Semen from scrapie-infected rams does not transmit prion infection to transgenic mice

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Abstract

Scrapie is the most common transmissible spongiform encephalopathy (TSE) in livestock. Natural contamination in sheep flocks is presumed to occur by maternal transmission to offspring. However, horizontal prion transmission from animal to animal exists and may be significant in sustaining and spreading contagion in the field. Artificial insemination is widely used in modern farming, and as large amounts of prion protein have been found in sheep sperm membrane, epididymal fluid and seminal plasma, horizontal transmission by this route was hypothesized since no clear information has been obtained on possible sexual transmission of TSE. We therefore tested the contamination levels of semen from scrapie-infected rams at different stages of incubation, including the clinical phase of the disease. We report here that under our experimental conditions ram semen did not transmit infectivity to scrapie-susceptible transgenic mice overexpressing the V¹³⁶R¹⁵⁴Q¹⁷¹ allele of the sheep prion (*PRNP*) gene. These results suggest that artificial insemination and natural mating have a very low or negligible potential for the transmission of scrapie in sheep flocks.

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Introduction

Transmissible spongiform encephalopathies (TSEs) or prion diseases are a group of neurodegenerative conditions, which include human Creutzfeldt–Jakob disease (CJD), bovine spongiform encephalopathy (BSE) and sheep scrapie (Prusiner 2001, Aguzzi & Miele 2004). These diseases are characterized by accumulation of an abnormal transconformed fibrillar form PrP^{Sc} of the host prion protein PrP^C in the brain. This PrP^{Sc} is partially resistant to proteinase K digestion (giving rise to PrP^{Res}) and it has been associated with infection. However, transmission of infectivity has also been demonstrated in the absence of detectable PrP^{Res} in brain samples (Lasmezas *et al.* 1997).

Natural contamination in sheep flocks seems to occur mainly via maternal transmission to offspring, but horizontal prion transmission from animal to animal has been demonstrated and may be important in sustaining contagion (Miller & Williams 2003, Lacroux *et al.* 2007). Among the horizontal routes of transmission, the oral route is strongly suspected (Andreoletti *et al.* 2000, Sales 2006), but other routes such as sexual transmission remain to be assessed since no clear

information is available (for a critical review see Wrathall 1997).

We have previously analysed sheep semen and demonstrated that it contains high levels of normal cellular prion protein (PrP^C) under different glycosylated and proteolytic isoforms, mainly derived from the epididymal fluid (Gatti *et al.* 2002, Ecroyd *et al.* 2004). PrP^C has also been found on the raft domain from the ovine sperm membrane (Gatti *et al.* 2002, Ecroyd *et al.* 2004), a result previously observed for cattle and human sperm (Shaked *et al.* 1999, Peoc'h *et al.* 2002). However, we were unable to detect PrP^{Res} after proteinase K treatment of scrapie-infected ram seminal plasma (Gatti *et al.* 2002), suggesting the absence of PrP^{Sc} and probably the absence of infectivity, which, however, remained to be proved. To rule out definitively any role of semen in scrapie transmission, we tested whether semen (i.e. seminal plasma and spermatozoa) could trigger the disease after inoculation to transgenic mice (tg338; overexpressing the permissive V¹³⁶R¹⁵⁴Q¹⁷¹ allele of the ovine prion protein), one of the most sensitive tests for scrapie infection (Vilotte *et al.* 2001).

Results

None of the tg338 mice injected with semen from the VRQ/VRQ genotype or from the ARR/ARR control ram showed any clinical signs during the period of the experiment (Table 1 and Fig. 1). The mice that were still alive at 749 days post-inoculation were killed because of ageing problems and their brains were removed and tested by Western blotting after proteinase K treatment. None of the brain suspensions from mice inoculated with semen showed any Western blot-positive reaction (Table 1).

In contrast, all tg338 mice injected with infected brain suspensions from the VRQ/VRQ and the ARQ/ARQ sheep rapidly showed clinical signs and were killed at the clinical stage of the disease between 156 and 166 days post-injection (Fig. 1). The mice brains were all tested positive for the presence of PrP^{Res} by Western blotting (Table 1).

Discussion

PrP^{Sc} can accumulate in various tissues other than the nervous tissue and the transmission of TSE can be achieved by ingestion or injection of infected organ or tissue homogenates as well as by blood transfusion (Houston *et al.* 2000, Andreoletti *et al.* 2006, Thomzig *et al.* 2007). We have previously demonstrated that a significant amount of PrP^C is present in sheep seminal plasma and epididymal fluid, but we failed to demonstrate the presence of PrP^{Res} after immunoprecipitation of the PrP from seminal plasma obtained from scrapie-infected animals (Gatti *et al.* 2002). It was, however, important to examine whether sheep semen could be infectious as the absence of PrP^{Res} does not always mean the absence of infectivity (Lasmez *et al.* 1997). To achieve this, we used a genetically modified mouse line that overexpresses the sheep VRQ allele and is considered to be highly effective in the detection of scrapie infectivity, demonstrated by the short incubation period observed after inoculation of brain homogenates

from infected VRQ/VRQ or VRQ/ARQ sheep from the same flock (Telling *et al.* 1995, Vilotte *et al.* 2001). The tg338 mouse line has been proved to be able to detect infectivity in a tissue with less than 1/5000 brain infectivity, which is equivalent to sensitivity within the picogram range of PrP^{Sc} per milligram of tissue (Andreoletti *et al.* 2004). Our results demonstrated that the transmission of scrapie did not occur with semen from infected rams at any point during scrapie incubation, even with a highly infected animal such as the oldest VRQ/VRQ ram that was sampled at the clinical stage and when spermatozoa and seminal plasma (and also cells and organelles that can be found within this fluid (Gatti *et al.* 2005, Sutovsky *et al.* 2007)) contain undetectable levels of infectivity. This absence of infectivity in sheep semen may reflect the lack of transconformation of the prion protein in the genital tract due to either the specific biochemical properties of the different glycosylated and truncated forms present (Shaked *et al.* 1999, Ecroyd *et al.* 2005) or the high level of protection formed by the testicular and epididymal blood barrier (Cyr *et al.* 2007). It could also be hypothesized that these tissues lack one or more (still unidentified) cofactors needed for the efficient conversion of PrP^C to PrP^{Sc} (Telling *et al.* 1995, Marc *et al.* 2007).

In order to match as closely as possible what happens in the field, semen was collected and treated as for insemination in this study. Only 20 µl raw semen could be injected into the brain of each mouse but they contained a quarter of the cells and seminal plasma of semen used for artificial insemination (AI) in sheep (4×10^8 spermatozoa per AI), and more than five times the quantity used for bovine insemination (20×10^6 spermatozoa per AI). Because ewes are only mated or inseminated a few times in their lives (between 5 and 10 times), we can confidently conclude that the semen used in artificial insemination is an unlikely vector for scrapie transmission in sheep, although our results would need to be confirmed with a larger number of infected rams or by injection of a larger volume of semen to mice by the

Table 1 Results of inoculation of tg338 mice with extracts of scrapie brain and semen.

Number	Donor sheep				tg338 mice	
	Genotype	Tissue	Age (months) at		Days to terminal (n/n ₀) ^a	Confirmed diagnosis ^b
			Sampling	Death ^c		
1	VRQ/VRQ	Brain	24	24	157 (5/5)	+
2	VRQ/VRQ	Brain	24	24	155 (6/6)	+
3	ARQ/ARQ	Brain	24	24	166 (6/6)	+
4	ARQ/ARQ	Brain	24	24	165 (6/6)	+
5	VRQ/VRQ	Semen	24 ^d	25	> 749 (0/14)	—
6	VRQ/VRQ	Semen	14	21	> 749 (0/13)	—
7	VRQ/VRQ	Semen	8	21	> 749 (0/13)	—
8	ARR/ARR	Semen	58	90	> 749 (0/15)	—

^aMean value for all animals in the set (number of diseased animals/number of animals inoculated). ^bResults of Western blotting performed on mouse brains. ^cAll animals killed at the terminal stage of the disease, except sheep #8. ^dSheep #5 showed clinical signs at time of sampling.

Figure 1 Survival curves of mice injected with brain extracts and semen from scrapie-infected sheep. Fourteen 9-week-old transgenic mice (tg338) were injected intracerebrally with either brain homogenates (0.2 mg brain equivalent per mouse) from scrapie-infected VRQ (filled circle) or ARQ (open circle; mean of two different experiments with two different sheep for each genotype) or with 20 µl semen from an ARR ram (triangle) and VRQ-infected rams of different ages (8 months, white square; 14 months, grey square; 24 months, black square). No statistical differences were observed in survival curves between groups of mice inoculated with semen from either susceptible or resistant sheep (Kaplan–Meier $\chi^2 = 2.313$, d.f. = 3, $P = 0.51$).

i.p. route, although this could be a less sensitive test than the intracerebral route.

Our results are important because millions of artificial inseminations are performed each year in sheep and various other species (Thibier 2005), and it is a widespread practice that has commercial and economical impact in the modern agriculture and also for the human reproduction.

Materials and Methods

Biological materials

Semen was obtained from adult Romanov rams and brain samples from adult Romanov sheep. All animals were born and raised in the chronically infected INRA Langlade experimental flock (Elsen *et al.* 1999). For semen, rams from two PrP genotypes were used: three scrapie-susceptible VRQ/VRQ rams (8, 14 and 24 months old) and one scrapie-resistant ARR/ARR ram (58 months old). In this flock, VRQ/VRQ rams are used for reproduction and fertility for the different sensitive genotypes is about 60% vs 72% for resistant genotypes. The 24-month-old VRQ/VRQ ram already showed classical signs of infection (weight loss, head tremor, scratching, etc.). The other two did not show any signs at the time of collection but developed symptoms later. For brain, 24-month-old VRQ/VRQ and VRQ/ARQ sheep (both sensitive genotypes of the same breed and reared in the same flock at the same time) were used. All VRQ/VRQ and VRQ/ARQ animals were killed at the

clinical stage and their brains were found to be positive for scrapie by immunohistochemistry (not shown).

Given the well-documented time-course of the disease in VRQ/VRQ sheep in this experimental flock, the positive result indicated that the younger rams were already contaminated at the time of semen collection (Andreoletti *et al.* 2000). As expected, the ARR/ARR ram did not show any clinical signs but was killed at 90 months of age and its brain was found to be negative for scrapie. One ejaculate was collected from each ram by artificial vagina and rapidly frozen at -20°C . After defrosting, ejaculates were tested for bacterial contamination before injection and mixed with an antibiotic cocktail (penicillin 2 µg; streptomycin 2 µg; kanamycin 2 µg per injection) that did not interfere with scrapie transmission.

Mouse bioassay

For each crude semen sample, 20 µl containing about 10^8 sperm cells were injected intracerebrally under anaesthesia to fourteen 9-week-old tg338 mice. As an infectivity control, 20 µl brain suspensions (10 mg/ml) from the infected VRQ/VRQ and the VRQ/ARQ sheep were injected intracerebrally to five or six tg338 mice (experiment performed twice).

Mice were then monitored each day for clinical signs, and either killed at the terminal stage of the disease or killed after 749 days. Any death arising during the experimental period was recorded and all mice, including those that died from intercurrent diseases, were necropsied for brain sampling. A 10% (w/v) solution was made for all brains, treated with proteinase K and tested for PrP^{Res} by Western blotting using the mouse monoclonal antibody 8G8 (Krasemann *et al.* 1999).

All animals were killed according to the requirements of the INRA Animal Care and Ethics Committee.

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