Stability of murine scrapie strain 87V after passage in sheep and comparison with CH1641 ovine strain

Journal of General Virology (2015), **96**, 3703–3714 **DOI** 10.1099/jgv.0.000305

Stability of murine scrapie strain 87V after passage in sheep and comparison with the CH1641 ovine strain

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Breed- and prion protein (*PRNP*) genotype-related disease phenotype variability has been observed in sheep infected with the 87V murine scrapie strain. Therefore, the stability of this strain was tested by inoculating sheep-derived 87V brain material back into VM mice. As some sheep-adapted 87V disease phenotypes were reminiscent of CH1641 scrapie, transgenic mice (Tg338) expressing ovine prion protein (PrP) were inoculated with the same sheep-derived 87V sources and with CH1641. Although at first passage in VM mice the sheep-derived 87V sources showed some divergence from the murine 87V control, all the characteristics of murine 87V infection were recovered at second passage from all sheep sources. These included 100 % attack rates and indistinguishable survival times, lesion profiles, immunohistochemical features of disease-associated PrP accumulation in the brain and PrP biochemical properties. All sheep-derived 87V sources, as well as CH1641, were transmitted to Tg338 mice with identical clinical, pathological, immunohistochemical and biochemical features. While this might potentially indicate that sheep-adapted 87V and CH1641 are the same strain, profound divergences were evident, as murine 87V was unable to infect Tg338 mice but was lethal for VM mice, while the reverse was true for CH1641. These combined data suggest that: (i) murine 87V is stable and retains its properties after passage in sheep; (ii) it can be isolated from sheep showing a CH1641-like or a more conventional scrapie phenotype; and (iii) sheep-adapted 87V scrapie, with conventional or CH1641-like phenotype, is biologically distinct from experimental CH1641 scrapie, despite the fact that they behave identically in a single transgenic mouse line.

Received 23 June 2015
Accepted 29 September 2015

INTRODUCTION

Scrapie is the archetype of the transmissible spongiform encephalopathies (TSEs) or prion diseases, which include among others bovine spongiform encephalopathy (BSE), chronic wasting disease of cervids and Creutzfeldt–Jakob disease of humans. Classical scrapie (which is distinct from atypical scrapie or Nor98) is a naturally occurring, infectious and contagious disease of sheep and goats, first reported more than 200 years ago (Schneider *et al.*, 2008). Unlike BSE, which is believed to be caused by a single, unique agent (Bruce *et al.*, 1994; 2002), the existence of strain variation in scrapie has long been documented from work performed in laboratory rodents (Bruce *et al.*, 1991). Thus, many strains of scrapie have been identified in mice, which differ in clinical (incubation period) and pathological (vacuolar lesion profile) characteristics (Fraser, 1976; Dickinson & Fraser, 1977) when isolated and passaged in the same line of inbred mice (Bruce *et al.*, 1991). Current methods of strain typing in mice additionally include: (i) assessment of the phenotype of disease-associated prion protein (PrP^d) accumulation in the brain by immunohistochemistry (IHC), histoblotting or paraffin-embedded tissue blotting (Bruce *et al.*, 1994; Jeffrey *et al.*, 2001; Lezmi *et al.*, 2006; Beck *et al.*, 2010); and (ii) the characterization of the

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Abbreviations: BSE, bovine spongiform encephalopathy; IHC, immunohistochemistry; p.i., post-inoculation; PrPd, disease-associated PrP; PrPres, protease-resistant core of PrPd; TSE, transmissible spongiform encephalopathy; WB, Western blotting; WT, wild type.

biochemical profile of the protease-resistant core (PrP^{res}) of PrP^{d}, usually by Western blotting (WB) (Somerville *et al.*, 1997; Kuczius *et al.*, 1998; Baron *et al.*, 2004).

One of the murine scrapie strains so far identified and characterized is 87V. It was originally isolated from a case of scrapie in a crossbred Cheviot/Border Leicester sheep after intracerebral primary injection and subsequent passage in VM mice homozygous for the prion allele *Prnp*^{b} (previously called *Sinc*^{p7}; Bruce & Dickinson, 1979). 87V is considered stable in terms of clinical and pathological phenotype when propagated in VM mice. However, inoculation of the same original sheep source in C57BL mice (homozygous for the *Prnp*^{a} or *Sinc*^{s7} allele) gave rise to a strain called 87A, which on subsequent passages in the same mouse line showed sudden and sporadic signs of instability resulting in two different strains, 87A itself and ME7 (originally designated 7D; Bruce & Dickinson, 1979). These findings suggest that either the original sheep scrapie case contained a mixture of strains, one of which was able to propagate only in one mouse line/genotype (7D/ME7 in C57BL mice), or that an originally single agent strain was capable of changing some of its properties as a result of passage in a particular mouse line, C57BL in this case (Bruce & Dickinson, 1979). Murine 87V PrP^{res} exhibits similarities with BSE- and CH1641-derived PrP^{res} on WB analysis, in terms of glycoprofile (predominance of the diglycosylated band), low molecular mass of the unglycosylated band and lack of reactivity with N-terminal antibodies such as P4 (Kuczius & Groschup, 1999; Baron & Biacabe 2007; Baron *et al.*, 2008). However, 87V PrP^{res} is more stable or resistant than BSE PrP^{res} to proteinase K treatment (Kuczius & Groschup, 1999).

The approach of natural sheep scrapie strain typing by bioassay in mice has some drawbacks. One is that some scrapie isolates do not transmit efficiently to wild type (WT) mice due to either the nature of the agent or to the so-called 'species barrier' (Dickinson, 1976; Bruce & Fraser, 1991; Bruce *et al.*, 2002). To try to overcome this problem, transgenic mice have been developed that express ovine PrP; among these are the TgOvPrP4 and Tg338 mice, which express in homozygosity the ovine $^{136}A^{154}R^{171}Q$ and $^{136}V^{154}R^{171}Q$ alleles, respectively (Crozet *et al.*, 2001; Vilotte *et al.*, 2001; Laude *et al.*, 2002). With this approach, Baron & Biacabe (2007) succeeded in transmitting the CH1641 experimental sheep scrapie source (Dickinson & Fraser, 1977) to transgenic TgOvPrP4 mice, when previous attempts to transmit this source to WT mice had failed (Foster & Dickinson, 1988; Baron *et al.*, 2004). Moreover, on transmission to TgOvPrP4 mice, similarities were found between sheep-derived CH1641 and mouse-derived 87V, in terms of: (i) attack rates and to a lesser extent incubation periods; (ii) WB glycoprofiles; (iii) mobility of the unglycosylated PrP^{res} band; and (iv) differential reactivity to the globular domain and N-terminal PrP antibodies in WB (Baron & Biacabe, 2007). A different report on the same transmissions indicated that similarities between CH1641-like cases and 87V extended to IHC-demonstrable PrP^{d} types in brain (mostly extracellular fine particulate and intraneuronal aggregates) and, to a lesser extent, vacuolar lesion profiles and distribution (Bencsik & Baron, 2011).

The present report addresses the transmission back into VM mice of 87V previously passaged in sheep (Sisó *et al.*, 2012) with the aim of assessing the stability of the original murine strain. Given the similarities between 87V and CH1641 that have already been expounded, which were also observed in the original murine 87V to sheep experiment (Sisó *et al.*, 2012), this report also covers the outcome of inoculations of Tg338 mice with the same sheep-derived 87V sources and CH1641 ovine scrapie with the aim of further comparison between these two strains.

RESULTS

Clinical aspects of disease in mice: attack rates (ARs) and survival times (STs)

All 10 VM mice inoculated with normal sheep brain homogenate were negative for PrP^{d} by IHC or by WB ($n=5$ in each case) when killed due to old age [674 ± 58 days post-inoculation (p.i.), mean $\pm$ SD] in the absence of clinical signs. Equally, the 10 mice of the second passage, which were inoculated with a brain homogenate from one negative VM mouse of the first passage, were all negative when killed at the end of the experiment (set at 498 days p.i.). Evidence of TSE infection was not detected by IHC ($n=6$) or WB ($n=4$) in any of the 10 VM mice inoculated with ovine CH1641 that either died or were clinically silent when culled at 627 ± 56 days p.i. (Table 1 and Fig. 1a).

ARs and STs for VM mice of the first and second passages are detailed in Table 1 and illustrated in Fig. 1. For first passages, ARs were 100 % for VM mice inoculated with the murine 87V positive control and for those challenged with Suffolk AA-derived inoculum (as all three sheep-derived 87V inocula were from $^{154}R^{171}Q$ homozygotes, these sources are identified by the codon 136 genotype throughout this report). However, STs were significantly shorter ($P<0.001$) for the murine control (304 ± 19 days p.i.) than for the Suffolk AA-passaged 87V (384 ± 32 days p.i.). For first-passage VM mice inoculated with Cheviot AA-derived 87V, ARs were significantly lower (58 %; $P<0.01$) when compared with the two previous groups, and STs were also significantly longer (440 ± 84 days p.i.; $P<0.001$ compared with murine 87V and $P<0.05$ compared with Suffolk AA-derived 87V). Only one mouse inoculated with Cheviot VV-derived 87V succumbed to infection with an ST of 391 days p.i. (AR 10 %, significantly lower than the three previous groups: $P<0.001$ compared with the murine 87V positive control and with Suffolk AA-derived 87V and $P<0.05$ compared with Cheviot AA-derived 87V). In the second passages, ARs were 100 % for all four 87V inoculations (three with inocula originally derived from sheep and one with murine control). STs were also very similar between the four groups, with the only significant difference ($P<0.01$) occurring between Cheviot AA-derived 87V (313 ± 13 days p.i.) and the murine control (298 ± 6 days

Table 1. Clinical aspects of the outcome of the experiment

AR, Attack rate in number of mice IHC or WB positive/number of mice surviving for long enough to be considered (see text) and percentage in parentheses; ST, survival time in days post-inoculation expressed as mean ± SD. For statistical significance of differences, refer to text; NA, not available.

Source of inoculum	VM mice				Tg338 mice	
	1st passage		2nd passage		1st passage	
	AR	ST	AR	ST	AR	ST
Suffolk AA 87V	15/15 (100 %)	384 ± 32	15/15 (100 %)	306 ± 13	12/12 (100 %)	157 ± 16
Cheviot AA 87V	7/12 (58 %)	440 ± 84	14/14 (100 %)	313 ± 13	12/12 (100 %)	149 ± 7
Cheviot VV 87V	1/10 (10 %)	391	14/14 (100 %)	317 ± 46	12/12 (100 %)	148 ± 9
CH1641 (AA)	0/10 (0 %)	627 ± 56	NA	NA	12/12 (100 %)	157 ± 3
Murine 87V	14/14 (100 %)	304 ± 19	10/10 (100 %)	298 ± 6	0/12 (0 %)	570 ± 169
NSB/NI*	0/10	674 ± 58	0/10 (0 %)	498 ± 0	0/12 (0 %)	588 ± 162

*NSB/NI, normal brain from an ARQ/ARQ sheep (VM mice) or non-inoculated (Tg338 mice).

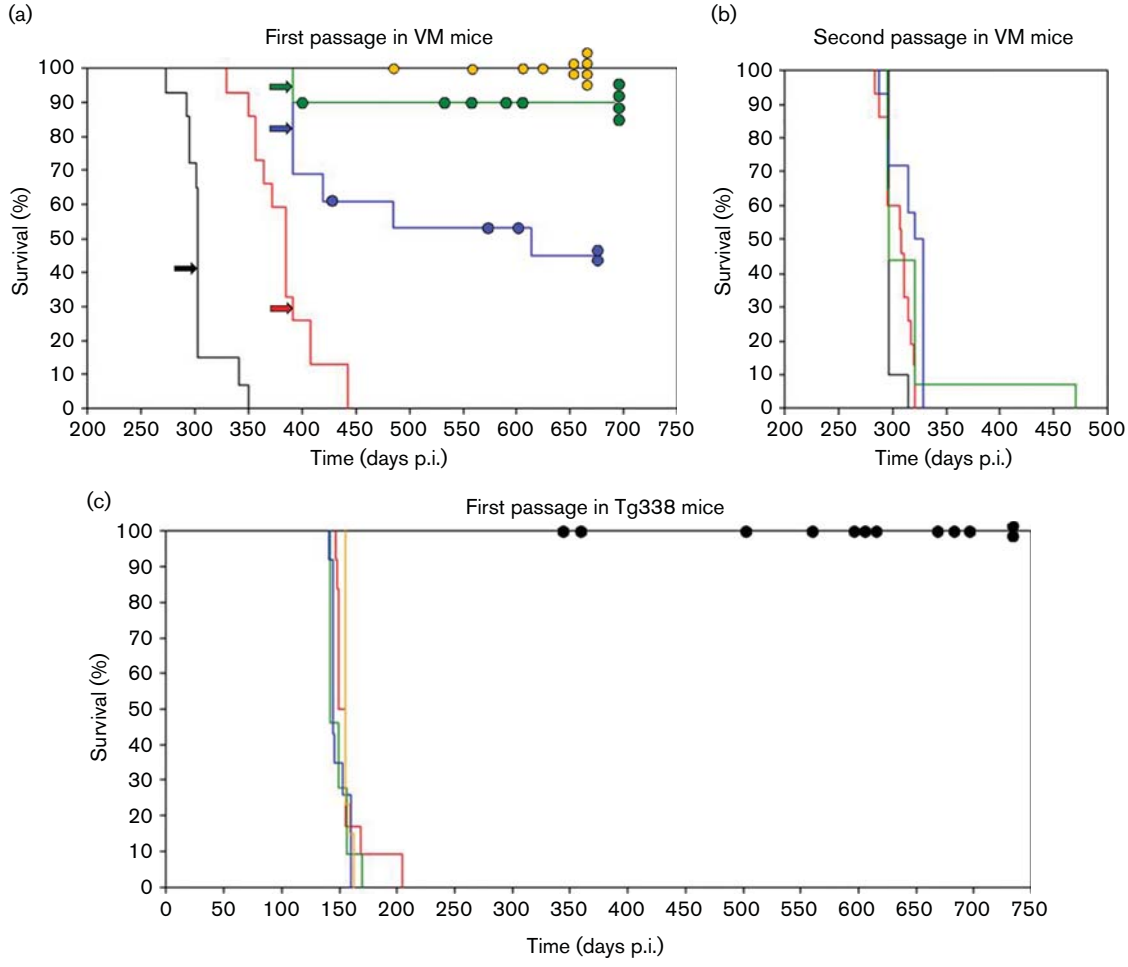

Fig. 1. Survival curves as percentages at various days p.i. of the different mouse groups [VM mice (a, b) and Tg338 mice (c)] challenged intracerebrally with different 87V sources: black, murine control; sheep-derived: red, Suffolk AA; blue, Cheviot AA; green, Cheviot VV; orange, CH1641. Arrows in (a) indicate mice selected for second passages in VM mice. Circles indicate the number of days p.i. of PrP^{d}/PrP^{res}-negative mice dying after the first positive mouse in each group, which are included in the calculation of ARs. A second passage in VM mice from the CH1641 inoculum was not attempted as all mice of the first passage were negative. Data for VM mice inoculated with normal sheep brain and for non-inoculated Tg338 mice are not illustrated.

p.i.). The lack of difference in ST between the murine control and the Cheviot VRQ-derived 87V (317 ± 46 days p.i.) appeared to derive from the fact that one of the mice inoculated with this source had a protracted ST (470 days p.i.; Fig. 1b), giving a high SD to this group.

Unlike VM mice, Tg338 mice did not succumb to inoculation with the murine 87V control, while all of the mice inoculated with CH1641 did, with STs similar (157 ± 3 days p.i.) to those of Suffolk AA-derived 87V (157 ± 16 days p.i.) and slightly but significantly longer ($P < 0.05$) than those of Cheviot AA-derived (149 ± 7 days p.i.) and Cheviot VV-derived (148 ± 9 days p.i.) 87V (Table 1 and Fig. 1c). ARs for all sheep-derived 87V were also 100 %. Non-inoculated Tg338 mice did not show any pathological or IHC evidence of infection when killed at 588 ± 162 days p.i.

Neuropathological features in mice inoculated with murine 87V, sheep-derived 87V and CH1641

Vacuolar lesion profiles obtained at first and, particularly, second passages in VM mice were very similar across all three groups of sheep-derived 87V and were also similar to those resulting from inoculation with the murine 87V positive control. In all cases, vacuolation was most prominent in the thalamus and in all three white-matter areas scored, and was least conspicuous in the cerebellum, hypothalamus and cerebral cortex (Fig. 2a, b). Similarly, although different from those in VM mice, vacuolar lesion profiles in Tg338 mice were almost identical among the three groups inoculated with sheep-derived 87V and also between these and mice challenged with CH1641 (Fig. 2c). In these mice, vacuolation was most pronounced in the hypothalamus, thalamus and medulla, while white-matter tracts were almost completely unaffected.

The profiles of PrP^d accumulation in the brains of VM mice at first and second passage were also very similar, in terms of both distribution and PrP^d types, among the three mouse groups inoculated with sheep-derived 87V and also between these and the murine 87V control. Accumulation of PrP^d was widespread in the brain and was particularly prominent in the septal area, hippocampus (with particular targeting of the CA2 sector and the stratum lacunosum moleculare), thalamus and hypothalamus, midbrain (particularly lateral

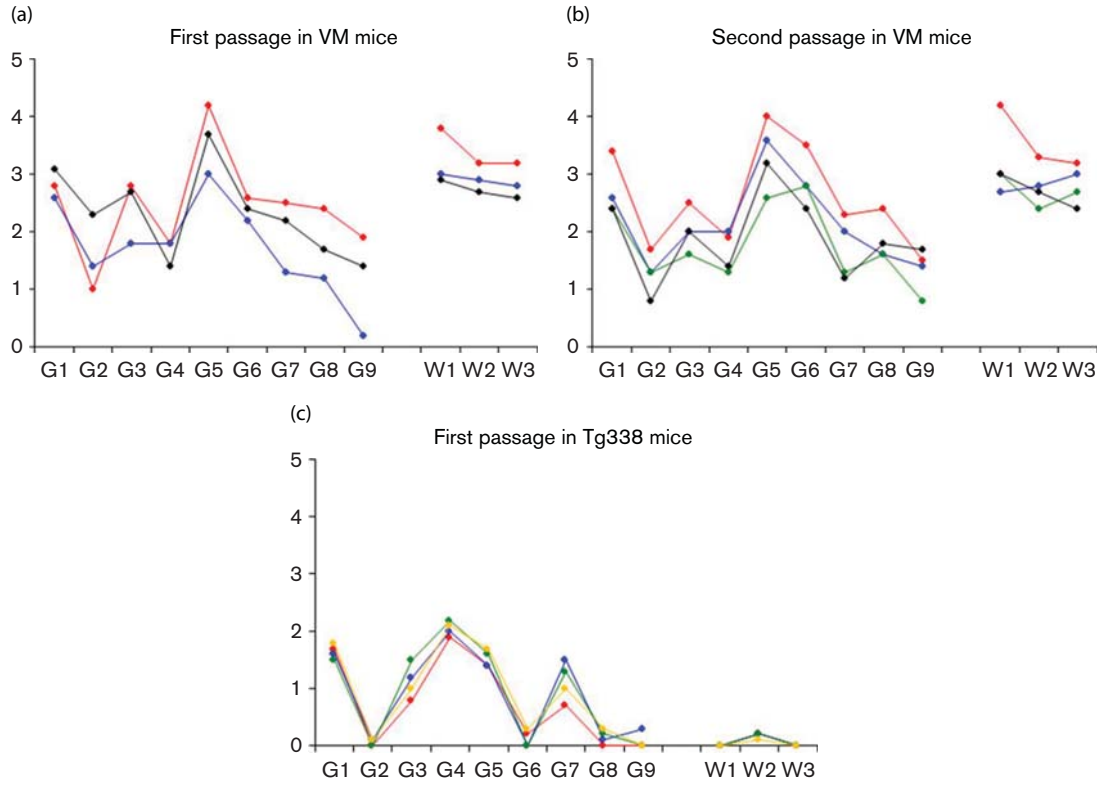

Fig. 2. Vacuolar lesion profiles in the different mouse groups [VM mice (a, b) and Tg338 mice (c)] challenged with the different 87V sources: black, murine 87V control; sheep-derived: red, Suffolk AA; blue, Cheviot AA; green, Cheviot VV; orange, CH164. There are no profiles for CH1641 in VM mice or for murine 87V in Tg338 mice as none of these mice developed any evidence of TSE. There is no profile for the first passage of Cheviot VV-derived 87V in VM mice, as only one mouse became clinically affected; this was confirmed by WB and the brain used for second-passage inoculation. Error bars are not illustrated due to the similarity of profiles. G1–G9, grey-matter areas; W1–W3, white-matter areas. The *y*-axis shows the evaluation of vacuolar lesions, which was scored from 0 to 5 (Fraser, 1976).

geniculate body, substantia nigra and pontine nucleus) and medulla (Fig. 3a). In the latter, fine particulate (sometimes adopting a perineuronal arrangement), intraneuronal and intraglial PrP^d types predominated. PrP^d aggregates were also conspicuous in the cerebral cortex and in the granular layer of the cerebellum, where they were mostly of the coalescing/immature plaque PrP^d type (Fig. 3a). In addition, vascular and non-vascular mature PrP^d plaques were present in association with the needle track and with the ventricles and subarachnoidal space. The similarity in PrP^d types throughout the brain was reflected in almost identical PrP^d profiles at both first (Fig. 3b) and second (Fig. 3c) passage.

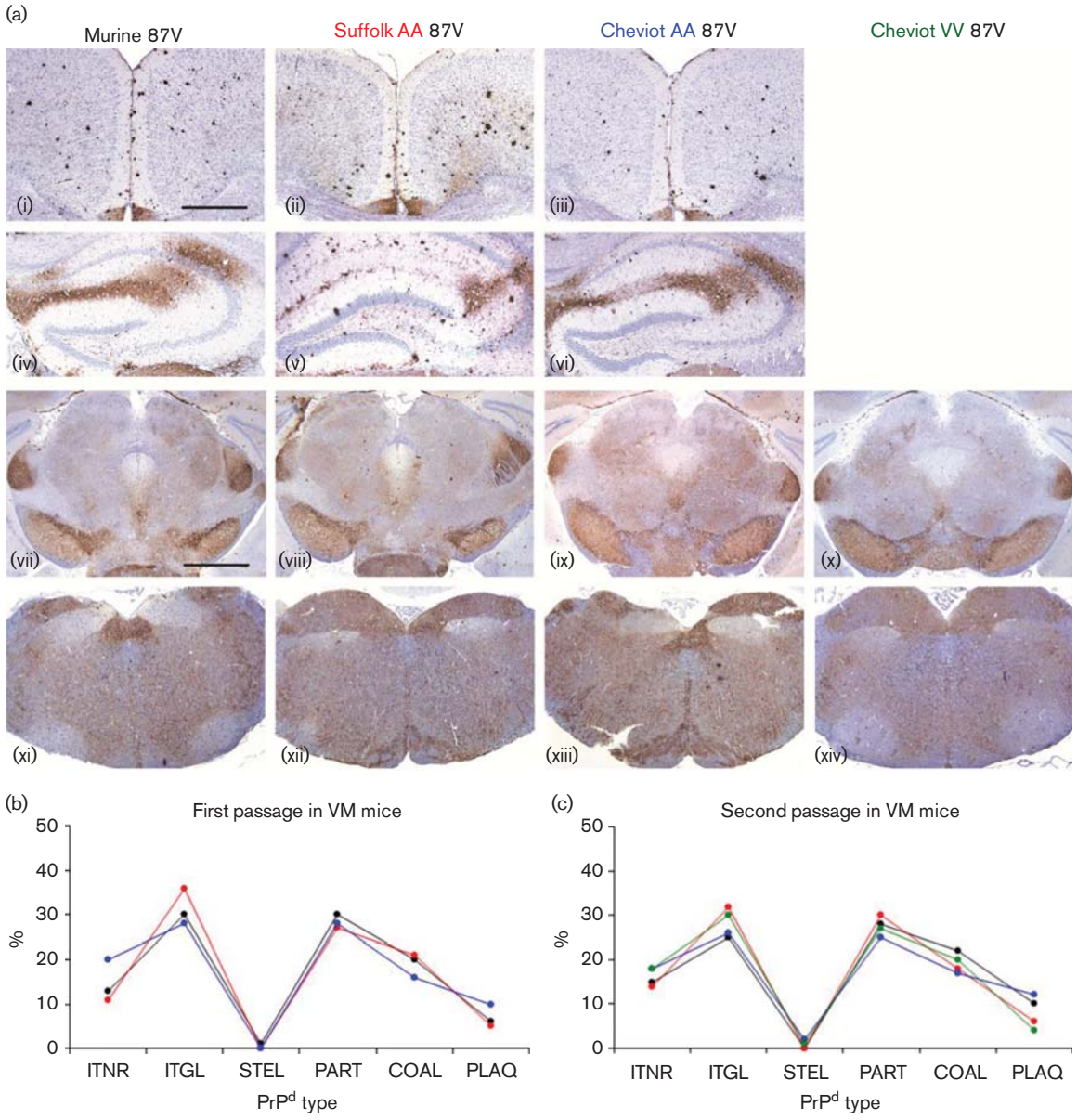

Fig. 3. Features of PrP^d accumulation in the brains of VM mice. (a) Similarity of PrP^d distribution and PrP^d types throughout the brain of mice at first (i–vi) and second (vii–xiv) passage regardless of the source of inoculum (as indicated in the column headings). Note the prominent coalescing type in the cerebral cortex (i–iii) and diffuse fine particulate PrP^d targeting the same areas of the hippocampus (iv–vi), midbrain (vii–x) and medulla (xi–xiv). Images show IHC with the 2G11 PrP antibody and haematoxylin counterstaining. (i) and (vii), 375 μm (i–vi); 750 μm (vii–xiv). (b, c) The marked similarity in PrP^d types is reflected in the PrP^d profiles both at first (b) and second (c) passage. Error bars are not illustrated due to profile similarities. There are no images (a) or a profile (b) for mice challenged at first passage with Cheviot VRQ-derived 87V, as only one mouse of this group was positive and was used for second-passage transmission. ITNR, intraneuronal; ITGL, intraglial (intra-microglial and intra-astrocytic combined); STEL, stellate; PART, particulate; COAL, coalescing; PLAQ, plaques (vascular and non-vascular combined).

In Tg338 mice, accumulation of PrP^d in the brain was of a markedly lower magnitude and had a more restricted distribution than in VM mice. Thus, the cerebral and cerebellar cortices, the septal area and the hippocampus were largely unaffected, and PrP^d types were only of the intraneuronal, intraglial and fine particulate types, with absence of coalescing deposits or plaques (Fig. 4a). These three types of PrP^d accumulation were most conspicuous in the habenula, hypothalamus, substantia nigra, vestibular complex, deep cerebellar nuclei and all nuclei of the caudal medulla, and were almost identical among the three sheep-derived 87V sources and CH1641 (Fig. 4b), although this last source resulted in generally lower PrP^d brain magnitudes.

Biochemical features of PrP^{res} from brains of mice inoculated with murine 87V, sheep-passaged 87V and CH1641

An illustration of representative WB results in VM mice is given in Fig. 5(a). Differences in PrP^{res} glycoprofiles were not found between two successive passages of murine 87V, while first and second murine passages of Suffolk AA-derived 87V differed ($P<0.05$) in the proportion of diglycosylated PrP. Differences between first and second passages could not be tested for the Cheviot-derived 87V as only one mouse brain was available for WB from primary passage of these two sources. When comparing the glycoform ratios of second passages, the murine 87V control and the Cheviot VV-derived 87V source produced significantly ($P<0.05$) lower diglycosylated ($\sim$49–53 %) and higher unglycosylated ($\sim$19–20 %) PrP^{res} than the two AA-derived (Suffolk and Cheviot) 87V sources ($\sim$59–60 % and 12–13 %, respectively for di-and unglycosylated PrP^{res}; Fig. 5b).

Differences in molecular masses of the three different WB bands in VM mice were not observed between the two successive passages of murine 87V or between the first and second passage of Suffolk AA-derived 87V (results not shown). Fig. 5(c) illustrates the absence of significant differences in molecular masses of the three bands when the four sources

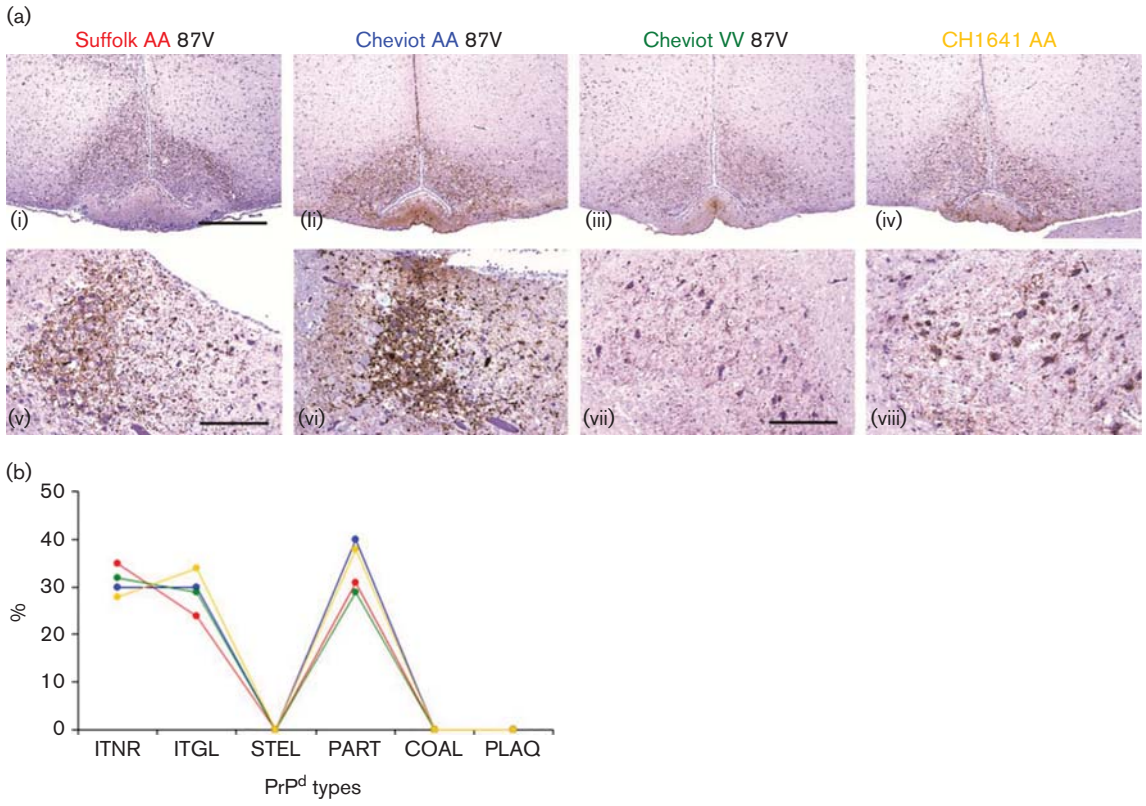

Fig. 4. Features of PrP^d accumulation in the brains of Tg338 mice. (a) Particulate PrP^d in the arcuate and ventromedial hypothalamic nuclei and the median eminence of mice challenged with the four sources (as indicated in the columns) (i–iv), and particulate, intraneuronal and intraglial PrP^d in the vestibular complex (v, vi) and deep cerebellar nuclei (vii, viii). (i), (v) and (vii), 375 μm (i–iv); 150 μm (v, vi); 75 μm (vii, viii). IHC with 2G11 PrP antibody and haematoxylin counterstaining. (b) High degree of similarity in PrP^d profiles among the three sheep-derived 87V sources (red, Suffolk AA; blue, Cheviot AA; green, Cheviot VV) and CH1641 (orange), with similar proportions of intracellular and fine particulate deposits. See Fig. 3 legend for abbreviations. Error bars are not illustrated due to profile similarities.

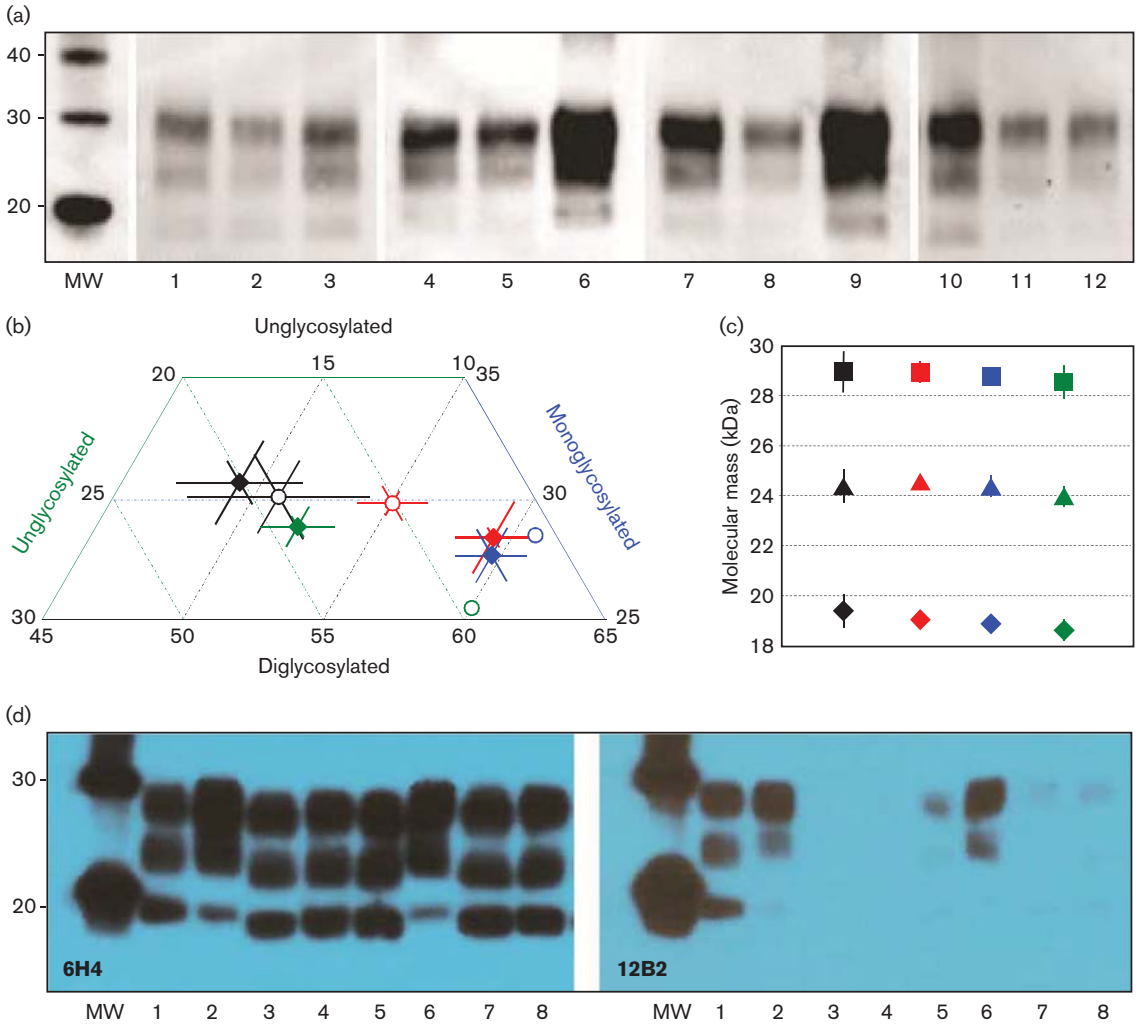

Fig. 5. WB characteristics of PrP^{res} from mouse brains. (a) WB with SAF84 from second-passage VM mice inoculated with the different 87V sources. Lanes: MW, molecular mass marker in kDa; 1–3, murine control; 4–6, Suffolk AA; 7–9, Cheviot AA; 10–12, Cheviot VV. (b) Tri-blot representation of the proportion of di-, mono- and unglycosylated PrP^{res} (results shown as means ± SEM) of VM mice challenged with the different 87V sources. Open circles represent first passage and solid diamonds second passages of: black, murine control (n=7 and n=5, respectively); red, Suffolk AA (n=7 and n=7, respectively); blue, Cheviot AA (n=1 and n=5, respectively); green, Cheviot VV (n=1 and n=7, respectively). Second-passage PrP^{res} from the murine control and Cheviot VV had significantly lower diglycosylated (~49–53 %) and higher unglycosylated (~19–20 %) PrP^{res} than second-passage PrP^{res} from Suffolk AA and Cheviot AA sources (~59–60 % di- and 12–13 % unglycosylated PrP^{res}). (c) Molecular masses of the diglycosylated (squares), monoglycosylated (triangles) and unglycosylated (diamonds) PrP^{res} glycoforms; results shown as means ± SEM for the different 87V sources (first and second passages combined): black, murine control (n=12); red, Suffolk AA (n=14); blue, Cheviot AA (n=6); green, Cheviot VV (n=8). (d) WB characteristics of PrP^{res} from brains of Tg338 mice (unless stated otherwise) inoculated with the different sources; left panel, 6H4 antibody; right panel 12B2 antibody. Lanes: MW, molecular mass marker in kDa; 1, classical scrapie from ARQ/ARQ sheep; 2 and 6, murine 87V control (in VM mice, as this source did not infect Tg338 mice); 3, 4 and 7, Cheviot VV-, Suffolk AA- and Cheviot AA-passaged 87V, respectively; 5 and 8, CH1641. Note the similar masses of the unglycosylated band and the absence of signal with 12B2 in the three sheep-passaged 87V sources and CH1641.

were compared (first and second passage combined; statistical analyses not shown), which on average were 28.6–29.2 kDa for the diglycosylated form, 24.0–24.4 kDa for the mono-glycosylated form and 18.7–19.3 kDa for the unglycosylated form.

In Tg338 mouse brains, WB with 6H4 antibody showed low molecular masses of the unglycosylated band of all three sheep-passaged 87V sources and CH1641 and also absence of the PrP^{res} signal with 12B2 antibody in common with these four inocula (Fig. 5d).

DISCUSSION

Inoculation of sheep-passaged 87V back into VM mice apparently resulted in reisolation of the original murine strain, irrespective of the sheep breed and *PRNP* genotype or the disease phenotype in such sheep. This interpretation is based on the clinical, histopathological and immunohistochemical similarities of the disease in VM mice at second passage between those inoculated with the three sheep sources and the murine positive control used in the experiment. ARs and STs obtained at second passage in this experiment also agreed with those for 87V in VM mice described in the literature (Bruce *et al.*, 1976, 1991; Fraser & Bruce, 1983). Additionally, the vacuolar lesion profiles were similar to those described elsewhere (Bruce & Dickinson, 1985; Kim *et al.*, 1990; Beck *et al.*, 2012; Thackray *et al.*, 2012), although white-matter vacuolation in the last three of these reports was less pronounced than in our experiment. Similarities with other studies extend also to IHC-detectable PrP^{d} aggregates, such as plaques/coalescing deposits [Bruce & Fraser, 1975; Fraser, 1976; Fraser & Bruce, 1983; Bruce & Dickinson, 1985; Gibson, 1986 (although these reports describe plaques based on histopathological and not IHC examinations); Beck *et al.*, 2012; van Keulen *et al.*, 2015] and fine particulate, intraneuronal and intraglial PrP^{d} types [Jeffrey *et al.*, 1994a, b, 1996 (although some of these observations were based on immunoelectron microscopy); Beck *et al.*, 2012; van Keulen *et al.*, 2015]. In terms of the biochemical features of PrP^{res} in the brains of VM mice inoculated at second passage with sheep-derived 87V, in some instances (Cheviot VV) the glycoprofile was indistinguishable from that of the murine control, while in others (AA sheep) it showed a higher proportion of diglycosylated PrP^{res} than the murine controls but was similar to that described by others (Somerville *et al.*, 1997; Beck *et al.*, 2012; van Keulen *et al.*, 2015). In this respect, it is perhaps noteworthy that 87V isolated from field cases in the report by Beck *et al.* (2012) and in another study (Thackray *et al.*, 2012) have all been from ARQ/ARQ sheep.

Reisolation of 87V at second passage in VM mice was achieved despite some phenotypic divergences within the three sheep groups, particularly in terms of the biochemical properties of brain PrP^{res} and in primary transmission results. Suffolk AA-adapted 87V, with a conventional scrapie phenotype, transmitted best to VM mice, showing only slightly longer STs than those in the murine 87V control, which could be attributable to the species barrier effect. The other two 87V-infected sheep groups, Cheviot AA and VV, which developed a CH1641-like phenotype, showed less-efficient transmission on primary passage back to VM mice, in terms of both AR and ST; of these two groups, the lowest transmissibility of the Cheviot VV-adapted 87V could be due to *PRNP* genotypic differences with VM mice, which are AA at codon 132 (homologous to ovine codon 136). This could suggest that the *PRNP* genotype of the sheep from which 87V was originally isolated (Bruce & Dickinson, 1979) was ARQ/ARQ (genotyping was not available at the time) rather than VRQ/VRQ, which would fit with the already-mentioned recent isolation of 87V from field cases of scrapie in ARQ/ARQ sheep (Beck *et al.*, 2012; Thackray *et al.*, 2012). It would also appear that isolation of 87V in VM mice is more readily achievable from sheep with conventional scrapie phenotype, but whether the above-mentioned field cases showed similar immunohistochemical and biochemical profiles to the Suffolk AA sheep of the present experiment is unknown, as such details are not included in these publications. This is another pitfall of classical strain typing by bioassay: the lack of phenotypic characterization of the original scrapie sources. Previous bioassay studies frequently define 'strains' by the outcome of the bioassay, which in itself is an interaction between the infectious agent from an outbred species and the murine host. Without data on the original donor phenotype, it is difficult or impossible to read back bioassay outcomes to the original donor strain.

An alternative explanation for the relatively low transmissibility of sheep-adapted 87V to mice on primary passage could be that such transmissions were not from the agent amplified during the ovine infection but were from residual murine inoculum. We believe that this possibility is highly unlikely as: (i) specific attempts to detect residual inoculum in sheep intracerebrally infected with sheep scrapie (Hamir *et al.*, 2002) or cattle BSE (Sisó *et al.*, 2009) gave negative results; (ii) the sheep inocula for intracerebral challenge were prepared from the medulla oblongata, while the donor sheep had been inoculated in the parietal cortex; and (iii) transmission to VM mice at primary inoculation was most successful from the Suffolk AA-derived 87V inoculum, i.e. from the donor sheep with the longest survival time of all three donors (see Methods), and was therefore least likely to contain residual inoculum.

Another notable point is the fact that a cloned strain, 87V in this case, which was later recovered, can produce disease with different PrP^{res} biochemical properties in sheep of the same ARQ/ARQ genotype but of a different breed, such as Suffolk and Cheviot (Sisó *et al.*, 2012). It is unlikely that the genetic difference at codon 141 (leucine homozygosity for Suffolk sheep and phenylalanine, either in homo- or heterozygosity for Cheviot sheep) can be responsible for the different IHC and WB reactivity with P4, as the amino acid sequence targeted by this mAb excludes codon 141 and as changes in another codon (AA or VV at codon 136 between the two groups of Cheviots) does not alter P4 immunoreactivity (Sisó *et al.*, 2012). Phenotypic variability of classical scrapie in sheep of the same ARQ/ARQ genotype has been documented and interpreted as indicative of the existence of different strains in the field (González *et al.*, 2010). This interpretation may need to be revised, as it would appear that genetic factors other than the *PRNP* gene can be responsible for some degree of phenotypic divergence of disease in sheep of the same ARQ/ARQ genotype infected with a cloned, presumably unique, strain.

The results of the transmissions to Tg338 mice were intriguing. On the one hand, all three sheep-derived 87V sources and CH1641: (i) transmitted with high efficiency and similar short incubation periods; (ii) gave rise to indistinguishable vacuolar lesion profiles and types and distribution of PrP^{d} in the brain; and (iii) provided PrP^{res} with identical biochemical properties. This would suggest some sort of relationship between 87V and CH1641, which is in sharp contradiction with CH1641 being unable to infect VM mice in this study but efficiently infecting Tg338 mice, while the opposite was true for the murine 87V control.

The very low rates or absence of transmission of the experimental CH1641 scrapie source to WT mice have been repeatedly stated (Foster & Dickinson, 1988; Hope *et al.*, 1999) and clearly documented for C57BL mice (*Prnp* $^{a/a}$; Baron *et al.*, 2004), which is in agreement with our results in VM mice (*Prnp* $^{b/b}$). When 87V was inoculated into Cheviot AA and Cheviot VV sheep, it caused disease with an immunohistochemical and biochemical 'CH1641-like' pattern (Sisó *et al.*, 2012), and on transmission to Tg338 mice these two sources were indistinguishable from experimental CH1641. However, Cheviot-derived 'CH1641-like' 87V is different from CH1641 because it transmits to WT VM mice, as do other 'CH1641-like' scrapie isolates from ARQ/ARQ sheep to C57BL/6 mice (Vulin *et al.*, 2012). This suggests that caution is required when interpreting the results of transmissions to Tg338 mice in so far as the definition of strains is concerned. Moreover, the failure of Tg338 mice to develop any evidence of infection when inoculated with murine 87V was surprising. This might be the effect of the 'species barrier', as these mice express ovine VRQ PrP. However, this is a weak argument because: (i) murine 87V can efficiently infect sheep of the VRQ/VRQ genotype, at least by a combined intracerebral/subcutaneous route (Sisó *et al.*, 2012); and (ii) other ovine transgenic mice such as TgOvPrP4 (expressing ARQ ovine PrP) are susceptible to 87V infection (Baron *et al.*, 2004; Baron & Biacabe, 2007).

Several 'CH1641-like' natural scrapie isolates transmitted to TgOvPrP4 mice have revealed biochemical similarities between these isolates, experimental CH1641 and murine 87V, which has allowed differentiation between them and experimental ovine and murine classical BSE and L-type BSE (Baron & Biacabe, 2007; Baron *et al.*, 2008). Similarities between CH1641, 'CH1641-like' and 87V also extend to patterns of PrP^{d}/PrP^{res} deposition in the brain and, to a lesser extent, the vacuolar lesion profiles (Bencsik & Baron, 2011). Transmission to C57BL and RIII mice was attempted from one of the 'CH1641-like' isolates with negative results (Baron & Biacabe, 2007); it is perhaps worth noting that this isolate came from a VRQ/VRQ sheep, as did the original CH1641 source (Jeffrey *et al.*, 2006).

In summary, the 87V murine scrapie cloned strain caused disease in sheep with two different phenotypes, a 'CH1641-like' phenotype in ARQ/ARQ and VRQ/VRQ Cheviot sheep and a more conventional classical scrapie phenotype in ARQ/ARQ Suffolk sheep. Although recovery at first passage back into VM mice was most successful from the Suffolk AA source, the original 87V strain was recovered from all three sources upon second passage, indicating the stability of the murine strain. However, although transmission to Tg338 mice would indicate that sheep-passaged 87V and CH1641 are the same agent, failure to transmit CH1641 to VM mice and murine 87V to Tg338 in this study indicates that CH1641 is not the origin of 87V and, together with other published evidence, that this murine strain may be related to isolates of ARQ/ARQ origin.

METHODS

Experimental design. The outcome of the first part of the study, in which sheep of two different breeds and *PRNP* genotypes were inoculated with murine scrapie strain 87V, has already been reported (Sisó *et al.*, 2012). Briefly, ARQ homozygous Suffolk and Cheviot sheep and VRQ homozygous Cheviot sheep succumbed to murine 87V infection with a 100 % AR after combined oral, intracerebral and subcutaneous inoculation. Two disease phenotypes were observed: (i) Suffolk AA sheep (n=3) developed a 'conventional' scrapie phenotype, with predominance of intracellular PrP^{d} in brain (Fig. 6a), which was demonstrable with C-terminal and N-terminal antibodies (Sisó *et al.*, 2012); on WB analyses, PrP^{res} was also detected with SAF84 and P4 antibodies (Fig. 6b); and (ii) Cheviot AA and Cheviot VV sheep (n=3 each) developed a 'CH1641-like' phenotype (Stack *et al.*, 2002; Jeffrey *et al.*, 2006), also with predominance of intracellular PrP^{d} in brain, which was not demonstrable with N-terminal antibodies (Sisó *et al.*, 2012); on WB analyses of brain extracts, PrP^{res} was detected with SAF84 but not with P4 (Fig. 6b) and the molecular mass of the unglycosylated band was lower than that observed in brains from Suffolk AA sheep (Fig. 6c).

In the experiment covered by the present report, the stability of the original 87V murine strain was tested by passage back into VM mice (Fig. 7). For this purpose, three sheep from the first part of the experiment were selected; they had the following characteristics in terms of *PRNP* genotype, ST and total magnitude of PrP^{d} in brain: (i) Suffolk AA with no other polymorphisms, 719 days and 7.9; (ii) Cheviot AA homozygous for phenylalanine at codon 141, 452 days and 5.3; and (iii) Cheviot VV with no other polymorphisms, 663 days and 11.5. The PrP^{d} IHC profiles and WB features of the three 87V-infected sheep selected for the mouse bioassay are given in Fig. 6, which is representative of all sheep within the three groups.

Homogenates of medulla from each of these three sheep were prepared as a 10 % (w/v) dilution in PBS, treated at 56 °C for 30 min and checked for sterility by routine microbiological techniques. Twenty microlitres of each sheep-derived inoculum was injected intracerebrally in three groups of 15 VM mice; another group of 15 VM mice was similarly challenged with murine 87V inoculum (positive control), and a group of 10 VM mice was inoculated intracerebrally with a 10 % (w/v) homogenate of normal brain from an ARQ/ARQ sheep (negative control). For the second passage, one mouse from each inoculation group was selected (normally a mouse with a survival time close to the mean of the group) and its brain prepared as a 10 % (w/v) homogenate in PBS, 20 µl of which was injected intracerebrally in groups of either 15 mice (for the three inocula originating from sheep-derived brain material) or 10 mice (for the positive and negative controls).

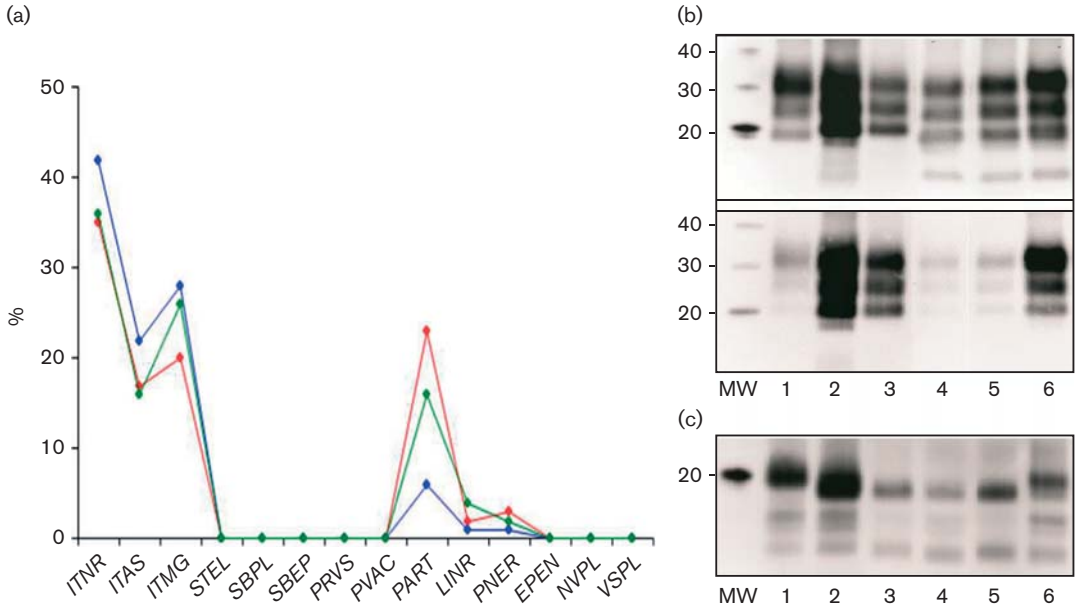

Fig. 6. Immunohistochemical and biochemical features of the three sheep-derived 87V inocula used for intracerebral injection in mice. (a) PrP^d profiles in the brain of Suffolk AA (red), Cheviot AA (blue) and Cheviot VV (green) sheep infected with 87V, expressed as percentage of each PrP^d type over total PrP^d. The types considered were: ITNR, intraneuronal; ITAS, intra-astrocytic; ITMG, intramicroglial; STEL, stellate; SBPL, subpial; SBEP, subependymal; PRVS, perivascular; PVAC, perivacuolar; PART, particulate; LINR, linear; PNER, perineuronal; EPEN, ependymal; NVPL, non-vascular plaques; and VSPL, vascular plaques. Note the predominance of intracellular PrP^d and almost complete restriction of extracellular PrP^d to fine particulate deposits. (b) WB profiles with PrP antibodies SAF84 (top panel) and P4 (bottom panel) of (lanes): 1, ARQ/ARQ sheep BSE; 2, ARQ/ARQ sheep natural scrapie; 3, VRQ/VRQ sheep natural scrapie; 4–6, Cheviot VV, Cheviot AA and Suffolk AA sheep, respectively, infected with 87V. Note the presence of a further lower-molecular-mass band in the three sheep-derived 87V sources, and also the lower molecular mass of the unglycosylated band and the greatly diminished signal with P4 for sheep BSE and the two Cheviot-derived 87V sources, which contrasts with the Suffolk-derived 87V and classical scrapie cases. (c) WB with SAF84 after PNGase treatment of brain homogenates of (lanes): 1, ARQ/ARQ sheep natural scrapie; 2, ARQ/ARQ sheep BSE; 3; ARQ/ARQ sheep CH1641; 4–6, Cheviot VV, Cheviot AA and Suffolk AA sheep, respectively, infected with 87V. Note the higher molecular mass of unglycosylated PrPres (~20 kDa) from 87V-infected Suffolk AA sheep compared with the 87V-infected Cheviot sources.

In view of the similarities in disease phenotype of some of the sheep-adapted 87V cases and CH1641 (see above), five groups of 12 Tg338 mice (Laude *et al.*, 2002) were each inoculated with the three sheep-derived 87V inocula, with the murine 87V control and with CH1641 from an ARQ/ARQ sheep. A further group of 12 non-inoculated Tg338 mice were monitored for their lifespan to assess the development of any disease phenotype from overexpression of the transgene. Inoculation of Tg338 mice was done only at first passage as was the intracerebral injection of a further group of 10 VM mice with CH1641 ovine scrapie (Fig. 7). Inoculations of Tg338 mice were done with the same inocula and followed the same methodology as for VM mice.

All experimental procedures were approved by the ethics committees of the Moredun Research Institute and the Roslin Institute and carried out under appropriate UK Home Office project and personal licences. Mice were monitored daily for clinical signs compatible with a TSE or intercurrent illness. At this point, or at the end of the study, mice were killed humanely following Home Office regulations. All mice were subjected to the same laboratory examinations.

Laboratory examinations. From each experimental group, brains from approximately half of the mice were fixed in 10 % buffered

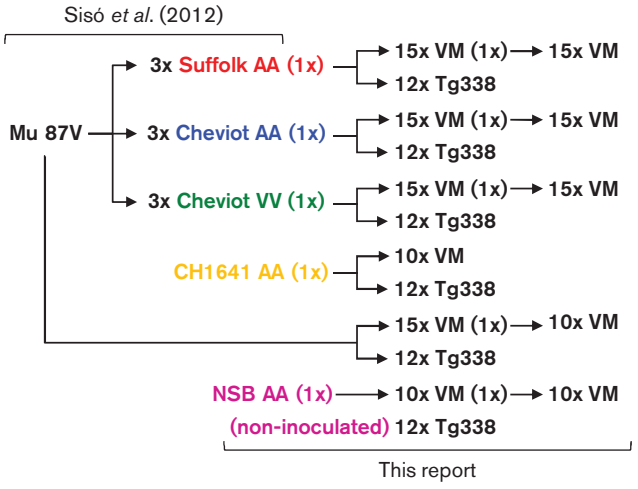

Fig. 7. Schematic representation of the experimental design. Mu, murine. NSB, normal sheep brain.

formalin for histopathological and IHC examinations and the other half was frozen at −80 °C for WB analyses.

Formalin-fixed brains were trimmed to obtain the standard areas for the assessment of vacuolar lesion profiles (Fraser, 1976), including nine grey-matter areas (G1–G9) and three white-matter areas (W1–W3). Trimmed tissue specimens were processed routinely, embedded in paraffin wax, cut to 4 µm thickness, dewaxed, rehydrated by standard procedures, and stained with haematoxylin and eosin for evaluation of vacuolar lesions, which were scored from 0 to 5 (Fraser, 1976).

For IHC demonstration of PrP^d accumulation, serial sections of the same wax blocks were subjected to antigen retrieval and quenching of hydrogen peroxide, as described previously (González *et al.*, 2002, 2005); non-specific antigen blocking was done with a Mouse on Mouse kit liquid protein concentrate solution (Vector Laboratories). Tissue sections were then incubated with the primary mAb 2G11 (Novus Biologicals); the subsequent steps of the IHC procedure were performed using a commercial immunoperoxidase technique (Vector-Elite ABC kit, Vector Laboratories) following the manufacturer's instructions and sections were finally counterstained with Mayer's haematoxylin. The magnitude of PrP^d accumulation was scored from 0 (absent) to 3 (severe) in the following brain areas: (i) frontal cortex, corpus striatum and basal ganglia; (ii) temporoparietal cortex, hippocampus, thalamus and hypothalamus; (iii) midbrain; and (iv) cerebellar cortex, deep cerebellar nuclei and medulla oblongata. The PrP^d types observed and considered to construct the PrP^d profiles were the intraneuronal, intraglial (intramicroglial and intra-astrocytic combined), stellate, fine particulate, coalescing and plaques (vascular and non-vascular combined). The histomorphology of these PrP^d types has been described elsewhere (González *et al.*, 2002, 2005); plaques were only considered as such when they showed a homogeneous core and a radiate periphery (mature plaques), while in the absence of these features the dense accumulations of PrP^d were termed 'coalescing', without prejudice to them actually corresponding to primitive plaques (Jeffrey *et al.*, 1994b, 1996). The overall score for each PrP^d type was calculated as the mean of the scores in the different brain areas and the total brain PrP^d as the sum of the mean scores for each PrP^d type; the PrP^d profile was then expressed as the percentage of each PrP^d type contributing to total brain PrP^d.

WB analyses on VM mouse brains were performed as described previously in detail for sheep brain samples (González *et al.*, 2012; Sisó *et al.*, 2012). Membranes were probed using the SAF84 mAb (R-Biopharm) and the relative intensities of the di-, mono- and unglycosylated bands of PrP^{res} and molecular masses of the unglycosylated band were measured for comparison between experimental groups. WB analyses on Tg338 mouse brains were carried out as described previously (Jeffrey *et al.*, 2006), and parallel membranes were blotted with 6H4 (Prionics AG) and 12B2 (from J. P. M. Langeveld, Central Veterinary Institute, Lelystad, The Netherlands) antibodies to allow discrimination in PrP^{res} truncation sites between different mouse groups.

Data analysis. In this report, AR figures were calculated disregarding those mice that, within each group, died earlier than the first mouse showing indication of TSE infection by either IHC or WB. Similarly, in the calculation of STs, only those mice with neurological signs of IHC- or WB-confirmed TSE were included.

Differences in ARs between the different mouse groups were analysed by Fisher's exact test and those in STs by unpaired *t*-tests (GraphPad Prism; GraphPad Software). Unpaired *t*-tests were also applied to the comparison of WB glycoprofiles and molecular masses of the di-, mono- and unglycosylated PrP^{res} bands between the different experimental groups.

ACKNOWLEDGEMENTS

This work was funded by the Department for Environment, Food & Rural Affairs grant SE1948 and Biotechnology and Biological Sciences Research Council (BBSRC) Institute Strategic Grant BB/J004332/1. The authors are indebted to the technical staff of the High Security Units at both the Moredun Research Institute (MRI) and the Roslin Institute for upkeep of the experimental mice, to Yvonne Pang (MRI) for preparation of WB samples, and to Anne Dunachie and Maria Oliva (APHA) for conducting IHC procedures. The authors would also like to acknowledge Aileen Boyle and the Roslin Institute histology staff for Tg338 mouse lesion profiles. Critical revision of the manuscript by Jim Hope (APHA) is also appreciated.

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