Comparative studies on the thermostability of five strains of transmissible-spongiform-encephalopathy agent

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The causal infectious agents of TSEs (transmissible spongiform encephalopathies or prion diseases) are renowned for their resistance to complete inactivation. Survival of TSE infectivity after autoclaving potentially compromises many procedures where TSE infectivity may be present, including surgical instrument sterilization. In the present study, the heat inactivation properties of five different TSE agents were tested in a variety of experiments by exposing them to a range of heat inactivation conditions. Although TSE infectivity was reduced after heating to 200 °C in a hot air oven, substantial amounts of infectivity remained. Unlike wet heat inactivation, no TSE strain-dependent differences were observed in the reduction in the amounts of infectivity produced by dry heat inactivation. However, the incubation periods of mice infected with one dry heated TSE strain, ME7, were substantially prolonged, whereas there was little or no effect for two other TSE models. Varying autoclaving conditions for three TSE strains between 132 and 138 °C, and times of exposure between 30 and 120 min, had little or no effect on the recovery of TSE infectivity. The results illustrate the limitations of TSE agent inactivation using heat-based methods. The results support the hypothesis that the structures of TSE agents are stabilized during heat-inactivation procedures, rendering them much more refractory to inactivation. This may occur through dehydration of the causal agents, specifically through the removal of the water of solvation from agent structures and hence stabilize interactions between prion protein and TSE agent-specific ligands.

Introduction

The TSEs (transmissible spongiform encephalopathies) are a group of infectious diseases that include scrapie in sheep and goats, BSE (bovine spongiform encephalopathy), chronic wasting disease in three species of North American deer and various forms of Creutzfeldt–Jakob disease in humans. The structure of the causal agent has yet to be determined [1]. The virino hypothesis suggests a two-component model for the agent, in which a host-independent informational molecule binds to and is protected by a host protein, probably PrP (prion protein) [2]. Diversity in the intrinsic thermostability properties of different TSE strains also suggests that they must contain at least two structurally dissimilar components. According to the virino hypothesis, one of the components is predicted to be structurally independent of the host and to vary in structure between TSE strains, while the other is likely to comprise dimers or higher multimers of the host protein, PrP [3]. By contrast, the prion hypothesis proposes that a single host-encoded protein PrP alters its conformation and becomes infectious [4].

The inactivation of TSE infectivity has been notoriously difficult. Although heat inactivation can be effective at destroying all TSE infectivity under optimal experimental conditions [5], in many cases residual infectivity can be recovered [6–8] and may compromise routine sterilization procedures. Dry heat treatments are less effective than steam heat [9]. When a series of five TSE strains each passaged in two mouse PrP genotypes were autoclaved, there were wide variations in the degree to which infectivity was reduced, depending on strain of the agent, but with little effect caused by variation of PrP genotype [10]. In some, but not all cases, heated samples had considerably prolonged incubation periods when compared with unheated control samples with the same dose of TSE infectivity [10–12]. Here, we report results from experiments in which heat inactivation has been carried out under various conditions. They were not originally planned as a comparative series, but were performed over several years to test various theoretical and practical aspects of TSE agent inactivation with heat. Conditions for experiments to test the efficacy of porous load and gravity displacement autoclaving (Tables 2 and 3) were chosen which reflected those used in practice and to provide a reasonable worst-case challenge to the system, as understood at the time the experiments were set up. It was

Key words: autoclave, dehydration, dry heat, heat inactivation, transmissible spongiform encephalopathy (TSE) infection and strain.

Abbreviations used: BSE, bovine spongiform encephalopathy; dpi, days post-injection; ID_{50}, infectious dose; PrP, prion protein; TSE, transmissible spongiform encephalopathy.

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possible to vary the conditions, as the machines allowed, to see whether improvements could be recommended. Other experiments were designed to test other aspects of heat inactivation. The collated results demonstrate the limitations of heat inactivation processes to destroy TSE infectivity and are consistent with a model in which 'fixation' of the proteinaceous component of the agent is correlated with resistance to inactivation. Although the 'fixation' of proteins can involve the loss of essential water molecules, it will be argued later that this might even occur during autoclaving, where there is an abundant presence of water. If TSE agents are fully hydrated, inactivation is thought to occur via a denaturation reaction that is TSE strain-dependent [3]. However, after dehydration, the denaturation reaction may not proceed and TSE agent inactivation can only take place through methods that destroy biological molecules. Inactivation may only occur at higher temperatures and with different kinetics through the chemical destruction of components of the agent [1], which are only effective under extreme conditions and show no TSE strain specificity.

Materials and methods

Tissue sources

Rodent passaged TSE strains were further passaged, with strain 263K in LVG hamsters, ME7 in SV mice, and 301V and 22A in VM mice [VM mice carry the *Prnp*^{bb} (*Sinc*^{p7p7}) and congenic equivalent SV mice carry the *Prnp*^{aa} (*Sinc*^{s7s7}) allele of the *PrP* gene]. When signs of TSE infection had reached a defined end point [13], animals were killed, brains removed and frozen at $-20\,^{\circ}$C. Cow BSE brain was from a field case of BSE, generously provided by the Veterinary Laboratories Agency (Weybridge, Surrey, U.K.). Brains were macerated with numerous scalpel cuts to obtain a uniform consistency or homogenized as 10% saline suspensions in Griffiths tubes.

Heat treatment of samples

Dry heat exposure was performed in a Gallenkamp Hotbox oven. For each TSE strain, three samples of 7–10 mg of macerate were weighed on to sterile glass slides and placed into preheated sterile glass Petri dishes. Samples were heated for 20 or 60 min after the oven returned to $200 \pm 2\,^{\circ}$C. Samples were rehydrated before injection into animals by placing dental plugs soaked in sterile water in the Petri dishes overnight.

For the autoclaving experiments, 50 or 375 mg samples of TSE-infected brain macerate were placed within the grinding area of Griffiths tubes. The tubes were angled to face away from each other to minimize risks of cross-contamination. Autoclaving was performed in a Motoclave porous-load autoclave (British Sterilizer Co., Hainault, Essex, U.K.). To ensure that the autoclave was performing efficiently, the experiments in Table 2 were independently monitored by the Common Services Agency (now Scottish Healthcare Supplies, Edinburgh, Scotland, U.K.). This involved performance checks on the autoclave before, during and after the experimental programme. These were all satisfactory. During the experiments, temperatures within the experimental samples and in other areas of the chamber were monitored using recently calibrated thermocouples. After appropriate modification of the controls by an engineer, the same autoclave was used to perform the gravity displacement autoclave run on 50 mg samples of macerate or 1 ml aliquots of 10% homogenate.

To test whether TSE infectivity survived a laboratory cleaning and sterilization regime, 301V infected brain was homogenized in a Griffiths tube, the homogenate was decanted and the empty but unwashed tube autoclaved at $136\,^{\circ}$C for 1 h in a porous load autoclave. Saline (0.9% NaCl; 1 ml) was then homogenized in the tube and tested for residual infectivity. The tube was then washed by soaking in Decon for 2 h, washed with a tube brush, rinsed nine times in cold water followed by continuous rinsing in running cold water for 1 h followed by a final rinse in distilled water, air-dried, packed in autoclavable nylon packaging (Portex; Surgical Supply Services, Cumbernauld, North Lanarkshire, Scotland, U.K.) and autoclaved again at $136\,^{\circ}$C for 1 h; all these steps are according to our standard laboratory procedures. Again saline was homogenized in the tube and tested for residual infectivity.

Inoculation of rodents

Samples were frozen at $-20\,^{\circ}$C until injection. On thawing, saline was added to obtain a 10% (w/v) solution, based on the original weight of the sample. Resuspension of the samples was facilitated and transfer losses avoided by prior placement of the samples into the Griffith tubes. The dry heat samples were homogenized with saline to produce a 1% solution and then serially diluted 10-fold in saline. ME7 was assayed in C57BL mice, 22A and 301V in VM mice, cow BSE in RIII mice, which were all injected intracerebrally with 20 μl of inoculum and 263K in LVG hamsters was injected intracerebrally with 50 μl of inoculum. All injections were performed under halothane general anaesthesia. When clinical signs of TSE infection had developed to a standard end point, animals were killed [13] and TSE diagnosis was confirmed histologically.

Assessment of survival of TSE infectivity

In the experiments reported in Tables 1 and 4, titrations were performed and titres calculated by the Karber method [14]. In the other experiments, a single dilution of inoculum was assayed. This approach does not allow the direct calculation of titre in most cases. However, if complete groups of animals succumb to the infection, the

Table 1 Effect of 200 °C dry heat on TSE infectivity

Brain macerates (7–10 mg) were heated in a dry oven; then, after rehydration, TSE infectivity was measured by titration. ME7 was assayed in C57BL mice, 301V assayed in VM mice and 263K assayed in LVG hamsters by intracerebral injection of serial dilutions into groups of 12 animals and titres calculated from the proportion of TSE-positive cases in each dilution by the Karber method [14].

TSE strain	Time	Titre (–log ID_{50}/g)	Titre reduction
ME7	Control	8.37	–
	20 min	5.35	3.02
	60 min	5.31	3.06
263K	Control	8.33	–
	20 min	5.36	2.97
	60 min	4.97	3.36
301V	Control	8.33	–
	20 min	6.03	2.30
	60 min	5.30	3.03

dose–response curve can be used to calculate titres from the average incubation period unless the dose–response relationship is altered, as is often the case after heating TSE infectivity at high temperatures. Nevertheless, the use of such an approach is often justified, since the number of animals used is reduced. In the experiment described in Table 2, the Poisson distribution could be used to calculate residual titre after autoclaving [15,16], since in all but one case only a proportion of each group succumbed to TSE infection.

Results

Exposure of TSE infectivity to 200 °C dry heat

To determine the effect of TSE inactivation by dry heat treatment on TSE agents, brain samples from three TSE models (ME7 in SV mice, 301V in VM mice and 263K in LVG hamsters) were heated at 200 °C in a 'dry heat' oven for 20 or 60 min, rehydrated and titrated by serial dilution in mice. Titres were reduced by approx. 3 log ID_{50} (infectious dose) after heating of all three samples for 20 min (Table 1), with little additional loss after 60 min (0–0.7 log ID_{50}). There was a marked prolongation of the incubation period of heated ME7 samples compared with the same equivalent doses in the unheated control ME7 sample, the effect being significantly greater after 60 min than after 20 min (Figure 1). There was only a small shift in the 263K dose–response curve on increasing the heating time and no effect on dose–response curves of heated 301V. The survival properties of TSE infectivity exposed to dry heat were not strain-specific, unlike the differences in heat-inactivation properties observed in aqueous solution [3]. However, the interaction with the host of the surviving subpopulation of TSE agent was altered in a TSE strain-specific fashion.

Porous-load autoclaving of TSE infectivity

In contrast with the experimental procedure used to test the effect of dry heat, modern autoclaving programmes are not designed for the conduct of experimental procedures, but are designed to optimally inactivate micro-organisms by exposure to superatmospheric steam. To determine whether TSE infectivity survived autoclaving and what parameters affected survival within the operating range of modern hospital autoclaves and near values recommended for autoclaving TSEs, the residual infectivity after autoclaving a 50 or 375 mg mass of brain macerate was measured for three TSE strains, at three temperatures and four times of exposure, giving a total of 72 autoclaved samples. Infectivity was measured for all samples by injection of a single dilution (10^{-1}) of each sample into groups of 18 mice. One sample (301V) caused TSE infection in all recipients. All other samples resulted in no TSE infection in any recipients or affected a proportion of the animals in the group, suggesting that the amounts of TSE infectivity surviving autoclaving were at or near the limits of detection (Table 2). Because of the structure of the results in this experiment, statistical analysis using binary logistic regression could be performed. The numbers of TSE-positive animals were compared with the total number injected, allowing overall trends from the data to be obtained. The conclusions from this analysis were as follows. Some infectivity was detected after autoclaving for all three strains. Most infectivity was detected in the 301V samples. Although least infectivity was detected in the autoclaved 22A samples, the initial titre was lower, so the measurable clearance was less. Statistical analysis showed that there was a significant difference in titre reduction with respect to the strain of agent (odds ratio 263K/22A = 7.6, 301V/22A = 44.6, 301V/263K = 5.8, $P < 0.001$ for all pairs). Significantly more infectivity survived in the 375 mg samples than in the 50 mg samples (odds ratio 3.8, $P < 0.001$). There was a trend towards a small but inconsistent reduction in residual infectivity with increasing time of exposure, but this was not significant. There was no significant effect owing to increasing temperature: temperature did not alter the effectiveness of autoclaving of 263K and paradoxically showed a slight reduction in effectiveness with increasing temperature with 301V, although this effect too, was not significant.

To determine whether autoclaving affected incubation periods in this experiment, the pooled survival curves for each TSE strain were analysed. They show that the 301V incubation periods are much more tightly distributed than those for the 263K and 22A strains. There was some prolongation of incubation periods in the 263K samples, but only a few incubation periods were markedly extended in the 301V samples (Figure 2). There were some very long incubation periods in the 22A samples, including several animals that were clinically normal at 600 dpi (days post-injection) but

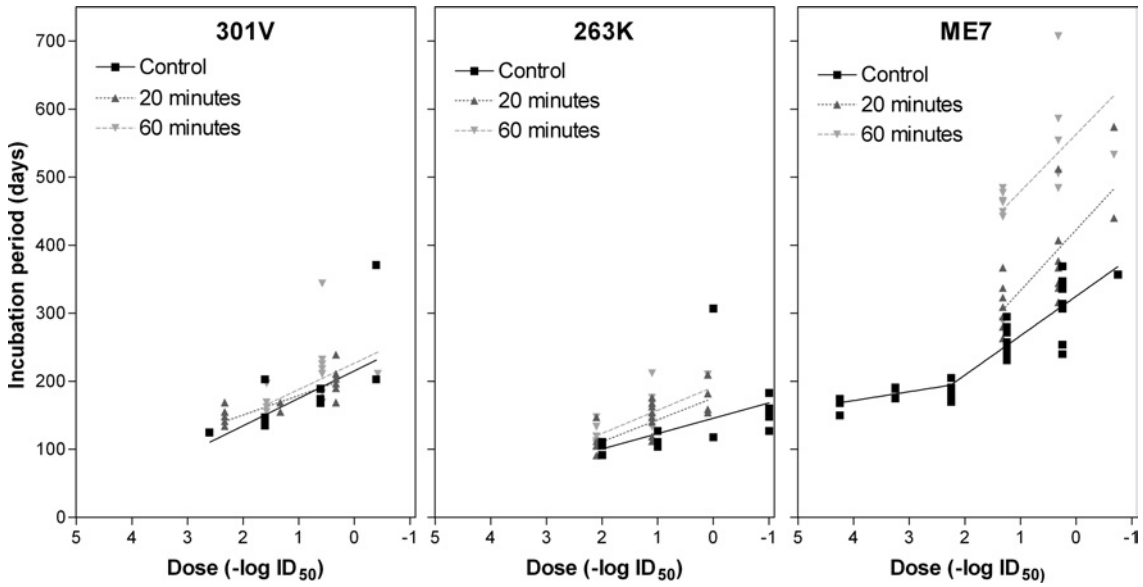

Figure 1 Effect of 200 °C dry heat on TSE dose–response curves

The amount of TSE infectivity in each dilution was calculated and plotted against the incubation period of that dilution for samples before and after exposure to dry heat for either 20 or 60 min. Linear regression lines are shown for each sample. A biphasic line has been fitted to the control ME7 sample.

Table 2 Effect of agent strain, weight of tissue, temperature and time on the survival of TSE infectivity after porous load autoclaving

Macerates of hamster brain infected with the 263K strain or VM mouse brains infected with the 301V or 22A TSE strains (with initial titres of 8.5, 8.5 and 7.0 –log ID_{50}/g respectively) were subjected to porous load autoclaving in lumps weighing 50 or 375 mg, at 134, 136 or 138 °C, for 9, 18, 30 or 60 min. Samples were resuspended in saline at a dilution of 10^{-1} with respect to the weight of the original brain and injected into groups of 18 hamsters or mice. TSE Pos, number of TSE-positive animals; total, number of positive animals plus those surviving beyond the incubation period of the last positive animal in that group. The experiments with the 263K strain were terminated at ⩾641 dpi (days post injection), 301V at ⩾414 dpi and 22A at ⩾471 dpi.

			301V		263K		22A	
Weight (mg)	Temperature (°C)	Time (min)	TSE Pos	Total	TSE Pos	Total	TSE Pos	Total
50	134	9	9	11	5	16	0	16
		18	9	14	2	16	0	15
		30	3	12	1	11	0	12
		60	2	13	2	16	0	12
	136	9	3	14	1	16	0	18
		18	10	17	2	18	0	18
		30	13	18	4	16	0	13
		60	1	17	2	17	0	16
	138	9	6	18	2	17	1	8
		18	18	18	3	15	0	12
		30	7	16	0	14	0	12
		60	10	17	0	16	0	17
375	134	9	14	18	3	13	0	16
		18	13	17	7	10	0	13
		30	8	16	10	14	0	13
		60	7	18	5	15	0	14
	136	9	12	17	10	16	0	12
		18	13	17	12	16	2	15
		30	15	18	5	16	5	13
		60	7	17	5	14	0	13
	138	9	17	17	0	16	4	13
		18	18	18	7	14	3	14
		30	15	18	1	13	0	11
		60	11	18	0	17	1	12

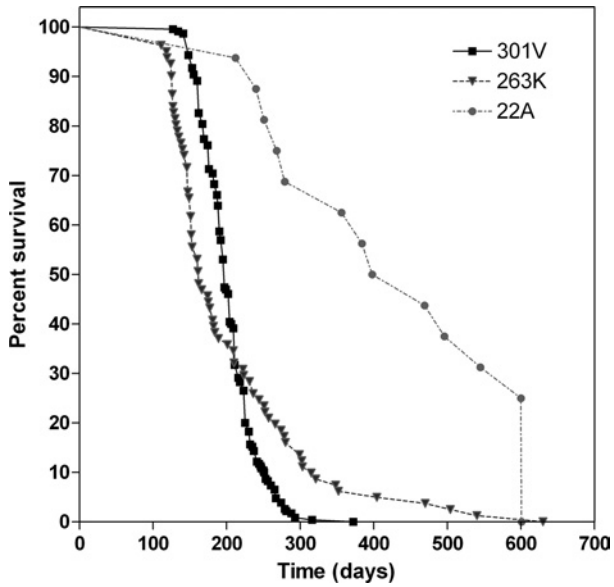

Figure 2 Effect of TSE strain on survival of TSE infectivity

The survival of TSE-positive cases after autoclaving (Table 2) of the 301V, 263K and 22A TSE strains is shown. (Three 22A cases were diagnosed by histological examination only after culling at 601 dpi.)

were diagnosed as TSE-positive from their pathological presentation.

In a separate experiment, the 22A TSE strain was heated as a macerate (titre 7.2 ID_{50}/g) in a hot air oven at 160°C for 1 h and then autoclaved in a porous load autoclave at 134°C for 18 min. Infectivity was detected after hot-air heating at the 10^{-1} dilution (18/18 positive for TSE infection, incubation period = 245.9 ± 6 days) and also after subsequent autoclaving, although the amount of infectivity was reduced (9/16 positive for TSE infection, incubation period = 333.4 ± 18.7 days).

Gravity displacement autoclaving of TSE infectivity

To determine how a typical gravity displacement autoclaving protocol affected TSE agent inactivation properties, the residual infectivity after gravity displacement autoclaving of samples from 301V-infected brain homogenates or macerates treated at 132 and 136°C for 60, 90 or 120 min was measured (Table 3). TSE infectivity was recovered from all samples with little difference in the numbers of cases or the average incubation periods. More infectivity was recovered in a similar macerate sample subjected to porous-load autoclaving, suggesting that gravity displacement may be preferable to porous-load autoclaving.

To determine whether prolonged exposure to autoclaving conditions at lower temperatures than normally used during autoclaving inactivated TSE infectivity, BSE-infected cow brain and 263K-infected hamster brain macerates were autoclaved once or twice at 72°C for 4 h and once or twice at 134°C for 18 min. The results indicate a small reduction in titre after autoclaving BSE at 72°C (Table 4). Analysis of the dose–response curves suggests a reduction of 0.5 logs after the first cycle and a total reduction of 0.6 logs after two cycles for the BSE samples. Since infectivity in the 10^{-4} dilution was not measured and therefore an end point dilution group was not available, the titres may have been slightly higher than the value obtained from the Karber calculation performed on the available data. However, comparison of the dose–response curves of untreated and heated samples shows no difference (results not shown), suggesting that the calculated titres are reasonably accurate and that there may have been a slight reduction in titre after exposure to 72°C. There was a similar small reduction estimated by incubation period comparisons in the 263K samples.

The maximum theoretical reduction in the BSE titre that could be measured after one autoclaving cycle at 134°C was $10^{2.5}$ ID_{50}. The actual measured reduction was $10^{1.9}$ ID_{50} after two sequential autoclaving cycles at 134°C, showing

Table 3 Gravity displacement autoclaving of 301V-infected mouse brain

Mouse brains infected with the 301V TSE strain with an initial titre of 8.5 –log ID_{50}/g were macerated or homogenized. The macerates (50 mg) and homogenates (1 ml of 10%) were subjected to gravity displacement autoclaving at either 132 or 136°C for 60, 90 or 120 min. The macerate samples were homogenized and made 10^{-2} dilution before assay in VM mice. All mice surviving beyond the last positive case in the group and diagnosed as negative for TSE infection were included in the total. Mice were observed until culled for welfare reasons, up to 945 dpi. Mean inc., mean of incubation period.

Tissue preparation	Autoclave technique	Temperature (°C)	Time (min)	TSE Pos	Total	Mean inc. (days)	S.E.M.
Homogenate	Gravity displacement	132	60	10	12	195.4	10.2
		132	90	8	9	186.9	18.5
		132	120	8	12	201.5	9.9
		136	60	6	12	187.8	1.2
Macerate	Gravity displacement	132	60	5	12	206.6	12.2
		132	90	5	12	196.8	16.1
		132	120	9	11	238.9	20.4
		136	60	5	9	247.2	29.1
Macerate	Porous load	136	60	12	12	178.2	10.0

Table 4 Autoclaving once or twice at 72 or 134 °C

Macerates (340 mg) of TSE-infected cow or hamster tissue were heated in a gravity displacement autoclave at 72 °C for 4 h or at 134 °C for 18 min. The macerate samples were homogenized at 10^{-1} dilution of the original brain sample, then subjected to serial dilution and assayed in groups of 12–18 RIII mice for cow BSE and up to 12 hamsters for 263K.

TSE source	Treatment	Titre–log ID_{50}/g	Reduction in titre (log)
Cow BSE	Control titration	4.8	
	72 °C		
	Once	4.3*	0.5
	Twice	4.2*	0.6
	134 °C		
	Once	2.4	2.5
	Twice	3.1	1.7
263K	Control titration	8.3	
	72 °C		
	Once	7.7*	0.57
	Twice	7.9*	0.4
	134 °C		
	Once	4.4	3.9
	Twice	3.2	5.1

*Titres estimated from incubation periods.

that the BSE agent can survive these conditions with relatively small losses in titre. The first exposure to porous-load autoclaving appeared to enhance survival of infectivity during the second run. This result is consistent with findings for the 263K hamster TSE model [8]. Overall the results from these experiments show, at most, very little reduction in titre after the exposure of BSE or 263K infectivity to 72 °C, even after very long exposure times. There is some loss of infectivity after porous-load autoclaving at 134 °C, but substantial amounts of infectivity could still be detected even though the initial measurable titre of BSE was low.

Treatment with ethanol

To assess whether ethanol exposure altered TSE infectivity survival during autoclaving, half brains from 22A-infected VM mice were immersed in saline or ethanol for 48 h, then either subjected to porous-load autoclaving at 134 °C for 18 min or held at room temperature and then assayed for residual TSE infectivity. Ethanol exposure at room temperature had no effect on the incubation period. No infectivity was recovered in the autoclaved sample after exposure to saline, but 17 out of 24 mice succumbed to TSE infection after ethanol exposure, showing that ethanol provides a degree of protection against heat inactivation during autoclaving (Table 5).

Demonstration of survival of infectivity on glassware

Accumulating data have suggested that TSE infectivity, particularly from the more thermostable TSE strains, which includes those derived from BSE, may not be completely inactivated (i.e. beyond the limits of detection) by standard autoclaving procedures. In practice, limitations to heat sterilization of TSEs may leave surgical and laboratory equipment contaminated with significant levels of TSE infectivity after autoclaving. Accordingly, an experiment was set up to test whether infectivity could be recovered from experimental equipment typically used in TSE experiments. 301V–infected brain was homogenized and the homogenizer washed and sterilized according to our standard laboratory procedures. One animal was diagnosed positive for TSE infection in the sample obtained from the homogenizer after autoclaving on the first occasion (Table 6). The diagnosis of this case was confirmed histologically both through the presence of vacuolation characteristic for TSEs and by immunostaining for TSE-specific deposition of PrP.

Table 5 Autoclaving 22A-infected brain after ethanol treatment

Two half brains, infected with the 22A TSE strain, were immersed in saline or 70% (v/v) ethanol for 48 h at room temperature (RT; 20 °C). The half brains were then irrigated for 12 h in continuously running water, then either autoclaved at 136 °C for 18 min or held at room temperature. They were then homogenized in saline at 10:1 dilution and injected intracerebrally into groups of 12 or 24 VM mice.

Sample	TSE pos	Total	Mean inc. (days)	S.E.M.
RT				
Ethanol	12	12	178	3.10
Saline	6	6	178	5.26
134 °C				
Ethanol	17	24	274	7.86
Saline	0	20	–	–

Discussion

Practical considerations

Autoclaves are designed to inactivate infectious organisms by imparting heat energy directly from steam to the surface of the sample. Heat transfer from steam is particularly efficient. The simpler mode of operation is gravity displacement, where steam is fed into the chamber, displacing the heavier air downwards. However, pockets of air may remain in the load. Porous-load autoclaving seeks to purge the air from the autoclave by first evacuating the chamber, then alternately pressurizing and depressurizing the chamber with steam to displace residual air pockets. These autoclaving regimes are highly effective for most micro-organisms. However, they may compromise inactivation of TSEs because the agents may be dehydrated during the evacuation stage and also may only be partially inactivated by the superatmospheric waves step. The residual infectivity may be stabilized and hence not further inactivated when the cycle progresses to the higher target temperature. Simply increasing the

Table 6 Recovery of TSE infectivity from a homogenizer

(a) Sterile saline was injected into VM or RIII mice. (b) Saline (1 ml) was pipetted into a Griffith homogenizer and homogenized as for tissue. It was then transferred to a Bijou bottle and subsequently injected into VM and RIII mice. (c) Mouse brain was homogenized in saline, at a concentration of 10% brain, in the same homogenizer transferred to a Bijou bottle and injected into VM mice. (d) After autoclaving the Griffith homogenizer at 136°C for 1 h, 1 ml of saline was added, homogenized as before and injected into VM mice. (e) After draining residual fluid, the homogenizer was subjected to local standard washing, drying, packing and re-autoclaving. Saline (1 ml) was added, homogenized and injected into VM mice. Inc, incubation period in days.

Sample	Mouse strain	TSE Pos	Total	Comment
Saline control (a)	VM	0	23	
Saline control (a)	RIII	0	22	
Saline wash of clean homogenizer (b)	VM	0	24	
Saline wash of clean homogenizer (b)	RIII	0	17	
301V Homogenate (c)	VM	4	4	Average Inc = 100 days
Autoclaved (d)	VM	1	24	Inc = 204 days
Autoclave + wash (e)	VM	0	23	

temperature e.g. from 134 to 138°C, the limiting temperature on many commercial autoclaves, or increasing the time of exposure is unlikely to substantially increase the degree to which TSE infectivity is inactivated.

Inactivation of TSEs by heating can sometimes be inefficient, as the results in the present paper illustrate. The results show that dry heat can stabilize some TSE infectivity and we suggest that the initial steps of a porous-load autoclave operate similarly. If heat is to be used for inactivating TSE infectivity, then conditions should be optimized to minimize dehydration of the sample before and during the procedure. In addition, the rate of heating may be important – rapid heating of the sample to the inactivation temperature may minimize the conversion of the TSE agents into the stabilized form [3]. The time and temperature obtained when the run conditions are finally attained may be of less importance if the status of the infective material has been previously compromised. Using higher temperatures (within the range normally achieved by hospital and laboratory autoclaves) and/or longer run times may be of little value.

The potential for infectivity to survive a cleaning and autoclaving regime is illustrated by the demonstration experiment reported above (Table 6). Although only one positive case was detected in this instance, the result shows that trace amounts of TSE infectivity can survive routine sterilization procedures, which could result in contamination of subsequent experiments, or the infection of patients during surgery. This result confirms the necessity of following our standard, universal practice that has been in place for many years, namely to only use new, unused equipment for the preparation of all TSE materials for injection. This policy covers post-mortem procedures to recover infected tissue for future passage and all equipment in direct contact with the inoculum during preparation.

Survival of infectivity

The remarkable survival of TSE infectivity under the extreme conditions reported here has suggested to some that normal mechanisms of inactivation of biological molecules may not operate for TSE agents [17]. However, we argue here that survival under even the most extreme conditions is compatible with the properties of biological molecules [3,18]. The results show that small amounts of TSE infectivity can survive temperatures that would be expected to inactivate most biological molecules. Under optimal conditions, TSE infectivity can be completely destroyed (to the limits of detection). For this to occur, the necessary conditions, including the hydration status of the infectious material, the rate of heating and the final temperature, should be optimal. However, under suboptimal conditions, some TSE infectivity often survives much higher temperatures, although usually only a very small percentage of the initial titre survives. Results from various sources show that some TSE infectivity can become stabilized by freeze-drying and dry heat, i.e. where the infective material becomes dehydrated; through wet heat/high pH where the rate of heating is slow; by exposure to organic solvents such as ethanol or where protein fixation occurs by other mechanisms; and in each case the infectively becomes resistant to heat denaturation [8,9, 19–21]. Prior exposure to dry heat for 1 h at 160°C stabilizes infectivity and makes it more resistant to inactivation by autoclaving. Collectively, these findings suggest that the TSE agents have changed their physicochemical state so that a subpopulation of infectivity becomes refractory to wet-heat-inactivation processes.

Each experiment reported here can be considered on the basis of the hypothesis that dehydration is affecting the degree of inactivation. In the dry heat experiment (Figure 1), the lump of brain macerate probably dehydrated as the sample warmed up over 6–7 min to 200°C. By the time temperatures were reached at which inactivation of hydrated infectivity takes place, a proportion of the agent would have been stabilized by dehydration. In all the autoclaving experiments the autoclave cycle provides a window of opportunity for sample dehydration. In the porous-load cycle, the autoclave chamber is first evacuated to remove air. On reaching a target vacuum, the autoclave chamber is alternately pressurized and depressurized with steam several times to ensure complete removal of air and replacement with superatmospheric steam. This results in a series of spikes in temperature, oscillating between approximately 100 and 120°C. Then more steam is introduced until the final target temperature is reached from whence the holding time is measured. The run is terminated by simultaneously

switching off the steam supply and application of a vacuum to dry the load. The surface of the tissue macerate is probably dehydrated during the initial air evacuation stage, an effect that would be minimized during gravity displacement autoclaving where steam simply displaces air from the chamber. However, gravity displacement autoclaving does not ensure complete removal of air from the sample. In samples exposed to ethanol, the tissue will also be dehydrated prior to autoclaving. Further conversion into a stabilized state may occur during the pressure spikes where TSE agents follow a postulated stabilization reaction, rather than a dissociation reaction on exposure to temperatures just above the point at which heat inactivation takes place.

We have previously argued that heat denaturation of TSE infectivity takes place biphasically with respect to both temperature and time. TSE agents are stable at low temperatures and remain stable until a critical temperature is reached. Above this temperature there is a rapid loss of infectivity with increasing temperature [3]. Above the critical temperature, inactivation takes place rapidly, but then reaches a plateau, so that with increasing time little additional inactivation takes place [1,22]. There are marked TSE strain-dependent differences in thermostability [3], and there is up to a 3 log difference in survival between the five TSE strains after autoclaving at 126 °C for 30 min [10]. Hence the denaturation pathway to inactivation involves a TSE strain-specific molecular component of the agent. By contrast, there was no detectable difference in the sensitivity of the three TSE strains to dry-heat inactivation. Once TSE agents are stabilized, the denaturation pathway to inactivation is presumably not available. Probably destruction of the stabilized particle occurs at significant rates only under more extreme conditions. For example, when autoclaving at 134–138 °C, most loss of infectivity probably occurs early in the cycle. Extending the exposure times to these high temperatures has only a small inconsistent effect (Table 2). This effect has also been observed by others for the 263K TSE strain autoclaved at 121 °C, where similar amounts of infectivity survived after 60 and 90 min; however, after autoclaving at 132 °C, residual infectivity was detected after 60 min but not after 90 min, a difference of 0.7 log ID_{50} or more [23]. It is suggested that any reduction in titre is due to the gradual destruction of protein and nucleic acid that occurs at these temperatures and that these processes occur monophasically through first-order reactions.

This rationale fits with the model of the TSE agent presented previously [1,3,10]. To account for the biphasic heat inactivation curves, it was proposed that the TSE agent comprised two or more discrete components. One component need not vary between TSE models, and indeed is likely to be the host protein PrP, present as a dimer or higher multimers [2]. The structure of the other component(s) is not known, but is presumed to be TSE agent-specific. High temperature may induce a protected form of the TSE agent, analogous to the dehydrated form. This leads to the hypothesis that the water of solvation, i.e. the water molecules intimately associated with the structure of the agent, is removed or displaced, stabilizing agent structure [1]. This hypothesis has recently gained support from the finding that PrP, operating through the disordered N-terminal region of the protein, can act as a nucleic acid chaperone, promoting binding to nucleic acids and the formation of nucleoprotein complexes [24]. Tompa and Csermely [25] have previously argued that intrinsically disordered proteins act as chaperones; they are highly hydrated and can bind to structurally heterogeneous substrates. These authors also suggested that the thermodynamics of binding include a large entropic factor due to an increase in order, which may be offset by increasing disorder in the substrate. Iterative transitions between disorder and order will lead to the most thermodynamically stable structure [25,26]. Such a process could operate for TSE agents, where iterative binding of PrP to a ligand may be accelerated by exposure to high temperatures and promote loss of the water of hydration and greater thermostability.

Changes in incubation periods

Incubation periods for partially inactivated samples are often longer than those for the equivalent control samples [11,12]. This property can compromise inactivation studies that rely solely on incubation periods to measure the degree of inactivation [27]. The effect is often most marked at the limiting dilution, where many mice infected with treated samples can have longer incubation periods than the control samples. Prolonged incubation periods were particularly evident in the dry heat experiment (Table 1). Some extended incubation periods were also observed in the porous-load autoclaving experiment (Table 2). Such data illustrate the necessity to observe all animals in inactivation experiments for extended periods to obtain reliably negative results [28]. Presumably the conditions used in these experiments altered the interaction between the infecting agent and the host in some models more than others, and this is particularly observed for the 301V/VM mouse model. However, extended incubation periods have also been found with 301V in other experiments (K. Fernie, D. M. Taylor and R. A. Somerville, unpublished work). These results suggest that there may be an intrinsic difference in properties between TSE strains, which results in greater diversity of response to very small numbers of infective units. The ME7 result in the dry heat experiment (Figure 1) may provide an additional insight. Extended heating for 60 min may have dehydrated the ME7 agent more greatly than after 20 min. Pathogenic mechanisms at or immediately after infection may be altered. The degree to which different strains are dehydrated may vary and hence differentially affect

pathogenesis. It is not known what pathogenetic steps may be affected. Possibly uptake of infectivity by the cells that are usually susceptible does not occur after heating, but infectivity is processed via alternative, longer cellular pathways. Alternatively, intracellular processing may be adversely affected, delaying the initiation of replication.

Conclusion

Overall, the accumulated data demonstrate the difficulties in destroying TSE infectivity using heat-based methods. Under optimal conditions, where the TSE agent and its surrounding tissue remain fully hydrated, TSE infectivity from even the most thermostable TSE strains can be destroyed. However, when any fixation of tissue occurs, e.g. through dehydration, infectivity becomes much more recalcitrant to efficient heat inactivation. In practical terms, this demonstrates the limitations of heat-based systems (e.g. autoclaving) for TSE sterilization. More fundamentally, these results support the hypothesis that the hydration state of the TSE agent is fundamental to their structural integrity.

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