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Natural scrapie in a closed flock of Cheviot sheep occurs only in specific PrP genotypes

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Summary. Natural scrapie in a closed flock of South Country Cheviot sheep has resulted in 45 deaths between 1986 and 1995. Of these cases, 35 sheep have been analysed for disease-linked PrP gene polymorphisms and all encode valine at codon 136 on at least one allele with 77% homozygous (VV_{136}) and 23% valine/alanine heterozygotes (VA_{136}). Mean survival time was 907 and 1482 days for VV_{136} and VA_{136} scrapie affected animals respectively. VV_{136} animals were all at great risk of disease if allowed to live long enough. However scrapie occurred only in a specific subgroup of VA_{136} sheep, survival advantage depending on VA_{136} animals being heterozygous for other polymorphisms at codons 154 or 171. The flock history has been recorded in great detail since its foundation in 1960 however there was no strong evidence for simple maternal or paternal transmission of disease other than inheritance of PrP genotype.

Introduction

A South Country Cheviot flock, founded in 1960, has been selected into two lines differing in their response to experimental challenge with scrapie [9]. Response to SSBP/1 (scrapie sheep brain pool/1) is under the control of a single gene, *Sip*, with two alleles, sA and pA [8], with sA being dominant [12]. Different sources of scrapie infected inocula have different transmission characteristics: SSBP/1 (termed an A group isolate) has shorter incubation periods in Sip^{sA} carriers (positive line) than in Sip^{pApA} (negative line) sheep, but CH1641 (a C group scrapie isolate) and BSE have shorter incubation periods in some negative line sheep than in positive line animals [10, 14, 15]. The flock has been completely closed (genetically isolated) since 1962 and is known as the Neuropathogenesis Unit (NPU)Cheviot flock to differentiate the sheep from others of the Cheviot breed.

Polymorphisms of the sheep PrP gene are so closely associated with incubation time differences (and the alleles of *Sip*) in NPU Cheviots [18, 21] that the

PrP gene and *Sip* are believed to be synonymous. In NPU Cheviot sheep, valine at codon 136 (V_{136}) is linked to Sip^{sA} whereas alanine at codon 136 (A_{136}) is linked to Sip^{pA}. Positive line animals with short incubation periods (167 days +/− 5) following subcutaneous (sc) inoculation with SSBP/1 are VV_{136} (Sip^{sAsA}), whereas those with longer incubation periods (322 days +/− 16) are VA_{136} (Sip^{sApA}). Negative line animals are all AA_{136} (Sip^{pApA}) and these resist sc challenge with SSBP/1. Inevitably, when crosses involve heterozygotes (Sip^{sApA}) in the positive line, a small number of Sip^{pApA} (AA_{136}) animals are produced and these also resist sc SSBP/1 inoculation. Challenge with CH1641 or BSE causes disease in only a proportion of both positive and negative lines and does not associate primarily with codon 136 variation. Instead, animals encoding glutamine at codon 171 (Q_{171}) on both PrP alleles (QQ_{171}) succumb to intracerebral (ic) inoculation whereas those having one allele with arginine (R_{171}) have much longer incubation period (RQ_{171}) and those with two (RR_{171}) are resistant [19, 20].

Although SSBP/1 targets animals carrying V_{136}, a minor influence of codon 171 is seen since VA_{136} animals which are also RQ_{171} have a longer incubation period (364 days +/− 17) than those which are QQ_{171} (260 days +/− 15). Similarly with CH1641 and BSE, although the major effect on incubation period depends on the codon 171 genotype, animals encoding V_{136} (VV_{136} or VA_{136}) have longer incubation periods than AA_{136} sheep [20]. Incidence of natural scrapie in other breeds has also been associated with variation at codons 136 and 171 [2, 4, 23, 26, 32].

PrP genotypes are now used to predict accurately the response of NPU Cheviot sheep to experimental challenge with SSBP/1, CH1641 and BSE [19] and the flock is thus a valuable resource for transmission and pathogenesis studies [13, 15]. However a low incidence of natural scrapie has been apparent for some time in the positive line (Sip^{sA} carriers) [14]. Accumulation of genotype information has now revealed that natural scrapie has occurred only in animals of specific PrP genotypes. This paper describes the natural scrapie outbreak within the NPU Cheviot flock and discusses hypothetical links with maternal or paternal transmission, PrP genotype and ovine lymphocyte antigens.

Materials and methods

Sheep and flock breeding strategy

All sheep in this study were from the NPU Cheviot flock. Detailed records held for every animal (> 5 000) since the foundation of the flock provided the pedigree data. The flock was formed in 1960 with 15 rams (born in 1960 and bought at auction) and 300 ewes (born 1957–1959 on three different farms believed to be scrapie-free). From 1962, when nine ewes (born 1957–1959) were introduced, the flock has been completely closed. All foundation animals were injected (usually sc) with SSBP/1 scrapie after mating (males) and after lamb weaning (females). The policy of challenging every animal was continued for many years to consolidate the selection lines and to study the genetics of control of susceptibility differences. Animals were mated up to three times prior to challenge but never following challenge, thus avoiding any possibility of maternal transmission of experimentally induced disease to

offspring. Experimentally challenged animals were kept completely separate from the breeding flock, thus avoiding any possibility of horizontal transmission of disease. Progeny lambs were mated as soon as they reached sexual maturity but challenged or culled only after the results of the parents' challenges were known. Experimental scrapie cases occurred in the foundation flock between 127–1607 days post inoculation (dpi) depending on the route of challenge but those succumbing to ic or sc challenge by around 500 dpi formed the basis of the positive line. Animals were assigned to the negative line if experimental scrapie did not develop within 2 years of sc inoculation.

Many of the animals described in this study had one or both parents challenged with experimental scrapie but only after mating (sires) or after weaning (dam). There was no association whatsoever between such experimental challenge of parents and the development of natural scrapie in progeny and no further details are presented.

Scrapie diagnosis and histopathology

Diagnosis of scrapie by clinical signs was confirmed or excluded in each case by histopathology. Clinical signs of natural scrapie in NPU Cheviots can last up to three or four months whereas for all cases of experimentally induced disease the course is much shorter – two to three weeks, and sometimes less. With natural scrapie, sheep invariably show signs of progressive pruritus, with hyperaesthesia and incoordination of gait. Histopathology gives some variation in intensity of vacuolation of neuro-anatomical regions but the brain stem is most frequently affected, e.g. dorsal vagus and reticular formation. Vacuolation of the cerebellum can occur as a focal lesion, but cortical vacuolation is not common. With experimentally induced disease, ataxia is always present but pruritus is seldom seen. The distribution of vacuolation can sometimes vary depending upon route of challenge and source of inoculum, however, following sc injection of SSBP/1 scrapie, vacuolation of the diencephalon is either very mild or absent altogether.

Genotype analysis

Genotypes are described in terms of PrP polymorphisms or as positive or negative line. Sometimes for sheep early in the history of the flock, the older *Sip* terminology is used because molecular analysis of the PrP gene was not available and the PrP genotype is not known. The relationship between the alleles of *Sip*, sA and pA, and the alleles of the PrP gene are as follows. There is only one Sip^{sA} related allele of the PrP gene, that encoding valine at codon 136 which has fixed linkage to other codon variants (see Table 1). It is not necessary therefore to give the full genotype of VV_{136} animals although in each case presented in this paper, it is known to be $RR_{154}\,QQ_{171}$. There are several Sip^{pA} related PrP alleles, all of which encode alanine at codon 136 but which vary at other codons, giving rise to several PrP genotype subgroups. It follows from this that Sip^{sAsA} animals form a single PrP genotype group and that Sip^{sApA} and Sip^{pApA} sheep consist of multiple PrP genotype groups.

High molecular weight DNA was extracted from sheep blood or tissues using an Applied Biosystems Nucleic Acid Extracter and genotypes established using polymerase chain reaction (PCR) products as described previously [23]. Genotypes at codons 136 and 154 were detected following digestion with *Bsp*HI or by sequencing [18, 23]. Codon 171 variants were detected by sequencing and by differential oligonucleotide hybridisation to PCR products immobilised on nylon membrane using a method adapted slightly from that previously described [6]. The oligonucleotide probes were 5′ TGGATCGGTATAGTA (arginine), 5′ TGGATCAGTATAGTA (glutamine), and 5′ TGGAGCATTATAGTA (histidine) and washing temperatures were 42 °C, 37 °C and ambient temperature, respectively.

Polymorphisms at other codons were detected by sequencing of PCR products and genotype frequency differences were assessed for significance using Student's t test.

Ovine lymphocyte antigen typing

Ovine lymphocyte antigen typing was carried out as previously described [29]. Ten lymphocyte antigens were assessed.

Results

History of natural scrapie in NPU Cheviots

Natural scrapie was first seen in the NPU Cheviot flock in 1970 [7] in 15 animals (1967 and 1968 birth cohorts) which had not been challenged with experimental scrapie. The natural disease continued to appear until the 1978 birth cohort with peak incidence in the 1970–73 birth cohorts. All 172 cases occurred in the positive line with survival times of 575 to 1790 days. (Survival time is defined as the time between birth and slaughter on welfare grounds following Home Office regulations.) A policy of slaughter of family lines of all scrapie cases was introduced and was apparently successful in eliminating scrapie in that there were no natural scrapie cases in the 1979, 1980 and 1981 birth cohorts. However in 1986, 2 animals, born in 1982 and 1984, died of natural scrapie. There have been in this second outbreak, between 1986 and May 1995, 45 cases in the positive line and none in the negative line. Lifespans of scrapie affected animals range from 497 to 2250 days and 35 of these sheep were PrP genotyped. Three out of the genotyped group of 35 scrapie animals had also been challenged sc with SSBP/1. These animals developed signs of scrapie less than 60 days after inoculation with SSBP/1 – far too short a time for experimental SSBP/1 scrapie to develop and with very long clinical courses and appropriate signs and pathology characteristic of natural scrapie. Scrapie cases have been excluded from this report if there was judged to be any chance of confusion between natural and experimental scrapie.

PrP genotypes in the NPU Cheviot flock

In 1990 routine PrP genotyping of all the animals in the NPU Cheviot flock began and now every lamb is genotyped within the first year of life. PrP genotypes of some animals born prior to 1990 are also available because DNA was prepared from stored tissues. Sheep PrP polymorphisms and haplotypes known to date are shown in Table 1a and 1b. All have been described previously except that at codon 141 which is new to this paper. At nucleotide 492 [17], a C to T transition leads to an amino acid codon change from leucine to phenylalanine and this occurs on the new haplotype M_{112}-A_{136}-F_{141}-R_{154}- Q_{171} with e3, h1 [22]. It occurs in both positive and negative line NPU Cheviots at approximately the same frequency as H_{154}. However after experimental sc challenge with SSBP/1, sheep differing at codon 141 appear to have similar incubation periods (manuscript in prep.).

Table 1. Sheep PrP variants

a Sheep PrP gene codon polymorphisms

Codon	amino acid change	breed	reference
112	methionine (M)		
	threonine (T)	Ile de France	26
136	alanine (A)		
	valine (V)	many	18
141	leucine (L)		
	phenylalanine (F)	NPU Cheviots	this paper
154	histidine (H)		
	arginine (R)	many	18
171	glutamine (Q)		
	arginine (R)	many	17
	histidine (H)	Texel/Lacaune	2, 4

b Sheep protein variants

Codon	112	136	141	154	171
	M	A	L	R	Q
	M	V	L	R	Q
	M	A	L	H	Q
	M	A	L	R	R
	M	A	L	R	H
	M	A	F	R	Q
	T	A	L	R	Q

Frequencies of PrP haplotypes in NPU Cheviot flock

Frequencies of PrPgenotypes in positive and negative lines have been influenced by selection for susceptibility and resistance to challenge with SSBP/1. This scrapie source targets animals according to codon 136 and, to a lesser extent, codon 171 genotype. V_{136}, which occurs on one haplotype with R_{154} and Q_{171}, does not occur in negative line sheep. All 258 negative line animals tested (1974–1994 birth cohorts) were AA_{136}. In positive line animals of the 1984–1994 (inclusive) birth cohorts (236 animals tested), V_{136} frequency was overall about 50%. However, reflecting the genotypes of the rams used, in the 1991 and 1992 birth cohorts V^{136} frequency rose to 59% and 63% respectively, and fell to 22% in the 1994 birth cohort. Birth cohorts between 1987 and 1992, had in total 12 sires, eight of which were VV_{136} and four were VA_{136}. In the period 1993–1995, rams have been chosen in order to decrease the frequency of VV_{136} homozygous lambs and this is reflected in the flock PrP genotype frequencies. VV_{136} homozygous animals currently form around 21% of the positive line, with 58%

VA_{136} and 22% AA_{136} – the latter being the result of heterozygote matings or positive/negative line crosses. These figures are slightly different from those published previously [19] because of changes in breeding strategy and increased numbers of animals tested.

The frequency of H_{154} (which occurs on only one haplotype with $A_{136}Q_{171}$) was 9% in the positive line and 39% in the negative line. R_{154} is encoded on several alleles and is more common (91% positive line, 61% negative line). Both allele and genotype frequencies have been variable over the last five years (454 animals tested) probably indicating that this codon has only a minor association with SSBP/1 susceptibility.

Variation in codon 171 haplotype frequencies has been greater in the negative than the positive line (403 animals tested), but the mean frequency of Q_{171} is 74% in the positive line and 62% in the negative line. H_{171} and T_{112} (Table 1) have not yet been found in NPU Cheviots.

Effect of genotype on incidence of natural scrapie

The genotypes of thirty-five natural scrapie cases occurring between 1986 and 1995 (Table 2) were 77% VV_{136} and 23% VA_{136}. All of these animals were also $RR_{154}Q_{171}$ – inevitable with VV_{136} animals as there is only one V_{136} allele (Table 1), but also indicating that only a subgroup of VA_{136} animals is being targetted. No $VA_{136}RQ_{171}$ animals have developed natural scrapie between 1986 and 1995, in observation periods of up to 3 534 days of age. This is despite the fact that, like all V_{136} carriers, this genotype is susceptible to experimental challenge with SSBP/1. There was no evidence of sex bias in scrapie cases which were 56% female, similar to the frequency of females in the flock. VV_{136} scrapie cases occurred from 1986 to 1995 in the 1982–1993 birth cohorts at mean age 907 days [standard deviation (SD) = 265]. Scrapie affected VA_{136} animals in the 1983–1991 birth cohorts had significantly longer survival times ($P<0.0005$) dying at mean age 1482 days (SD = 426). VA_{136} scrapie cases always had longer lifespans than VV_{136} cases of the same birth cohort (Fig. 1). There is also a clear shortening of lifespan of scrapie affected animals from the 1982 and 1983 birth cohorts up to cases occurring in the1986 birth cohort; thereafter lifespans in the two groups remain essentially constant (Fig. 1). VV_{136} and VA_{136} cases in the last few years have occurred at 700–900 and 1 100–1 200 days of age respectively, and this is now taken to be the standard "at risk" age range for sheep of these genotypes.

No natural scrapie cases have ever been recorded in AA_{136} animals from either the positive or negative lines. AA_{136} deaths are only the results of experimental challenge with scrapie (CH1641 and BSE), intercurrent illiness or culling for welfare or flock management reasons. These animals can have long lifespans, for example 18 positive line AA_{136} animals (born 1980–1990) survived beyond 1 900 days (mean age 2 451 days).

Sixteen VV_{136} animals (born 1981–1992), which were not recorded as having developed natural scrapie, were found amongst the genotyped animals. Of these,

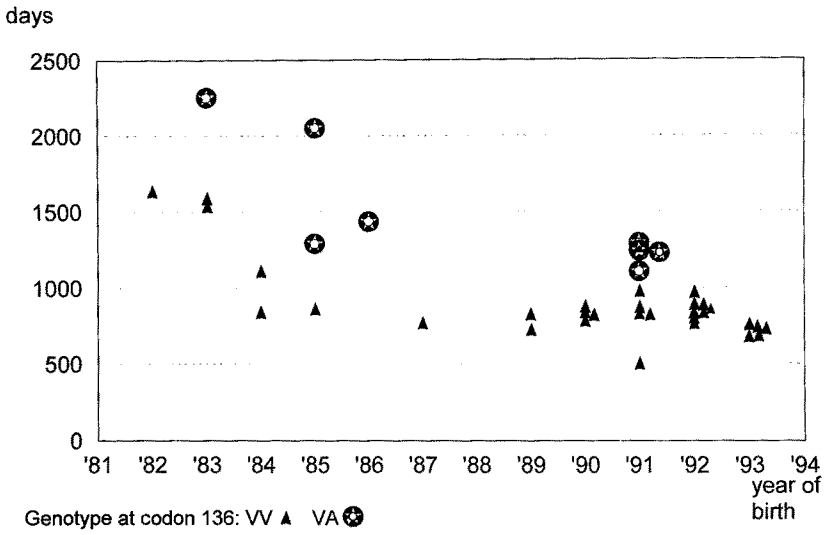

Fig. 1. Birth cohort and age at death from scrapie. Lifespan (days) for each individual animal plotted against year of birth

Table 2. PrP genotypes of 35 natural scrapie cases (died 1986–1995; birth cohorts 1982–1993)

PrP genotype	n[b]	%	Age at death (days)[a] mean	range	SD[c]
$VV_{136}RR_{154}QQ_{171}$	27	77	907	497–1631	265
$VA_{136}RR_{154}QQ_{171}$	8	23	1462	1107–2250	426

[a]Ages at death of VV_{136} and VA_{136} animals differ significantly ($P < 0.0005$)
[b]Number of animals
[c]Standard deviation

13 died, having been used in experiments, at less than 500 days of age, before natural scrapie signs would be expected. One died clinically negative and neuropathology negative at 820 days. Two animals survived to 1000 and 1550 days when they were challenged with SSBP/1 and died with experimental scrapie-like signs at 1185 and 1750 days respectively. These latter two animals were from the 1985 and 1981 birth cohorts in which signs of natural scrapie tended to take longer to develop (Fig. 1) than in more recently born cohorts. Since the 1985 birth cohort, every VV_{136} animal has developed natural scrapie if allowed to live long enough, none surviving more than 971 days.

Fifty VA_{136} animals (born 1985–1992) survived beyond 1107 days (the earliest VA_{136} scrapie case) and up to 3435 days without developing natural scrapie. (Table 3). The majority (68%) of the VA_{136} survivors were also RQ_{171}, but 11 of these 34 animals were still within the concurrent heterozygote scrapie "at risk" age range, 1100–1200 days. The other survivors were QQ_{171} and thus

Table 3. PrP genotypes of 50 Val/Ala$_{136}$ animals surviving beyond 1107 days (birth cohorts 1985–1992) which either died of non-scrapie causes or are alive at time of writing

PrP genotype	Number (alive)	%	Mean age (days)	Range	[a]Number (alive) within 1100–1250d age range
$VA_{136}RR_{154}RQ_{171}$	34 (8)	68	1961	1125–3435	11 (4)
$VA_{136}HR_{154}QQ_{171}$	10 (7)	20	1647	1180–2610	5 (3)
$VA_{136}RR_{154}QQ_{171}$	6 (4)	12	1679	1090–2735	3 (1)

[a]Number of animals which either died between 1 100–1 250 days of age or are alive and at 1 100–1 250 days of age and which are therefore within the age range at risk of scrapie

the same codon 171 genotype as the VA_{136} scrapie cases, however 10 of these survivors were HR_{154} whereas all VA_{136} scrapie cases were RR_{154}. A further six survivors were $VA_{136}RR_{154}QQ_{171}$, a genotype susceptible to developing natural scrapie (Table 2). One of these six animals is alive at 1 210 days and two were culled for flock management reasons at 1 090 and 1 185 days – all three therefore would be within the "at risk" age for natural scrapie. The other animals are still alive at 1 915, 1 940 and 2 735 days of age and thus beyond the age at which they would be expected to have developed natural scrapie.

Effect of scrapie in the parents on scrapie incidence in offspring

Pedigree information was searched for any evidence of natural scrapie simply transmitting from preclinically affected parents to their offspring, regardless of genotype. Both parents and offspring of scrapie affected animals were considered. Twenty-three scrapie sheep produced no offspring and are therefore uninformative, however out of the females, four scrapie ewes (three VV_{136}, one VA_{136}) each produced one VV_{136} offspring, which went on to develop scrapie itself, out of a total of 14 lambs. In each case, the scrapie lamb was the last surviving lamb produced by its scrapie dam. Three of these scrapie dams died at 365 days (two VV_{136}, one VA_{136}) and one at 820 days (VV_{136}) after birth of their scrapie offspring. Most of the other 10 progeny of the scrapie dams were culled or died or are still alive at less than 730 days, however one VV_{136} scrapie ewe also produced a lamb which lived 1 915 days (genotype unkown).

Three VV_{136} rams (A, B and C) produced in total 49 live offspring. (Part of the family tree of rams A and B is shown in Fig. 2 (line IIIa). Ram A, mated with 11 ewes (10 in Fig. 2, line IIIb, one – his mother – in Fig. 2, line II), had seven progeny which went on to develop scrapie (four VV_{136} and three VA_{136}), seven progeny which survived 1 125–1 790 days (six $VA_{136}RR_{154}RQ_{171}$ and one $VA_{136}HR_{154}QQ_{171}$) and one which died too young for scrapie to have developed (Fig. 2, line IV). Ram B had two scrapie affected progeny (one VV_{136} and one VA_{136}), two progeny (both $VA_{136}RR_{154}RQ_{171}$) which survived 1 215 and 1 615

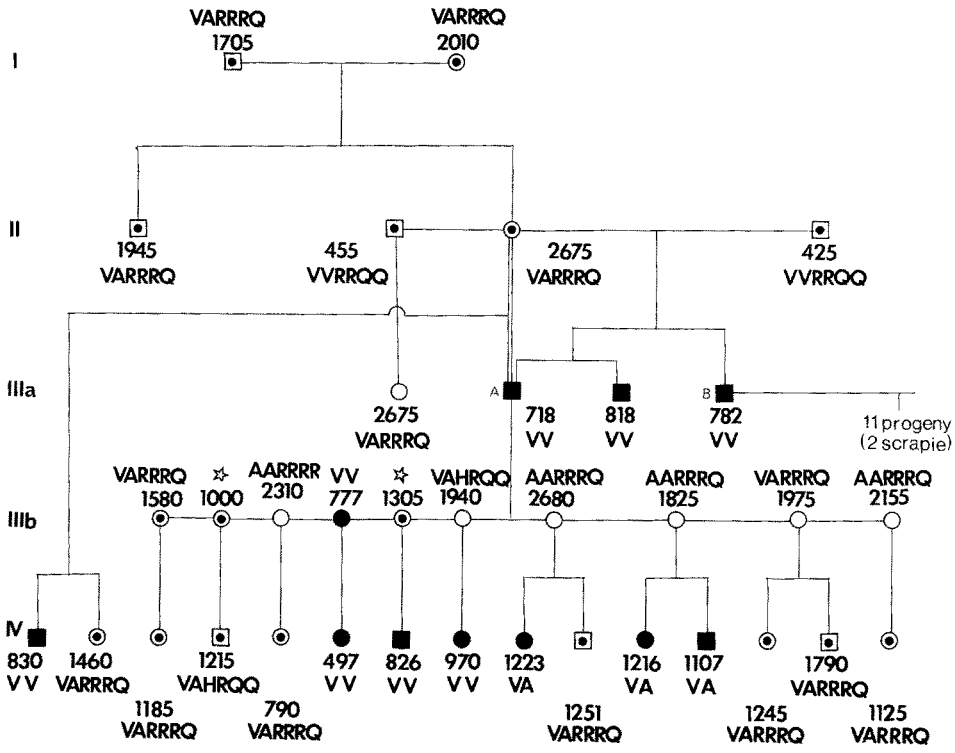

Fig. 2. Section of the NPU Cheviot sheep pedigree. Lifespan (days) and genotype of each animal in a family tree which includes scrapie animals. Genotypes of non-scrapie sheep are given in codon order 136, 154, 171. Scrapie affected sheep have only the 136 genotype shown as they are all $RR_{154}QQ_{171}$. ☆ Genotype unknown, □ male (▣ dead, not scrapie, ■ dead, scrapie), ○ female (◉ dead, not scrapie, ● dead, scrapie), || mother/son mating. I, II and III indicate generations. A and B identify the scrapie affected sires discussed in the text. Ram A (line IIIa) mated with ewes in IIIb and with his mother (line II) to produce progeny in line IV. Ewes mated with ram B not shown

days (the latter still alive) and seven others (all VA_{136} heterozygotes) which were challenged or culled early (not shown). Ram C (not shown) had seven lambs which were VV_{136} and developed natural scrapie, six VA_{136} lambs that lived 1 090–1 210 days and of which five are still alive (three $VA_{136}RR_{154}RQ_{171}$, two $VA_{136}RR_{154}RQ_{171}$ [one dead] and one $VA_{136}HR_{154}$ QQ_{171}) and ten lambs which are uninformative because of challenge or early culling.

Both dam and sire of one scrapie case (VV_{136}) were affected with natural scrapie and were themselves both genotyped as VV_{136}. This animal (Fig. 2, line IV), with two affected parents, had by far the shortest survival time of all the scrapie cases in the flock (497 days, 175 days shorter than the next shortest case).

Amongst the genotyped positive line AA_{136} survivors of natural scrapie,the dam of one animal was affected by natural scrapie which developed a few months after the birth. The 18 AA_{136} surviving animals themselves produced 88 offspring and natural scrapie was recorded in three of these (all VA_{136}) at 1 107,

1 216 and 1 223 days. Twenty-eight offspring were culled, used in experiments or died early but 27 of the others lived either into, or beyond, the age of highest scrapie incidence (1 095–3 620 days). The genotypes of 20 of this group were 14 $VA_{136}RR_{154}RQ_{171}$, one $VA_{136}HR_{154}QQ_{171}$, three $AA_{136}RR_{154}RQ_{171}$ and two $VA_{136}RR_{154}QQ_{171}$. The latter two animals lived for 1 215 and 1 095 days so are still within the scrapie "at risk" age range.

Of the 50 VA_{136} survivors (Table 3), 14 had sires which subsequently developed natural scrapie (three sires, all VV_{136}, the same animals A, B and C as above). Two of this group of 14 survivors lived 1 615 and 1 790 days.

The 50 VA_{136} survivors themselves produced 101 offspring including 17 VV_{136} scrapie cases. Thirteen of their offspring lived between 1 185–2 675 days without developing natural scrapie (ten $VA_{136}RR_{154}RQ_{171}$ and three $VA_{136}HR_{154}QQ_{171}$). As an example of this data, one animal ($VA_{136}RR_{154}$-RQ_{171}) which itself lived 2 675 days, produced 6 live lambs, four of which developed natural scrapie (all VV_{136}) and two of which survived 1 525 and 2 675 days (both $VA_{136}RR_{154}RQ_{171}$).

Six scrapie affected sheep are survived by their mothers, all alive at the time of writing. Three of these mothers are AA_{136} (aged 1 945, 1 945 and 3 041 days), one is $VA_{136}RR_{154}RQ_{171}$ (1 585 days of age) and two are $VA_{136}HR_{154}QQ_{171}$ (both aged 1 945 days). Five scrapie affected sheep are survived by their fathers, two rams of genotype $VA_{136}RR_{154}RQ_{171}$ and alive at ages 1 585 and 1 945 days.

This data strongly suggests that it is PrP genotype, rather than parental scrapie status, which is of most importance in determining a sheep's risk of developing scrapie.

Effect of parental line of origin on scrapie in offspring

Because natural scrapie has only occurred in the positive line, it was thought possible that only positive line animals were transmitting scrapie to their offspring and that having a negative line (AA_{136}) parent might in some way protect a sheep from scrapie. The line of origin, positive or negative, of each parent of all sheep involved in this study was therefore investigated but no firm conclusions could be drawn.

Scrapie in twins – potential for common uterine exposure

If scrapie is transmitted from parent to offspring *in utero* with no influence resulting from PrP genotype, it might be expected that if one twin developed scrapie, the other twin would also develop scrapie. There have been 19 sets of twins (38 lambs) with at least one in each set going on to develop scrapie. Nine twins of scrapie affected sheep were culled early or were stillborn and are therefore uninformative. The remaining 10 sets of twins are described in Table 4a. Six twins of scrapie animals also developed natural scrapie (Table 4a): 4 sets of VV_{136}twins, 1 set of $VA_{136}RR_{154}QQ_{171}$ twins and 1 set with lifespans of 1287 and 1 619 days (one $VA_{136}RR_{154}QQ_{171}$ and one of unknown genotype). Four twins of scrapie sheep lived longer than 1 000 days (Table 4b) and died or were

Table 4. PrP genotype and fate of twin pairs

Genotype Twin 1	Fate[a] Twin 1	Genotype Twin 2	Fate Twin 2
a Both twins affected by natural scrapie			
$VV_{136}RR_{154}QQ_{171}$	NS 718d	$VV_{136}RR_{154}QQ_{171}$	NS 818d
$VV_{136}RR_{154}QQ_{171}$	NS 777d	$VV_{136}RR_{154}QQ_{171}$	NS 832d
$VV_{136}RR_{154}QQ_{171}$	NS 837d	$VV_{136}RR_{154}QQ_{171}$	NS 971d
$VV_{136}RR_{154}QQ_{171}$	NS 827d	$VV_{136}RR_{154}QQ_{171}$	NS 886d
$VA_{136}RR_{154}QQ_{171}$	NS 1287d	unknown	NS 1619d
$VA_{136}RR_{154}QQ_{171}$	NS 1107d	$VA_{136}RR_{154}QQ_{171}$	NS 1216d
b One twin affected by natural scrapie			
unknown	NS 850d	$VA_{136}RR_{154}RQ_{171}$	culled, 2950d
$VV_{136}RR_{154}QQ_{171}$	NS 830d	$VA_{136}RR_{154}RQ_{171}$	culled, 1460d

[a] Fate either NS (natural scrapie) or culled (not scrapie) with lifespan in days

culled without developing natural scrapie (3 heterozygous at two codons, ie 136 and either 154 or 171, one AA_{136} discussed below).

Thirteen positive line AA_{136} survivors were each part of a set of twins. Most of these were used in challenge experiments or were culled before the natural scrapie age range, however one AA_{136} sheep which lived 2 555 days had a twin of genotype VV_{136} which developed natural scrapie at 833 days (Table 4b).

VA_{136} survivors included 37 animals which were each from a set of twins and two which were from triplet sets. Of these, 11 twins and all triplets were culled, used in experiments or died very young and are therefore not informative. Of the animals where the genotypes are known, the vast majority of twins have the same genotype at codons 136, 154 and 171 but were not necessarily monozygotic as many of them comprised a female plus male pair. Twentytwo twins of VA_{136} survivors lived (some still alive) between 730 and 3 040 days. The genotypes of six animals (not shown) which lived 1 245–2 250 days are all $VA_{136}RR_{154}RQ_{171}$ and two $VA_{136}HR_{154}QQ_{171}$ twins survived 1 215 days (one still alive). Three VA_{136} animals had twins which developed natural scrapie at 830 days (VV_{136}), 832 days (VV_{136}) and 850 days (genotype unknown) with survivor twins ($VA_{136}RR_{154}RQ_{171}$, $VA_{136}HR_{154}QQ_{171}$ and $VA_{136}RR_{154}RQ_{171}$) culled at 1460, 1180 and 2950 days respectively (Table 4b). The twin data therefore suggests that either uterine exposure does not play a part in scrapie transmission or that PrP genotype of the lamb is the primary determining factor in whether the animal will subsequently develop natural scrapie.

Effect of ovine lymphocyte antigen type on natural scrapie incidence

Twenty animals were analysed for variation in ten Class I lymphocyte antigens: five positive line animals (three VV_{136} scrapie cases and one each of $VA_{136}HR_{154}QQ_{171}$ and AA_{136} survivors) and 15 AA_{136} negative line sheep. All

20 sheep had the class I antigen G13b with no evidence of any other antigen [30]. The simplest explanation is that all 20 sheep are homozygous for this antigen and that the G13b gene has become fixed in this population.

Discussion

Incidence of natural scrapie in a closed flock of NPU Cheviot sheep appears to be linked to the PrP genotype of the sheep and not to be solely the result of simple transmission of infection from parents to offspring. Despite the finding in Ile-de-France sheep of linkage of scrapie susceptibility to variation in the ovine lymphocyte antigen complex [26], NPU Cheviots appear remarkably invariant in the Class I region. This is probably as a result of in-breeding as the flock has been genetically isolated since 1962. In the flock of Blackface sheep in which were described the ten antigens tested in the NPU flock, G13b has a frequency of 24.1% [30]. Although polymorphism at Class I and Class II loci is associated with variation in response to a variety of organisms [31], there seems to be no such association, at least with Class I antigens, with scrapie in NPU Cheviot sheep.

The primary factor associated with incidence of disease in this natural scrapie outbreak was (and is) the PrP genotype at codon 136. V_{136} was found to be an absolute requirement and all homozygotes are currently at high risk of developing scrapie. Most $VA_{136}RR_{154}QQ_{171}$ animals also succumbed to natural scrapie but not all VA_{136} animals were susceptible – the $VA_{136}RQ_{171}$ subgroup is apparently resistant – so any direct effect of the V_{136} allele is not completely dominant. There seemed also to be some survival advantage in the HR_{154} rather than RR_{154} genotype, an effect also observed in experimental SSBP/1 challenge (unpublished). Similar heterozygote advantage has been described for the human PrP gene and sporadic CJD [5]. No scrapie cases occurred in either negative or positive line *Sip*^{pApA} (or PrP:AA_{136}) sheep.

The NPU Cheviot outbreak of natural scrapie is thus unlike those described in Lacaune and Romanov sheep in France [4] where codon 171 variation seemed to be of primary importance in determining scrapie incidence. There was little association of scrapie with V_{136} and no survival advantage noted with H_{154}. However the association of R_{171} or H_{154} with reduced susceptibility has also been reported in Ile-de-France sheep, another breed in which susceptibility is strongly associated with V_{136} [26]. In scrapie affected Netherlands Texel sheep [2], scrapie occurred in $AA_{136}RR_{154}QQ_{171}$ sheep, not seen in the NPU Cheviot flock outbreak (this paper). However in Texels, H_{154} also seemed to have survival advantage. Codon 136 has limited polymorphism in Suffolk sheep [24, 32] and in Lacaune sheep [4] and in these breeds the most important codon is clearly 171. However as more information becomes available about different scrapie outbreaks, there are suggestions that natural scrapie sources may target different PrP codon variants in a similar manner to that shown for experimental scrapie sources [20] which "favour" either codon 136 or 171 or both. Most studies so far have agreed on the protective effect associated with R_{171}, especially when

homozygous [2, 4, 23, 26, 32] however there is a recent report from Japan of a single scrapie affected Suffolk sheep of RR_{171} genotype [25]. It may be that Japanese scrapie targets PrP genotypes differently from European and USA scrapie but the sheep may also be different genetically. The same study [25] reported a link of R_{171} with a particular series of restriction fragment length polymorphisms (RFLP) not found so far in British Suffolks (unpubl.). The implications of this finding are not clear at the moment.

Maternal or paternal transmission of disease (as opposed to PrP genotype) does not seem to be the primary factor controlling scrapie incidence in the NPU Cheviot flock. The likelihood of a twin developing scrapie was not influenced by whether the other twin developed scrapie – it depended entirely on its own PrP genotype. Scrapie sheep were more likely to produce lambs which went on to develop scrapie than were their positive line surviving flockmates, however this was again dependent on PrP genetics alone as scrapie sheep also produced offspring which lived longer than 1 107 days. In both groups (scrapie cases and positive line survivors) there were instances of animals with sire or dam having pre-clinical natural scrapie before mating or birth. Although there was no absolute link between scrapie in the parent and in the offspring, there was one exception to this in the VV_{136} scrapie case which died at 497, 175 days shorter than any other case. This sheep was the only case with both parents affected by natural scrapie and suggests that experiments designed to investigate the influence of parental scrapie on transmission of infection would be worthwhile.

Nearly all of the animals of the two susceptible genotypes have developed scrapie within defined times of birth. This raises the possibility that scrapie could be a disease which is entirely genetic in origin as proposed by Parry [28] and others. The survival to old age of some $VA_{136}RR_{154}QQ_{171}$ animals may mean that there is incomplete penetrance or expressivity of the genetic effect or that some other polymorphism may distinguish these survivors from those which develop scrapie. There is no link with any of the other known polymorphisms but the existence of others cannot be ruled out and will continue to be investigated.

However, because natural scrapie is also experimentally transmissible, possibilities other than the genetic disease idea must be considered. The shortening of lifespans of scrapie animals in the first few years of the outbreak suggests that there may have been a slow build up of infection in the environment. A relatively conventional infectious agent (as yet uncharacterised) would have to be both widespread within the NPU Cheviot flock and efficiently transmitted to all sheep of the susceptible genotypes – perhaps to all sheep. A low level of scrapie infection may be tolerated in the resistant animal, allowing limited multiplication without the appearance of disease but with the potential of acting as a source of infection of more susceptible sheep. The susceptible animal on the other hand may be unable to limit multiplication and spread of scrapie infection, producing disease more quickly in the homozygote VV_{136} than in the heterozygote $VA_{136}RR_{154}QQ_{171}$ sheep. If infection is usually established in peripheral tissues,

it is possible that the rate-limiting step is neuroinvasion without which infection could not spread to the brain to cause illness and death.

Differentiating between the two etiologies of genetic trait or genetic susceptibility in an environment where every animal becomes infected is extremely difficult. However three VV_{136} NPU Cheviot sheep may help resolve the issue. These animals are part of an experiment to investigate embryo transfer (ET) as a means to circumvent putative transmission of scrapie from ewe to lamb and were born in as clean conditions (eg pens sprayed with 20% sodium hypochlorite) as could be devised on a normal farm. Details of the animal husbandry methods will be published elsewhere [16], however, out of 13 VV_{136} ET lambs, 10 died of apparently natural scrapie (mean lifespan 902 days) and three are still alive at 1 473 days of age at the time of writing. The extremely stringent animal husbandry methods may have either reduced or prevented infection of these highly susceptible animals which will be closely observed for lifespan.

If scrapie is an infection, how and when could sheep be infected? Until recently the same lambing facilities were used for birth of all sheep in the main NPU Cheviot breeding flock (not the ET animals). Infectivity may have remained in the pasture in contact with infected placentae thus acting as a source of disease in the next batch of lambs. However, the most recent NPU Cheviot scrapie case (survival times 672 days) was born in a new lambing shed (first used during 1993 lambing) and did not come into contact with the old and possibly contaminated lambing areas. The interesting possibility has been suggested [4] that nematode parasites may act as a transmission vector for scrapie. Although all animals in the present study were treated to control nematode infection each year, they would all still be infected. This and other ideas will be further investigated as it remains unclear how an infection could be transmitted.

In conclusion, the NPU flock history suggests that the earlier (1970's) scrapie outbreak, coupled with the severe culling used to control it, simply removed the most susceptible PrP genotypes from the flock and resulted in scrapie vanishing temporarily. The deliberate increase in frequency of susceptible sheep for use in experiments in the 1980's allowed the disease to reappear. The future prospects for this flock are exciting. On the one hand, sheep of certain genotypes are resistant to natural scrapie but can be used to study both experimental scrapie and the possibility that such animals may act as carriers of infection. On the other hand, $VV_{136}RR_{154}QQ_{171}$ and $VA_{136}RR_{154}QQ_{171}$ sheep will almost certainly develop scrapie naturally, thus allowing controlled investigations into modes of transmission.

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References

1. Albritton LM, Tseng L, Scadden D, Cunningham JM (1989) A putative murine ecotropic retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus infection. Cell 57: 659–666
2. Belt PBGM, Muileman IH, Schreuder BEC, Bos-de Ruijter J, Gielkens ALJ, Smits MA (1995) Identification of five allelic variants of the sheep PrP gene and their association with natural scrapie. J Gen Virol 76: 509–517
3. Chatelain J, Millot P, Bourdon R, Callebert J, Fandon R, Grolleau D, Trouillet D (1992) Diverse biological parameters in clinically healthy sheep from a flock with scrapie: variations and correlations with OLA antigens. Exp Clin Immunogenet 9: 86–94
4. Clouscard C, Beaudry P, Elsen JM, Milan D, Dussaucy M, Bounneau C, Schelcher F, Chatelain J, Launay JM, Laplanche JL (1995) Different allelic effects of the codons 136 and 171 of the prion protein gene in sheep with natural scrapie. J Gen Virol 76: 2097–2101
5. Collinge J, Palmer MS, Dryden AJ (1991) Genetic predisposition to iatrogenic Creutzfeldt-Jakob disease. Lancet 337: 1441–1442
6. Deslys JP, Marce D, Dormont D (1994) Similar genetic susceptibility in iatrogenic and sporadic Creutzfeldt-Jakob disease. J Gen Virol 75: 23–27
7. Dickinson AG (1974) Natural infection 'spontaneous generation' and scrapie. Nature 252: 179–180
8. Dickinson AG, Outram GW (1988) Genetic aspects of unconventional virus infections: the basis of the virino hypothesis. In: Bock G, Marsh J (eds) Novel infectious agents and the central nervous system. Wiley, Chichester, pp 63–83 (Ciba Symposium, no. 135)
9. Dickinson AG, Stamp JT, Renwick CC, Rennie JC (1968) Some factors controlling the incidence of scrapie in Cheviot sheep injected with a Cheviot-passaged scrapie agent. J Comp Pathol 78: 313–321
10. Foster JD, Dickinson AG (1988a) The unusual properties of CH1641 a sheep passaged isolate of scrapie. Vet Rec 126: 5–8
11. Foster JD, Dickinson AG (1988b) Genetic control of scrapie in Cheviot and Suffolk sheep. Vet Rec 123: 159
12. Foster JD, Hunter N (1991) Partial dominance of the sA allele of the *Sip* gene for controlling experimental scrapie. Vet Rec 128: 548–549
13. Foster JD, McKelvie WAC, Mylne MJA, Williams A, Hunter N, Hope J, Fraser H (1992) Studies on vertical transmission of scrapie in sheep using embryo transfer. Vet Rec 130: 341–343
14. Foster JD, Hope J, McConnell I, Bruce M, Fraser H (1992) Transmission of bovine spongiform encephalopathy to sheep goats and mice. Ann New York Acad Sci 724: 300–303
15. Foster JD, Hope J, Fraser H (1993) Transmission of bovine spongiform encephalopathy to sheep and goats. Vet Rec 133: 339–341
16. Foster JD, Hunter N, Williams A, Mylne MJA, McKelvey WAC, Hope J, Fraser H, Bostock C (1996) Further observations on the transmission of scrapie in experiments using embryo transfer. Vet Rec (in press)
17. Goldmann W, Hunter F, Foster JD, Salbaum JM, Beyreuther K, Hope J (1990) Two alleles of a neural protein gene linked to scrapie in sheep. Proc Natl Acad Sci USA 87: 2476–2480
18. Goldmann W, Hunter N, Benson G, Foster JD, Hope J (1991) Different scrapie associated fibril proteins (PrP) are encoded by lines of sheep selected for different alleles of the *Sip* gene. J Gen Virol 72: 2411–2417

19. Goldmann W, Hunter N, Smith G, Foster JD, Hope J (1994a) PrP genotypes and the *Sip* gene in Cheviot sheep form the basis for scrapie strain typing in sheep. Ann New York Acad Sci 724: 296–299
20. Goldmann W, Hunter N, Smith G, Foster J, Hope J (1994b) PrP genotype and agent effects in scrapie: change in allelic interaction with different isolates of agent in sheep, a natural host of scrapie. J Gen Virol 75: 989–995
21. Hunter N, Foster JD, Dickinson AG, Hope J (1989) Linkage of the gene for the scrapie-associated fibril protein (PrP) to the Sip gene in Cheviot sheep. Vet Rec 124: 364–366
22. Hunter N, Foster JD, Benson G, Hope J (1991) Restriction fragment length polymorphisms of the scrapie-associated fibril protein (PrP) gene and their association with susceptibility to natural scrapie in British sheep. J Gen Virol 72: 1287–1292
23. Hunter N, Goldmann W, Benson G, Foster JD, Hope J (1993) Swaledale sheep affected by natural scrapie differ significantly in PrP genotype frequencies from healthy sheep and those selected for reduced incidence of scrapie. J Gen Virol 74: 1025–1031
24. Hunter N, Goldmann W, Smith G, Hope J (1994) The association of a codon 136 PrP gene variant with the occurrence of natural scrapie. Arch Virol 137: 171–177
25. Ikeda T, Horiuchi M, Ishiguro N, Muramatsu Y, Kai-Uwe GD, Shinigawa M (1995) Amino acid polymorphisms of PrP with reference to onset of scrapie in Suffolk and Corriedale sheep in Japan. J Gen virol 76: 2577–2581
26. Laplanche JL, Chatelain J, Westaway D, Thomas S, Dussaucy M, Brugere-Picoux J, Launay JM (1993) PrP polymorphisms associated with natural scrapie discovered by denaturing gradient gel electrophoresis. Genomics 15: 30–37
27. Millot P, Chatelain J, Dautheville C, Salmon D, Cathala F (1988) Sheep major histocompatibility (OLA) complex: linkage between a scrapie susceptibility/resistance locus and the OLA complex in Ile-de-France sheep progenies. Immunogenet 27: 1–11
28. Parry HB (1962) Scrapie: a transmissible and hereditary disease of sheep. Heredity 17: 75–105
29. Stear MJ, Pokorny TS, Fryda-Bradley S, Lie O, Bull RW (1990) Genetic analysis of the antigens defined at the third international *Bola* workshop. J Immunogenet 17: 21–28
30. Stear MJ, Bairden K, Bishop SC, Buitkamp J, Epplen JT, Gostomski D, McKellar QA, Schwaiger F-W, Wallace DS (1996) An ovine lymphocyte antigen is associated with reduced faecal egg counts in four month old lambs following natural, predominantly *Ostertagia circumcincta* infection. Int J Parasitol (in press)
31. Wakelin D, Blackwell JM (eds) 1988 Genetics of resistance of bacterial and parasitic infection. Taylor & Francis, London
32. Westaway D, Zuliani V, Mirenda Cooper C, Da Costa M, Neuman S, Jenny AL, Detwiler L, Prusiner SB (1994) Homozygosity for prion protein alleles encoding glutamine-171 renders sheep susceptible to natural scrapie. Genes Dev 8: 959–969

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