Different allelic effects of the codons 136 and 171 of the prion protein gene in sheep with natural scrapie

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Scrapie is a transmissible degenerative disease of the central nervous system occurring naturally in sheep. It belongs to the group of prion diseases also affecting man in which an abnormal isoform of the host-encoded prion protein (PrP) accumulating in the brain is responsible for neuronal death. Three main polymorphisms have been described in the sheep PrP gene, at positions 136, 154 and 171. A strong association between susceptibility/resistance to natural scrapie and a dimorphism at codon 136 of the ovine PrP gene has been reported in several breeds, including Romanov. This dimorphism, however, is not found in all scrapie-affected breeds. We have compared the PrP genotypes of Lacaune sheep obtained from enzootically affected flocks with those of apparently healthy sheep. A third variant at codon 171 was also evidenced. The results were compared with those obtained in a single experimental Romanov flock orally challenged with nematode parasites in which scrapie suddenly appeared and killed 80% of the sheep. We present evidence that, even in different epizootological circumstances, the major genetic factor controlling the susceptibility/resistance to natural scrapie in sheep, is represented by codon 171 genotype of the PrP gene. We also suggest that a modification of the allelic effects of codon 136 can occur in heavily infected animals.

Scrapie is an enzootic fatal neurodegenerative disorder of unknown aetiology affecting sheep. It is one of the transmissible subacute spongiform encephalopathies, also named prion diseases, which are diseases affecting both humans and animals. Scrapie was the first spongiform encephalopathy whose transmissibility was demonstrated (Cuille & Chelle, 1938). The biochemical hallmark of these diseases is an accumulation of an abnormal protease-resistant form of a host-encoded protein, the prion protein (PrP), in the central nervous system of affected humans and animals (see Prusiner, 1993 for a review). This pathological accumulation could result from a protein conformational change under the influence of unknown factors (Pan et al., 1993). Experimentally inoculated or ingested brain fractions containing this protein transmit the disease but the nature of the pathogen naturally present in the field is still unknown. For many years, several lines of evidence have indicated that sheep susceptibility to scrapie was under genetic control. Sip has been defined as the major gene controlling the susceptibility of Cheviot sheep, either experimentally challenged by the scrapie source SSBP/1 (Dickinson et al., 1968) or naturally affected (Foster & Dickinson, 1988). A close association between experimental incubation times in Cheviot and flanking polymorphisms of the sheep PrP gene suggested that Sip and the PrP gene were tightly linked, if not identical (Hunter et al., 1989). Examination of the sheep PrP coding sequence revealed a high degree of polymorphism, mainly located at codons 136 (Ala/Val), 154 (Arg/His) and 171 (Gln/Arg) (Goldmann et al., 1990, 1991; Laplanche et al., 1993a). The 136V variant was shown to be strongly associated with Cheviot and Swaledale sheep susceptibility to experimental scrapie after inoculation of the scrapie isolate SSBP/1 (Goldmann et al., 1991; Hunter et al., 1993). This association, also found with natural scrapie in Romanov, Ile-de-France, Swaledale, Shetland, Scottish Halfbred and Bleu du Maine breeds (Laplanche et al., 1993a; Hunter et al., 1993, 1994) led to the identification of the homozygous 136VV or heterozygous AV phenotypes as major risk factors for scrapie in these breeds. Nevertheless, the effect of codon 136 dimorphism in determining the susceptibility of

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Cheviot sheep appeared to be modified when other scrapie isolates such as BSE or CH1641 were used (Foster & Dickinson, 1988; Goldmann et al., 1994).

Natural scrapie-affected breeds are not all polymorphic at codon 136. This is the case in the indigenous French breeds Lacaune, Manech and Préalpes (Laplanchère et al., 1993b) as well as Suffolk sheep (Westaway et al., 1994). In Suffolks, the homozygous genotype 136AA/171QQ was strongly correlated with the development of the disease (Westaway et al., 1994). In our study, the PrP genotypes of scrapie-affected Lacaune sheep obtained from several flocks over 2 years were compared with a sample of apparently healthy control rams in order to define susceptibility-associated polymorphisms. The results were then compared with those obtained in a single Romanov flock in which scrapie suddenly appeared (after the sheep were orally challenged with nematode parasites) and killed 80% of the animals. This indicates that in extremely different epizootological conditions and in different breeds, the same PrP polymorphism plays a major role in the control of scrapie susceptibility in sheep.

The French Lacaune breed is located in the south of the Massif Central region. Ewes bred for milk production form the major part of the flocks in which nearly all replacements are obtained through artificial insemination. The rams are kept in two insemination centres in which scrapie has never occurred. Between September 1991 and October 1993, one scrapie-affected ram and 57 ewes, all from 10 different flocks were recorded. The incidence of scrapie in each flock was low (about 5%). Approximate age at disease onset was 17.5 months (range: 12–42 months). PrP genotypes of the 105 most frequently used rams from the two insemination centres were determined. Rams’ allelic frequencies were considered as representative of the allelic frequencies of the whole Lacaune population because they were at the origin of half of its genes. Furthermore, eight pairs of twin sheep from eight different flocks, one affected the other apparently healthy, all living in pairs but remaining in their respective flocks, were also investigated. The Romanov sheep included in this study came from a single experimental flock bred for various scientific purposes. Natural scrapie seemed to be absent from the initial flock since its creation in 1973/74. In 1990, some sheep from this flock were used for the study of genetic resistance to common nematode parasites. Seventy-four sheep born in October 1991 from parents previously selected for their susceptibility or resistance to parasites were orally challenged three times with large amounts of third-stage larvae obtained in vitro from eggs of Teladorsagia circumcincta and Trichostrongylus colubriformis between May and August 1992. A scrapie-like disease unexpectedly appeared in the subflock in April 1993 and killed 60 sheep (81%) in 14 months. This incidence was the highest reported so far and sheep susceptible and resistant to the parasite infestation were affected. DNA sequence and other data from 43 sheep were available for analysis (29 affected and 14 survivors). Median age at death was 726 days (range 687–968; n = 29). Approximate age at onset was 691 days (range 590–735). At the time of writing, survival time of unaffected sheep was up to 1125 days. Neuropathological examination revealed in the medulla oblongata the presence of spongiosis and gliosis compatible with the diagnosis of scrapie in all cases. The detection of the abnormal form of PrP in the brain of sheep selected at random firmly established the diagnosis of scrapie in both breeds.

Sheep DNA was extracted from peripheral blood leukocytes using standard procedures. Two fragments of the sheep PrP gene coding sequence (P2-P3 and P5-P6) were amplified using polymerase chain reaction (PCR) and analysed for variability by denaturing gradient gel
electrophoresis (DGGE) according to Laplanche et al. (1993a). PCR products to be sequenced were subcloned then sequenced using previously described procedures. Codons 136 and 171 genotypes were typed using BspH1 digestion or allele-specific oligonucleotide (ASO) hybridization as previously described (Maciulis et al., 1992; Laplanche et al., 1993a). The ASO sequence for 171H was 5’ CAGTGGATCATTATAGTAA 3’. Hybridization and washing temperatures in standard hybridization conditions were 43 °C and 51 °C, respectively. Comparisons were performed with the χ² test and the non-parametric Kolmogorov–Smirnov test.

During the DGGE analysis, a new variant was detected in 13 out of 171 Lacaune sheep (Fig. 1a). It corresponded to a new G → T substitution at nucleotide 584 (numbering according to Goldmann et al., 1990) leading to a CAG (Gln) to CAT (His) change at codon 171. This new polymorphism was confirmed by ASO hybridization and taken in account for the next step of genotyping (Fig. 1b). Genotyping of a sample representative of the Lacaune population revealed only four haplotypes 136A/171Q (0.55), 136V/171Q (0.38) and 136A/171R (0.07) as previously published (Laplanche et al., 1993a). No significant difference with the Hardy–Weinberg expected values was observed. As in the Lacaune breed, the genotypes of scrapie-affected Romanov sheep were distinct from those of the survivors (P < 0.01). Table 2 shows that affected sheep were all homozygous 171QQ and represented the three possible genotypes at codon 136 which was known to be polymorphic in this breed. In particular, 136AA/QQ sheep represented 38% of the affected sheep. Scrapie susceptibility seemed to be more pronounced in the 136VV parasitized animals as shown by a 100% disease incidence in this group with a noticeably narrow distribution of ages at death. It is noteworthy that the seven heterozygous 171RQ parasitized sheep are still alive at age 1125 days, regardless of their codon 136 genotypes.

For comparison, genotypes at codons 136 and 171 were also established for the parasitized Romanov flock. We found three haplotypes 136A/171Q (0.55), 136V/171Q (0.38) and 136A/171R (0.07) as previously published (Laplanche et al., 1993a). No significant difference with the Hardy–Weinberg expected values was observed. As in the Lacaune breed, the genotypes of scrapie-affected Romanov sheep were distinct from those of the survivors (P < 0.01). Table 2 shows that affected sheep were all homozygous 171QQ and represented the three possible genotypes at codon 136 which was known to be polymorphic in this breed. In particular, 136AA/QQ sheep represented 38% of the affected sheep. Scrapie susceptibility seemed to be more pronounced in the 136VV parasitized animals as shown by a 100% disease incidence in this group with a noticeably narrow distribution of ages at death. It is noteworthy that the seven heterozygous 171RQ parasitized sheep are still alive at age 1125 days, regardless of their codon 136 genotypes.

The data presented here show that in both Lacaune and Romanov flocks with different scrapie incidence, the disease developed predominantly in sheep harbouring the 171QQ genotype of the PrP gene, irrespective of the epizootological context. This association appears to be very strong in the Lacaune breed in which codon 136 has limited polymorphism. Homozygosity for glutamine at codon 171 has also been recently evidenced in scrapie-affected Suffolk sheep by Westaway et al. (1994). Sheep susceptibility seems highly dependent on the amino acid encoded by codon 171 but is not restricted to glutamine. Clearly, 171QH heterozygous Lacaune sheep seemed to be susceptible to scrapie as 171QQ animals. This was not the case in sheep with an arginine at codon 171. In our two breeds, 171RQ, RR or RH sheep remained apparently healthy. This protective effect associated with the 171R variant was also observed in Suffolk sheep (Westaway et al., 1994). Therefore, an arginine at position 171 in PrP seems to markedly reduce scrapie susceptibility and suppress or delay the onset of the
disease. This association is supported by the fact that in contaminated Lacaune flocks, 171RQ or RH animals remained apparently healthy when the disease appeared in their siblings. This effect of the 171R variant in disease prevention seems to be quite dominant as shown by the Romanov flock data. In spite of the 80% scrapie incidence in the whole parasitized flock, none of the 171R carriers developed the disease in the 18 months after the onset of the first cases. Similarly, no experimental disease occurred in Cheviot sheep carrying the 171R variant after an oral challenge with a bovine spongiform encephalopathy (BSE) isolate or inoculation with the scrapie source CHt641 (Goldmann et al., 1994). However, according to our previous observations of 136AV/171RQ Ile-de-France sheep developing the natural disease at 7 years (Laplanche et al., 1993a) and 136AV/171RQ Cheviot sheep susceptible to SSBP/1 after intracerebral inoculation (Goldmann et al., 1994), an arginine at codon 171 cannot be considered as fully protective. It is possible that the 171Q and 171H variants of the PrP gene are in linkage disequilibrium with the functional alleles of a recessive gene responsible for the disease. Nevertheless, numerous studies have shown that in several species, including man, mutations or polymorphisms in the PrP gene coding sequence are linked or associated with the development and the phenotype of the disease (Prusiner, 1993). Therefore, arginine, instead of glutamine or histidine, encoded by codon 171 might delay or inhibit the conformational change of PrP thought to be induced by the scrapie agent (Pan et al., 1993). In 171RQ or RH heterozygous sheep, the amount of the abnormal isoform would remain below the threshold of the neurotoxic concentration required to induce neuronal death.

The potential effect of the codon 136 dimorphism in relation to the 171QQ genotype could only be studied in the Romanov flock. Surprisingly, we found that all codon 136 genotypes were significantly represented in the scrapie-affected sheep, whereas genotype 136AA was thought to be partially protective on the basis of its low frequency (0 to 5%) found in diseased animals from enzootically affected flocks with a low incidence of the disease (< 5%) (Laplanche et al., 1993a; Hunter et al., 1993, 1994). The high incidence of the disease in the whole Romanov flock (up to 80% of mortality in 14 months) does not seem to be related to the genetic selection for resistance to parasites and rather suggests that a massive infection by the scrapie agent and/or by an unusual agent with a modified tropism has occurred in the infested group. The minor modulation of the incubation period in this flock, compared with that of flocks where a low level of infection apparently remains (Laplanche et al., 1993a), suggests that the allelic effects of codon 136 in 171QQ animals become slight when the level of infection increases or the agent changes. A different allelic effect may be also observed in experimental scrapie when the scrapie source varies. Cheviot sheep orally challenged with brain tissue from BSE-affected animals are affected, regardless of their codon 136 genotype whereas another scrapie source (SSBP/1) did not induce scrapie in 136AA/171QQ animals (Goldmann et al., 1994).

As the outbreak of scrapie, after an oral contamination of the sheep with nematode larvae, suggested a response similar to that observed in experimental scrapie, this prompted us to revisit the hypothesis of scrapie transmission through parasites. In 1968, Fitzsimmons & Pattison tried without success to transmit scrapie using nematode worms and larvae isolated from experimentally scrapie-affected sheep which they inoculated in the brain of healthy animals. The oral route is thought to be contaminating in natural scrapie (Hadlow et al., 1982) but it seems very unlikely that in the Romanov experiment, scrapie infectivity could remain strongly associated with the washed parasite eggs, or with any of the three successive stages of larvae obtained in vitro. We tried to detect the abnormal form of PrP in the third-stage larva extracts used in this infestation experiment but negative results were inconclusive. Another hypothesis is that the scrapie agent had been present in the ground for several years but at a very low level of infectivity, insufficient to promote the disease. The parasitic infestation would suddenly increase the animals' susceptibility to scrapie by facilitating the penetration by the agent through the lesions created by the large amount of larvae introduced in the sheep gastrointestinal tract.

As long as the exact nature of the scrapie agent remains unknown, thus precluding effective prevention, attempts to define genetic factors enhancing the sheep resistance will help to contribute to reduce the incidence of natural scrapie. However, the effect of these factors must remain constant throughout various epizootological circumstances. Allelic effects of codon 136 (when polymorphic) have an impact on incubation time in flocks with a low scrapie incidence and seem to be modified when the conditions of a high disease incidence emerged. At present, our main conclusion is that the major genetic factor controlling the susceptibility/resistance to scrapie in sheep, regardless of the infectivity or the agent, could be the amino acid encoded by the 171 codon of the PrP gene. Scrapie is not observed in mice artificially devoid of PrP (Büeler et al., 1992, 1993) and whether a single amino acid change in the protein may have the same effect in sheep has to be considered. Progress in the identification of such crucial residues in the protein would help to clarify the relationship between PrP and the scrapie agent.
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