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Pathogenetical significance of porencephalic lesions associated with intracerebral inoculation of sheep with the BSE agent

S. Sisó¹, M. Jeffrey¹, S. Martin¹, F. Houston²*, N. Hunter² and L. González¹

¹Department of Pathology, Veterinary Laboratories Agency, Pentlands Science Park, Bush Loan, Penicuik, Midlothian EH26 0PZ. ²University of Edinburgh, Royal (Dick) Veterinary School, Roslin Institute Neuropathogenesis Unit, Ogston Building, West Mains Road, Edinburgh, EH9 3JF, UK.

*Current address: Faculty of Veterinary Medicine, University of Glasgow, Bearsden Road, Glasgow, G61 1QH.

Corresponding author:

Silvia Sisó i Llonch

VLA-Lasswade, Pentlands Science Park

Bush Loan, Penicuik, Midlothian EH26 0PZ, Scotland, United Kingdom

Tel. +44 (0) 131 445 6074. Fax. +44 (0) 131 445 6166

mailto:s.siso@vla.defra.gsi.gov.uk

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Decreased rates of transmission of transmissible spongiform encephalopathies (TSEs) to sheep have been attributed to some polymorphisms of the prion protein (PrP) and to a “species barrier” on inter-species experiments. In addition, the blood brain barrier may be a further impediment to TSE neuroinvasion. The intracerebral (I/C) route is generally considered the most efficient for TSE transmission, as it may help to by-pass those factors. Therefore, susceptibility of particular species to specific TSE agents are conducted by this route.

Aims: This study (1) characterises the traumatic brain lesions associated with the I/C injection of the bovine spongiform encephalopathy agent in sheep, (2) assesses the relevance of such lesions in the outcome of clinical disease, and (3) provides insight into the mechanisms of PrP\textsuperscript{d} conversion and amplification following I/C challenge.

Methods: A total of 27 hemi-brains have been macroscopically and immunohistochemically examined to investigate the presence of lesions compatible with the needle track and the PrP\textsuperscript{d} distribution, respectively.

Results: No residual inoculum was found and the extension and severity of the traumatic brain lesions were unrelated to the clinical outcome. Sheep with PrP\textsuperscript{d} accumulation in the brain also showed conspicuous focal aggregates in the porencephalic lesions and in the circumventricular organs. In contrast, sheep without PrP\textsuperscript{d} deposits in the brain were also negative in the traumatic lesions.

Conclusion: Overall these findings suggest that the efficiency of the I/C route is due to effective absorption and blood re-circulation of infection, rather than to primary amplification at the site of injection.

Key words: Sheep, intracerebral, porencephalia, residual inoculum, prion protein
INTRODUCTION

Transmissible spongiform encephalopathies (TSEs) of humans and several animal species are characterized by the abnormal post-transcriptional conversion of the cellular prion protein (PrP\(^c\)) into a partially protease-resistant (PrP\(^\text{res}\)) and disease-associated isoform (PrP\(^d\)), which accumulates in the brain and is associated with neurodegeneration and onset of neurological signs. Sheep are experimentally susceptible to TSEs following challenge by a variety of routes of inoculation. These include oral [1,2,3], subcutaneous [4], dermal or ocular scarification [5], intralingual [6], conjunctival, intranasal and intraperitoneal [7], intravenous [8, 9, 10], and intracerebral [(I/C) 2,11] routes. However, the efficiency of these different routes is not the same, the I/C route being the one widely considered as the most efficient based on: 1) higher attack rates and shorter incubation periods than those observed by most other routes when sheep of the same PrP genotype are compared, even if the dose is lower for intracerebrally inoculated animals [2,6,7,11], 2) induction of lesions and even clinical disease in sheep of resistant ARR/ARR [12], or semi-resistant ARQ/ARR (N. Hunter, unpublished observations) genotypes, and 3) higher magnitude of accumulation and wider distribution of PrP\(^d\) in the brain [11]. These last authors reported that sheep I/C challenged with the bovine spongiform encephalopathy (BSE) agent accumulated more PrP\(^d\) in forebrain areas (frontal cerebral cortex and striatum) than did orally or intravenously inoculated ones, and hypothesized that this could respond to the proximity of these areas to the injection site.

In the course of a large experiment [12] aiming to determine the susceptibility of sheep of different breeds and PrP genotypes to I/C challenge with the BSE agent, and as a result of the routine pathological examinations undertaken, focal areas of cavitation or porencephaly were detected in the cerebral cortex of the right brain hemisphere of a few sheep. Because of their location, they were considered to result from the experimental injection procedure. In sheep showing widespread PrP\(^d\) accumulation in the brain these areas were also strongly positive for PrP\(^d\) in immunohistochemistry (IHC). Thus, it was considered that infectivity could spread from these
sites and account for the differences previously observed in PrP\(^d\) distribution [11] and progressive accumulation [13] between sheep challenged I/C and by other routes. Therefore, detailed examinations were carried out in the brains of 27 sheep in order to 1) characterize the pathological features of those injection-induced lesions and determine their prevalence after I/C challenge, 2) assess their influence on the generalization of PrP\(^d\) accumulation in the brain and the outcome of clinical signs, and 3) gain insight on the pathogenetic mechanisms that make I/C challenge more efficient than other oral or parenteral infections.

**MATERIALS and METHODS**

The animals included in this study belonged to an experiment briefly described previously [12], in which 87 sheep of three different breeds (Suffolk, Cheviot and Poll-Dorset) and six PrP genotypes (ARQ/ARQ, ARQ/ARR, ARR/ARR, VRQ/VRQ, VRQ/ARQ and VRQ/ARR, where polymorphisms are indicated by the amino acid single letter code at codons 136, 154 and 171) were I/C challenged with 0.05g of cattle BSE brain homogenate into the right forebrain, following the procedure described by Foster et al. [1]. The experiments were reviewed and approved by the appropriate Ethical Review Committee and carried out under a current Home Office Licence. Sheep were closely monitored for neurological signs and euthanized once clinical disease was evident; some of the infected sheep died from intercurrent conditions and some others were culled at the end of the experiment -between 2,200 and 2,500 days post-inoculation- in the absence of clinical signs of TSE. Details of the patterns and neuroanatomical distribution of PrP\(^d\) in the brains of clinically affected sheep from this experiment have been described previously [11]. Brains were removed at post-mortem, sliced sagitally, and one hemi-brain immediately immersed in formaldehyde. In the present study, a total of 27 fixed hemi-brains were macroscopically assessed for the presence of traumatic lesions compatible with the needle track by examining transversal sections of frontal, temporal and occipital cortices; 22 of the examined cases corresponded to the inoculated right hemi-brains and 5 to the contra-lateral non-inoculated hemi-brain. In addition, representative
sections of other brain areas (corpus striatum, thalamus/hypothalamus, midbrain, cerebellar vermis, and medulla oblongata at the obex) and of circumventricular organs [(CVOs) 13] were trimmed, embedded in paraffin-wax, and processed for histology and IHC examinations in order to 1) characterize the histological appearance and assess the extension of the lesions caused by the injection, and 2) determine the correlation between deposition of PrP\(^d\) in those lesions, in the brain parenchyma and in the CVOs.

Serial sections of those tissue specimens in which injection-derived lesions were observed after routine histological examinations were stained or immunolabelled by:

- a modified Van Gieson staining technique (Merck KGaA, Darmstadt, Germany) to highlight collagen and elastic fibres that could be part of the scarring process and neovascularization. Briefly, sections were immersed in Miller’s elastic stain solution for 1h, rinsed in running tap water for 2 min and immersed in Weigert’s solution for 5 min. After another 2 min rinse, picrofuchsin solution was applied for 2 min.

- a Pearl’s Prussian blue method in order to ascertain the presence of hemosiderin. Briefly, sections were immersed in acid ferrocyanide solution for 30 mins, rinsed in 3 changes of distilled water and then counterstained in 0.5% neutral red for 2 minutes.

- a luxol fast blue technique in order to assess the severity of myelin loss. Briefly, sections were immersed in 95% ethanol immediately removed and incubated at 60°C for 2 hours in luxol fast blue solution then quickly washed in 70% ethanol followed by differentiation in 0.05% lithium carbonate then 95% ethanol and counterstained in cresyl fast violet. Sections were then differentiated in cresyl violet.
- an immunohistochemical (IHC) method according to the protocol described by González et al. [13] which aims to detect the disease-associated prion protein (PrP\textsuperscript{d}) – a form of the prion protein partially protease-sensitive but not found in uninfected animals and therefore different from PrP\textsuperscript{os} or PrP\textsuperscript{c}, respectively. For that purpose, slides were incubated overnight at 24°C with R145 PrP rat monoclonal antibody (VLA Weybridge, UK, 1:4000 dilution) following antigen retrieval by immersion in 98% formic acid for 15 min at 24°C and autoclaving in 0.2% citrate buffer (pH 6.2) at 121°C for 30 min. The IHC procedure was completed by an immunoperoxidase method (Vector-elite ABC kit; Vector Laboratories, Peterborough, UK) with DAB (Sigma-Aldrich Company Ltd, Gillingham, UK) as a substrate, and sections were finally counterstained with Mayer’s haematoxylin. The magnitude of PrP\textsuperscript{d} accumulation was subjectively scored from 0 to 3 in each of the neuroanatomical sites above mentioned – focal traumatic lesions, CVOs and representative areas of the brain parenchyma-, by methods reported previously [11, 13, 14]. The topographical distribution of PrP\textsuperscript{d} was assessed for each animal.

- an IHC method with Z0334 polyclonal GFAP antibody (Dako, Ely, UK, 1:8000 dilution), to reveal astrocyte proliferation in association with the needle track lesion, by the same IHC protocol as for PrP\textsuperscript{d}.

- an IHC method with M0718 monoclonal CD68 antibody (Dako, 1:400 dilution), to investigate the eventual involvement of macrophages and activated microglia, by the same IHC protocol as for PrP\textsuperscript{d} with the exception of the antigen retrieval; this was performed by immersion of sections in 0.25% trypsin/α-chymotrypsin 1/1 v/v, pH7.8 (Sigma) for 5 minutes at 37°C, followed by washing in 3 changes of PBST and rinsing in running tap water for 10 minutes.

- a double IHC method for PrP\textsuperscript{d} with antibodies KG9 (IAH, Compton, UK, 1:4000), which recognises bovine but not ovine PrP, and Bar224 (CEA, Saclay, France, 1:32000), which recognises ovine but not bovine PrP. This double immunolabelling approach aimed to achieve differentiation
between residual cattle BSE inoculum and “de novo” converted ovine PrP\(^d\), and to compare PrP\(^d\) patterns and distribution at the injection site with those obtained in sections labelled with R145, which recognises both sheep and cattle PrP\(^d\). The procedure was basically the same as for single IHC with R145: sections were incubated first overnight with KG9 antibody and developed with DAB substrate (brown), and then incubated for 1 hour with Bar224 and developed with VIP substrate (purple). Double IHC was also performed for GFAP and PrP\(^d\) by incubating sections overnight with Z0334 antibody (developed with DAB), followed by 1 hour incubation with R145 (developed with VIP).

Sections from positive-control and negative-control tissue blocks were included in each IHC run to ensure consistency in the sensitivity and specificity of the IHC procedure, respectively. In addition, the specificity of the PrP\(^d\) immunodeposits observed at the injection-induced lesions was reassessed by incubating sections without the primary antibody or with a rat immunoglobulin of the same IgG1 isotype as R145. At the end of all staining or IHC procedures sections were dehydrated and mounted by routine procedures.

RESULTS

Occurrence and characteristics of the traumatic lesions resulting from the intracerebral injection

Gross lesions compatible with needle injury were observed in 15 out of 22 sheep in which the right hemi-brain was examined (Table 1). The contra-lateral, left hemi-brain was examined in a further 5 sheep, and no such lesions were observed. For sheep in which right hemi-brains were examined, those showing traumatic lesions were of three different breeds: Cheviot (1/4), Poll-Dorset (4/6) and Suffolk (10/12), and three different PRNP genotypes: ARQ/ARR (10/11),
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...no such lesions were found in the right hemi-brain from one ARQ/ARQ and two VRQ/ARR animals. Lesions consisted of a single elongated cyst of approximately 0.5-1cm length and 1-2mm width in the frontal aspect of the anterior lateral gyrus, mainly in the transition between grey and white matter (Figure 1). In seven cases, cavities extended from frontal to temporal cortical areas, and more occasionally involved the internal capsule (Table 1).

The needle injury within the brain did not appear to result in permanent overt clinical disease, as 11 out of 15 cases showing such lesions in the cerebral cortices were culled at the end of the experiment, around six-and-half years after inoculation, in the absence of clinical signs (Table 1). This is not surprising if we consider the small extent of the traumatic lesions, their location within the cerebral cortex, and the absence of inflammatory changes that could have resulted from needle contamination.

Haematoxylin-eosin stained sections showed rarefaction of grey matter and pallor of white matter surrounding cortical cavities; myelin loss was confirmed in the latter in luxol fast blue-stained sections (Figure 1). In most cases, lesions appeared as cysts partially filled with round cells of abundant cytoplasm containing yellow to brownish-orange granules of apparent blood breakdown products (Figure 1); they were confirmed as haemosiderin-loaded macrophages, as they showed CD68-positive reaction and the granules were positive in the Pearl’s Prussian blue stain. The cavities were delimited by a repair or scarring tissue formed by GFAP-positive fibrillary astrocytes and sparse microglial cells, in which marked proliferation of swollen neo-capillaries was observed (Figure 1). Numerous GFAP-positive but CD68-negative cells containing haemosiderin (Figure 1) were common in this repair tissue and in cortical grey matter areas adjacent to the cystic lesion, indicating that astrocytes were also involved in phagocytic removal of extravasated red blood cells.
Overall, the appearance of the lesions and the reactive changes were suggestive of an original vascular insult leading to focal haemorrhage. In a few cases with no grossly apparent porencephalia or little cavitation, a severe reactive fibrillary astrocytosis completely or almost completely repaired the old cavitating lesion (Figure 1). Exceptionally, severe fibrosis of the meningeal stroma similar to repair granulation tissue was revealed by Van Gieson staining; in these cases the needle appeared to have caused severe meningeal damage, although meningitis was never observed.

Deposition of PrP<sup>d</sup> associated with the traumatic lesions, the CVOs and elsewhere in the brain

As shown in table 1, in four of 15 cases in which traumatic lesions were found, PrP<sup>d</sup> was absent both in the injection-induced cystic area and in the rest of the brain parenchyma. Three of these were of the ARR/ARR PrP genotype and one was ARQ/ARR; none of them had shown signs of disease.

In three sheep (two ARQ/ARR and one ARR/ARR sheep) without PrP<sup>d</sup> accumulation in any other area of the brain parenchyma, intracellular, single or multiple R145-positive granules restricted to the periphery of the cystic lesion were observed. Those granules were also immunolabelled, albeit more faintly, with Bar224 (Figure 2). Omission of the primary PrP antibody or substitution by a rat IgG1 (Figure 2) resulted in loss of such granular labelling; these granular deposits were therefore interpreted as PrP-specific. Double immunolabelling was used to discriminate between PrP of cattle origin (using KG9) and that of sheep origin (using Bar224). No labelling was observed with KG9 antibody indicating an absence of cattle derived PrP<sup>d</sup> from the inoculum. However, using the Bar224 antibody, PrP<sup>d</sup> was recognised within the granules indicating local de novo generation of ovine PrP<sup>d</sup>. In order to ascertain the association between those granular PrP-immunoreactive deposits and the experimental BSE challenge, tissue samples with similar porencephalic lesions were sourced from archive cases of non-TSE conditions, such as...
swayback or chronic polyoencephalomalacia. Immunolabelling of such sections with R145 and Bar224 showed similar PrP positive granules (data not shown).

Finally, in another eight of those 15 cases, PrP\textsuperscript{d} immunodeposits, positive with R145 or Bar224 but negative with KG9 were present both in the traumatic lesion and in several other areas of the brain confirming again that PrP\textsuperscript{d} as no residual inoculum (cattle BSE). Accumulation of PrP\textsuperscript{d} in the porencephalic lesions varied from mild deposits restricted to the margins of the cysts, shown by double IHC to be often associated with fibrillary astrocytes, to prominent, sometimes plaque-like aggregates in the repair tissue surrounding the traumatic lesion, which appear to spread along white matter tracts to neighbouring areas of the cerebral cortex (Figure 2) and even to the internal capsule. The amount of PrP\textsuperscript{d} in the focal traumatic lesions appeared to be related to the extent or severity of the lesion itself rather than to the total magnitude of PrP\textsuperscript{d} accumulation in the whole of the brain. However, accumulation of PrP\textsuperscript{d} in the cerebral cortex of right hemispheres showing traumatic lesions was more prominent than in those hemi-brains, either right or left, without evidence of traumatic lesion (Table 1, figure 3). As far as deposition in brainstem areas and cerebellum is concerned, right hemi-brains without discernible traumatic lesions (n=7) and left hemi-brains (n=5), showed a magnitude and distribution of PrP\textsuperscript{d} that was similar to that observed in right hemi-brains in which injection-derived cystic lesions were found (Figure 3). Altogether, these findings suggest that PrP\textsuperscript{d} accumulated specifically in association with the traumatic lesions when these persisted after intracerebral inoculation, in those cases in which generalized PrP\textsuperscript{d} aggregates were also present, and that infection spread from those focal lesions to neighbouring forebrain areas. As shown in table 1 and figure 4, all PrP\textsuperscript{d}-positive sheep showed widespread and marked immunoreactivity in the CVOs, even in those cases where the total PrP\textsuperscript{d} magnitude in the brain was low.
Lack of intracellular immunolabelling with the P4 monoclonal antibody [3] confirmed that all generated PrP\(^d\), either in the traumatic lesion or in the brain parenchyma, was of the BSE characteristics (data not shown).

**DISCUSSION**

Sheep with long incubation periods or culled at the end of the experiment were deliberately selected for this study in order to ascertain the persistence of traumatic lesions associated with I/C injection. As expected, most of them were of PRNP genotypes associated with resistance to infection by the oral route [2] and with relatively low susceptibility by the I/C route (N. Hunter, unpublished observations). Also, they mostly showed low overall magnitudes of PrP\(^d\) in the brain, even if clinically affected as previously described [11], which allowed comparison between the amounts of PrP\(^d\) deposited in the traumatic lesions, in the CVOs, and elsewhere in the brain parenchyma.

As no significant clinical signs were reported immediately after challenge, the initial lesions probably resulted in minimal oedema and increased intracranial pressure. Focal cavities or cysts persisted for years and were presumably stable in the cerebral cortex of some sheep after I/C injection of BSE. The fact that those focal lesions were not discernible in some other sheep subjected to the same inoculation procedure, indicates variability in the damage initially caused: in some cases such damage would be insignificant and readily healed, while in other cases, accidental puncture of blood vessels would lead to focal haemorrhage followed by a slow, incomplete and imperfect repair. The presence of haemosiderin-loaded macrophages and astrocytes argues for an initial vascular damage followed by a macrophage-associated removal of erythrocytes and later by reactive astrogliosis and neovascularisation. It seems clear from the results here presented that these porencephalic lesions, even when they persist, have no long-term repercussion for neurological
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disease, probably because of the limited size of the cavities formed and their location within the cerebral cortex. In fact, sheep with no discernible traumatic lesions in the cerebral cortex displayed more commonly neurological signs with generally shorter incubation periods, even though they accumulated less PrP\text{d}, not only in forebrain areas but also elsewhere, than sheep with porencephalic lesions (Table 1).

The second point addressed by this study was the potential impact of the traumatic cerebral lesions on the efficiency of the I/C route of infection and on the relatively distinct PrP\text{d} distribution after I/C inoculation when compared to other routes. It has been reported that, in oral scrapie and BSE transmissions to sheep, PrP\text{d} progressively accumulates in the brain in a caudo-rostral manner, with early involvement of the dorsal motor nucleus of the vagus and of the CVOs, and relatively late involvement of forebrain areas in pre-clinically affected animals [13]. PrP\text{d} deposition was also less severe in the frontal cerebral cortex and corpus striatum of clinically affected BSE-infected animals when challenged by the oral or intra-venous routes [11]. In contrast, sheep inoculated with BSE by the intracerebral route, either in pre-clinical stage [13] or clinically affected [11] showed an earlier or more severe involvement of forebrain areas, respectively. We believe that those differences are the result of the focal injury, as PrP\text{d} accumulation in the forebrain was less pronounced in those sheep in which focal traumatic lesions were not discernible, or when examining the contra-lateral, left brain hemisphere, while PrP\text{d} depositions in other areas like thalamus, brainstem or cerebellum were bilaterally distributed (Figure 3). However, focal accumulation of PrP\text{d} in the injection-derived lesions and its further spread to neighbouring areas of the brain do not appear to result from primary conversion of inoculum-derived PrP\text{d} at the site of injection. Thus, remains of the cattle-derived inoculum were not detected in those lesions. Also, sheep that did not show PrP\text{d} accumulation elsewhere in the brain were also negative in the focal traumatic lesions, and the magnitude of PrP\text{d} accumulation in those lesions, when it occurred, was never significantly higher than in the whole of the brain. Furthermore, sheep of the same breed and PRNP genotype could develop moderate to severe PrP\text{d} accumulation in the brain, and even clinical
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signs, in the absence of discernible traumatic lesions. These findings do not support the notion that

generalized PrP\textsuperscript{d} accumulation in the brain of I/C infected sheep arises from extension of primary
conversion at the inoculation site.

More problematic to interpret are the intracellular PrP granules observed at the periphery of
cysts of three sheep, in which no PrP\textsuperscript{d} deposition was found elsewhere in the brain (Figure 2). Once
their residual bovine inoculum origin is eliminated, two alternative hypothesis can be proposed: 1) that they originated from early and inefficient \textit{in situ} conversion of ovine PrP\textsuperscript{c} into PrP\textsuperscript{d}, which was
latter phagocytised by reactive macrophages and astrocytes, or 2) that they correspond to
endocytosed PrP\textsuperscript{c}, following its earlier focal over-expression as a result of the traumatic injury. The
facts that our IHC protocol for PrP\textsuperscript{d} did not include enzymatic pre-treatment (i.e. digestion with
proteases), the findings of PrP\textsuperscript{c} upregulation in hypoxic human brains with no associated prion
pathology [15], and the detection of similar immunoreactive granules in cerebral cortices of sheep
with chronic porencephalic lesions unrelated to prion disorders support this second possibility.

We therefore postulate that after intracerebral inoculation with BSE-infected brain material,
the inoculum (cattle BSE) is reabsorbed before any conversion \textit{in situ} takes place, circulates in the
cerebrospinal fluid (CSF) and is drained to the blood stream through the dural venous sinuses
and/or to the lymphatic system through the cribriform plate, following the lymphatic cerebrospinal
and interstitial fluids absorption pathways [16, 17]. Once in the circulatory system, infectivity
would again reach the brain by portals of entry such as the CVOs [13]. However, because of the
rich and fenestrated neovascularization [18] of the repaired focal traumatic lesions, or because of
their intimate contact with CSF from meningeal wounds, infectivity will also gain rapid access to
those lesions, resulting in focal accumulation of PrP\textsuperscript{d} and later spread to related forebrain areas. The
consistent and conspicuous PrP\textsuperscript{d} deposits observed in the CVOs –which also have fenestrated
capillaries- of these same sheep argues in favour of this hypothesis. It remains to be elucidated if a
local up-regulation of PrP\textsuperscript{c}, as a result of the traumatic and vascular insult, contributes to the focal accumulation of PrP\textsuperscript{d} in the porencephalic areas.

This notion of rapid absorption of the intracerebral inoculum is in agreement with the findings of Hamir et al. [19], in I/C scrapie-infected sheep. These authors found similar lesions in association with the injection, though more of an acute nature, as sheep were killed shortly after inoculation; residual inoculum was detected up to three days post-inoculation, but not for the following six weeks. Similarly, no PrP\textsuperscript{res} or infectivity were detected in the brain of mice inoculated with the agent of Creutzfeldt-Jakob disease during a four-week post-inoculation period, which was attributed to rapid absorption of the inoculated material [20]. It has been noticed for viruses, that both strain and dose at the site of inoculation have an influence on the rate of viral transport through neural circuity [21]. It is plausible then that after a high dose I/C inoculation, cells at the site of inoculation are the initial site at which infectivity is established. However, such a neural circuity hypothesis following the needle puncture could not explain how PrP\textsuperscript{d} deposition in extra-cortical brain areas remained bilateral and symmetrical. According to the recently described role of the CVOs as portal of entry of TSE agents in the brain of sheep [13], it looks more plausible that PrP\textsuperscript{d} from the inoculum in these animals is reabsorbed, ending in the CSF, lymph and/or blood to re-enter the brain through the blood supply, CSF and CVOs.

According to this hypothesis of blood recirculation of infectivity after I/C injection, other routes of infection should be as efficient as the I/C. Indeed, Hamir et al. [6] have reported similar efficiency of the I/C and the intralingual routes, although in this case, rapid access of the infectious agent via nerves could not be excluded. Although proper comparisons between the I/C and the intravenous routes have not been performed, results of a recent blood transfusion experiment [14] indicated that the intravenous route is more efficient than oral dosing. In that experiment, five ARQ/AHQ sheep dosed orally with cattle BSE did not show any evidence of PrP\textsuperscript{d} accumulation in any tissue when culled 300-400 days post-dosing, whereas another five sheep of the same genotype
developed clinical disease at around 700 dpi, when infected intravenously with a lower dose of the same inoculum. This incubation period is just slightly longer than that observed after intracerebral injection of cattle BSE in sheep of a more susceptible ARQ/ARQ genotype, which is readily infected by the oral route [11].

Therefore, these observations suggest that the dogma of the I/C route being the most efficient, and the reasons of its efficacy in terms of lesion severity and incubation period, have to be re-considered. Improved efficacy of the I/C route reported previously may be connected more to rapid and efficient delivery of infectivity to blood circulation, rather than to accelerated local replication and avoidance of the immune system, as originally suggested [22,23].

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REFERENCES


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Table 1. Details of the sheep examined, the occurrence of porencephalic lesions associated with the needle injury, and the accumulation of PrP\textsuperscript{d} in those lesions and elsewhere in the brain.

<table>
<thead>
<tr>
<th>ID No</th>
<th>Breed</th>
<th>Genotype</th>
<th>PM</th>
<th>Traumatic lesion</th>
<th>PrP\textsuperscript{d} in</th>
<th>Forebrain</th>
<th>Brain</th>
<th>CVOs</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>DPI</td>
<td>CS</td>
<td>Location</td>
<td>PrP\textsuperscript{d}</td>
<td>Forebrain</td>
<td>Brain</td>
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<tr>
<td>C533</td>
<td>Suffolk</td>
<td>ARQ/ARR</td>
<td>2275</td>
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<td>F/T</td>
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<tr>
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<td>Suffolk</td>
<td>ARR/ARR</td>
<td>2241</td>
<td>N</td>
<td>T</td>
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<td>Cheviot</td>
<td>ARR/ARR</td>
<td>2151</td>
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<td>ARR/ARR</td>
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<td>N</td>
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<tr>
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<td>ARR/ARR</td>
<td>2418</td>
<td>N</td>
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ID No, sheep identification number; PM, at post-mortem; DPI, days post-inoculation; CS, clinical signs; F, frontal cortex; T, parieto-temporal cortex; S, corpus striatum; Y, yes. N, no; nf, not found; R, right hemibrain; L, left hemibrain; na, not applicable. Averages shown in bold for DPI, PrP\textsuperscript{d} magnitude in forebrain and in the whole brain. *Average score for clinically-affected sheep only; note that hemibrains with focal traumatic lesions showed proportionally higher magnitude of PrP\textsuperscript{d} accumulation in forebrain with respect to the whole brain (23%) than right hemibrains without
traumatic lesions (17%). Fractions within the CVO column show the number of positive CVOs out of the number examined.

**Figure 1. Characteristics of the lesions induced by the needle in the cerebral cortex.** Lesions (green arrow) could be grossly identified as small cavities in the right cerebral cortex at the transition between white and grey matter of the anterior lateral gyrus (A; 1, sulcus splenialis; 2, sulcus lateralis). Histologically, needle-induced lesions appeared as areas of white matter rarefaction (B; haematoxylin and eosin [H-E] x10) or cystic cavities between the white and grey matter (C; H-E x2), in which macrophages filled with hemosiderin-like granules were observed (C inset; H-E x60). Moderate to severe myelin loss was associated with the porencephalic lesion (D; luxol fast blue x4). The repair tissue surrounding the cysts consisted mostly of proliferation of neo-capillaries (E; Van Gieson x4) and reactive astrocytes (F; GFAP x10). In some cases, the traumatic lesions were almost healed by the repair tissue (G; H-E x4). GFAP-positive protoplasmic astrocytes were also present in the neighbouring grey matter (H; GFAP x20) and also contained hemosiderin (I, Pearl’s Prusian blue x 20).

**Figure 2. Accumulation of PrP\(^d\) in the traumatic lesions caused by the intracerebral injection.**

Most de novo created, sheep derived PrP\(^d\) accumulated in the cerebral white matter, within or in close association with the repair tissue (A; IHC with R145 x4). PrP\(^d\) immunodeposits varied in magnitude being occasionally very prominent and plaque-like (B; IHC with R145 x60) specially when associated with florid plaques (B inset; H-E x60). Reactive astrocytes forming part of the repair tissue also contained PrP\(^d\) (C; double IHC for GFAP [brown] and with R145 [purple] x10; left inset x60). Over-expression of PrP\(^c\) shown as brownish granules was observed in the focal traumatic lesions (D; IHC with R145 x20) of three sheep that did not show generalized PrP\(^d\) accumulation in the brain; they were seldom intraneuronal (D; lower inset x20), and more frequently intra-astrocytic (D; upper inset x20); these granules were also labelled with another PrP antibody (E; IHC with Bar224 x60), but not with a rat IgG monoclonal (F; x60) or with KG9 PrP
Porencephalia after intracerebral BSE

antibody (G; x60). The bluish-black cytoplasmic granules (F and G) represent pigment and not PrP
immunoreactivity.

Figure 3. Topographical distribution of PrP\textsuperscript{d} accumulation in hemi-brains of sheep infected
with cattle BSE by the intracerebral route. Right (R) forebrain areas of I/C challenged sheep
with porencephalic lesions showed diffuse and more abundant PrP\textsuperscript{d} deposits than did left hemi-
brains (L), while PrP\textsuperscript{d} was symmetrically distributed in the diencephalon and brainstem. Note that
PrP\textsuperscript{d} accumulated in the site of injection (arrow). Colours indicate magnitude of PrP\textsuperscript{d} accumulation
in those areas: not coloured (0, absence), yellow (0-1, mild), orange (1-2, moderate), and red (2-3,
severe).

Figure 4. Immunolabelling of PrP\textsuperscript{d} deposits in CVOs. An asterisk (*) highlights the specific
location of the CVO when the picture has been captured at low magnification. AP, Area postrema,
x2; PG, Pineal gland, x4; ME, Median eminence, x2; SCO, Subcommisural organ, x20; OVLT,
Organum vasculosum of the lamina terminalis, x2; SFO, Subfornical organ, x2. Note the close
proximity between some of the CVOs and the ventricles (III and IV), the Silvio’s aqueduct (SAq.),
the dorsal motor nucleus of the vagus (DMNV), the optic nerve (ON), the fornix, the anterior white
commissure (AWC) and the posterior white commissure (PWC).