Phenotype of disease-associated PrP accumulation in the brain of bovine spongiform encephalopathy experimentally infected sheep

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In view of the established link between bovine spongiform encephalopathy (BSE) and variant Creutzfeldt–Jakob disease and of the susceptibility of sheep to experimental BSE, the detection of potential cases of naturally occurring BSE in sheep has become of great importance. In this study, the immunohistochemical (IHC) phenotype of disease-associated prion protein (PrPd) accumulation has been determined in the brain of 64 sheep, of various breeds and PrP genotypes, that had developed neurological disease after experimental BSE challenge with different inocula by a range of routes. Sheep BSE was characterized by neuron-associated intra- and extracellular PrPd aggregates and by conspicuous and consistent deposits in the cytoplasm of microglia-like cells. The stellate PrPd type was also prominent in most brain areas and marked linear deposits in the striatum and midbrain were distinctive. Sheep of the ARR/ARR and ARQ/AHQ genotypes displayed lower levels of PrPd than other sheep, and intracerebral BSE challenge resulted in higher levels of PrPd accumulating in the brain compared with other routes. The PrP genotype and the route of challenge also appeared to affect the incubation period of the disease, giving rise to complex combinations of magnitude of PrPd accumulation and incubation period. Despite these differences, the phenotype of PrPd accumulation was found to be very consistent across the different factors tested (notably after subpassage of BSE in sheep), thus highlighting the importance of detailed IHC examination of the brain of clinically affected sheep for the identification of potential naturally occurring ovine BSE.

INTRODUCTION

Sheep are the natural hosts of scrapie, the first transmissible spongiform encephalopathy (TSE) to be described, and are also susceptible to experimental infection by other TSE agents, such as those causing transmissible mink encephalopathy (Hadlow et al., 1987) and bovine spongiform encephalopathy (BSE) (Foster et al., 1993). The susceptibility of sheep to BSE, together with the widely accepted theory of BSE being the origin of variant Creutzfeldt–Jakob disease (vCJD; Collinge et al., 1996; Bruce et al., 1997; Hill et al., 1997; Scott et al., 1999), have raised considerable concern. In Great Britain in particular, the size of the BSE epidemic and management practices suggest that sheep could have been exposed to natural infection through contaminated feedstuffs (Schreuder & Somerville, 2003). Moreover, in experimental ovine BSE, disease-associated prion protein (PrPd) and infectivity are widespread throughout tissues of the lymphoreticular system (LRS), the gastrointestinal tract (Foster et al., 2001; Jeffrey et al., 2001b) and blood (Houston et al., 2000; Hunter et al., 2002). In this respect, sheep BSE resembles sheep scrapie more closely than cattle BSE and, thus, the possibility exists that it could be transmitted between infected and uninfected sheep and maintained even in the absence of the original source of infection. Whether natural BSE in sheep is merely a hypothetical possibility is open to debate (Schreuder & Somerville, 2003) but, in any case, the search for methods that may allow differentiation between scrapie and BSE in sheep is a current priority.

The classical method of TSE strain typing involves transmission and serial passage of infectious material (‘isolate’) in a panel of inbred mouse lines and its characterization by the incubation period of the disease in mice and the vacuolar lesion profile in brain (Fraser & Dickinson, 1973). This method has allowed discrimination between BSE and several sheep scrapie strains, leading to the conclusion that...
the BSE agent is a unique, single strain that is stable after passage in different species, including sheep (Bruce et al., 1994, 1997; Foster et al., 1996).

Either after passage in mice or directly in original tissues, assessment of the biochemical profile of TSE isolates by immunoblotting (glycotyping) attempts the characterization of TSE strains by differences in the proportions of the three glycoforms of protease-resistant PrP (PrPres) and in the molecular mass (MM) of the glycosyl moiety after protease digestion. Despite some variability in the results of the studies carried out so far (reviewed by Schreuder & Somerville, 2003), the general pattern is that the aglycosyl fraction runs more slowly (higher MM) in most scrapie sources tested than in experimental ovine BSE (lower MM). This probably reflects differences in the cleavage site within the N terminus of abnormal PrP, a notion that is confirmed by the absence or marked reduction of the bands when sheep BSE gels are incubated with mAb P4 (Stack et al., 2002), which recognizes the 93–99 PrP epitope (Thuring et al., 2004). However, immunoblot patterns similar to those of sheep BSE have been described for CH1641, a classical experimental scrapie source (Hope et al., 1999), and the biochemical properties of PrPres may depend not just on the TSE agent, but also on the tissue type and even area within the brain (Somerville, 1999).

The concept of differential truncation of the N terminus of sheep BSE-derived PrP was first proposed by Jeffrey et al. (2001a), who used immunohistochemistry (IHC) with a panel of PrP antibodies to distinguish between experimental ovine BSE and several sheep scrapie sources. Further studies indicated that, unlike scrapie, the site of truncation within the flexible tail of ovine BSE PrP was tissue- and even cell-type-dependent (Jeffrey et al., 2003) and that differentiation between the two infections could be approached by examining neural and non-neural tissues, particularly those of the LRS. Whilst the initial studies were restricted to sheep of the ARQ/ARQ PrP genotype, recent extended examinations have pointed out that the pattern of immunolabelling is identical in experimental BSE of sheep of other genotypes (Martin et al., 2005).

We have previously reported the possible usefulness of the ‘PrPd profile’ for characterization of scrapie strains (González et al., 2002). Unlike the ‘lesion profile’, which addresses the magnitude of neuropil vacuolation in specific brain areas, the method is based on IHC recognition and scoring of different morphological and cell-associated types and patterns of PrPd accumulation in the brains of affected sheep. The PrPd profile appears to be mainly determined by the TSE agent or strain, with other factors, particularly the PrP genotype, producing only minor effects (González et al., 2003a). It has been hypothesized that these distinct profiles can reflect differences in cellular tropism and in PrP processing by different TSE strains (González et al., 2003b). In the present study, we have used a similar IHC-profiling method to characterize the phenotype of PrPd accumulation in the brains of sheep affected experimentally with BSE, and have assessed the effects of several factors. Our intention was to provide further tools for discriminating between scrapie and ovine BSE and to contribute to the understanding of the pathogenesis of sheep TSEs.

**METHODS**

**Animals and experimental procedures.** IHC examination for PrPd accumulation was performed in the brains of 64 sheep, all of which showed neurological signs after experimental infection with the BSE agent. These animals were gathered from several ongoing and completed experiments and were grouped according to breed and PrP genotype, source and type of inoculum and route of challenge, as detailed in Table 1.

Sheep were of four different breeds [Cheviot (n = 27), Poll-Dorset (n = 7), Suffolk (n = 12) and Romney (n = 18)] and five different PrP genotypes [ARQ/ARQ (n = 42), ARQ/ARQ (n = 6), ARQ/ARQ (n = 5) and ARQ/VRQ (n = 8)], which are indicated as polymorphisms at codons 136, 154 and 171 for the two alleles (the single-letter amino acid code is used). PrP genotyping was performed by sequencing of PCR-amplified products (Baylis et al., 2000). The animals had been exposed experimentally to BSE infection by one of three routes [intracerebral (IC, n = 40), intravenous (IV, n = 11) or oral (n = 13)], using either blood (n = 5) or brain homogenates (n = 59) from either of two host sources [cattle (n = 40) or sheep (n = 24)].

Details of experimental procedures have been given elsewhere [for IC challenge by Foster et al. (1993), for oral challenge by Jeffrey et al. (2001b) and for IV challenge by Hunter et al. (2002)] and were also summarized by Martin et al. (2005). The protocol for inoculation of sheep-passaged BSE inoculum was identical to that described for cattle inoculum.

All sheep were monitored closely and were killed humanely once clinical signs were considered to be highly suggestive of TSE (Table 1). The clinical period extended from 1 to 10 days in approximately 50% of the sheep, from 11 to 30 days in another 25% and from 1 to 5 months in the remaining sheep. Three of the eight ARR/ARR sheep succumbing to BSE IC challenge were those reported by Houston et al. (2003) and the other five belonged to the same experimental series.

**IHC examinations and PrPd profile.** Brains were fixed in formaldehyde, trimmed and embedded in paraffin wax according to standard procedures. A detailed account of the IHC protocol, including antigen retrieval and blocking steps, was given previously (González et al., 2002). Primary antibody R486 was used in 21 animals and PrP antibody R145 in the remaining 43; ten sheep were examined with both antibodies to ensure comparability of results. R486 and R145 are, respectively, a rabbit anti-PrP polyclonal antiserum and a rat mAb that recognize bovine PrP amino acid residues 217–231 (R. Jackman, personal communication). Sections from positive-control tissue blocks were included in each IHC run to ensure consistency in the sensitivity of the method. Apart from internal negative controls of the IHC technique (substitution of primary antibody by normal rabbit serum or normal rat IgG), each run also included negative-control tissues from TSE-unexposed sheep.

Brains were examined at six different neuroanatomical sites: frontal cerebral cortex, corpus striatum, thalamus/hypothalamus, midbrain, cerebellum at the vermis and medulla oblongata at the obex. Most of the PrPd types and patterns considered at these sites corresponded to those already described in previous publications (González et al., 2002, 2003b). Intracellular PrPd included intraneuronal and intraglial granular immunodeposits in the cell cytoplasm. Two types of intraglial PrPd were recognized: one as single or a few large granules...
Table 1. Animals used for the study, incubation period of the disease and magnitude of total PrPd accumulation

For description of genotypes, see text.

<table>
<thead>
<tr>
<th>Group</th>
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<th>Genotype</th>
<th>Age*</th>
<th>Inoculum</th>
<th>Number</th>
<th>Clinical course*</th>
<th>Incubation period*</th>
<th>PrPd score†</th>
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*Age at challenge, clinical course (from first signs to cull) and incubation period (from challenge to cull) in days (mean±SEM).
†The different inocula are identified by different superscripts: a, ic, brain pools of BSE-affected cattle titrated IC/IP in RIII mice (titres were 10±5, 10±6 and 10±7 LD50 g−1, respectively); b, brain pool of Cheviot sheep (titre in RIII mice of 10±5 LD50 g−1) that developed clinical TSE after oral challenge with cattle BSE inoculum; c, brain pool from Romney sheep (titration pending) that developed clinical disease after being dosed orally with inoculum d; e, non-titrated individual blood aliquots (either 400–450 ml whole blood or buffy coat extracted from 50 ml whole blood) from sheep of group 7.
§This group of sheep comprised five, five and three animals challenged with 10−3, 10−4 and 10−5 dilutions of inoculum b, respectively.

RESULTS

Negative-control brain samples and normal serum-treated tissue sections produced no immunoreactivity, whereas positive-control sections gave specific immunolabelling of comparable intensity in all IHC runs performed.

Topographical description of the PrPd profile

None of the animals investigated showed PrPd accumulation in the choroid plexus or in the form of vascular plaques. Detection of PrPd associated with oligodendrocytes was attempted in the corpus callosum and in the cerebellar white matter; although some immunolabelling was observed at these points, it was unclear whether it was associated with oligodendrocytes or with intermingled astrocytes. Therefore, no separate quantification of oligodendroglial PrPd was performed. Subependymal and ependymal PrPd deposits were generally mild and inconsistent and, except for a few IC-challenged sheep in which lateral ventricles were involved, they were restricted to the third ventricle.

Cerebral cortex. The magnitude of PrPd accumulation in the cerebral cortex was in general lower than in other
areas. The predominant types were the subpial and stellate in the grey matter and the perivascular in the white matter. The latter tended to appear at the margins of the gyral white-matter tips, subjacent to the grey-matter junction (Fig. 2a), and was often associated with perivascular PrPd accumulations that seemed to be made of coalescing perivascular deposits (Fig. 2b). Intra-astrocytic, intraneuronal and intramicroglial PrPd accumulation in the grey matter was of low grade and inconsistent.

**Corpus striatum.** The pattern of PrPd accumulation at this site was characteristic (Fig. 2c, d). Intraneuronal deposits were moderate in the caudate nucleus and more prominent in the globus pallidus and putamen nuclei and, in many instances, PrPd accumulated in neurons that also showed perineuronal deposits. The main type of neuropil accumulation of PrPd was in the form of thick linear threads, which often connected with the perineuronal aggregates (Fig. 2e). Intramicroglial PrPd accumulation was consistent and prominent, with affected cells appearing scattered in the neuropil and occasionally also within intraneuronal vacuoles (Fig. 2f). Extracellular PrPd of the stellate type was conspicuous (Fig. 2c, d), whilst the presence of intra-astrocytic, perivacuolar and perivascular PrPd was negligible. Because of the lack of the latter two types, the white-matter bundles of the external and internal capsules were characteristically devoid of PrPd (Fig. 2d).

**Fig. 2.** Topographical description of the phenotype of PrPd accumulation in the brain of BSE-affected sheep. (a) Cerebral cortex of an ARQ/ARQ Cheviot sheep challenged IC with cattle-brain BSE (group 1) showing stellate PrPd in grey matter (left half) and perivascular PrPd in white matter at the junction with the grey matter. Bar, 170 μm. (b) Detail of perivascular accumulation of PrPd, made up of coalescing perivascular aggregates in the white matter of the cerebral cortex of another sheep of group 1. Bar, 85 μm. (c) Corpus striatum of a VRQ/VRQ Cheviot sheep challenged IC with cattle-brain BSE (group 5) showing conspicuous stellate and linear types of PrPd accumulation in the caudate nucleus. Bar, 420 μm. (d) In another sheep of group 5, the same PrPd types as in (c) are prominent in the grey matter of the corpus striatum, but white-matter bundles lack immunodeposits. Bar, 420 μm. (e) ARQ/ARQ Romney sheep challenged IC with sheep-brain BSE (group 4) showing intraneuronal PrPd deposits coexisting with perineuronal and linear aggregates in a single neuron of the globus pallidus. Bar, 28 μm. (f) Corpus striatum of another sheep of group 5; phagocytic cells appear to be removing PrPd from the remains of the cytoplasm of a neuron (intramicroglial PrPd), whilst PrPd also accumulates around the perikaryon and neurites. Bar, 28 μm. (g) Conspicuous intramicroglial PrPd and miniature plaque-like deposits in the neuropil (coalescing PrPd type) of the ventrolateral thalamic nucleus; although the image corresponds to an ARQ/ARQ Suffolk sheep challenged IC with cattle-brain BSE (group 3), these plaque-like deposits were most frequent and prominent in ARR/ARR sheep of group 6. Bar, 28 μm. (h) Midbrain of an ARQ/ARQ Poll-Dorset sheep challenged IC with cattle-brain BSE (group 2) showing prominent linear and particulate PrPd deposits in the substantia nigra and marked stellate PrPd accumulation in the tegmentum. Bar, 420 μm. (i) Cerebellum of a Cheviot sheep of group 1; prominent stellate PrPd accumulation in the molecular layer, mild subpial aggregate and marked extracellular, mesh-like deposits in the Purkinje and molecular layers, resembling the profiles of Bergmann glial cells. Bar, 85 μm. (j) Detail of intra-astrocytic (arrows) and intramicroglial (arrowheads) PrPd granules in the cerebellar white matter of a Poll-Dorset sheep of group 2. Bar, 17 μm. All images were obtained after IHC with R145 antibody and haematoxylin counterstaining.
Phenotype of PrP^d accumulation in sheep BSE
**Thalamus and hypothalamus.** In most sheep, PrP\(_d\) accumulation at this level was more prominent in the hypothalamus than in the thalamus. Granular PrP\(_d\) aggregates in neuronal perikarya were consistently present and the most frequent and prominent PrP\(_d\) type in the neuropil was the particulate/coalescing, followed by the linear. Occasionally, coalescing PrP\(_d\) aggregates had a miniature plaque-like appearance (Fig. 2g), but were devoid of a homogeneous central core, and Congo red staining of semi-serial sections provided negative results. The predominant extracellular PrP\(_d\) type relating to glial cells was again the stellate, whilst the magnitude of perivascular PrP\(_d\) never reached high levels. Intra-astrocytic PrP\(_d\) accumulation was generally low or negligible, but intramicroglial PrP\(_d\) was very prominent and consistent (Fig. 2g).

**Midbrain.** The overall magnitude of PrP\(_d\) accumulation was greatest at this site. The predominant intracellular types were the intraneuronal, particularly in the red nucleus, and the intramicroglial. The intra-astrocytic type, in contrast, was seldom found and only in low amounts. Marked and consistent PrP\(_d\) deposits were found in the neuropil, particularly in the substantia nigra, where the linear and the particulate/coalescing types were characteristic (Fig. 2h). Stellate PrP\(_d\) accumulation was also very prominent and frequent, whereas perivascular PrP\(_d\) was seldom conspicuous.

**Cerebellum.** This site also displayed significant amounts of PrP\(_d\). Almost all sheep showed very prominent intraneuronal PrP\(_d\) in the deep cerebellar nuclei, but none did so in the Purkinje cells. Intramicroglial PrP\(_d\) was conspicuous both in the white matter and in the granular and Purkinje cell layers, coinciding with extracellular PrP\(_d\) collections. Perivascular PrP\(_d\) accumulation in the white matter was frequently marked, but, unlike the cerebrum, these aggregates involved the core of white-matter bundles and were not associated with perivascular deposits, which were almost completely absent. Perineuronal PrP\(_d\) around deep cerebellar nuclei was mild and inconsistent. Subpial PrP\(_d\) was not as conspicuous as in the cerebrum, being generally negligible or low. Moderate or high stellate PrP\(_d\) deposits were often found in the cortical molecular layer, but more marked and frequent were extracellular, coalescing collections of PrP\(_d\) in the granular and Purkinje cell layers (Fig. 2i). Multigranular deposits of PrP\(_d\) in the cytoplasm of astrocyte-like cells were evident in the white-matter tips of most sheep (Fig. 2j).

**Medulla oblongata (obex).** The three most prominent and consistent PrP\(_d\) types in this area were the intraneuronal, the particulate/coalescing and the intramicroglial. Although there were some individual variations, all neuronal nuclei were affected to practically the same extent. Particulate PrP\(_d\) aggregates were most evident in the dorsal motor nucleus of the vagus (DMNV) and in the spinal tract of the trigeminal nerve. Linear deposits of PrP\(_d\) were only occasionally substantial, and perineuronal PrP\(_d\) was confined to the ventral border of the DMNV. Perivascular and stellate accumulations of PrP\(_d\) were inconsistent and sparse. Intramicroglial PrP\(_d\) was very prominent throughout and intra-astrocytic deposits, without reaching the same levels as in the cerebellum, were often found in the spinocerebellar tracts.

**Effect of different factors on the phenotype of PrP\(_d\) accumulation**

Three aspects or parameters were considered when comparing the phenotype of PrP\(_d\) accumulation between sheep groups: the magnitude of total PrP\(_d\), its topographical distribution and the PrP\(_d\) profile. All groups were similar in terms of topographical distribution and, particularly, relative proportions of the different PrP\(_d\) types and patterns (PrP\(_d\) profile); phenotypic differences mainly involved the magnitude of total PrP\(_d\) accumulation.

The dose of inoculum did not affect any of these parameters. Within group 4 (Table 1), the incubation periods for the animals challenged with 10\(^{-3}\), 10\(^{-4}\) and 10\(^{-5}\) dilutions were 520 ± 19-4, 554 ± 35-0 and 756 ± 48-0 days, respectively (two sheep out of five of the latter group were still alive at 1550 days post-infection). In spite of these differences, the PrP\(_d\) phenotypes of the three subgroups were almost identical (results not shown). The absence of correlation between PrP\(_d\) phenotype and incubation period extended to all sheep groups studied (see next section). The magnitude of total PrP\(_d\) was also unrelated to the duration of the clinical disease, the incubation period or the age at challenge (Table 1).

The breed of sheep did not affect either the magnitude of total PrP\(_d\) accumulation (Table 1; compare groups 1, 2 and 3), its profile (Fig. 3a) or its topographical distribution (data not shown). The host source of inoculum (cattle or sheep) did not influence the PrP\(_d\) profile, but appeared to have a slight contradictory effect on the magnitude of total PrP\(_d\) (Fig. 3b, c). Thus, whilst sheep challenged IC with infected cattle brain accumulated slightly more PrP\(_d\) than those of the same genotype infected with sheep BSE inoculum (Table 1; compare groups 1–3 with group 4), the opposite was observed with animals challenged orally (groups 8 and 9). For these comparisons, sheep of different breeds were grouped, but, as described above and in comparisons made within group 9 (data not shown), the breed did not seem to have an effect on the PrP\(_d\) accumulation phenotype.

An unambiguous effect of the PrP genotype was found when ARR/ARR sheep were compared with other groups, but no differences were observed between ARQ/ARQ and VRQ/VRQ sheep (Fig. 4a). Sheep bearing the AHQ allele showed levels of PrP\(_d\) similar to those in ARR homozygotes, but the influence of histidine at codon 154 was problematic to evaluate, as other coincidental factors may have influenced the PrP\(_d\) phenotype of these nine sheep (three were AHQ homozygotes, dosed orally, and six were...
ARQ/AHQ, infected IV). The eight ARR/ARR affected sheep (group 6) and the six ARQ/AHQ sheep challenged IV (group 11) showed little or no subpial, subependymal, perivascular, perineuronal or intra-astrocytic PrP\(_d\). Perivascular PrP\(_d\) was virtually confined to the cerebellar white matter and the stellate type was largely restricted to the striatum and midbrain. Linear and particulate PrP\(_d\) accumulations in these 14 sheep were substantial only in the hypothalamus and midbrain. They displayed little intramicroglial PrP\(_d\) anywhere in the brain, and intraneuronal PrP\(_d\) was inconspicuous except for the deep cerebellar nuclei and the DMNV. Despite these differences in total magnitude (Table 1), the neuroanatomical distribution and profile of PrP\(_d\) in these animals were similar to those of sheep of other PrP genotypes (Fig. 4b), although miniature pseudoplaques in the neuropil, often arranged in rows, were most conspicuous and widespread in ARR/ARR sheep.

The route of inoculation influenced the magnitude of total PrP\(_d\) accumulation and, to a lesser extent, the PrP\(_d\) profile (Fig. 5a, b). Sheep challenged by the IC route accumulated almost 50% more PrP\(_d\) than those dosed orally and more than twice than animals inoculated IV (Table 1). Within the IV group, however, ARQ/ARQ animals receiving sheep

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**Fig. 3.** Effect of breed of sheep and source of inoculum on the PrP\(_d\) profile. (a) Similarity of profiles (mean ± SEM) between sheep of different breeds with all other factors in common (all ARQ-homozygous sheep inoculated IC with cattle brain, groups 1, 2 and 3). Breeds: ▲, Cheviot; ■, Poll-Dorset; ○, Suffolk. (b) The PrP\(_d\) profiles (mean ± SEM) of sheep inoculated IC with cattle (groups 1–3 merged) and sheep (group 4)-brain homogenates are similar. (c) Similarity of profiles (mean ± SEM) between groups dosed orally with cattle (group 8)- and sheep (group 9)-brain inocula. Observe that the relative magnitudes of PrP\(_d\) accumulation for cattle and sheep inocula are different depending on the route of challenge. Host sources of inoculum: ▲, cattle; ■, sheep. PrP\(_d\) types: ITNR, intraneuronal; ITAS, intra-astrocytic; ITMG, intramicroglial; STEL, stellate; SBPL, subpial; SBEI, subependymal; PRVS, perivascular; PVAC, perivacuolar; PRCO, particulate/coalescing; LINR, linear; PNER, perineuronal; EPEN, ependymal.

**Fig. 4.** Effect of PrP genotype on the PrP\(_d\) profile. (a) Profiles (mean ± SEM) of sheep groups of different genotypes with most other factors in common (all sheep inoculated IC with cattle brain: groups 1–3 merged, 5 and 6) are similar, despite differences in magnitude. (b) Similarity of the profiles (mean ± SEM) between sheep groups of different genotypes is also remarkable when other concurrent factors intervene (all 64 sheep examined: groups 1–4 merged, 5, 6, 7, 8–10 merged, and 11). ▲, ARQ/ARQ; ■, VRQ/VRQ; ○, ARR/ARR; △, AHQ/AHQ; ∇, ARQ/AHQ. For description of PrP\(_d\) types, refer to Fig. 3.
blood reached PrP\textsuperscript{d} levels similar to those infected by the oral route, whereas those infected with cattle brain (ARQ/AHQ sheep) were in the ranges of the ARR/ARR sheep, as already mentioned. The differences in magnitude of PrP\textsuperscript{d} deposition were not distributed evenly throughout the brain. Sheep inoculated IC showed similar levels of PrP\textsuperscript{d} to those challenged by other routes in and caudal to the midbrain, the differences being particularly noticeable in the cerebral cortex (Fig. 5c). This was reflected in the PrP\textsuperscript{d} profile, so that orally and IV-infected sheep showed lower levels of the PrP\textsuperscript{d} types found commonly in the forebrain (subpial, stellate, perivacuolar and perivascular) than did IC-challenged sheep (Fig. 5a, b).

Effect of PrP genotype and route of infection on incubation period and magnitude of total PrP\textsuperscript{d} accumulation

Sheep of the ARQ/ARQ genotype and those carrying the AHQ allele had significantly shorter incubation periods than ARR/ARR and VRQ/VRQ allele-bearing animals (Fig. 6). Amongst the ARQ/ARQ animals, those challenged IC and IV had shorter incubation periods than sheep dosed orally, among which one sheep showed a very protracted infection (1132 days). Conversely, AHQ homozygotes challenged orally had a shorter incubation period than ARQ/AHQ sheep inoculated IV with cattle brain. The incubation period, however, was not the factor determining the magnitude of total PrP\textsuperscript{d} accumulation in the brain, as ARR sheep showed much lower levels of PrP\textsuperscript{d} than did VRQ animals. Also, AHQ sheep showed a lower magnitude of PrP\textsuperscript{d} accumulation than ARQ homozygotes, a difference that was clear when groups challenged by the same route were compared (Fig. 6).
DISCUSSION

After passage in inbred mice, the ‘BSE signature’ can be recognized by incubation period, vacuolar lesion profile (Fraser et al., 1992; Bruce et al., 1994) and glycotyping (Collinge et al., 1996; Hill et al., 1997). This signature is the same regardless of the source of the original isolate, i.e. cattle, sheep or other species, including humans (Bruce et al., 1997; Hill et al., 1997). With this study, we show that, by detailed IHC examination, a similar signature can be identified in BSE-infected sheep irrespective of breed, PrP genotype, route of inoculation or source, type and dose of inoculum.

PrP\textsuperscript{d} phenotype of BSE in sheep

In spite of differences in overall magnitude and, to a lesser extent, topographical distribution of PrP\textsuperscript{d} deposition in the brain, the PrP\textsuperscript{d} profile was remarkably similar in all BSE-infected sheep examined. In our series, PrP\textsuperscript{d} accumulated at the highest levels in the brainstem, thalamus/hypothalamus and cerebellum, and at the lowest levels in the cerebral cortex. After IC challenge, however, PrP\textsuperscript{d} deposits in the cerebral cortex and particularly in the striatum were conspicuous (presumably reflecting its proximity to the injection point), whereas aggregates in the cerebellum were mild following IV infection (Fig. 5c). As a result, the magnitude of total PrP\textsuperscript{d} deposition in the brain was highest in IC-challenged sheep and lowest in IV-inoculated animals. Another factor, the PrP genotype, also influenced the overall amount of PrP\textsuperscript{d} in the brain, its effect being evident in ARR homozygotes, which showed low PrP\textsuperscript{d} levels. Similarly, ARQ/AHQ sheep accumulated little PrP\textsuperscript{d}, but, in this case, a combined effect of the route of challenge (IV) and the source and type of inoculum (cattle brain) could not be analysed separately.

The PrP\textsuperscript{d} profile of BSE-affected sheep was characterized by conspicuous intraneuronal, intramicroglial and extracellular stellate and neuropil aggregates, relatively low or moderate astrocyte-associated PrP\textsuperscript{d}, either intra- or extracellular, inconsistent ependymal PrP\textsuperscript{d} deposits and absence of PrP\textsuperscript{d} in choroid plexus cells or in the form of vascular plaques. Very characteristic was the appearance of PrP\textsuperscript{d} deposition in choroid plexus cells or in the form of vascular plaques. PrP\textsuperscript{d} profile of BSE-affected sheep was different from that seen previously in cases of natural scrapie in sheep of various breeds and genotypes and in experimental SSBP/1 infection (González et al., 2002, 2003b; Fig. 7). It was also very different from that generated by infection of sheep with the CH1641 scrapie strain (Fig. 7; M. Jeffrey & L. González, unpublished observations), a finding of particular relevance in view of the reported biochemical similarities between both agents (Hope et al., 1999; Stack et al., 2002). Furthermore, the features of sheep BSE reported here are indistinguishable from those described following infection of Lacaune sheep with a French isolate of cattle BSE (Lezmi et al., 2004), but very different from atypical scrapie cases, such as the recently reported Nor98 type (Benestad et al., 2003; M. Jeffrey & L. González, unpublished observations).

Our results suggest that a systematic IHC assessment of PrP\textsuperscript{d} accumulation in the brain would be suitable for identification of naturally occurring BSE in sheep. This is supported by several findings: firstly, the consistency of the profile across sheep breeds (see Fig. 3a) and, allowing for magnitude differences, PrP genotypes (Fig. 4a, b); secondly, the absence of effect of the host source of the inoculum on the PrP\textsuperscript{d} profile (Fig. 3b, c), which agrees with the stability of cross-species-passaged BSE found in mice (Bruce et al., 1994) and with the similarity of glycoprofiles and IHC-labelling properties between cattle-derived and sheep-passaged ovine BSE (M. Stack and others, unpublished observations); thirdly, the homogeneity of PrP\textsuperscript{d} profiles of individual sheep (data not shown) challenged with sheep-derived BSE inoculum by either IC, oral or IV routes. Overall, this phenotypic consistency of PrP\textsuperscript{d} accumulation

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Fig. 7. PrP\textsuperscript{d} profiles of sheep BSE and four scrapie sources obtained by IHC with R486/R145 antibodies. There are similarities between the profiles of BSE in two different genotypes (●, VRQ/VRQ, n=5, group 5; ○, ARQ/ARQ, n=14, groups 1, 2 and 3, this study) and differences between these and two experimental scrapie sources (□, SSBP/1 in VRQ/VRQ Cheviot sheep, n=5, adapted from González et al. (2002); ▲, CH1641 in ARQ/ARQ and ARQ/AHQ Cheviot sheep challenged IC, n=4; M. Jeffrey & L. González, unpublished observations) and two natural scrapie sources (●, in ARQ/ARQ Suffolk sheep, n=5; ■, in VRQ/VRQ Cheviot sheep, n=5; L. González, unpublished observations). For description of PrP\textsuperscript{d} types, refer to Fig. 3. VASC, Vascular plaques.
in the brain points towards a 'BSE signature' in sheep that would persist after a potential recirculation of BSE in sheep following the removal of the original source of infection.

**PrPd phenotype of BSE in other species**

The consistency of the BSE phenotype in sheep does not parallel the variation detected when different species are compared. Thus, the abundance of PrPd plaques in mice carrying the p7 *Sinc* allele (Fraser *et al*., 1992; Brown *et al*., 2003), in experimentally infected macaque monkeys (Lasmézas *et al*., 1996) and in vCJD patients (DeArmond & Ironside, 1999) contrasts with the almost-complete absence of amyloid/PrP plaques in other species. In addition to sheep, as reported above, neither pigs (Ryder *et al*., 2000), cats (Wyatt *et al*., 1991) nor exotic ungulates (Jeffrey & Wells, 1988) develop such plaques, and they are absent or very rare in BSE cases affecting British cattle (Wells & McGill, 1992; Wells & Wilesmith, 1995). Assessing similarities or differences for PrPd types other than plaques proves difficult, due to lack of detailed descriptions of the PrPd-accumulation patterns in other species. From the literature, however, accumulation of PrPd in cattle BSE targets grey matter rather than white matter and includes granular or particulate, linear and perineuronal types in the neuropil, as well as intraneuronal and stellate types (Wells & Wilesmith, 1995). These descriptions are coincident with our observations on sheep BSE, as are the findings of intraneuronal, neuropil-associated and stellate PrPd accumulations in the brainstem and corpus striatum of experimentally infected pigs (Ryder *et al*., 2000).

**Influence of PrP genotype on the PrPd phenotype of BSE**

The phenotype of PrPd accumulation in the brain of BSE-infected mice is influenced by PrP genotype, so that plaque formation is only observed in homo- or heterozygous *Sinc* p7, but not in *Sinc* s7s7, mice, which accumulate PrPd in a sparse, diffuse form (Fraser *et al*., 1992; Brown *et al*., 2003). With the exception of the miniature pseudoplaques, which were found almost exclusively in ARR/ARR sheep, we have not observed similar qualitative effects of the PrP genotype in sheep, but rather one on the magnitude of PrPd accumulation. This effect was unambiguous in ARR/ARR sheep and probably also in those carrying the AHQ allele. In our study, the host PrP genotype also appeared to influence the incubation period, which was very lengthy for ARR and VRQ homozygotes and VRQ allele-bearing sheep (Table 1; Fig. 6). In contrast, ARQ homozygotes had much shorter incubation periods, although this was apparently modulated by the route of challenge. The two groups of sheep carrying the AHQ allele also had short incubation periods, but the route of infection appeared to have an opposite effect; this, however, could also reflect genotype differences (homo- and heterozygotes) or result from interaction between source and type of inoculum (cattle brain) and route of challenge.

Overall, four combinations of incubation period and magnitude of PrPd accumulation have been observed: (i) short incubation period and high PrPd levels in ARQ/ARQ sheep; (ii) short incubation period and low PrPd levels in AHQ sheep; (iii) long incubation period and high PrPd levels in VRQ sheep; and (iv) long incubation period and low PrPd levels in ARR homozygotes. This situation raises questions about the dynamics of accumulation of PrPd in the brain following BSE agent infection. In *vitro* studies have shown that PrP polymorphisms modulate the conversion of cellular PrP into its abnormal counterpart, which is more efficient for allotypes linked to highly susceptible genotypes and vice versa (Bossers *et al*., 1997, 2000). These findings might explain our observations in ARQ/ARQ and ARR/ARR sheep, but not the inverse relationship between incubation period and PrPd levels found in VRQ and AHQ sheep. We hypothesize that conversion/accumulation of VRQ PrP is efficient, hence the high PrPd levels found in these sheep, but starts late after infection, hence the long incubation period, and that just the opposite situation (low efficiency but early start) could happen in AHQ sheep.

Another intriguing question, derived from the low PrPd levels found in ARR and AHQ sheep, regards the significance of PrPd to clinical disease, as it seems clear from the evidence shown that they are not proportionally related. This finding is not unique to sheep BSE and has also been described when comparing SSBP1 with natural scrapie (González *et al*., 2002). We think that at least two explanations can be considered: firstly, that only some morphological types of PrPd give rise to neurological disease when accumulating in the brain, and, secondly, that PrPd of different polymorphisms has different damaging potential or toxicity for the brain. In the first case, intraneuronal PrPd and extracellular deposits in the neuropil would be the likely candidates, as these are the only types that reached moderate levels in ARR and AHQ sheep. In the second case, less ARR and AHQ PrP of any cellular or morphological type would be needed to trigger the neurological manifestations than when accumulating PrP is of the ARQ or VRQ polymorphisms. A third possibility would be that PrPd accumulation is either unrelated to or not the main event propitiating neurological deficit and disease.

**Conclusion**

Detailed assessment of the morphological features and neuroanatomical distribution of PrPd in the brain of sheep displaying TSE-like clinical signs is a useful means of approaching identification of BSE in sheep. The consistency of the IHC phenotype of PrPd accumulation after sheep-to-sheep passage and across a range of sheep breeds, routes of challenge and PrP genotypes shows the stability of the BSE agent, without having to resort to experimental bioassay methods. Whilst not a unique or definitive method, study of the PrPd phenotype, in conjunction with other IHC, biochemical and biological approaches, offers a realistic possibility for the confirmation of naturally occurring BSE in sheep.
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