

Experimentally induced bovine spongiform encephalopathy did not transmit via goat embryos

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Goats are susceptible to experimental challenge with bovine spongiform encephalopathy (BSE). This study set out to investigate whether the transmission of BSE could occur in goats following the transfer of embryos from experimentally infected donor females into uninfected recipient females. The results showed no evidence of transmissible spongiform encephalopathy disease in any of the offspring which developed from embryos from infected donors, nor indeed in any of the recipient females used as surrogate dams. In addition, there was no indication of experimental BSE spreading as either a venereal infection to males used in mating or by maternal transmission to offspring born naturally to experimentally infected donors, although numbers were small.

Introduction

In goats, scrapie can be transmitted experimentally by intracerebral (i.c.) inoculation (Pattison & Millson, 1960), after oral dosing (Pattison & Millson, 1961) and following subcutaneous (s.c.) challenge (Hadlow *et al.*, 1974). A spongiform encephalopathy has also been induced in goats after i.c. inoculation of brain homogenate from humans affected by Creutzfeldt–Jakob disease (Hadlow *et al.*, 1980). Natural scrapie in sheep has been shown to transmit laterally to goats if they are closely confined for a long time with a succession of sheep natural scrapie cases (Brotherston *et al.*, 1968). It is now thought probable that scrapie is an endemic disease of goats, which apparently propagate the infection at least partly by maternal transmission (Hourigan *et al.*, 1979). The possibility that scrapie could be transmitted via ovine germ cells has been investigated in experimental models of the disease using the technique of embryo transfer. A study from the USA produced results which seemed to show that scrapie could not transmit via the embryo (Foote *et al.*, 1993). However, the lack of information about PrP genotypes of the donors and their embryo progeny, and hence whether they were susceptible to

scrapie, made interpretation of the results difficult. Other studies, at the Neuropathogenesis Unit (NPU), indicated the complex nature of this type of work in high-incidence natural scrapie flocks and also served to highlight the lack of compelling evidence on this possible pathway of transmission of scrapie in sheep (Foster *et al.*, 1992, 1996).

The purpose of the present goat experiment was to examine whether bovine spongiform encephalopathy (BSE) could be transmitted via the caprine embryo following BSE experimental infection of the mothers who donated embryos. It was already known that goats are susceptible to BSE following i.c. inoculation and oral dosing (Foster *et al.*, 1993) and that there were no recorded cases of natural scrapie in the NPU goat herd which might otherwise have compromised results.

Methods

■ **Goats.** Goats used as embryo donors were of mixed breed, comprising British Alpine, Anglo-Nubian and Toggenburg. Recipients were mostly British Sanaan, while the sires were Siberian (Gorno-Altai). Previous studies at NPU suggest breed of goat is of little importance in transmissible spongiform encephalopathy (TSE) transmissions. The donors and recipients were 3–6 years old at the time of embryo transfer, while the sires were between 1 and 2 years old at mating.

■ **PrP genotyping.** This was performed for a dimorphism at codon 142 [isoleucine (Ile₁₄₂) to methionine (Met₁₄₂)] using the protocol set out in Goldmann *et al.* (1996). In short, the PrP coding region was amplified

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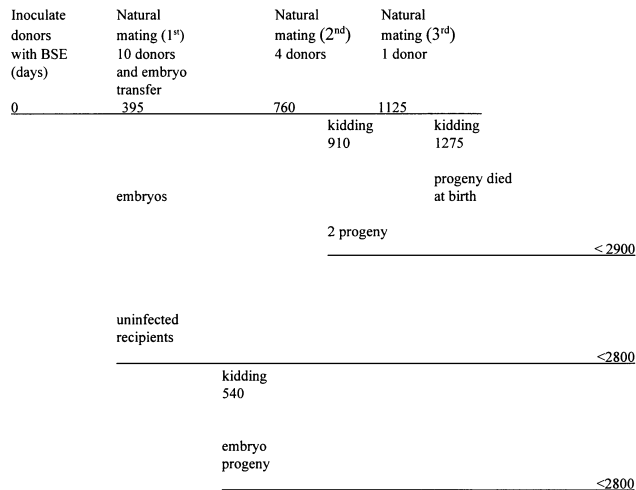


Fig. 1. A schematic of events from the inoculation of the embryo-donor goats with BSE at day 0 and their subsequent natural matings, until the culling of their embryo progeny, the recipient mothers and two offspring born a year after the transfer of embryos.

by PCR on genomic DNA isolated from lymphocytes and the resulting DNA fragment was digested with restriction enzyme *Nsi*I. The presence or absence of this restriction enzyme site reveals the methionine or isoleucine codon, respectively. PrP genotyping for a variation in the number of octapeptide repeats, a five-repeat or a three-repeat allele, was done by PCR amplification between PrP codons 2 and 185, followed by size separation on agarose gels of the generated 550 bp or 502 bp DNA fragments, respectively.

Challenge. Ten goats destined to be embryo donors were challenged with BSE, six by i.c. and four by s.c. injection 389 days prior to the transfer of embryos. The former challenge was performed using 0.5 ml of a 10% BSE cattle brain homogenate in a sterile saline solution inoculated into the parietal cortex of anaesthetized goats (halothane and nitrous oxide) via a 2 mm drill hole. Subcutaneous challenge comprised 5 ml of a 10% BSE homogenate solution equally divided between four inoculation sites, i.e. the inner, rostral surface of each leg.

Goat mating procedures. Five males were used for sequential natural mating of embryo-donor females for embryo recovery with a contact time of approximately 30–45 min over a period of 24 h. At 389 days following their challenge with BSE the donor females were mated in two groups by supervised rotation to the males, which meant that every male had contact with each donor. One of these males (46 × 93) was also mated over approximately 3 weeks for two successive years following embryo transfer, firstly with four of the longest lived donors (45 × 39, 45 × 40, 45 × 46 and 45 × 47) and then, in the second year, with 45 × 39 (Fig. 1). In the years following collection of embryos for transfer, the donors were allowed to kid naturally.

Embryo-transfer procedures. Donor and recipient female goats were treated for 17 days with fluorogestone acetate-impregnated intravaginal pessaries. The donors were superovulated using ovine follicle stimulating hormone (Ovagen) for 4 days prior to supervised natural mating with individual males over 24 h (see above). Embryos were collected from anaesthetized (halothane and oxygen) BSE-challenged donors 6 days post-mating and were immediately transferred, unwashed, by laparoscopy into anaesthetized (as above), unchallenged recipients which had been synchronized for oestrus with the donors.

Kidding and goat husbandry. Recipient goats were penned individually in disinfected housing (sprayed with 20% chlorox) 2–3 days prior to kidding. They were kept in their pens after kidding for 3–4 days and then allowed to mix with the other females and kids in a large open pen. During the period of housing (approximately 2 weeks) goats were offered only hay to eat. They were then turned out onto reseeded pasture and remained there for the duration of the experiment.

Hay was on offer throughout the winter as well as a non-ruminant, vegetable-based protein supplement. Husbandry procedures were performed at pasture with dedicated equipment.

Histology and PrP^{Sc} detection. Brain tissue was recovered for fixation and freezing from all goats, from both those which died of intercurrent disease and end-of-experiment culls. Formalin-fixed tissue was sectioned at 8 µm for haematoxylin and eosin staining.

Frozen tissue was used for disease-related PrP^{Sc} extraction (Hope *et al.*, 1986) and analysis by polyacrylamide gradient gel and Western blotting using a rabbit anti-PrP polyclonal antibody (1B3). Brain tissue from a goat experimentally challenged with scrapie was used as a positive control.

Immunocytochemistry. Formol-fixed tissue was pre-treated with formic acid and sections were subjected to hydrated autoclaving (120 °C for 15 min) followed by a 20 min incubation at 37 °C in a 0.1% trypsin solution designed to aid PrP^{Sc} recognition (Foster *et al.*, 1996).

Endogenous peroxidase was blocked by treating sections with 1% hydrogen peroxidase in methanol. Sections were immunostained by the indirect two-step method using a monoclonal antibody (BG4; diluted 1:11) raised in mice against recombinant bovine PrP protein N-terminal sequence (supplied by Chris Birkett, Institute for Animal Health, Compton). The secondary antibody was rabbit anti-mouse IgG conjugated to peroxidase and the chromagen was AEC (amino-ethyl carbazole). In control slides, normal mouse serum was substituted for monoclonal antibody, and sections of brainstem from a natural scrapie sheep were included as positive controls.

Results

Development of BSE in donor goats

All ten donors of goat embryos developed TSE-like disease following experimental challenge with BSE, confirmed by neurohistology and PrP^{Sc} blotting. Incubation periods ranged from 547 to 1284 days post-challenge (Table 1) and the length of incubation period was linked to a polymorphism at codon 142 of the PrP gene. This showed partial dominance of Met₁₄₂ on lengthening of incubation periods and has been published previously (Goldmann *et al.*, 1996). In addition, a variation in the number of octapeptide repeats (five or three) in the caprine PrP coding region has been identified (Goldmann *et al.*, 1998). The results reported here do not show any association between PrP genotype and survival of the embryo-transfer offspring or the recipients.

Clinical duration of disease in seven of the affected donor female goats lasted for less than 7 days (Table 1), but in the three other donor goats, clinical signs were present for 17 days (45 × 43, s.c. challenge), 30 days (45 × 50, i.c. challenge) and 60 days (45 × 48, i.c. challenge). In all but one of the goats clinical signs comprised predominantly ataxia and trembling, with some accompanying slight pruritus, especially in goats with a

Table 1. Embryo-donor female goats challenged either by i.c. or s.c. inoculation with homogenates of BSE and the sires involved in matings

Donor female	Incubation period (days)	Disease duration (days)	PrP ^{Sc}		Vacuolation	PrP genotype	
			Western blotting	ICC		Repeat	Codon 142
Intracerebral challenge							
45 × 45	547	≤ 7	+	+	+	5/5	Ile/Ile
45 × 46	985	≤ 7	+	+	+	5/5	Ile/Met
45 × 47	982	≤ 7	+	ND	+	5/5	Ile/Met
45 × 48	608	60	+	+	+	5/5	Ile/Ile
45 × 49	547	≤ 7	+	+	+	5/5	Ile/Ile
45 × 50	573	30	+	ND	+	5/5	Ile/Ile
Subcutaneous challenge							
45 × 39	1284	≤ 7	+	+	+	5/5	Met/Met
45 × 40	889	≤ 7	+	+	+	5/5	Ile/Ile
45 × 42	748	≤ 7	+	ND	+	5/5	Ile/Ile
45 × 43	760	17	+	ND	+	5/5	Ile/Ile

Sire	Survival time (days)	Clinical duration (days)	PrP ^{Sc}	Vacuolation	PrP genotype
46 × 93	2947	0	ND	ND	5/3 Ile/Ile
46 × 94	3136	0	–	–	5/5 Ile/Ile
46 × 95	3287	0	–	–	5/3 Ile/Ile
46 × 96	Alive	–	ND	ND	5/5 Ile/Ile
46 × 97	2729	0	–	–	5/3 Ile/Ile

ND, Not done.

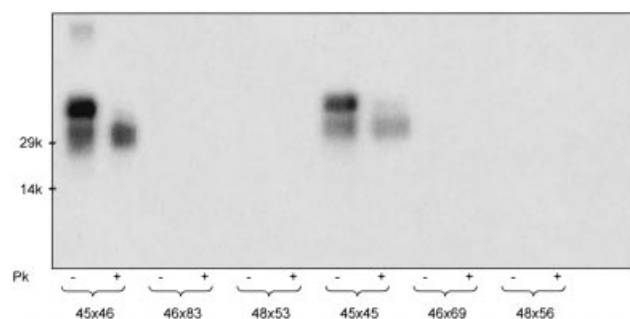


Fig. 2. PrP immunoblotting in selected goat brain samples from embryo donors, recipients and embryo-transfer progeny using proteinase K (Pk) treatment (+) or not (–). 45 × 46 and 45 × 45, Goat embryo donors inoculated i.c. with BSE (see Table 1); 46 × 83 and 46 × 69, goat embryo recipients culled with nil signs of disease; 48 × 53 and 48 × 56, goat embryo-transfer progeny culled with nil signs of disease.

shorter clinical duration. One goat (45 × 40, s.c. challenge) became recumbent within 2 days of manifesting signs of ataxia.

Vacuolation in the six goats challenged i.c. was intensive in the thalamus/hypothalamus and nuclei of the basal ganglia, and present to a lesser extent in the optic tectum of the

midbrain. Brainstem nuclei showed intermittent levels of vacuolation with foci occurring in the cuneate nucleus, olivary nuclei, pontine nuclei and in the raphe.

The cerebellum was spared from vacuolation in all but one animal (45 × 49). Low levels of vacuolation were observed in the parietal and frontal cortex of only two goats (45 × 46 and 45 × 47). The four goats inoculated s.c. showed much less intensive vacuolation of the thalamic nuclei and basal ganglia. Brainstem nuclei, the midbrain and cortical areas demonstrated equivalent or perhaps slightly lower levels of vacuolation compared with the same areas from i.c.-challenged goats.

PrP^{Sc} was detected by Western blotting in the brains of all ten BSE-challenged animals (Fig. 2). Immunocytochemistry (ICC; Fig. 3) was performed on four i.c.-challenged donor goats (45 × 45, 45 × 46, 45 × 48 and 45 × 49) and in two challenged s.c. (45 × 39 and 45 × 40) and large deposits of PrP were observed in every case. Levels of PrP immunostaining were elevated in the thalamic nuclei and basal ganglia of i.c.-challenged goats compared to nuclei of the medulla oblongata such as the dorsal vagus, and in the reticular formation. In contrast to the i.c.-challenged goats, the s.c.-challenged goats

had much less intensive immunodecoration by accumulated PrP^{Sc} in the thalamus and basal ganglia.

Lack of transmission of BSE to the male goats used in matings

The donors were mated 389 days after inoculation with BSE and their embryos were transferred 6 days later into synchronized recipients which were uninfected at the time of transfer. There was an interval of between 164 and 901 days from the time that the males were removed after mating to the time that the donors were culled following the appearance of TSE clinical disease (Table 2). The males were all aged approximately 500 days at mating. One male remains healthy at the time of writing (3325 days old) while four died of intercurrent causes at 2729, 2949, 3136 and 3318 days of age, showing no clinical signs of TSE prior to death. Three of these goats were confirmed as negative for TSE by histology and PrP^{Sc} Western blotting. Tissue was not collected from one male (46 × 93) aged 2949 days at death.

Lack of transmission of BSE to the female recipient goats

Twenty-two female goats aged between 730 and 1460 days old were used as recipients for transferred embryos. Seventeen were culled 5½ years later at between 2730 and 3460 days of age with no signs of clinical disease. The remaining five died of intercurrent disease at between 1945 and 2216 days of age, when they showed neither clinical nor histopathological evidence of disease. PrP^{Sc} could not be detected by Western blotting in brain samples from any of these recipients, nor could accumulations of PrP^{Sc} be found in three of them by ICC (Figs 1 and 2). The PrP genotypes of the recipients were Ile/Ile₁₄₂, with one exception, which was Ile/Met₁₄₂.

Lack of transmission of BSE to the progeny of embryo transfer

Recipient female goats gave birth to 37 kids (Table 2) from 57 transferred embryos, of which 15 died from, or were culled due to, intercurrent illness unrelated to scrapie-like disease at ages of less than 560 days (nine goats) or between 941 and 1630 days old (six goats). The remaining 22 goat progeny, which were derived from embryos collected from both i.c.- and s.c.-challenged donor goats, were culled at between 2030 and 2130 days of age with no clinical signs of TSE disease. Brain samples recovered from all progeny goats, including those which died from or were culled with intercurrent disease, showed negative for scrapie histopathology and for PrP^{Sc} by Western blotting, and in the four goats tested by ICC (48 × 47, 48 × 53, 48 × 56 and 48 × 66). Scrapie control samples were always positive in these analyses. PrP gene analysis of these progeny showed that 35 were Ile/Ile₁₄₂ and two were Ile/Met₁₄₂.

Lack of transmission of BSE to the progeny of natural birth

The male 46 × 93 was used to mate naturally with four of the longest surviving BSE-challenged donor females after their embryos had been harvested, and the last surviving donor (45 × 39) the following year (Fig. 1). The four embryo-transfer donor goats had incubations of 982 (i.c.), 985 (i.c.), 889 (s.c.) and 1284 (s.c.) days respectively (Table 1) and were mated again naturally approximately 300 days after the first embryos had been flushed for transfer into synchronized recipients. Two of these re-mated donors produced one surviving offspring each, both with the Ile/Ile₁₄₂ genotype (Table 3), one of which died suddenly at 1700 days of age and the other of which was culled at 1962 days of age with chronic lameness. Neither of the goat progeny had any apparent clinical signs associated with scrapie-like disease and both were negative for PrP^{Sc} by Western blotting and ICC, and for histological signs of a TSE (Fig. 3), even though their BSE-challenged mothers both developed TSE-like disease, which was confirmed histologically and by Western blotting as PrP^{Sc}-positive. The longest incubation donor (1284 days) was mated naturally again the following year, but produced no surviving offspring. The interval between final contact of the male with this donor and the death of the male was approximately 1630 days.

Discussion

The transfer of 6-day-old embryos from BSE-infected donor female goats, some of which were fully two-thirds into their incubation periods, into uninfected recipients and the subsequent survival of all offspring from TSE-like disease is of significance. It demonstrates that maternal transmission via the caprine germ line as a means of initiating BSE as a persistent infection in goats did not occur in this study.

All ten of the BSE-infected donors developed unequivocal TSE disease following one of either of two routes of challenge. Intracerebral inoculation of TSE in goats is known from other studies (Foster & Dickinson, 1988; Foster *et al.*, 1993) to induce a high incidence of disease with relatively short incubation periods and was used for that purpose in this study. The s.c. route was used to initiate a peripheral disease, more akin to a 'natural' type of infection, and generated longer incubation periods. It was subsequently shown that the length of incubation period with either route is influenced by an amino acid polymorphism in the goat PrP gene (Goldmann *et al.*, 1996). Differences in the overall intensity of vacuolation between these two routes of challenge, with the i.c. route producing more intensive lesions, has also been observed in rodents, where vacuolation was more widespread following i.c. rather than intraperitoneal challenge of mice of defined PrP genotypes with selected scrapie strains (Fraser, 1976).

The progeny resulting from the flushed embryos of the goat donors were culled at over 2000 days of age at the end of

Table 2. Number of progeny born to BSE-infected embryo-donor female goats, the length of time into the incubation periods of their mother before embryo transfer/birth and how long the progeny survived

All progeny were negative for clinical and histopathological signs of disease, and for PrP^{Sc} by Western blotting.

Donor	No. of progeny	Percentage of incubation at ET	Interval between ET and disease onset (days)	Survival of progeny (days)
Intracerebral challenge				
45 × 45	2	71	158	4, 559
45 × 46	4	39	596	166, 1630, 2073, 2075
45 × 47	0	40	593	–
45 × 48	8	64	219	0, 1497, 1741, 2030, 2031, 2045, 2073, 2094
45 × 49	0	71	158	–
45 × 50	7	68	184	161, 941, 1497, 2079, 2092, 2094, 2130
Subcutaneous challenge				
45 × 39	2	30	895	559, 2113
45 × 40	6	44	500	183, 187, 2080, 2080, 2107, 2109
45 × 42	4	52	359	198, 2081, 2081, 2130
45 × 43	4	51	371	1275, 2031, 2072, 2073

ET, Embryo transfer.

Table 3. Data from progeny born naturally to BSE-infected embryo-donor female goats and the sire involved in matings

Donor female	No. of progeny	Percentage of incubation at mating (birth)	Interval between mating (birth) and disease onset [days]	Offspring survival time (days)	Genotype
45 × 39	1	59 (71)	522 (372)	1962	Ile/Met ₁₄₂
45 × 47	1	76 (93)	220 (70)	1700	Ile/Met ₁₄₂

Sire	Age at mating (days)	Interval between mating and cull (days)	Age at cull (days)	Genotype
46 × 93	940	1988	2949	Ile/Ile ₁₄₂

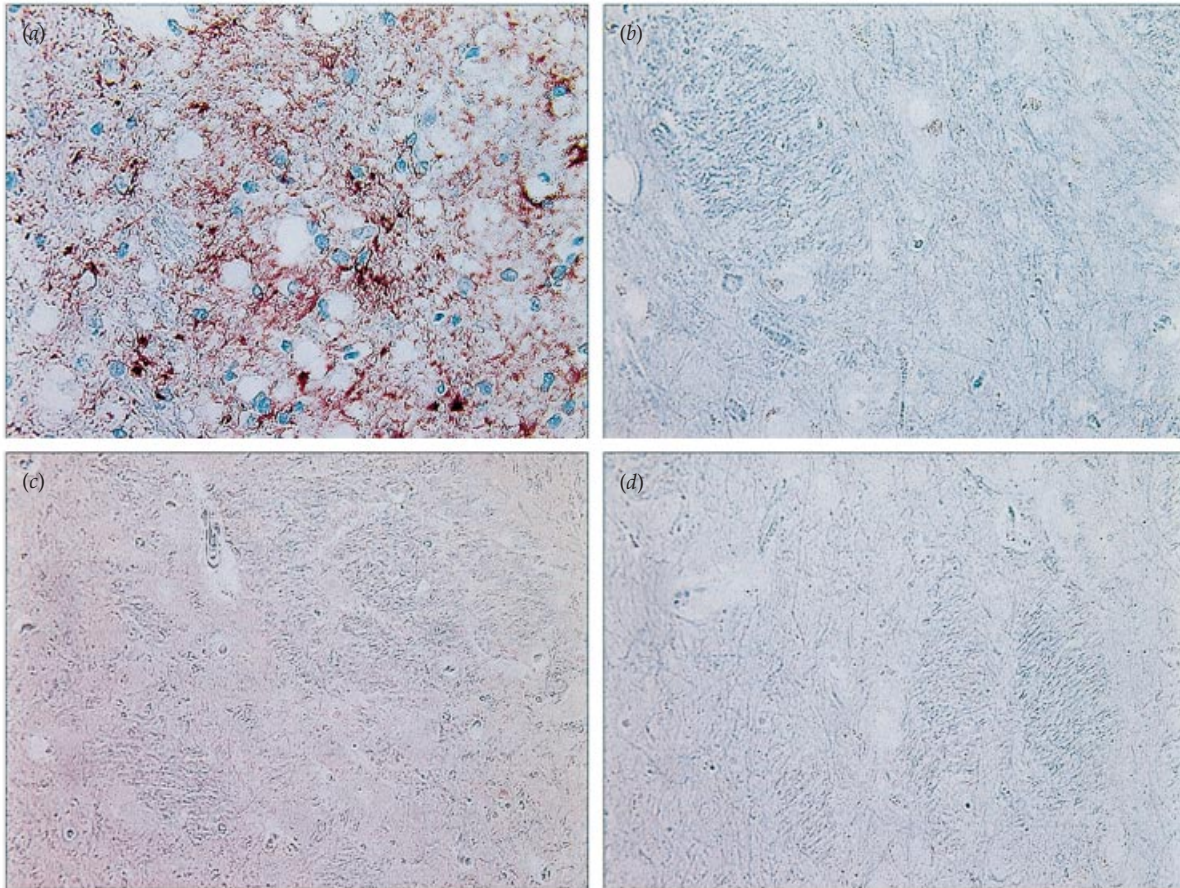


Fig. 3. (a) PrP^{Sc} immunostaining in the thalamic nuclei of embryo-donor goat 45 × 46 (Table 1; Fig. 2), which developed clinical disease 985 days following i.c. challenge with BSE (× 400). (b)–(c) Nil PrP^{Sc} immunostaining in the thalamic nuclei of embryo-transfer recipient goat 46 × 69 (b) and embryo-transfer progeny goat 48 × 56 (c) (Fig. 2), both of which were culled with no sign of clinical disease (× 400). (d) Nil PrP^{Sc} immunostaining in the thalamic nuclei of goat 50 × 80 (Table 2), which was born naturally to BSE-challenged embryo-transfer donor goat 45 × 47, and culled with no sign of clinical disease (× 400).

the experiment and none showed any evidence of TSE infection. This was based on a number of criteria, which included clinical signs of disease, histological assessment for vacuolar neurodegeneration and detection of the disease-associated isoform of the PrP protein (PrP^{Sc}) by Western blotting and ICC. Twenty-six of the embryo-transfer progeny were of the most susceptible, homozygous Ile₁₄₂ genotype, suggesting that the disease should have manifested eventually in at least these adult offspring provided that they had been exposed to an infective dose of BSE in their BSE-infected donor mothers.

In addition, BSE does not appear to have been transmitted between infected female goats and uninfected male goats during contact at mating, because none of the five males used during this experiment developed any signs of disease or indication of infection. One of the male goats is still alive, over 2500 days after first contact with the infected embryo-donor females, while three males died from causes other than TSE, after periods of between 2100 and 2600 days. The remaining

male which was used to mate naturally with five of the longer incubation period donors in successive years following the transfer of their embryos, also died with no apparent clinical signs related to TSE disease, approximately 1630 days after last contact with the infected donor females. Whether the males were ever exposed to any level of BSE infectivity for the varying periods of contact during mating of the infected donors is impossible to estimate. However, two of the donors developed clinical disease just over 150 days after contact with all five males and so were in the later stages of incubating BSE.

Two of the four donor females which were re-mated naturally to one of the males produced one offspring each. The offspring survived for 1700 and 1962 days, respectively, before dying or being culled for reasons unrelated to TSE, again suggesting the lack of BSE transmission by the maternal route. The shortest of the survival times of the two progeny (1700 days), is 199 days longer than the longest incubation period for direct BSE infection of NPU goats by any of the i.c., s.c. or oral routes of experimental challenge (Goldmann *et al.*,

1996). The 1700-day-old goat progeny reached that age in spite of its mother being in the terminal stages of disease and developing clinical signs when it was 70 days old (Table 3), which meant that it had to be weaned about 30 days earlier than normal.

The apparent non-transmission of disease to this goat progeny is interesting because it indicates that its BSE-infected mother may not have been excreting infectivity, regardless of the stage in her incubation of the disease when she was carrying/nursing her offspring. If she had been, it may not have been at a level sufficient to trigger a disease pathogenesis in her offspring. It could be that the offspring became infected, but that the disease never progressed beyond the subclinical phase. Whatever the reason, it is known that a proportion of neonatal mice can survive intraperitoneal challenge with scrapie, and that a similar biological mechanism(s) may have been operating in the case of this surviving goat progeny (Outram *et al.*, 1973).

There is a clear probability of maternal transmission of natural sheep scrapie occurring from an infected ewe to her lamb (Dickinson *et al.*, 1974; Hourrigan *et al.*, 1979). Natural scrapie also appears to be transmissible between sheep and goats from infected to uninfected animals, if they are reared in close proximity (Greig, 1950; Brotherston *et al.*, 1968). Evidence for the transmission of BSE from an infected cow to her calf is not conclusive, and if it does occur it only does so at a frequency of about 10% (Wilesmith *et al.*, 1997). The findings from the present study have shown that the transmission of BSE from experimentally infected embryo-donor goats did not occur in any of their embryo-derived progeny, with 22 of the 37 goat progeny surviving until culling at over 2000 days of age. If 10% of these progeny were to have succumbed to BSE, at least two cases would have been expected to manifest. With no cases occurring from the 22 surviving progeny this represents a prospective incidence of disease of less than 5%.

BSE also failed to transmit by contact with the sires used during the natural mating of these infected donors when they were in the later stages of infection, nor was there any evidence of the maternal transmission of the disease to two goat offspring born naturally to, and reared by, their BSE-infected mothers. Certainly, previous studies designed to examine the extent of scrapie infectivity in goats affected experimentally (Hadlow *et al.*, 1974) and in those with natural scrapie (Hadlow *et al.*, 1980) failed to show scrapie infection in the ovary or uterus, respectively. Nor has BSE infectivity been detected in the uterus (Fraser & Foster, 1994) from cattle affected with BSE. Whether infection with BSE also induces a nil or minimal level of infectivity in these goat reproductive tissues remains unresolved.

In conclusion, if goats became infected with BSE through the feeding of contaminated meat and bone meal, our evidence, although selective, does not support the possibility of the disease being maintained as a natural infection.

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