Characterization of the effect of heat on agent strains of the transmissible spongiform encephalopathies

Robert A. Somerville and Nicola Gentles

Neuropathogenesis Division, The Roslin Institute and Royal (Dick) Veterinary School of Veterinary Studies, University of Edinburgh, Roslin, Midlothian, EH25 9PS, Scotland, UK

The causal agents of the transmissible spongiform encephalopathy (TSE) diseases, sometimes called prion diseases, are characterized by high resistance to inactivation with heat. Results from thermal inactivation experiments on nine TSE strains, seven passaged in two PrP genotypes, showed differences in sensitivity to heat inactivation ranging over 17 °C. In addition, the rate of inactivation with increasing temperature varied between TSE models. In some cases passage in an alternative PrP genotype had little effect on the resulting inactivation properties, but for others the infectious agent was inactivated at lower temperatures. No strain with higher thermostability properties was selected. The effect of mixing two TSE strains, to see whether their properties were affected through interaction with each other, was also examined. The results showed that both strains behaved as expected from the behaviour of the unmixed controls, and that the strain responsible for inducing TSE disease could be identified. There was no evidence of a direct effect on intrinsic strain properties. Overall, the results illustrate the diversity in properties of TSE strains. They require intrinsic molecular properties of TSE agents to accommodate high resistance to inactivation and a mechanism, independent of the host, to directly encode these differences. These findings are more readily reconciled with models of TSE agents with two separate components, one of which is independent of the host and comprises a TSE-specific nucleic acid, than with models based solely on conformational changes to a host protein.

INTRODUCTION

The transmissible spongiform encephalopathies (TSEs) include as their archetype scrapie in sheep and goats, bovine spongiform encephalopathy (BSE), chronic wasting disease (CWD) affecting three North American cervids and various forms of Creutzfeldt–Jakob disease (CJD) in man. Their causal infectious agents remain poorly characterized, although a structural role for the host glycoprotein PrP is generally accepted (Somerville, 2002). TSE agents are characterized by high resistance to inactivation with heat and other physical and chemical treatments. This is manifest by survival of TSE infectivity after autoclaving (Kimberlin et al., 1983; Taylor et al., 1998, 1994, 2002) and dry heat treatments (Brown et al., 1990; Taylor et al., 1996). TSE-agent strains differ in their heat inactivation properties i.e. their thermostability. The causal agent of BSE may be particularly thermostable, allowing it to survive rendering and end up in meat and bone meal that was incorporated into cattle feed (Taylor et al., 1995, 1997; Wilesmith et al., 1988, 1991).

Characterization of the kinetics of heat inactivation with respect to time (Kimberlin et al., 1983; Somerville, 2002) shows rapid reduction of infectivity, followed by survival of a subpopulation which declines little with increasing time. The rate of inactivation with respect to increasing temperature under optimal experimental conditions shows little inactivation at temperatures below approximately 70–90 °C, depending on TSE strain; above these temperatures the amount of infectivity declines rapidly (Dickinson & Taylor, 1978; Kimberlin et al., 1983; Rohwer, 1984; Somerville et al., 2002). These findings led to the development of a two-component model of TSE agents (Somerville et al., 2002). This complements and supports the virino hypothesis (Farquhar et al., 1998), which proposes that TSE agents comprise a host-independent informational molecule, presumably a nucleic acid, bound to and protected by a host protein molecule, PrP. By contrast these findings are not yet adequately accommodated by current versions of the prion hypothesis, which proposes that PrP assumes abnormal conformations, some of which become infectious. However recent demonstrations of apparent de novo generation of TSE-like infectivity from recombinant PrP and minimal other non-agent-specific cofactors, have provided strong support to the

Six supplementary figures, two supplementary tables and supplementary materials and methods are available with the online version of this paper.
protein-only hypotheses (Deleault et al., 2007; Makarava et al., 2010; Wang et al., 2010).

Here we report a survey of heat-inactivation properties of nine mouse-passaged TSE strains passaged in two congenic mouse lines carrying the Prnp<sup>aa</sup> or Prnp<sup>bb</sup> genotypes of PrP. We also examined how mixtures of two TSE-agent strains behaved and could be distinguished after thermal inactivation.

**RESULTS**

**Thermostability properties of TSE models**

The TSE strains used in these experiments were derived from four sources: natural scrapie sources, an experimental scrapie sheep brain pool source (SSBP/1), the drowsy goat source (Dickinson, 1976) and BSE (Bruce et al., 1994). Their derivations are shown in Fig. 1 and described in detail in Supplementary Materials (available in JGV Online). Eighteen combinations of TSE strain and mouse strain (PrP genotype) were heated over a range of temperatures, resulting in reduced amounts of TSE infectivity, which was measured by observing the increase in incubation period and converting this into equivalent titres (Fig. 2a–d). These data were fitted to curves from which the initial titre (top), mid point of the reaction (X<sub>c</sub>) and rate of inactivation (k) at X<sub>c</sub> are calculated (Fig. 2e).

**Strains derived from natural scrapie: ME7 and 87A.** ME7: two passage lines of ME7 (ME7-S pass line (thermo-Stable) and ME7-L pass line (thermo-Labile) were established from ME7 exposed to dry heat at 160 °C and used as sources of inocula for this series of experiments (Fig. 1a). Five ME7 samples (samples 1–5) from the same passage line (line ME7-S) have similar thermostability properties, whether passed in SV or VM mice (Fig. 2a). This includes the original experiment in which ME7/SV and ME7/VM were assayed, and ME7 in the brains of three mice from these assays that were re-heated: an ME7/SV sample heated to 95 °C, an ME7/VM sample heated to 95 °C and an unheated ME7/SV control (Fig. 3a). These results demonstrate the reproducibility of the heat inactivation curves. Furthermore, no change in thermostability properties was detected by analysing heat-treated samples. However ME7 from passage line ME7-L had significantly lower thermostability (P<0.003 for all four pairwise comparisons; Supplementary Table S2a, available in JGV Online). ME7 samples in the mixture experiment all behaved similarly (see below). The rates of inactivation appear similar.

A proportion of animals had incubation periods beyond the end point of the dose–response curve for both ME7-S and ME7-L. Incubation periods of heated samples that were longer than those of the equivalent control doses have previously been observed with ME7 (Dickinson & Fraser, 1969; Taylor et al., 2002) and other strains (Taylor & Fernie, 1996). Whether this is because of the presence of a strain with high thermostability and long incubation period but low titre in the donor brain, or results from the change(s) in properties of the agent such that it becomes more thermostable but with longer incubation periods remains to be determined. Passage of ME7-S in VM mice did not affect thermostability measurements significantly, i.e. there was no measurable effect because of PrP genotype (Fig. 2a).

**Strains derived from SSBP/1: 22C, 22A and 22F.** 22C: the data from the 20, 65 and 70 °C samples from the 22C/VM experiment are not available. Nevertheless, the curve obtained from the data above 75 °C is similar to that obtained from 22C/SV, albeit with very wide confidence intervals. No effect arising because of the PrP genotype was found (Fig. 2b). No differences in lesion profiles between heated and unheated samples were found (Supplementary Fig. S3, available in JGV Online).

**Strains derived from the drowsy goat source.** 79A and 139A: the four models using 79A and 139A in SV and VM mice were the most thermostable examined (Figs 2c and 2e). 79A/SV and 139A/SV were very similar. The 79A/VM model was significantly more thermolabile than 22A/VM (P<0.02). 139A/VM was thermostable but with longer incubation periods remains to be determined. Passage of ME7-S in VM mice did not affect thermostability measurements significantly, i.e. there was no measurable effect because of PrP genotype (Fig. 2a).

22F: there is very little change in titre up to 95 °C but a substantial decrease at higher temperatures, giving a very sharp drop in the inactivation curve; the steepest drop observed (Fig. 2b).

**Strains derived from the drowsy goat source.** 79A and 139A: the four models using 79A and 139A in SV and VM mice were the most thermostable examined (Figs 2c and 2e). 79A/SV and 139A/SV were very similar. The 79A/VM model was significantly more thermolabile than the other three models (P<0.003). 139A/VM was inactivated at a significantly slower rate than 79A/SV and 79A/VM (P<0.02). No differences in lesion profiles between heated and unheated samples were found (Supplementary Fig. S4, available in JGV Online).
Fig. 1. Summary of the passage histories of: (a) Natural scrapie, (b) drowsy goat, (c) SSBP/1 and (d) BSE-derived TSE strains. Serial passages were usually performed in C57BL or VM mice at low or high dilutions of inoculum (HD and LD, respectively). Most passage lines included a step in which the inoculum was exposed to 160 °C dry heat or to 3.8 Mrad of ionizing radiation. C57: C57BL/Dk mice, BG: C57BL mice carrying the Beige mutation; VM: VM/Dk mice; i.c., intracerebral.
Strains derived from BSE. 301C and 301V: 301V/VM and 301C/SV were the most thermostable TSE models characterized (Figs 2d and 2e). Samples from the same pass line of 301V (alone or in mixtures) showed similar properties in five separate VM assays (see below); prior digestion with proteinase K had little effect. However, both 301C and 301V strains were significantly more thermostable when passed in the alternative PrP genotype (Bruce et al., 1994), i.e. 301C passed in VM mice (P<0.001) and 301V in C57BL mice (P<0.01) (Fig. 3b). In the mixture experiment (Fig. 4) the 100% 301V/VM sample assayed in VM mice exhibited a similar thermostability curve to that shown in Fig. 3. However, the assay in SV mice showed much more thermostable properties. The lesion profiles of the higher temperature samples are lower than those from lower-temperature samples (Supplementary Fig. S5, available in JGV Online). The effect is most clearly seen in the pooled data for 301C/VM. Similar lower lesion profiles were found in higher dilutions of titrations of both 301C and 301V (data not shown), suggesting that the reduction in lesion profile is because of lower amounts of infectivity in the higher-temperature samples. However, it remains possible that differences in agent strain may contribute to the difference.

Comparison of the thermostability properties of 18 TSE models. In total, the thermostability properties of 18 TSE models have been measured (Fig. 2e). 79A/VM was most thermostable, with $X_u=79 \, ^\circ\text{C}$ and 301V/VM was most thermostable, with $X_u=97 \, ^\circ\text{C}$. Fig. 2(e) illustrates, firstly, that the major difference in thermostability between TSE models is related to strain of TSE agent; differences in $X_u$ between individual models were significant (P<0.05) in 51% of the individual comparisons of the 18 models. Secondly, in some cases there was little or no difference in thermostability properties because of the PrP genotype of the host in which the TSE strain has been replicated (139A, 22C, 22A, ME7-L, ME7-S), but in other cases there is a substantial and significant effect (79A, 301C, 301V).

Thirdly, the length of the bar represents the rate of inactivation with increasing temperature, with shorter bars indicating more rapid inactivation rates. The highest rate of inactivation was found with 22F, while slower rates of inactivation were apparent for several models. Differences in $k$ between individual models were significant (P<0.05) in 27% of the individual comparisons. Significant differences at $P<0.0004$ (135 pairwise comparisons were made; therefore a significance level for individual pairs is $P<0.0004$ according to the Bonferroni test) were found between models for 10% of the comparisons of $X_u$ and 1.5% for $k$. The comparisons between individual models of $X_u$ and $k$ are shown in Supplementary Table S2.

Heat inactivation of mixtures of two TSE strains

The aim of this experiment was to investigate how mixtures of two TSE strains would be inactivated, and whether their properties could be distinguished. ME7-L and 301V were selected because 301V is more thermostable than ME7-L, and they can be distinguished by their incubation periods and lesion profiles in SV and VM mice. ME7 has shorter incubation periods in SV mice but longer incubation periods in VM mice (about 170 days and 350 days for $10^{-2}$ dilution of brain homogenate injected intracerebrally) than 301V (approximately 230 days and 115 days).

Inactivation of mixtures and assay in VM mice. All inactivation curves from the three mixtures are similar to those for 100% 301V when assayed in VM mice, although the 10% 301V sample has a slightly lower thermostability (Fig. 4b). They differ substantially from the curve of 100% ME7-L assayed in VM mice. There is no evidence of ME7-L contributing to the inactivation curve or to the pathology. The lesion profiles are similar to those for 301V (Supplementary Fig. S6, available in JGV Online).

Inactivation of mixtures and assay in SV mice. In this case unheated ME7-L should have a shorter incubation period in SV mice than 301V, and therefore should have dominated the incubation period and pathology until sufficient ME7 was heat-inactivated for 301V to be detectable. The inactivation curves for the mixtures of 90, 50 and 10% ME7-L follow those of 100% ME7-L (Fig. 4b).

Some SV mice (seven in the 90% ME7-L sample, 12 in the 50% ME7-L sample, 16 in the 10% ME7-L sample and 15 in the 0% ME7-L sample) had longer incubation periods than TSE mixtures heated at >100 $\, ^\circ\text{C}$, whereas only two mice succumbed from the 100% ME7-L sample. These groups tended to have lesion profiles similar to those of 301V in SV mice (Supplementary Fig. S6). The lesion profiles of the 100 and 105 $\, ^\circ\text{C}$ groups in the 50% ME7 mixture and the 95, 100 and 105 $\, ^\circ\text{C}$ groups in the 10% ME7 groups resemble those for 100% 301V in SV mice rather than the ME7/SV profiles seen in lower-temperature groups, suggesting that 301V, but not ME7, caused clinical disease in the mice. These data are interpreted as follows: at $\leq 90 \, ^\circ\text{C}$ ME7 survived and because it has a shorter incubation period in SV mice than 301V, an ME7 infection was established. However, little or no ME7 survived at >90 $\, ^\circ\text{C}$, but 301V did survive and infected the mice. Nevertheless it should be borne in mind that ME7 can survive at temperatures above 90 $\, ^\circ\text{C}$ with very long incubation periods and may still be present in these samples.

It is concluded that the relative survival of two TSE strains could be distinguished according to their relative incubation periods and lesion profiles. Although ME7 was identified in the SV mice at $\leq 90 \, ^\circ\text{C}$, 301V is presumed to be present since it survived at higher temperatures. ME7 will have masked 301V because of its shorter incubation period.

DISCUSSION

The remarkable resistance of TSE agents to heat inactivation challenges hypotheses about their structure and compromises methods for sterilization of TSEs. It has been
known for many years that some TSE infectivity can survive autoclaving (Taylor et al., 2000). Differences in thermostability between five TSE strains passaged in SV and VM mice were implied by differences in survival of infectivity after autoclaving (Taylor et al., 2002), and were previously characterized for three TSE models (Somerville et al., 2002). The degree of thermostability reported here for each TSE strain correlates with the differences in survival after autoclaving (ME7-L was used in the autoclaving experiment) and with another autoclaving experiment in which 301V and 22A were compared with the 263K-hamster passaged strain; more 301V survived autoclaving than 22A or 263K (Fernie et al., 2007). Differential survival of TSE agents after partial inactivation or alteration in properties might have implications for the interpretation of field cases of TSEs with respect to understanding the source of infection and differential strain diagnosis.

**Origins of diversity in TSE strain thermostability**

Although TSE strains vary in thermostability, it is theoretically possible that individual strains do not change their thermostability properties. However, it is much more probable that, like other infectious organisms, strains of increased or decreased thermostability can be isolated under appropriate selective conditions. For example, BSE may have arisen in part because a particularly thermostable strain survived rendering. Diversity in heat-inactivation properties is considered here in the context of the derivation of the experimental strains from a variety of sources: a natural sheep-scrapie source, an experimental sheep-scrapie source, an experimental goat-scrapie source and cattle BSE. From all these sources, mouse models were derived by serial passage in two mouse PrP genotypes. Their diversity has been characterized previously by measuring relative incubation periods in a panel of mice differing in PrP genotype, by the amount and distribution of vacuolar pathology in the brain (Bruce et al., 1991), the degree to which PrPSc in the brain is glycosylated and the apparent molecular mass of non-glycosylated PrPSc (Somerville et al., 2005). The present study documents for the first time the great diversity in thermostabilities and demonstrates that differences in thermostability could be used as an additional tool to distinguish between TSE sources.
The results show not only that TSE strains differ in thermostability but that in some cases subpopulations of TSE agent may exist or arise within a single source. Change in properties or the selection of TSE strains with different thermostabilities can also be inferred from the passage histories of each source. This is illustrated in the following four comparisons.

First, SSBP/1 has given rise to 22C, 22A and 22F, which differ considerably in their thermostability. Results from the 22A/VM model suggest that there is a thermolabile, short incubation-period component present. After its inactivation (at $80{\degree}C$) the more thermostable, longer incubation period component is detected and determines the inactivation properties. The thermolabile component is not detected in 22A after passage in SV mice, so inactivation properties are less complex. In contrast to the 22A/VM model, 22F, which was derived from 22A and subjected to high dilution cloning, shows little diversity in properties, suggesting that only one component is involved in determining inactivation properties.

Second, there are three examples (301C, 301V and 79A) in the dataset where change of properties has occurred on passage in an alternative PrP genotype. That this is not due to an intrinsic difference in the thermostability of the different PrP molecules is demonstrated by the reduction in thermostability in 301C on passage in VM mice and 301V on passage in SV mice. Moreover, several of the other strains showed no change on passage in the alternative PrP genotype. In the case of 301V, one explanation for the difference between the 301V/VM and 301V/SV passages is...

**Fig. 4.** Heat inactivation of mixtures of two TSE strains. (a, b) Heat-inactivation curves of five mixtures of ME7 and 301V models assayed in SV or VM mice. (c) Heat-inactivation ranges of the mixtures. Boxes show the range over which 30 to 70% inactivation was measured. Error bars indicate 95% confidence limits. Colour coding for each mixture indicated in (a) and (b) also applies to (c).
the maintenance of different subpopulations of TSE agent, or their formation by mutation; one subpopulation replicates more readily, has a shorter incubation period in VM mice and is more thermostable than another population that is more thermolabile but replicates more readily in SV mice.

Third, in the case of 79A and 139A, there is evidence from other experiments which indicates that one strain can be derived from the other under certain selective conditions (M. E. Bruce, unpublished data); for instance, passage of 139A through spleen led to the selection of 79A (Carp et al., 1997). In the present experiment, 79A/SV and 139A/SV have similar thermostabilities. The population of 79A that had replicated in VM mice had a lower thermostability than the population of 79A in SV mice. With 139A there appears to be a mixture of a high-titre population with low thermostability and a lower-titre population with high thermostability. Other models with shallow inactivation slopes might also indicate the presence of more than one population of agent with differing thermostabilities.

Fourth, it has been reported previously that the first mouse-to-mouse passage using inoculum from a mouse infected with the K.Fu. strain of CJD that had been heated to 80°C was more thermostable than the primary source of infection from human brain (Walker et al., 1983). We attempted to see whether a more thermostable TSE strain could be selected from heated ME7. No change was detected in thermostability after heating. However, serendipitously, we found two pass lines of ME7, which had been established since the ME7 source had been exposed to 160°C dry heat, differed in thermostability. An experiment measuring the thermostability of an ME7 passage prior to the passage of the heated agent strain gave an intermediate \( X_c \) value (Somerville et al., 2002). The simplest explanation is that the 160°C-heat step destroyed most of the infectivity and that the thermostability of ME7 from these two brains may have differed. A more thermolabile clone survived the dry-heat passage to establish the ME7-L line, but a more thermostable clone established the ME7-S line. Alternatively, the source of ME7 used in the mixture experiment had been passaged through mice more often than the ME7 source used in the original experiments, thereby giving an opportunity for a more thermolabile strain to emerge where thermostability was not a selection criterion.

**Implications for the structure and function of TSE agents**

The existence of numerous TSE strains has been used to support the hypothesis that TSE agents contain genetic information encoded by an informational molecule that is independent of the host (Farquhar et al., 1998; Somerville, 2002). In two respects the present data support this hypothesis. They provide a new biophysical measure of intrinsic, structural differences between strains, thereby providing additional evidence of their diversity. The diversity of properties implies the need for a molecular mechanism for encoding such diversity. Current protein-only (prion) hypotheses of TSE agents propose that differences in conformational change to the abnormal conformation that PrP adopts in TSE-infected tissue are sufficient to distinguish strain properties. There are differences in PrP\textsuperscript{Sc} structure, those revealed by Fourier transform–infrared (FT-IR) spectroscopy for example (Caughey et al., 1998; Thomzig et al., 2004). Mechanisms dependent on protein-only replication mechanisms are postulated to be based on the growth of amyloid fibrils whose tertiary structures can vary, with such structures being able to be propagated faithfully and also change to alternative structures, for example when a different polypeptide sequence is available for conversion to the fibrillar structure. There is, therefore, a developing theoretical argument based on the prion hypothesis and on in vitro work with PrP and other amyloid-forming proteins (Baskakov, 2009). However, fundamental questions about how these structures carry discrete, mutable genomic information, interact with the host and maintain their structural integrity during replication and partial denaturation remain to be answered. If it were accepted that there is a host-independent molecule carrying the genetic information (Farquhar et al., 1998; Somerville, 2002), mechanisms for encoding diversity can readily be envisaged. Efforts to identify candidate nucleic acids continue. Recently it has been reported that RNA extracted from TSE-infected hamsters could be combined with recombinant PrP and cause disease (Simoneau et al., 2009). In a separate development, small circovirus-like DNA sequences have been isolated and characterized from three TSE sources (Manuelidis, 2010).

It was established many years ago that TSE strains can mutate (Angers et al., 2010; Bruce & Dickinson, 1987; Kimberlin et al., 1989; Li et al., 2010), so strains with different properties found in the same source might have existed from the origin of the isolate or have arisen by mutation. The data in the present study suggest that subtle changes in properties might occur more often than previously thought. Indeed, rather than assuming high strain stability with mutational change occasionally occurring, it may be more appropriate to consider circumstances in which mutational change occurs at high rates (Kimberlin et al., 1989). Mutational change might affect only thermostability or it might occur concomitantly with changes to other biological measures of strain properties, incubation period and distribution of pathological lesions. The quasispecies hypothesis (Domingo et al., 1997; Vignuzzi et al., 2006) proposes that RNA viruses have a higher mutation rate than that found in other organisms, allowing them to adapt rapidly to different host environments (e.g. different tissues) and have different opportunities for new infection. As an infection proceeds within a host, populations with different pathogenetic functions evolve. High rates of mutational change can be found even...
in the infectious organisms with the smallest RNA genomes, the viroids (Matousek et al., 2004). In the current experiments TSE strain identity is preserved experimentally by maintaining identical conditions for its propagation, thereby continually selecting for constant properties. Opportunities for selection of different subpopulations occurred during serial passage of each TSE strain when samples were passed at high dilution to select only the highest titre subpopulations, when each strain was passed through the alternative PrP genotype and after heating to select the subpopulations which were most thermostable.

Artificial mixtures

Concerns have been raised about the consequences of co-infection with two unrelated strains. For example, what would be the likely fate of a co-infection of sheep with natural scrapie and BSE, and would it be possible to recognize BSE in a background of natural scrapie? Hence the mixture experiment reported above was performed to determine whether such a mixture would lead to confounding biophysical properties of the agent after heating or to biological properties that would be uninterpretable. In the experimental system used, it was clear that the two strains behaved as predicted, with no confounding results. Co-infection of artificial mixtures of TSE strains results in no detectable interference in the replication of the faster strain, although infection at different times with two strains can lead to interference (Dickinson et al., 1972; Nishida et al., 2005). The data here indicate that, as predicted, ME7 in high proportions and at low temperatures induced clinical disease and was responsible for the predominant pathology if ME7 and PrP genotypes (i.e. scrapie incubation period gene) combined as a short incubation period model. However, at higher temperatures 301V can be detected if present in a sufficient concentration in the original mixture. Specifically, the properties of the more thermostable strain, 301V, predominated when passed in VM mice to give the shorter incubation period. No evidence of any effects from ME7 were detected, although it must be presumed that some ME7 was present at the lower temperatures used. When the mixtures were assayed in SV mice where ME7 has the shorter incubation period, ME7-like properties were detected, except for samples in which ME7 had been inactivated, in which case 301V-like properties were detected according to the lesion profiles. Incubation periods were also prolonged, consistent with the presence of 301V. These results suggest that a highly thermostable agent would survive a heating event and could infect animals, but the infection could be masked by co-infection with a TSE agent from another source.

Survival of TSE infectivity when exposed to high temperatures

Very little infectivity was detected after heating to temperatures >115 °C for even the most thermostable of the 18 TSE models characterized. (Data from other experiments where samples were heated to greater temperatures showed only occasional cases.) This finding is consistent with biological properties of other thermostable biological structures and organisms, e.g. thermostable enzymes from thermophilic bacteria (Somerville et al., 2002), but contrasts with the significant amounts of infectivity of some TSE strains that can survive autoclaving, notably those derived from BSE (Taylor et al., 2002). The current experiments were conducted under conditions presumed to be optimal for thermal inactivation and, in particular, where opportunities for dehydration of the sample were avoided. Although there is evidence of relatively highly thermostable subpopulations of agent being present in certain sources in the current experiments, much higher titres of infectivity have survived autoclaving at higher temperatures than used here. Therefore it must be concluded that the structure of the agent might be altered by heat and converted into more thermostable structures so that a much higher proportion of infectivity survives heat under non-optimal inactivation conditions such as autoclaving or exposure to dry heat (Fernie et al., 2007).

Conclusion

The results illustrate, in a novel way, the diversity of TSE strains. They require molecular properties of TSE agents to accommodate high resistance to inactivation and a mechanism, independent of the host, which encodes these differences. It is difficult to imagine how a host protein might accommodate these requirements by itself. Specifically, there is no detailed model based on the prion hypothesis of how conformational differences are structurally defined, replicated conservatively or thermodynamically sustained, e.g. after partial denaturation. The prion hypothesis also does not explain how and why this unique postulated mechanism of information transmission could and should evolve – information that is independent of the host but uniquely dependent on host structures for TSE agent transmission. In contrast, models of agent structures containing two components, one of which is a TSE-specific nucleic acid, are compatible with these data and their interpretation proposed above.

METHODS

Heating and assay of samples. TSE-agent strains (ME7, 22A, 22C, 79A, 139A, 301C and 301V) were passed in SV (Prnp<sup>wa</sup>) or VM (Prnp<sup>wa</sup>) mice. Two strains, 87A and 22F, were passed in C57BL (Prnp<sup>wa</sup>) mice. The passage history of each TSE agent strain is summarized in Fig. 1 and the details given in Supplementary Materials (available in JGV Online). Brains were collected and stored at −70 °C when animals showed clinical signs of TSE disease at a defined end point (Dickinson et al., 1968). Brain homogenates (10% w/v) were made in PBS using a Dounce homogenizer and stored at −70 °C. For heating, samples diluted at 1% (v/v) with PBS, in 0.5 ml aliquots, were transferred to glass test tubes and overlaid with five drops of mineral oil to limit evaporation during heating. Samples were placed in a Multiclav (Baskerville), which was pressurized to 5...
bar (5 x 10^5 Pa) before heating to prevent boiling above 100 °C, and then heated for 10 min at the target temperature. Depending on the target temperature, heating from RT took approximately 2 min, and cooling from the target temperature to below RT using an ice/water bath took <1 min. Samples for the infectivity assay were selected at six temperatures with intervals of 5 °C from between 65 °C and 110 °C, according to the predicted inactivation properties of the TSE model. A RT control was included. Temperature was monitored using a thermocouple in a sample containing control brain homogenate. Groups of six SV or VM mice were injected according to the PrP genotype in which the TSE strain is normally passaged. For example, ME7/SV and ME7/VM were assayed in SV mice; ME7 in the brains of three mice from these assays which were reheated: an ME7/SV sample heated to 95 °C (ME7/SV→heat 95 °C→SV→heat 25–95 °C→SV), an ME7/VM sample heated to 95 °C (ME7/VM→heat 95 °C→SV→heat 25–95 °C→SV), and an unheated ME7/SV control (ME7/SV→heat 25 °C→SV→heat 25–95 °C→SV). ME7 and 301V brain homogenates were also mixed in five ratios: 100:0, 90:10, 50:50, 10:90 and 0:100. Aliquots of each mixture were heated and injected into both SV and VM mice. Mice were injected intracerebrally under general anaesthetic with 20 μl of inoculum without further dilution. They were scored for clinical signs of disease and culled at a defined clinical end point (Dickinson et al., 1968). TSE infection was confirmed histologically. Brain sections were scored to obtain lesion profiles (Fraser & Dickinson, 1967).

Titre calculations. Dose–response curves were obtained from NPU archival data for each TSE model and used to establish biphasic linear regressions using the computer program GraphPad Prism (see Supplementary Fig. S1, available in JGV Online). Individual titres were calculated from incubation periods by using the equations derived from fitted curves.

Data analysis: the data were analysed using GraphPad Prism to obtain heat-inactivation curves. Titre and temperature data were fitted to the inverted form of the logistic equation, known as the Fermi function: 

$$Y = \frac{top}{1 + \exp[k(T - Tc)]}$$

(Peleg, 1996), where top=top of the curve. The bottom value was set to a nominal titre of zero –log10 ID50 as a nominal minimum dose of infectivity.

Very extended incubation periods, which were occasionally found in heated samples, were set to a nominal zero –log10 ID50. For groups in which only a proportion of the animals succumbed to TSE infection, the proportion of TSE-positive animals was used as an approximate estimate of titre instead of incubation period. The equation calculates the top of the curve (top) to estimate the initial titre in unheated samples, the infection point in the curve (Tc) – the temperature at which the TSE strain is inactivated and the slope of the curve (k) – the rate of inactivation with respect to increasing temperature. GraphPad Prism allows pairwise statistical comparison of output parameters. Accordingly Xc and k for each pair of curves was compared and compiled (Supplementary Table S2, available in JGV Online). In the mixture experiment, to compare TSE models with different incubation periods where the TSE strain responsible for determining incubation period was not known, the incubation periods for unheated samples were calculated and subtracted to create relative incubation period curves. These values were used as above to calculate heat inactivation curves.

ACKNOWLEDGEMENTS

The contributions of Karen Fernie and Philip Steele, the NPU animal facility and pathology group and Jill Sales for statistical advice are gratefully acknowledged. Funding from UK Department of Health and Department of the Environment and Rural Affairs is also gratefully acknowledged.

REFERENCES


