Digestion and transportation of bovine spongiform encephalopathy-derived prion protein in the sheep intestine

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Bovine spongiform encephalopathy (BSE) is acquired orally and the mechanisms involved in the absorption and transportation of infectivity across the gut wall are therefore critical. Isolated gut loops were created in lambs, massaged to remove intestinal contents (flushed) or left non-flushed, inoculated with cattle BSE homogenate and excised at different time-points. Gut loops were examined by immunohistochemistry (IHC) for disease-associated prion protein (PrPd), and the contents were analysed by Western blotting (WB) to determine the degradation rate of protease-resistant PrP (PrPres). The contents of scrapie-inoculated gut loops from a previous experiment were analysed by WB, and these in vivo digestion results were compared with those of an in vitro experiment on the same transmissible spongiform encephalopathy homogenates. BSE-inoculum-derived PrPd was detected by IHC in the gut lumen between 15 min and 3.5 h. It was found in the intestinal lymphatic system from 30 min onwards and was present at the highest frequency at 120 min post-inoculation.

In vivo degradation of PrPres in the BSE inoculum had a significantly (P<0.006) different pattern compared with scrapie-derived PrPres, with the BSE PrPres degrading more rapidly. However, the overall amount of degradation became similar by 120 min post-challenge. The results of the in vitro digestion experiments showed a similar pattern, although the magnitude of PrPres degradation was less than in the in vivo environment where absorption could also take place. BSE-derived PrPres is less protease resistant than scrapie PrP over a short time-course and the disappearance of detectable PrPres from the gut lumen results from both absorption and digestion by intestinal contents.

INTRODUCTION

Bovine spongiform encephalopathy (BSE) and scrapie are neurodegenerative diseases that affect primarily cattle and sheep, respectively. They belong to the transmissible spongiform encephalopathies (TSEs), a group of invariably fatal diseases (Prusiner, 1998) that also includes, among others, sporadic Creutzfeldt–Jakob disease (CJD) and variant CJD (vCJD) of humans (Will, 2003) and chronic wasting disease (CWD), which affects several deer species (Collinge, 2001). TSEs are characterized by the abnormal post-transcriptional conversion of the cellular prion protein (PrPc) into disease-associated isoforms (PrPd), which accumulate mainly in the central nervous system and, depending on the host species and the TSE agent involved, also in the lymphoreticular system (Collins et al., 2004). Immunohistochemical (IHC) detection of PrPd in tissues, or by laboratory methods including a protease digestion step [to detect protease-resistant PrP (PrPres)], are currently the most common approaches for a definitive diagnosis of any TSE (Gavier-Wilde´n et al., 2005).

TSEs can often be transmitted experimentally between species by parenteral inoculation, oral dosing or both (Manson et al., 2006), although natural BSE, scrapie and CWD are presumably acquired by the oral route (Kimberlin & Wilesmith, 1994; Hoinville, 1996; Press et al., 2004; Will, 2003). Similarly, ingestion of BSE-contaminated bovine products is accepted as being the cause of vCJD (Bruce et al., 1997). Therefore, a critical step in transmission during natural infections is likely to be the uptake and translocation of the infectious agent from the alimentary tract prior to its
dissemination through the different systems and tissues of the host. We have shown previously by IHC that sheep scrapie brain homogenate introduced into isolated gut loops of genetically susceptible sheep resulted in PrPres entering the villous lacteals and submucosal lymphatics within 15 min, and remained detectable in those structures up to 3.5 h post-inoculation (p.i.) (Jeffrey et al., 2006). Additionally, PrPres was detected in association with dendritic-like cells in the subcapsular sinuses of the draining lymph nodes for up to 24 h post-challenge.

It was shown in the same study that, when samples of the sheep scrapie inoculum were successively incubated in vitro with ruminal, abomasal, biliary and small intestinal fluids for a total of approximately 20 h, only trace amounts of PrPres could be detected by Western blotting (WB). This suggested that, under in vivo circumstances, the majority of abnormal PrP within the inoculum would readily be digested. Previous studies that have investigated the effect of alimentary tract fluids on the levels of PrPres have been contradictory. One study, in which brain homogenate from a sheep infected with scrapie was exposed to bovine ruminal fluid for 24 h, found no reduction in PrPres signal when examined by WB (Nicholson et al., 2007). Conversely, Scherbel et al. (2006) found almost complete loss of WB PrPres signal when a homogenate of scrapie-positive hamster brains was exposed to either bovine ruminal or colonic contents for 20 h. Similarly, in rodents, a loss of 95% of PrPres has been detected on transit of scrapie-infected whole-brain homogenate through the alimentary tract (Krüger et al., 2009). These different results could be due to the variability between experiments with respect to the species of origin of the inocula, the gastrointestinal fluids used for digestion, the species challenged, the use of different antibodies to detect abnormal PrP, or other factors.

Scrapie has been recognized for over 200 years (M’Gowan, 1914), but there is no evidence that it transmits naturally to any species other than sheep and goats (Wells, 2003), which suggests that there is a strong species barrier preventing infection spreading from sheep to humans. Scrapie in sheep also has a well-defined genetic basis of susceptibility to natural infection (Goldmann, 2008). However, BSE, apart from infecting cattle, has crossed several species barriers and naturally infected humans, cats and several ruminant species (Jeffrey et al., 2001; Kirkwood & Cunningham, 1994; Will, 2003). Why BSE is different from scrapie in this regard is uncertain, but how the agent crosses mucosal barriers and initially enters the body may be critical. The aims of this study were to investigate: (i) the early alimentary pathogenesis of BSE infection in respect of uptake and transport across the ileal mucosa of genetically susceptible sheep; and (ii) the resistance of cattle BSE agent to digestion by intestinal contents in vivo, and the validity of mimicking this digestion in vitro. This paper also benefits from the comparison between the BSE and scrapie agents by extending some of the experiments already published for scrapie infection in sheep (Jeffrey et al., 2006).

RESULTS

Contents of gut loops (four adjacent gut loops of ileum/distal jejunum were created in each animal as shown in Fig. 1) inoculated with normal brain homogenate and analysed by WB (as outlined in Methods) were all found to be negative for PrPres. As loops containing TSE homogenate and normal brain were adjacent to one another, this showed that the surgical techniques used were effective in preventing transit of the inocula between loops, giving confidence that both IHC examinations for PrPres and WB calculations of residual PrPres were valid.

Translocation of PrPres from the intestinal lumen to the intestinal mucosa

Mild oedema of intestinal villi and fluid and cellular infiltration of lymphatics were observed on examination of histological sections of gut stained with haematoxylin and eosin (H&E). These changes were similar to those described in a previous study (Jeffrey et al., 2006) using scrapie homogenate.

Specific immunolabelling for PrPres using R145 antibody showed the presence of non-macrophage or dendritic cell-associated, granular immuno-reactive material in the lumen of the BSE-inoculated gut loops. However, it did appear to be adherent to the surface of villous enterocytes (Fig. 2a). Specific labelling was also present within villous lacteals of the lamina propria and submucosal lymphatics (Fig. 2b, c) at different time-points p.i. in both flushed (i.e. massaged to remove intestinal contents) and non-flushed gut loops (Table 1). Inoculum containing PrPres in the gut lumen was detected with a similar frequency throughout the experiment. However, detection of PrPres in the lymphatic compartments began at 30 min p.i., was most frequent at 2 h p.i. and was still detected at 210 min (Table 1).

![Fig. 1. Ileal gut-loop compartments were produced using double ligatures at 10 cm intervals and inoculated in the order shown with BSE or normal bovine brain (NB) homogenates.](image-url)
At the time-points studied, PrP<sup>d</sup> was not seen in any of the Peyer’s patch compartments (dome, secondary follicles or interfollicular T-cell areas) or in the draining jejunal lymph nodes. All gut compartments from loops inoculated with negative-control homogenate were devoid of PrP<sup>d</sup> immunolabelling.

**Kinetics of degradation of PrP<sup>res</sup> in gut contents incubated with cattle BSE inoculum**

To investigate the kinetics of degradation of PrP<sup>res</sup> in gut contents incubated with cattle BSE inoculum, BSE and control inocula were incubated within the gut loops for either 15 min (four loops; see Methods for details), 30 min (two loops), 120 min (four loops) or 210 min (two loops) before surgical removal and analysis (see Methods). The reduction in WB PrP<sup>res</sup> signal in the contents of non-flushed gut loops varied between 0 and 98 % when compared with ‘time zero’ (0 min) samples. The individual gut sample results were: (i) at 15 min, 34, 86, 96 and 98 %; (ii) after 30 min, 64 and 96 %; (iii) at 120 min, 0, 83, 98 and 98 %; and (iv) after 210 min, 0 and 94 % (Figs 3a and 4a). In the contents of flushed gut loops, the reduction of PrP<sup>res</sup> signal varied between 40 and 77 %, compared to ‘time zero’ samples. Individual results were: (i) at 15 min, 42, 61 and 65 % (one sample of gut-loop contents was unsuitable for analysis); (ii) at 30 min, 40 and 57 %; (iii) at 120 min, 58, 62, 65 and 77 %; and (iv) at 210 min, 63 and 70 % (Figs 3b and 4b).

Flushed gut-loop contents appeared to show lower PrP<sup>res</sup> signal reductions on average than non-flushed gut loops (Fig. 4). However, because the variability in PrP<sup>res</sup> signal reduction was substantial, there was no evidence of any statistically significant difference between flushed versus non-flushed, the time of sampling or the interaction of these two parameters with respect to the amount of PrP<sup>res</sup> present.

**Residual PrP<sup>res</sup> in gut loops incubated with the scrapie agent**

When WB analyses were conducted on the contents of scrapie-challenged non-flushed gut loops, there appeared to be little or no reduction in PrP<sup>res</sup> signal at either 30 min (0, 0, 28 and 25 %) or 90 min (0 and 28 %). For samples taken 210 min p.i., PrP<sup>res</sup> signal reductions were 0, 59, 61, 70, 71 and 77 % (Figs 3c and 4a). Unlike the BSE-challenged animals, each of these gut-loop samples was from an individual sheep. Similarly, PrP<sup>res</sup> signal reductions in flushed gut-loop contents were: (i) at 15 min p.i., 0, 0, 3, 19 and 21 %; and (ii) at 120 min, 0, 55, 73, 76 and 91 % (Figs 3d and 4b). The two samples obtained 24 h (1140 min) p.i. showed complete elimination of signal (Fig. 3d).

Unlike BSE experiment-derived samples, the contents of gut loops inoculated with scrapie brain homogenate showed progressive reduction of PrP<sup>res</sup> signal over the incubation time, the degree of which appeared to be independent of the flushing or non-flushing treatment (Fig. 4). Although there was no statistically significant difference between flushed and non-flushed gut loops, there was strong evidence (P<0.01) of differences in mean

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**Fig. 2.** R145 IHC labelling (brown pigment) of BSE-challenged loops of ileum. (a) A single cell attached to the surface of a villous enterocyte shows intracellular PrP labelling. Bar, 60 μm. (b) Cell debris and loose amorphous material is present within mucosal lymphatics. A dispersed, granular PrP labelling is present within this material. Bar, 40 μm. (c) Submucosal lymphatic surrounding Peyer’s patches showing PrP labelling. Bar, 22 μm.
response over time, with the first three sampling times having low estimated mean percentage reductions (9, 13 and 14 %, respectively) and the two later time-points having much higher percentage reductions (59 and 56 %, respectively; Fig. 4).

**Combined analysis of BSE versus scrapie PrP<sup>res</sup> depletion from gut loops**

There was strong evidence \( P<0.006 \) of a difference in the pattern of change over time with respect to the amount of PrP<sup>res</sup> within gut loops between the BSE and scrapie groups. The estimated mean percentage reduction in the BSE group was around 65 % at all time-points considered, whereas the mean percentage reductions in the scrapie group were lower at the first two sampling times (9 and 13 %, respectively) compared with the last two sampling times (59 and 56 %, respectively), suggesting that BSE disappears from the gut lumen more rapidly than scrapie within the initial 30 min.

**Direct comparison of BSE versus scrapie gut-loop samples at 15 min versus 120 min**

In general, for the BSE-inoculated animals, the percentage reductions in PrP<sup>res</sup> in gut-loop samples were similar at 15 and 120 min. In contrast, four out of the five scrapie-inoculated animals showed a substantial change between the two time-points. However, two animals showed atypical results [lamb ID 45 in the BSE group (Table 1) and lamb ID 1287 in the scrapie group (Jeffrey et al., 2006)]. There was evidence \( P<0.05 \) that the changes in percentage reduction of PrP<sup>res</sup> between 15 and 120 min differed significantly between the groups, with the scrapie group generally having larger changes in percentage

**Table 1. Synopsis of methods and IHC and WB results on gut loops inoculated with BSE homogenates**

GLum, Gut lumen; LPLc, lamina propria lacteals; SMLm, submucosal lymphatics. IHC results are scored as: +, positive; −, negative; ?, inconclusive. WB results are expressed as percentage signal reduction compared with ‘time zero’ equivalent; NA, not available.

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**Fig. 3.** Western immunoblots of BSE non-flushed (a), BSE flushed (b), scrapie non-flushed (c) and scrapie flushed (d) in vivo samples. Left panels show ‘time zero’ (0 min) titrations and right panels show the corresponding percentage reduction of PrP<sup>res</sup> signal of gut-loop contents over the various time-points. Only some individual gut-loop samples are shown.
reduction between these two times compared with the two BSE groups. This again suggests that BSE disappears from the gut lumen more rapidly than scrapie within the initial 30 min.

**WB analysis for PrPc**

During extraction of PrP from the samples of gut-loop contents, detergents such as N-lauryl sarcosine and NP-40 were used to dissociate proteins from organic matter. Even prior to proteinase K (PK) treatment, WBs of these extracts revealed a profile and molecular mass identical to that of PrPres. This suggests that all PrPc within the samples of gut-loop contents had either been solubilized by the detergents used in the extraction process or digested by gut enzymes. Therefore, total PrP analysis (PrPc + PrPres) was not possible even in ‘time zero’ samples.

**In vitro degradation of PrPres from cattle BSE, ovine BSE and sheep scrapie brain homogenates**

The cattle BSE PrPres showed faster degradation after 15 min of in vitro incubation with sheep-gut contents compared with sheep scrapie (46 and 14 %, respectively; Fig. 5a, b). While the cattle BSE inoculum did not show any further PrPres signal reduction, the sheep scrapie homogenate did show increased degradation, to 37 % reduction after 210 min incubation (Fig. 5a). These results were similar to those of the in vivo study, although overall the levels of degradation were lower under in vitro conditions. The ovine-derived BSE homogenate showed little or no PrPres degradation over the same periods of time (0 % at 15 min and 6 % at 210 min; Fig. 5c).

**DISCUSSION**

The process of translocation and removal from the sheep-gut lumen of PrPd present in a cattle BSE homogenate observed in this study is consistent with that found previously using a sheep scrapie inoculum (Jeffrey et al., 2006). In both experiments, PrPd from the inoculum appears to be absorbed rapidly, reaching the lacteals and submucosal lymphatics within 30 min, despite some residual inoculum being still detectable in the lumen by 2–3.5 h. The lymphatic localization of the absorbed inoculum suggests a transit through or between the villous epithelial cells, as has been shown for other aggregated amyloid products such as βA4 protein (Ano et al., 2008). From those lymphatic vessels, PrPd, and presumably infection, would be distributed systemically via the haemolymphatic system.
Neither BSE- nor scrapie-derived PrP<sup>d</sup> appeared to be translocated preferentially across the follicle-associated epithelium (FAE) of the dome, as no detectable PrP<sup>d</sup> was found in M cells, other epithelial cells of the FAE, dendritic cells in the dome or cells of the Peyer’s patches during the first hours after intra-intestinal inoculation. This is in contrast with the results of some studies in rodents orally dosed with the 263K scrapie strain; in these, early replication of infectivity in secondary follicles of the Peyer’s patches was observed (Bergström et al., 2006), suggesting that the M cell/dendritic cell/germinal centre pathway might provide an important portal of infection. However, other studies performed with the same rodent-adapted scrapie strain did not show early involvement of the Peyer’s patches (Beekes & McBride, 2000, 2007) or showed involvement of M cells only at late stages of disease (Krüger et al., 2009). These differences could be due primarily to the dose of agent, so that high doses might overwhelm the gut mucosal barrier and result in unnatural routes of absorption or transcytosis. In cattle BSE, for example, Peyer’s patches are not involved in natural infection and only accumulate PrP<sup>d</sup> after oral administration of a high dose of infectious brain homogenate (Terry et al., 2003). This suggests that, after natural exposure with a ‘normal’ infectious dose, infectivity may enter lacteals following a non-selective transportation pathway across the villous epithelium.

The results of the WB analyses also provide broad support for previously published data, insofar as most experiments confirmed digestion of PrP<sup>res</sup> by alimentary tract enzymes (Krüger et al., 2009; Scherb et al., 2006). In our experiments, PrP<sup>res</sup> digestion was significantly faster and more complete at early time-points in BSE homogenates than in scrapie ones; these findings are consistent with previous studies that suggest that BSE PrP<sup>res</sup> is more protease sensitive than scrapie-derived PrP<sup>res</sup> (Kuczius & Groschup, 1999). This notion is supported further by the IHC results, as both the frequency and magnitude of subjectively assessed IHC signals for transcytosed cattle BSE PrP<sup>d</sup> in this study were substantially lower than the signals detected in similar previous scrapie experiments (Jeffrey et al., 2006). The in vitro digestion results again confirm this notion, as similar degrees of BSE PrP<sup>res</sup> degradation (46%) were found at 15 and 210 min p.i., being higher than those of scrapie PrP<sup>res</sup>, which increased from 14% at 15 min to 37% at 210 min. In a previous study, scrapie-derived PrP<sup>res</sup> was seen to degrade almost completely on prolonged exposure (48 h) to a cocktail of alimentary fluids in vitro (Jeffrey et al., 2006), so it is likely that similar results would have been achieved in the present experiments had the samples been incubated for longer than 210 min. The large variability in reduction of PrP<sup>res</sup> at different time-points from individual gut loops seen on WB may have been due to variation in the nature of digestive juices or bacterial flora within the alimentary tract of individual animals. Although some variability of our results may be explained by the use of an in vivo model in which some factors might not be equally controlled between animals, or even between loops of the same animal, variations might also be partially explained by the intrinsic, strain-specific properties of the abnormal PrP and by the interaction of PrP with the gut environment over time.

The lower levels of BSE PrP<sup>res</sup> (highest degradation degrees) found in gut loops incubated in vivo compared with the same homogenates incubated in vitro does not necessarily mean that the in vivo system is more efficient, as the disappearance of PrP<sup>res</sup> in this environment is also due to absorption, as demonstrated by IHC.

The persistence of residual PrP<sup>res</sup> in gut loops incubated for more than 3 h could be due to some of it binding to minerals within the alimentary contents, and therefore not being available for digestion or for transcytosis, resulting in its detection by WB after different incubation times. This would agree with previous studies showing that infectivity readily binds to metals (Flechsig et al., 2001) and that both infectivity and PrP<sup>res</sup> also bind minerals present in soil (Johnson et al., 2006). Hypothetically, PrP<sup>res</sup> might also bind to plant-derived digesta or cellulose in a way that may stabilize some forms of BSE PrP<sup>res</sup>. In some rodent scrapie models, it has been shown that intestinal bacteria may digest PrP<sup>res</sup> to below biochemically detectable levels without impacting on infectivity (Scherbel et al., 2007), and recent experiments (Beekes & McBride, 2007) have concluded that faeces contribute to horizontal contamination. Whether digestion of abnormal PrP has an impact on faecal infectivity or whether hypothetically stabilized PrP<sup>res</sup> forms are biologically relevant are not addressed within this study. However, Jeffrey et al. (2006) showed that, despite high levels of digestion of PrP<sup>res</sup>, two out of two sheep challenged with scrapie brain homogenate and subsequently allowed to develop disease did so with relatively short incubation periods. This again suggests that digestion of PrP<sup>res</sup> by alimentary fluids does not have any effect on infectivity, which may also be the case with BSE.

**METHODS**

**Animals, surgical procedures and inocula.** New Zealand-derived, 2–3-month-old scrapie-free Suffolk lambs (n=12) of the susceptible PRNP genotype ARQ/ARQ (genotypes are expressed as the single-letter amino acid code at codons 136, 154 and 171 of PrP) were starved overnight before induction and maintenance of general anaesthesia with halothane gas. A mid-line laparotomy incision allowed exposure of the gut and the location of the ileo-caecal junction. In some lambs, the small intestine was massaged gently to remove the gut contents (flushed) such that the walls of the intestine stuck to each other, showing that nearly all liquid and solid material was absent. In others, the gut contents were retained (non-flushed) such that at the end of preparation (see below) each gut loop appeared half full (approx. 10 ml content). Four adjacent gut loops of ileum/distal jejunum were created in each animal by placing double ligatures at 10 cm intervals, in a way that the arterial blood supply and the venous and lymphatic drainage via the mesenteric vessels were minimally compromised, as described previously (Jeffrey et al., 2006) and shown in Fig. 1. All experimental protocols were approved.
In order to extract proteins from the contents of both BSE- and scrapie, provided the 'time zero' equivalent. Once prepared, these samples were then either treated with proteinase K (PK) (BDH) or not treated in order to further determine total PrP versus PrP\textsuperscript{\textnormal{res}}. Briefly, 300 μl supernatant was aspirated into each of two tubes [one tube contained 6 μl PK (2 mg ml\textsuperscript{-1}) to give a final concentration of 50 μg ml\textsuperscript{-1} when the sample was added] and incubated at 37 °C for 1 h. The duplicate tube without PK was kept at 4 °C. To both sets of samples 3 μl Pefabloc (100 mM; Roche) was added to inhibit any further PK activity and maintain the respective dilutions. Samples were subjected to centrifugation at 22 000 g for 30 min at 4 °C and the resultant pellets were resuspended in 30 μl 0.1% N-lauryl sarcosine. An aliquot (5 μl) was removed to allow determination of total protein concentration (see below) and the remaining sample was denatured by diluting 1:1 with SDS sample buffer (Invitrogen) and incubated at 100 °C for 20 min.

To optimize samples for comparison of levels of PrP, the total protein concentration was determined using a BCA protein assay kit (Pierce) and equalized by dilution prior to addition of sample buffer. Protein levels were adjusted to the same level as the appropriate ‘time zero’ sample (flushed or non-flushed, BSE or scrapie, as required). This allowed loading of the same amount of protein into each well of a 12% SDS-PAGE gel (NuPage system; Invitrogen) to enable quantification of PrP signal.

Finally, WB and densitometric analyses were performed as follows. The gut-loop samples (test samples) and the ‘time zero’ samples were titrated by making double dilutions and separated by 12% SDS-PAGE. The resultant gels were immunoblotted and probed with antibody 6H4 (anti-PrP monoclonal antibody; Prionics) diluted 1:5000 with Tris-buffered saline with 0.1% Tween 20. Signal was detected using Super Signal West Femto maximum sensitivity substrate (Pierce) and a Kodak IS440 image station (Labtech International). The intensity of labelling of each PrP\textsuperscript{\textnormal{res}} band was measured by determining the area under each curve produced by the PrP\textsuperscript{\textnormal{res}} signal using Kodak Digital Science 1D Software (Labtech International). A standard curve of the titrated ‘time zero’ sample was plotted (Microsoft Excel 2002 XP) and the correlation coefficient (R²) was rejected if it was less than 0.94. The signal intensities of the test samples from the 15, 30, 90, 120 and 210 min and 24 h time-points were determined from the standard curve and expressed as a percentage reduction in signal from the ‘time zero’ sample value.

**In vitro model of intestinal PrP digestion.** Gut contents were collected from the ileum of a scrapie-free sheep and mixed 1:1 with 10% brain homogenates of either cattle BSE, ovine-derived BSE or natural sheep scrapie in polystyrene bijouxs (Sterilin). A ‘time zero’ sample was taken immediately after the mixture was prepared and samples were then incubated at 39 °C with gentle agitation and aliquots were taken at 15, 30, 120 and 210 min. All samples were stored at −20 °C until analysed by WB, which was performed by methods identical to those described above.

**Statistical analyses.** Because of the different data structure between the BSE and the scrapie groups, linear mixed models were initially fitted separately to the BSE and scrapie groups. Time, treatment (flushed/non-flushed) and the time \times treatment interaction were included in the models as categorical fixed effects, and the animal was included as a random effect. The parameters in each model were estimated using the REML directive in Genstat (12th edn; VSNI). These estimates are based on a small number of animals and the data contain outliers (the zero observations), so the results of the analyses should be interpreted with care. However, there was little evidence in either the BSE- or the scrapie-inoculated groups of any marked effect of flushed versus non-flushed and a linear mixed model was fitted to the combined data (omitting the two observations from 90 min p.i. for the scrapie animals as no BSE samples were taken at this time). Challenge, time and the challenge \times treatment interaction were included in
REFERENCES


