Comparative titration of experimental ovine BSE infectivity in sheep and mice

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Titration studies of the infectivity of experimental bovine spongiform encephalopathy (BSE) in sheep are necessary to assess the risk for human health posed by the ovine infection relative to the original cattle disease. Here, a comparative titration was performed of sheep-passaged BSE infectivity in Romney sheep and RIII mice, by the intracerebral (i.c.) and i.c. plus intraperitoneal (i.p.) routes, respectively. The sheep-to-mouse species barrier was lower than anticipated, as similar titres were obtained for both sheep [1 × 10⁵.⁴ (i.c.) ID₅₀ g⁻¹] and mice [1 × 10⁶.⁰ (i.c. + i.p.) ID₅₀ g⁻¹]. Moreover, sheep of the ARR/ARR PrP genotype all succumbed to i.c. challenge with a 10⁻³ dilution of 0.5 g of a brainstem pool from BSE-affected sheep, indicating that resistance to natural infection in sheep of this genotype must reside in some mechanism of peripheral pathogenesis.

Bovine spongiform encephalopathy (BSE) is a neurological disease of cattle first recognized in Great Britain in 1985 (Wells et al., 1987). It is the cause of human variant Creutzfeldt–Jakob disease (Bruce et al., 1987; Will et al., 1996), presumably through exposure to contaminated food. The zoonotic nature of BSE prompted titration experiments to determine the minimum infectious dose for cattle and laboratory mice in an attempt to evaluate the risk for humans. Whilst entry of brain tissue into the human food chain is not intentional, titration studies are always performed on this inoculum, as it contains the highest infectivity levels overall, to provide a worst-case scenario. Transmission of BSE to laboratory RIII mice by intracerebral (i.c.) inoculation was first achieved by Fraser et al. (1992), due to a shortage of inoculum, the 10⁻⁶ dilution of 0.5 g of a brainstem pool from BSE-affected sheep, indicating that resistance to natural infection in sheep of this genotype must reside in some mechanism of peripheral pathogenesis.

The inoculum titrated within this comparative experiment was derived from the brainstem of three AHQ/AHQ Romney sheep that developed confirmed TSE after oral dosing with a brainstem homogenate from six cows clinically affected with natural BSE. These three brainstems were pooled and stored as a 10 % (w/v) homogenate (10⁻¹ dilution) in PBS, pH 7.2.

Six groups of five 12-month-old Romney sheep, all of the ARQ/ARQ prion protein (PrP) genotype, were i.c. challenged with 0.5 ml of the above inoculum at dilutions of between 10⁻³ and 10⁻⁸. Five ARR/ARR sheep of the same breed and age were i.c. injected with a 10⁻³ dilution of the same homogenate, and a further five ARQ/ARQ sheep were inoculated with PBS. Details of the i.c. injection technique for sheep have been published elsewhere (Foster et al., 1993).

Six groups of 20 RIII mice (PRNP genotype s7s7) were inoculated at 6–8 weeks of age with the same inoculum as that used for the sheep, at dilutions ranging from 10⁻¹ to 10⁻⁶. Infection was carried out by a combined i.c. (20 µl) and i.p. (100 µl) route using procedures described elsewhere (Fraser et al., 1992); due to a shortage of inoculum, the 10⁻¹ group only received the i.c. injection. Another 20 mice were...
injected with PBS by the same routes and were used as a negative control.

Sheep and mice were monitored clinically for the development of neurological signs; once these were considered characteristic of TSE, animals were euthanized either by barbiturate overdose (sheep) or by carbon dioxide (mice). Some mice were culled due to intercurrent illness or to old age [approx. 640–650 days post-infection (p.i.)]. Infectious titres for both species were estimated as ID$_{50}$ using the Spearman–Kärber method (Hamilton et al., 1977). Differences in ID$_{50}$ titre between sheep and mice and in incubation periods between different dilution groups, in both sheep and mice titration experiments, were assessed for statistical significance using unpaired t-tests (two-tailed P value).

Histopathological (HP) and immunohistochemical (IHC) examinations were performed on representative brain areas from sheep and mice using standard procedures. Tissue sections (5 µm) were stained with haematoxylin and eosin and examined for the presence of spongiform changes. Consecutive sections were subjected to antigen-retrieval procedures, as described elsewhere (González et al., 2002), and to IHC labelling for disease-associated PrP (PrP$^{\beta}$) using primary antibodies R145 for sheep tissues (González et al., 2005a) and 1A8 for murine specimens (González et al., 2005b).

All sheep challenged with the $10^{-3}$ and $10^{-4}$ dilutions succumbed to BSE, confirmed by HP and IHC, as did three out of five sheep challenged with the $10^{-5}$ dilution. All of the remaining ARQ/ARQ Romney sheep were still alive at 2200 days post-challenge, i.e. >3 years after the last BSE casualty. Based on these attack-rate figures, the infectious titre of the inoculum was estimated as $10^{5.4}$ ID$_{50}$ g$^{-1}$, with a 95% confidence interval of $10^{4.8}$–$10^{5.8}$. Incubation periods ranged from 470 to 562 days (mean 520 days) for the group inoculated with $10^{-3}$ dilution, from 483 to 667 days (mean 554 days) for the $10^{-4}$ group and from 644 to 828 days (mean 749 days) for the three sheep of the $10^{-5}$ group that developed the disease (Fig. 1). Differences in mean incubation periods were not significant between correlative dilutions (Fig. 1), but were significant between the $10^{-5}$ and $10^{-3}$ dilution groups ($P<0.05$).

Four out of five ARR/ARR sheep challenged with a $10^{-3}$ dilution of the same inoculum developed clinical disease (incubation periods of 1342–1709 days) and their brains showed severe spongiform change and were strongly positive for PrP$^{\beta}$ by IHC. The remaining sheep of this PrP genotype died from intercurrent listerial encephalitis at 834 days post-challenge; its brain showed weak intra- and extracellular PrP$^{\beta}$ deposits in the dorsal motor nucleus of the vagus nerve and other areas of the brain.

The phenotype of PrP$^{\beta}$ accumulation in the brain of the 13 ARQ/ARQ clinically affected sheep has been described in detail previously (González et al., 2005a) and no differences in this respect were observed between the different dilution groups. The PrP$^{\beta}$ profiles were very similar between ARQ/ARQ and ARR/ARR sheep, although the latter showed more abundant plaque-like accumulations of PrP$^{\beta}$ (Fig. 2).

After discounting those mice dying of old age, animals were euthanized either by barbiturate overdose (sheep) or by carbon dioxide (mice). Infectious titres for both species were estimated as ID$_{50}$ using the Spearman–Kärber method (Hamilton et al., 1977). Differences in ID$_{50}$ titre between sheep and mice and in incubation periods between different dilution groups, in both sheep and mice titration experiments, were assessed for statistical significance using unpaired t-tests (two-tailed P value).

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Accumulation of PrP<sup>d</sup> was most pronounced in the CA2 sector of the hippocampus, the hypothalamus and the deep cerebellar, inferior olivary and vestibular nuclei. Only three types of PrP<sup>d</sup> deposition were observed: diffuse punctuate in the neuropil, intraneuronal and intramicroglial (Fig. 2).

The lesion profile, as determined by the vacuolar degeneration score in nine selected brain areas (Fraser & Dickinson, 1973), was similar in all dilution groups. Spongiform change was most conspicuous in the dorsal nuclei of the medulla and in the hypothalamus, and was least pronounced in the cerebral and cerebellar cortices (Fig. 3).

RIII mice are susceptible not only to cattle BSE (Fraser et al., 1988, 1992) but also to sheep-passaged BSE, and develop clinical disease with pathological features indistinguishable from those of direct cattle-to-mouse transmission (see Fig. 3). Whether or not an adaptation of the BSE agent to RIII mice has occurred as a result of passage in sheep is difficult to ascertain due to the diversity of inocula used in different studies. Thus, the inoculum used by Fraser et al. (1992) appeared to be more infectious than the one in our study, at least in terms of incubation period (around 40–50 days longer in our study), particularly if we consider that their results were after i.c. injection, whilst ours were after a combined i.c./i.p. injection. This combined route results in shorter incubation periods after high-dose infection (Fraser et al., 1992), explaining why, in our experiment, the 10<sup>−1</sup> group (i.c. only) had a slightly longer incubation period than the 10<sup>−2</sup> group (i.c. + i.p.). In contrast to these experiments, the cattle inoculum used in the study by Bradley (2001) gave a titre in RIII mice of only 10<sup>3.3</sup> (i.c. + i.p.) ID<sub>50</sub> g<sup>−1</sup>, which is clearly lower than in our study, whilst Fraser & Foster (1994) reported a titre of 10<sup>5</sup>–10<sup>6</sup> ID<sub>50</sub> g<sup>−1</sup>, which is similar to ours. Other titrations of BSE-infected sheep brains have recently been carried out in RIII mice with resulting infectious titres of 10<sup>4</sup> and 10<sup>3.5</sup> (i.c. + i.p.) ID<sub>50</sub> g<sup>−1</sup> (S. J. Bellworthy, unpublished results). It would appear, therefore, that the infectiousness of different cattle and sheep brains can be variable, even if these all originate from animals at clinical disease stage.

The commonly accepted notion (Fraser et al., 1992) that the i.p. route makes little contribution at high dilutions enables a certain degree of confidence in the comparison between infectious titres in sheep and mice. The fact that the PrP genotype of the donor sheep (AHQ/AHQ) was different from that of the recipients probably had little relevance in the final sheep titre, as the ARQ/ARQ genotype is considered the most susceptible to BSE, regardless of the origin of the inoculum.
inoculum. Therefore, in this experimental model of sheep-passaged BSE, the effect of the species barrier appeared to be negligible, as the difference of less than half a logarithm between sheep and mouse titres was not statistically significant. This is in contrast to the much higher (approximately 500-fold) difference observed previously between cattle and mice (Bradley, 2001); the precise explanation of these differences is not known.

Adaptation of the BSE agent to sheep as a result of previous passage in sheep does not seem to have occurred. The eight cases of bovine-derived BSE in ARR/ARR sheep described by González et al. (2005a) had an incubation period of 1333 ± 86 days (mean ± SEM) after i.c. inoculation of 0.5 ml of a 10^{-1} brain homogenate with a titre in RIII mice of 10^{5.4} (i.c. + i.p.) ID_{50} g^{-1}. The two ARQ/ARQ sheep described in this paper developed clinical BSE after a statistically similar (1469 ± 82 days) incubation period when inoculated with 0.5 ml of a 10^{-3} (100-fold more diluted than the cattle inoculum) sheep brainstem homogenate with a titre in RIII mice of 10^{4.0} (i.c. + i.p.) ID_{50} g^{-1} (more than 100-fold higher than the cattle inoculum). The incubation periods of ARQ/ARQ sheep challenged with those two inocula were also closely similar: 558 ± 11.4 days for 17 sheep inoculated with cattle BSE (González et al., 2005a) and 520 ± 19.4 days for the five sheep inoculated with the 10^{-3} dilution of the sheep BSE homogenate (P = 0.12).

An exact figure of the relative resistance of ARR/ARR sheep compared with ARQ/ARQ animals cannot be provided, as all four ARR/ARR sheep challenged with the 10^{-3} dilution succumbed to BSE (and the fifth presumably would have done so had it not died from an intercurrent infection) and it is not known whether some would eventually have succumbed to a 10^{-4} dilution. However, because only three out of five of the ARQ/ARQ sheep inoculated with a 10^{-5} dilution developed clinical TSE, the maximum susceptibility ratio between ARQ/ARQ and ARR/ARR would be 60:1. This ratio might, however, only apply to the i.c. route, as ARR/ARR sheep appear to be much more resistant to infections by other more natural routes such as the oral route, which so far has provided negative results (S. J. Bellworthy, personal communication). The resistance of ARR/ARR sheep to oral challenge is not due to blocked absorption through the gut epithelium (Jeffrey et al., 2006), but to some other aspect of peripheral pathogenesis.

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References


