattern of individual neurons in the formatio reticularis (IT21 and IT61, Figure 3A) and sparse, but clear, intraneuronal staining of PrP^{Sc} in the nucleus tractus solitarii and nucleus motorius nervi trigemini of the animal IT25. The immunolabeling simultaneously seen in the spinal cord was consistently confined to the substantia intermedia centralis and lateralis of the intermediolateral column (Figure 3B).

The immunolabeling pattern of animals that displayed high amounts of PrP^{Sc} in the brain stem and the spinal cord was characterized by an intense intracytoplasmatic (intraneuronal and intraglial) and cell surface–associated (linear and particulate to coalescing) PrP^{Sc} reaction pattern throughout the obex region and in the gray matter of the spinal cord.

Somatic Nervous System

The trigeminal ganglion was examined by IHC exclusively. PrP^{Sc} was initially found in a few satellite cells of animals displaying a mild accumulation at the obex (IT25, 40 mpi). At later stages in the pathogenesis, increased PrP^{Sc} deposition levels in the brain frequently corresponded with higher PrP^{Sc} levels most pronounced in satellite cells of this ganglion (Figure 2E).

Naturally Infected BSE Field Case (R09/06)

To compare the PrP^{Sc} distribution in naturally and experimentally infected animals, we examined the corresponding samples of a clinically affected field case, applying the same IHC procedure. The brain stem at the level of the obex showed a severe PrP^{Sc} accumulation. The analysis of the severely autolytic samples from the gut did not provide any positive results. In contrast, clear specific immunolabeling indicated PrP^{Sc} depositions in the CMGC. Furthermore, single neurons of the sympathetic nervous system were labeled, in particular in the GCC

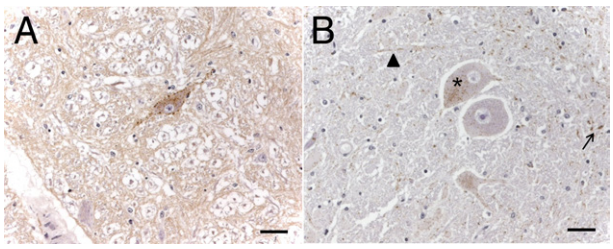

Figure 3. PrP^{Sc} accumulation in the CNS. **A:** Mild deposition of PrP^{Sc} in the formatio reticularis of the medulla oblongata at the level of the obex (IT61, 32 mpi); one single neuron reveals a clear intraneuronal and perineuronal staining pattern. **B:** Moderate PrP^{Sc} accumulation in the substantia intermediolateralis at the lumbal segment of the spinal cord with intraneuronal (**asterisk**), intraglial (**arrow**), and linear (**arrowhead**) immunolabeling (IT38, 44 mpi). IHC: mAbs L42 (**A**) and mAb 12F10 (**B**) are used. Scale bars: 50 μm (**A** and **B**).

and the stellate ganglion. The involvement of the parasympathetic nervous system was represented by a clear PrP^{Sc} accumulation in the vagus nerve. No samples were available for the spinal cord, the splanchnic nerve, or the ganglion mesenteriale caudale.

Discussion

Through a comprehensive oral BSE challenge experiment, the goal of this study was to clarify the neural pathway of the BSE agent in its natural host from early preclinical to terminal stage of the disease. Samples from the CNS, ANS, somatic nervous system, and gut-associated lymphoid tissue were taken from two sets of randomly selected animals that were serially sacrificed at 4-month intervals beginning at 16 mpi and ending at 44 mpi. Extremely thorough TSE sterile tissue sampling and preparation procedures were used to obtain high-quality target tissue samples. These tissues were analyzed by IHC and biochemical assays to detect PrP^{Sc} and by transgenic mouse bioassay to detect BSE infectivity. Because previous studies[9] revealed that the PTA-immunoblot and rapid tests have a slightly lower sensitivity compared with IHC and mouse bioassay, we did not include these methods to investigate the bovine tissue samples.

One of the major questions in studying the early pathogenesis of TSEs is the classification of the obtained results into a centripetal (from periphery to the CNS) or a centrifugal (from CNS to the periphery) spread. It is commonly accepted that a severe accumulation of PrP^{Sc} and/or high amounts of infectivity in the brain stem are associated with a centrifugal spread of the infectious agent in the peripheral nervous structures, preventing the determination of the structures involved in the early pathogenesis. On the other hand, brain stem material without any infectivity/accumulation of PrP^{S} indicates an early exclusively centripetal spread of PrP^{Sc} from the gut to the brain. Therefore, we used a highly sensitive mouse bioassay to differentiate three groups of cows: those with no involvement of the CNS, those with only minor involvement of the CNS, and those with moderate to severe involvement of the CNS. Interestingly, there is a distinct variability concerning the length of the incubation periods of cows within one group. For example, IT24 sacrificed at 24 mpi and IT25 sacrificed at 40 mpi are both in the same group, revealing only mild deposition of PrP^{Sc}. These differences in the incubation periods are most probably due to the tremendous individual variability between the cows and their genotypes. Furthermore, the first set of positive results in the trigeminal ganglia is always associated with an accumulation of PrP^{Sc} in the obex region. Therefore, to differentiate between a centripetal and a centrifugal spread of the BSE agent, we examined the trigeminal ganglion. The positive results obtained for this ganglion were interpreted most likely as the beginning of a centrifugal spread back into the peripheral neural structures. This seems to occur after a minor involvement of the obex region (IT25, 40 mpi).

Because it was known that the BSE pathogen spreads mainly via the nervous system in cattle,[8,13,18] in this study, we focused on the distribution of PrP^{Sc}/infectivity in the ANS. The involvement of the Peyer's patches and ENS was comprehensively elucidated earlier.[9] Our findings indicate that the BSE prions ascend along both sympathetic and parasympathetic projections into the CNS, in addition to an initial phase of local amplification in the small intestine (essentially in the distal ileum). In preclinical cattle sacrificed before 32 mpi, ascension was only detectable by bioassay in Tgbov XV mice, but not by IHC, most likely because of the superior sensitivity of the transgenic mouse bioassay compared with IHC. A spurious amount of PrP^{Sc} restricted to a discriminate number of cells in peripheral tissues could also cause differential sensitivity of the techniques. Furthermore, samples used for these two methods did not originate from identical (but adjacent) sites and may, thus, have contained slightly different amounts of PrP^{Sc} and/or infectivity.

The parasympathetic and the sympathetic spinal cord circuitry have already been discussed as the centripetal propagation routes for TSE agents in scrapie-infected hamsters and sheep[19–24] and in BSE-infected bovines.[8] PrP^{Sc} deposits in the dorsal motor nucleus of the vagus nerve of the obex imply an involvement of the parasympathetic route along the vagus nerve. A specific role of the vagus nerve has exclusively been described in clinically affected cattle by infectivity/PrP^{Sc} detection.[12] Another possibility is the distribution along the splanchnic nerves and the intermediolateral column of the spinal cord.[22,24] Enteric and abdominal ganglia, such as the CMGC, are involved in both the parasympathetic and sympathetic centripetal routes. Concerning the involvement of the different parts of the ANS during the early spread of the infectious agent, the most interesting results were found in the youngest animals of our study. Both animals at 16 mpi showed BSE infectivity in sympathetic projections (GCC and splanchnic nerves). In addition, one of these cows revealed infectivity in the spinal cord most likely as a result of the sympathetic spread. The parasympathetic tissues were free of infectivity in

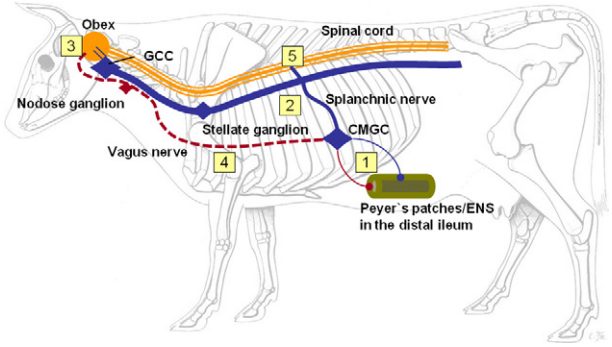

Figure 4. Schematic representation of the BSE prion pathway in cattle after oral exposition. Orange, CNS; blue, peripheral nervous system, sympathetic parts; red, peripheral nervous system, parasympathetic parts. 1, After passing the intestinal mucosa, the agent spreads via sympathetic and parasympathetic projections to the CMGC. 2, Along the splanchnic nerve, BSE prions enter the sympathetic ganglia chain. 3, The infection of the obex is modulated by the GCC. 4, An alternative pathway is the spread along the vagus nerve. The transmission into the brain occurs via the dorsal motor nucleus of the vagus nerve or through the GCC, which exchanges fibers with the vagus nerve. 5, As an additional dissemination, the infection of the spinal cord via the splanchnic nerve is possible.

these animals, whereas both cows sacrificed at 20 mpi contained BSE prions only in the parasympathetic nervous system (cervical vagus nerve and nodose ganglion) but not in purely sympathetic projections or in spinal cord. To our knowledge, this is the first report describing the presence of infectivity in the ANS before the involvement of the CNS in several peripheral neural tissues between 16 and 20 mpi in BSE-infected bovines. These results clearly indicate that both pathways are involved in the early pathogenesis of BSE, but not necessarily simultaneously. Our theory is supported by the coexistence of either none or only spurious amounts of infectivity in the CNS and a remarkable involvement of the sympathetic fibers in cows slightly later in the incubation period.

In addition to the more frequent involvement of the sympathetic samples, a higher transmission rate compared with the parasympathetic samples is obvious. Considering all these results, it is tempting to assume a more dominant and crucial role of the sympathetic nervous system in the pathogenesis of BSE in cattle. The delayed onset of disease in sympathectomized mice infected with scrapie is indicative for this pathway as well.[25] Although our results are in accordance with previous studies showing a spread along the CMGC and the splanchnic nerves, notably, our data favor a further distribution via the sympathetic ganglia chain, including the stellate ganglia and the GCC. The occurrence of a mild PrP^{Sc} accumulation in the brain stem in association with infectivity solely in sympathetic structures (IT24, 24 mpi) supports the importance of the sympathetic spread. Hence, a mandatory involvement of the intermediolateral column of the spinal cord is not observed. The detection of infectivity in the spinal cord of one animal at 16 mpi indicates a third, additional pathway to the brain as a result of the dissemination along the sympathetic splanchnic nerve. Moreover, we suspect a critical role of the comprehensively involved GCC, because the location close to the brain provides sympathetic fibers to almost all cranial nerves and possibly results in an effect on the brain in uncommon (formatio reticularis and nucleus motorius nervi trigemini) or parasympathetic-related areas (dorsal motor nucleus of the vagus nerve and nucleus tractus solitarii), as the initial PrP^{Sc} depositions in the CNS shown herein.

Nevertheless, our results also support the previously postulated early parasympathetic route of the BSE agent along the vagus nerve and the nodose ganglion, although to a lesser degree than the sympathetic structures. Furthermore, infectivity of the nodose ganglion before CNS involvement presumes a centripetal spread along sensory fibers of the vagus nerve, which are in contrast to the assumption that the vagus-associated nodose ganglion might be affected via the centrifugal spread.[26] Taken together, our results indicate that there are three independent possible neuronal ascension routes for BSE prions (in order of putative importance): sympathetic > parasympathetic > spinal cord projections.

Results from scrapie studies in hamster and sheep determined CMGC to be a part of the sympathetic circuitry, which also contains parasympathetic fibers[21,22,24] as a possible route of ascension of the agent to the CNS. Our analysis revealed infectivity in the CMGC already from 16 mpi for both possible centripetal routes, leading to the question of where the initial determination of infected fibers takes place. We assume that the route toward the brain likely depends on the nerve type initially infected at the ENS. Sensory fibers of the vagus nerve were found widely distributed in the gut.[27,28] Furthermore, vagal efferent synapses in intrinsic ganglia of the ENS innervate the intestinal wall, including the mucosa and submucosa.[29] However, a more extensive sympathetic presence in the ENS[30] and the CMGC[31] and wide innervations of lymphoid structures by the sympathetic nervous system[32–34] were reported. These facts could explain the wider and earlier involvement of the sympathetic fibers, as previously discussed. Furthermore, it cannot be excluded that the CMGC may influence the route of infection by the close contact of both sympathetic and parasympathetic nerve types within this ganglion as an operating center of the ANS.

In summary, our data prove an early and widespread distribution of infectivity in the ANS in preclinical cattle before an infection of the CNS. This study is able to confirm the assumed spread via sympathetic and parasympathetic structures for the BSE agent (Figure 4), but we determined a more crucial role of the sympathetic nervous system in the initial neuronal distribution. However, both pathways could be separately involved. According to our results, the spinal cord seems to represent an additional route of ascension, in addition to the more prominent pathways of the ANS.

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