An enzyme–detergent method for effective prion decontamination of surgical steel

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Prions, transmissible agents that cause Creutzfeldt–Jakob disease (CJD) and other prion diseases, are known to resist conventional sterilization procedures. Iatrogenic transmission of classical CJD via neurosurgical instruments is well documented and the involvement of lymphoreticular tissues in variant CJD (vCJD), together with the unknown population prevalence of asymptomatic vCJD infection, has led to concerns about transmission from a wide range of surgical procedures. To address this problem, conditions were sought that destroy PrPSc from vCJD-infected human tissue and eradicate RML prion infectivity adsorbed onto surgical steel. Seven proteolytic enzymes were evaluated individually and in pairs at a range of temperatures and pH values and the additional effects of detergents, lipases and metal ions were assessed. A combination of proteinase K and Pronase, in conjunction with SDS, was shown to degrade PrPSc material from highly concentrated vCJD-infected brain preparations to a level below detection. When RML prion-infected wires were exposed to the same enzymic treatment, intracerebral bioassay in highly susceptible hosts showed virtually no infectivity. The prion-degrading reagents identified in this study are readily available, inexpensive, non-corrosive to instruments, non-hazardous to staff and compatible with current equipment and procedures used in hospital sterilization units.

INTRODUCTION

Prion diseases such as Creutzfeldt–Jakob disease (CJD) and kuru in humans and scrapie and bovine spongiform encephalopathy (BSE) in animals are transmissible by inoculation with, or dietary exposure to, infected tissues (Cuillé & Chelle, 1936; Gajdusek et al., 1966; Gibbs et al., 1968). According to the protein-only hypothesis, the transmissible agent, or prion, is composed principally or entirely of abnormal isoforms of a host-encoded glycoprotein, prion protein (PrP) (Prusiner, 1982). The disease-related isoform, PrPSc, is derived from its normal cellular precursor, PrPC, by a post-translational process that involves a conformational change (Pan et al., 1993). PrPSc can be distinguished biochemically from PrPC by its partial protease resistance and detergent insolubility (McKinley et al., 1983).

Classical CJD (sporadic and iatrogenic CJD) is rare and the infectious agent is mainly found in the central nervous system. These factors have made the risk of iatrogenic infection from surgical instruments rather low. Even so, iatrogenic transmission of classical CJD via neurosurgical instruments, despite their routine sterilization, has been reported (Bernoulli et al., 1977). Recent epidemiological evidence suggests that classical CJD may also be transmitted by other surgical procedures (Collins et al., 1999).

The appearance of variant CJD (vCJD) in the UK from 1995 onwards, and the experimental confirmation that it is caused by the same prion strain that causes BSE in cattle (Bruce et al., 1997; Collinge et al., 1996; Hill et al., 1997), raised concerns that an epidemic of vCJD will occur as a result of the extensive exposure of the UK population to BSE prions between 1980 and 1996 (Cooper & Bird, 2002a, b, c). BSE has now been recognized in cattle herds from most countries in the European Union, the USA, Japan and Canada and the possibility remains of BSE prion infection in sheep flocks. vCJD has been reported from France, Italy, Ireland and the USA. The majority of vCJD cases have occurred in the UK and, while the number of cases remains relatively small (~150), it continues to rise. Recent estimates based on mathematical modelling and the clinical cases of vCJD to date suggest that the total epidemic may be relatively small (Ghani et al., 2003), but key uncertainties, notably with respect to major genetic effects on incubation period (Lloyd et al., 2001), suggest the need for caution and also such models cannot estimate the
number of asymptomatic, infected individuals. It is the latter that is most relevant to assessing risks of iatrogenic transmission and the number of currently infected individuals is unknown. Human prion incubation periods, as evidenced by kuru, may span decades. Also, in addition to pre-clinically infected individuals, the possibility of sub-clinical carrier states of prion infection in humans, as recognized in several animal models, must also be considered (Asante et al., 2002; Hill et al., 2000). A retrospective study of archived surgical lymphoreticular specimens (largely appendix) has recently reported 3 out of 12,674 positive for PrP immunoreactivity and estimated prevalence of infection at 237 per million (95% confidence interval 49–692 per million) (Hilton et al., 2004). Appendix appears to be a less sensitive reporter of vCJD prion infection than tonsil (Joiner et al., 2002) and this retrospective study may therefore have underestimated prevalence. A prospective national-scale anonymous screen of discarded tonsillectomies for PrPSc was announced by the Chief Medical Officer of the UK and is now under way to try to estimate better the prevalence of asymptomatic infection with vCJD prions in the community. There is therefore a significant potential risk of secondary transmission of vCJD via medical and surgical procedures performed on asymptomatic individuals incubating the disease. A possible transfusion-associated case of vCJD has indeed already been reported (Llewelyn et al., 2004).

Importantly, the pathogenesis of prion diseases varies with different host species and prion strain combinations. It is clear that the pathogenesis of vCJD differs substantially from that of other forms of CJD in that, unlike classical CJD, lymphoreticular tissues, including lymph nodes, are extensively (and uniformly) involved, with PrPSc levels in tonsil up to 10% of those seen in brain (Wadsworth et al., 2001). PrPSc is also readily detectable in retina and optic nerve and, at lower levels, in some other tissues, including rectum, in vCJD (Wadsworth et al., 2001). Such tissue distribution means that many common surgical procedures pose a potential risk of iatrogenic transmission, including abdominal surgery, tonsillectomy and gastrointestinal and rectal biopsy (Hill et al., 1999; Wadsworth et al., 2001). Even in classical CJD, the infectivity has been reported from a range of tissues from primate transmission studies, albeit at lower levels than in the central nervous system (Brown et al., 1994). PrPSc is also detectable at low levels in skeletal muscle and other tissues in some sporadic CJD patients (Glatzel et al., 2003).

It is established that prions are highly resistant to conventional chemical and physical procedures designed to inactivate viruses and bacteria, including treatment with organic solvents, formaldehyde and detergents and sterilization at 121°C (Taylor, 1989, 1991; Taylor et al., 1994). The degree of resistance varies between different strains of prions, with differences in the relative protease resistance of PrPSc (Groschup et al., 2000) and thermal inactivation of prion infectivity (Taylor et al., 2002). It is well established that PrPSc is an excellent surrogate marker of prion infectivity but in certain circumstances infectivity and PrPSc respond differently to treatment. Heating of some TSE strains has been shown to reduce infectious titre significantly without reducing the level of PrPSc quantifiable within a Western blot (Somerville et al., 2002). Decontamination methods that demonstrate activity against PrPSc must therefore be verified by bioassay for infectivity itself.

Surgical stainless steel is capable of binding prions avidly and acting as a remarkably efficient reservoir of infectivity (Flechsig et al., 2001; Zobeley et al., 1999). Indeed, a contact time as short as 5 min with prion-infected mouse brain, despite subsequent exhaustive washing, is sufficient for high titres of infectivity to be adsorbed to steel wire (Flechsig et al., 2001). Insertion of such wires into the brains of indicator mice for only 30 min was enough to transmit disease. Infectivity bound to metal was found to persist far longer in the brain than when injected as a homogenate, which may explain the high efficiency of infection mediated by a prion-coated, steel surface (Flechsig et al., 2001).

A combination of the widespread tissue distribution, the potentially high prevalence of clinically silent vCJD infection and the robustness of the infectious agent prompted a risk assessment by the UK government and the introduction of measures aimed at reducing the risk of iatrogenic transmission via blood and blood products and contaminated instruments. An audit of, and subsequent improvements to, hospital sterilization facilities was carried out and single-use instruments were introduced for all tonsil surgery in the UK in 2001, but later suspended in England because of concerns about a higher surgical complication rate.

In view of the prion transmission risk, the development of effective human prion decontamination protocols, validated for metal-bound prions, remains a key public health priority in the UK and elsewhere. The currently available decontamination protocols are based on studies with infected brain homogenates and macerates, not metal-bound prions, and are limited to animal not human prion strains. Although strains of mouse-passaged BSE are available, the nature of passage through rodent hosts results in a mouse-adapted strain that no longer behaves identically to the original bovine inoculum. To address this problem, we have sought readily available and inexpensive agents that destroy prion infectivity without damaging the instruments being decontaminated. Conditions normally used for inactivating prions — typically, powerful oxidizing agents or strong alkalis — are highly corrosive both to the instruments and the washing equipment used in hospital sterilizing units and present a hazard to those involved in their handling.

Previous studies have indicated that proteolytic methods could be used for prion decontamination. Using mouse-adapted BSE prions (strain 3O1V), McLeod et al. (2004) found that treatment with Properase was inefficient, leading only to a modest increase in incubation time rather
than a reduction in the number of animals infected. Using SDS at 100 °C followed by proteinase K (PK) treatment, Langeveld et al. (2003) found that PrPSc present in brain stem samples of bovine BSE and sheep scrapie could be degraded to a level below the sensitivity of standard Western blot analysis. However, the technique is 25-fold less sensitive than the phoshatungstate precipitation method used here and no material was assayed in animal models.

To find both efficient and practical methods, we investigated an extensive matrix of chemical and enzymatic treatments for their ability to eradicate PrPSc from vCJD prion-infected human brain tissue as assessed by the most sensitive available in vitro methods.

To assess such methods on metal-bound prions, we then applied a mouse model system using prion-contaminated metal wires as surrogate surgical instruments (Fleischg et al., 2001) to compare these novel methods with existing reagents and sterilization procedures. For these wire experiments, we used the most sensitive bioassay available to us, namely RML prions assayed in Tg20 and CD-1 mice.

**METHODS**

**Source of homogenates.** Strict biosafety protocols were followed and all work with human prions was carried out in a microbiological containment level 3 facility. Homogenates of histopathologically confirmed vCJD brain tissue, for which informed consent and ethical approval had been obtained for this research, were prepared. Cortex tissue was homogenized as a 20 % (w/v) preparation and each was pre-warmed to a different reaction temperature. An aliquot of 20 ml of 10 % brain homogenate was vortexed to ensure even suspension. The homogenate was vortexed to mix (the final concentration of SDS was pre-warmed to 100 °C) and the homogenate was vortexed to mix. The buffers used were: for trypsin, 400 mM Tris/HCl, pH 8.5; for bromelain, 80 mM CaCl2; and for papain, 400 mM NaH2PO4, pH 6-0; for pepsin, 400 mM NaH2PO4, pH 2-2; for trypsin, 400 mM Tris/HCl, pH 8-5, 80 mM CaCl2; and for Pronase, subtilisin and PK, 400 mM Tris/HCl, pH 7-5. The homogenate was split into three aliquots of 165 ml of each and was pre-warmed to a different reaction temperature. An aliquot of 10 ml was then removed into 10 ml 2 × SDS-loading buffer [125 mM Tris/HCl (pH 6-8), 20 % (v/v) glycerol, 4 % (v/v) SDS, 4 % (v/v) 2-mercaptoethanol, 0-02 % (v/v) bromophenol blue] containing 8 mM 4-(2-aminoethyl)-benzene sulfonyl fluoride (AEBSF) (Pefabloc SC; Roche) as a zero time point control. To the remaining 155 ml homogenate, 51 ml enzyme stock was added and the homogenate was vortexed to mix (the final concentration of enzyme with respect to units of activity is given in Table 1). The enzyme/homogenate mixtures were incubated with agitation at the given temperatures and aliquots of 20 ml were removed at designated time points and quenched by the addition of 20 ml 2 × SDS-loading buffer and heating to 100 °C for 10 min. The samples were then subjected to centrifugation in a microfuge (15 000 g) for 1 min and 27 ml of each supernatant (except the zero time point control, which was 20 ml) was applied to a 16 % Tris/glycine gel (Novex; Life Technologies) according to the manufacturer’s instructions. Gels were electrophoresed onto PVDF membrane (Immobilon-P; Millipore) and subsequently blocked in PBS containing 0.05 % (v/v) Tween 20 (PBST) and 5 % (w/v) non-fat milk powder for 60 min. After washing in PBST, the membranes were incubated with anti-PrP mAb ICSM35 (D-Gen Ltd) diluted to 0.2 µg ml⁻¹ in PBST for at least 60 min before washing in PBST (30 min) and incubation with an alkaline phosphatase-conjugated goat anti-mouse antibody (Sigma) diluted 1:10 000 in PBST for 60 min. Following washing in PBST (30 min), the membranes were developed using AttoPhos reagent (Promega) and visualized on a Molecular Dynamics Storm 840 (Amersham).

For serial binary enzyme digests, 20 µl aliquots of 15 % homogenate in PBS were mixed with 5 µl 12-5 % SDS to give a final concentration of 2-5 % SDS. The mixture was pre-warmed to the reaction temperature of 40 °C and a 5 µl aliquot of the first enzyme solution was added to the 25 µl solution of detergent-treated homogenate and incubated at 40 °C for 30 min. A 0-5 µl aliquot of the second enzyme stock solution was added to the 30 µl solution of SDS– and enzyme-treated homogenate produced by the serial procedures above. The mixture was then incubated at 40 °C for a further 30 min. The final enzyme concentrations used were 6-7 µg PK ml⁻¹ plus 32 µg Pronase ml⁻¹ and 83 µg Pronase ml⁻¹ plus 6-6 µg papain ml⁻¹. Reactions were quenched by the addition of an equal volume of 2 × SDS-loading buffer and heating to 100 °C for 10 min. Samples were then subjected to electrophoresis and Western blotting according the above protocol.

| Table 1. PrP destruction activity of single proteases |
|---|---|---|---|
| Protease | Activity (U mg⁻¹) |
| | 30 °C | 40 °C | 50 °C |
| Pepsin | 0-00083 | 0-00025 | 0-00014 |
| Trypsin | 0-59 | 13-0 | 0-49 |
| Bromelain | 0-021 | 0-029 | 0-085 |
| Pronase | 9-2 | 220 | 54 |
| Subtilisin | 270 | 47 | 68 |
| Papain | 0-17 | 0-33 | 0 |
| PK | 781 | 2124 | 2082 |

Effective prion decontamination of surgical steel
**In vivo bioassay of decontamination efficacy.** In order to assay the efficacy of successful in vitro methods in vivo, 5 x 0.15 mm steel wires cut from monofilament steel suture material (Sterelex; B. Braun GmbH) were exposed to PBS-brain homogenates [20% (w/v) in Tables 2 and 4 and 10% (w/v) in Table 3] prepared from mice terminally sick with RML prion infection. The wires and homogenate were then incubated for 30 min on an Eppendorf thermomixer at 37 °C and 800 r.p.m. Following incubation, the homogenate was pipetted off (and later diluted in PBS and injected into positive control animals, as a 1% homogenate in PBS). The wires were rinsed briefly in PBS, to remove loosely adherent macroscopic particles of homogenate, and left to dry for at least 30 min to mimic residue dried onto surgical instruments. Wires prepared as above were then subjected to various disinfection procedures, before being inserted into the brains of anaesthetized Tg20 transgenic mice (Fischer et al., 1996) (Tables 2 and 3; Fig. 4a, b) or inbred, wild-type CD-1 mice (Tables 4; Fig. 4c). The transgenic mouse line Tg20 has multiple copies of the Prn-p gene and consequently overexpresses the prion protein (Fischer et al., 1996). This high level of expression of PrP dramatically reduces the incubation periods for mouse-adapted prion strains and, as a result, Tg20 mice are a widely used reporter strain for prion infectivity. Wild-type CD-1 mice were also used to verify that the effects were not specific to the transgenic Tg20 line.

In the case of red heat, wires were heated in a Bunsen flame until incandescent and, to assess the effectiveness of NaOH, wires were incubated in 2 M NaOH for 1 h before autoclaving as indicated.

As a positive control, wires were incubated with homogenate as above, but were not subjected to any decontamination procedure prior to insertion. As a negative control, wires were prepared identically in terms of rinsing and drying, but were incubated with PBS, rather than RML brain homogenate.

Care of mice was according to institutional guidelines. Mice were examined daily and were culled if they exhibited signs of distress or once a diagnosis of clinical prion disease was established (Carlson et al., 1986). All brains from implanted mice were analysed by Western blotting, and/or neuropathological examination, including animals culled for intercurrent illness or found dead (with rare exceptions where brains were autolysed). The data in Tables 2–4 report attack rate as the total number of clinically affected mice as a proportion of the total number of implanted mice. Animals were scored positive if they exhibited clinical signs and/or neuropathological evidence of prion infection, or PrP Sc on Western blot analysis.

**Western blotting.** Brain homogenates (10%) were prepared in Dulbecco’s PBS (lacking Ca 2+ or Mg 2+) by serial passage through needles of decreasing diameter. PK digestion (100 μg ml −1 final protease concentration, 1 h, 37°C), electrophoresis and Western blotting were performed as described previously (Wadsworth et al., 2001). Blots were blocked in PBS containing 0-05% (v/v) Tween 20 and 5% non-fat milk powder and probed with anti-PrP mAb ICSM35 (Asante et al., 2002) in conjunction with appropriate alkaline phosphatase-conjugated secondary reagents and chemiluminescent substrate CDP-Star (Tropix Inc.) as described previously (Asante et al., 2002, Wadsworth et al., 2001).

**Neuropathological analysis of experimental animals.** Mouse brains were fixed in 10% buffered formalin, immersed in 98% formic acid for 1 h, formalin post-fixed and embedded in paraffin wax. Serial sections of 4 μm nominal thickness were pre-treated with Tris/citrate/EDTA buffer for antigen retrieval in a pressure cooker for 10 min. PrP deposition was visualized using mAb ICSM35 (1:3000) (Joiner et al., 2002). Gliosis (data not shown) was detected with anti-GFAP rabbit polyclonal antiserum (1:1000; DAKO), using an automated immunostaining system (http://www.ventanamed.com). Haematoxylin-eosin stains were routinely done to assess spongiosis. Sections of brains were examined by the same person (S.B.), blinded to the identity of the animal and treatment group. All sections were scored for spongiosis, neuronal loss, gliosis and PrP immunoreactivity.

**Autoclaving.** All autoclaving experiments were performed in a Getinge GE661ER2 porous load steam autoclave set to operate at either 121 or 134 °C as appropriate.

**RESULTS**

A panel of readily available and inexpensive proteolytic enzymes was assessed for their ability to degrade total PrP (PrP C and PrP Sc) in human vCJD brain tissue. Shown in Fig. 1 are time-courses of digestion of PrP using Pronase (Fig. 1a, b) and PK (Fig. 1c, d). Western blots (Fig. 1a, c), developed using the anti-PrP mAb ICSM35, were quantitatively scanned and the combined intensities of the three

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**Fig. 1.** Proteolytic digestion kinetics of PrP from vCJD brain tissue. (a, c) Western blots of aliquots removed from a Pronase (a) or PK (c) digestion of vCJD brain homogenate at various time points. Each lane contains 10 μl of a 10% (w/v) homogenate and is visualized using ICSM35 as primary antibody. (b, d) Total immunoreactivity was determined for each time point in the Western blot of Pronase (b) or PK (d) digestion by densitometry. The values were then plotted as a percentage of the starting material with respect to time. Superimposed upon the data is a curve representing a fit of the data to a double exponential decay.
PrP glycoform bands at each time-point were plotted (Fig. 1b, d). With both enzymes, the progress curves are complex and cannot be described by simple zero- or first-order kinetics. This complexity is likely to result from a number of factors, including differential rates of PrP<sup>C</sup>/PrP<sup>Sc</sup> digestion, non-uniform susceptibility of PrP<sup>Sc</sup> in different environments and enzyme autolysis during the incubation. An additional complication is an initial increase in immunoreactivity following the first 5 min digestion with proteases. This is likely to result from breakdown of aggregates normally resistant to SDS denaturation so that, following proteolysis, this PrP can enter the gels. Despite these limitations, these data showed a close fit to a double-exponential function (with a relatively protease-sensitive component and a second, much more resistant, fraction) and this was used phenomenologically to analyse the data. In each case, the digestions were performed in 2 % SDS, the omission of which reduced the effectiveness of the digestion by at least an order of magnitude and led to poor reproducibility of results (data not shown).

Seven enzymes were tested in this assay system and the data are summarized in Table 1. We defined a destruction unit as the enzyme activity required to degrade half of the most resistant PrP fraction in 1 ml of a 10 % brain homogenate in a 1 h incubation and ranked the enzymes for their ability to digest vCJD PrP<sup>Sc</sup>.

In addition to testing individual proteolytic enzymes, we investigated various sequential incubations of enzyme pairs in an effort to identify complementary activities. In many cases, autolysis and reciprocal proteolysis of the paired enzymes reduced the effectiveness of PrP degradation (data not shown). However, two pairings of enzymes proved to have enhanced activity against PrP (Fig. 2). Sequential digestion with Pronase and then papain degraded more PrP than either enzyme alone, but by far the most effective treatment was sequential digestion with PK followed by Pronase. In this case, only trace amounts of PrP remained to be visualized by Western blotting.

A significant further enhancement of degradative activity is afforded by incubating vCJD homogenate at 100 °C in SDS prior to proteolysis. Variation in SDS concentration between 2 and 6 % (w/v) showed no significant difference in the efficiency of destruction during subsequent proteolysis (data not shown), a finding consistent with the SDS effect being saturated at 2 % (w/v). In an attempt to quantify the level of destruction that can be achieved using a detergent and enzyme treatment, we exploited the ability of phophotungstic acid to precipitate PrP<sup>Sc</sup> specifically (Wadsworth <em>et al</em>, 2001). Following heating to 100 °C for 15 min in the presence of 4 % (w/v) SDS, 3 ml equivalents of 10 % (w/v) vCJD brain homogenate were treated sequentially with PK and Pronase. Visualization on a Western blot (Fig. 3) showed only residual amounts of PrP remaining, compared with saturating signal levels seen with 20 µl of 10 % vCJD brain homogenate in the control and SDS-only lanes. The Western blotting protocol employed can detect 5 nl equivalents of 10 % (w/v) vCJD brain homogenate (Wadsworth <em>et al</em>, 2001). The level of signal we observed from 3 ml homogenate was less than that seen from 50 nl equivalent of 10 % brain homogenate, giving a rough estimate of the level of destruction of PrP<sup>Sc</sup> as five orders of magnitude.

![Western blot detection of PrP following treatment with combinations of proteases](http://vir.sgmjournals.org)

**Fig. 2.** Western blot detection of PrP following treatment with combinations of proteases. The activity of sequential proteases following incubation with SDS was determined by Western blotting an equivalent of 10 µl of a 10 % (w/v) vCJD brain homogenate. Detection was with the anti-PrP mAb ICSM35 as primary antibody. The zero-time material was not loaded on the Western blots shown here, since the very high level of protease-sensitive material that is degraded in the first 2 min reduces the dynamic range and sensitivity of visualization of the PrP<sup>Sc</sup> signal.

![Western blot detection of PrP concentrated from vCJD brain homogenate using phosphotungstic acid precipitation](http://vir.sgmjournals.org)

**Fig. 3.** Western blot detection of PrP concentrated from vCJD brain homogenate using phosphotungstic acid precipitation. The control (lane 1) contained 20 µl of a 10 % (w/v) vCJD brain homogenate and lane 2 contained 20 µl of 10 % (w/v) vCJD brain homogenate material treated with SDS alone. Lane 3 contained the equivalent of 3 ml of a 10 % (w/v) vCJD brain homogenate (concentrated by phosphotungstate precipitation; see Methods) following the three-phase treatment with SDS, PK and Pronase. The neighbouring panel (lanes 4–6) shows a similar Western blot performed with the same materials diluted 100-fold in 1 × SDS loading buffer. Detection was with mAb ICSM35.

http://vir.sgmjournals.org
In the above, in vitro, experiments, PrPSc was used as a surrogate marker for infective material. Although this is a widely accepted argument for most experimental systems (McKinley et al., 1983), there are some data that show there is not an exact correlation in all circumstances (Somerville et al., 2002). Hence, although the enzymatic degradation described above represents a very significant reduction in the level of detectable PrPSc, the question remained as to what effect there was upon infectivity for metal-bound prions, as assessed by mouse bioassay. We have previously reported that infectivity bound to steel wires is capable of transmitting infection, with attack rates and incubation periods similar to those seen following intracerebral (i.c.) injection of infectious brain homogenates (despite the much smaller amount of surface-bound material compared with a homogenate injection) (Weissmann et al., 2002). Previous work had also confirmed that untreated steel wires are not toxic when inserted intracerebrally (data not shown). In this study, we chose to use RML prions, owing to the fact that there is no animal model for the bioassay of vCJD that fully lacks a species or transmission barrier, thereby considerably limiting the sensitivity of such assays. Hence, RML prion assays in Tg20 and CD-1 mice, which are species-barrier-free and which have been very extensively characterized, were performed to assess prion inactivation on a metal surface.

When exposed to a 20 % (w/v) homogenate of RML prion-infected brain, as described above, implanted wires produced clinical prion disease in 100 % of Tg20 indicator mice, with a mean incubation period of 67 days (Table 2; Fig. 4a). This was a shorter incubation period than that produced by direct i.c. inoculation of 30 μl of a 1 % (w/v) homogenate of RML. By contrast, uninfected wires produced no disease by criteria of clinical signs, neuropathology or Western blot detection of PrPSc.

To establish the effectiveness of current procedures, three proprietary disinfectants alongside two autoclaving regimes were investigated using these methods. Disturbingly, we found that none of the reagents provided an acceptable degree of disinfection, as shown in Fig. 4(a). Indeed, two of the reagents, LPH and Endozyme Plus, still resulted in 100 % disease transmission, with only a moderate reduction in infectious titre as evidenced from the slight increase in incubation period (Table 2). Disinfection with the third reagent, LPHse, still resulted in clinical disease in 3 of 5 indicator mice, with short incubation periods. This result is in contrast to that recently reported by others (Fichet et al., 2004), where LPH was seen to be completely effective in the assays used. One possible explanation for this is that the titre of the material used was low, as evidenced from the relatively long incubation period of 90 days for strain 263K in Syrian hamsters. High-titre material would be expected to produce an incubation period of 72 days.

While autoclaving appeared to be effective, histological examination of clinically unaffected animals revealed signs of infection in 2 of 6 animals from the 121 °C group and 1 of 4 for the 134 °C group. A binary combination of proteolytic enzymes, Pronase and papain, was also assessed for efficacy against prions and there was no detected transmission of disease in the three mice tested.

Having established a standard protocol for assessing the effectiveness of decontamination procedures, we subjected wires exposed to a 10 % (w/v) homogenate of RML prion-infected brain to the detergent and protease treatment (SDS–PK–Pronase) we developed for destroying PrP in tissue homogenates. Larger groups of 20 indicator mice were used. As before, infected, untreated wires produced a 100 % clinical attack rate with a short incubation period of 62 ± 0–7 days (Table 3; Fig. 4b). Direct i.c. inoculation of a 1 % (w/v) RML homogenate also produced a 100 % clinical attack rate, with a similar incubation period (63 ± 0–5 days) to the infected wires.

The encouraging data from the in vitro studies on vCJD brain homogenate, described above, were supported by almost complete destruction of detectable surface-bound infectivity when assayed in the indicator mice (Table 3; Fig. 4b). Indeed, of 18 mice successfully implanted following the three-stage decontamination regime with SDS, PK and Pronase, only one succumbed to disease and with a considerably prolonged incubation period of 101 days. In distinction to standard mouse titrations using serial dilution of tissue homogenates, it is more difficult to quantify titre of infectivity in these metal wire experiments (Flechsig et al., 2001); however, these bioassay results are consistent with the findings of the in vitro studies (Fig. 3).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Clinically affected*</th>
<th>Incubation time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected wire</td>
<td>0/5</td>
<td>–</td>
</tr>
<tr>
<td>1 % RML i.c.</td>
<td>5/5</td>
<td>75 (1–0)</td>
</tr>
<tr>
<td>Infected wire, no sterilization</td>
<td>5/5</td>
<td>67 (1–5)</td>
</tr>
<tr>
<td>LpH (10 %; 90 min RT)</td>
<td>5/5</td>
<td>91 (2–6)</td>
</tr>
<tr>
<td>LpHse (10 %; 90 min RT)</td>
<td>3/5*</td>
<td>70 (0)</td>
</tr>
<tr>
<td>Endozyme Plus (10 %; 90 min RT)</td>
<td>5/5</td>
<td>81 (1–0)</td>
</tr>
<tr>
<td>Enzyme-treated (40 °C; 60 min)</td>
<td>0/5</td>
<td>–</td>
</tr>
<tr>
<td>Autoclaved (121 °C; 20 min)</td>
<td>0/6b</td>
<td>–</td>
</tr>
<tr>
<td>Autoclaved (134 °C; 20 min)</td>
<td>0/4c</td>
<td>–</td>
</tr>
<tr>
<td>Enzymes + autoclaved (121 °C)</td>
<td>0/5d</td>
<td>–</td>
</tr>
<tr>
<td>Enzymes + autoclaved (134 °C)</td>
<td>0/4</td>
<td>–</td>
</tr>
</tbody>
</table>

*aSome animals that did not display clinical signs and survived to the end of the experiment were nevertheless scored as infected following neuropathological examination. Total numbers infected were: a, 4/5; b, 2/6; c, 1/4; d, 1/5.
Fig. 4. Kaplan–Meier survival curves of mice intracerebrally implanted with prion-contaminated surgical steel wires. In (a) and (b), short-incubation-period Tg20 transgenic mice were used while, in (c), conventional CD-1 mice were used. (a) Wires coated with RML prions and not subjected to any decontamination procedure produced clinical disease with short incubation periods in 100% of the animals (○). Similarly, i.c. inoculation with 30 μl of a 1% (w/v) RML-infected brain homogenate produced short incubation periods (●). The disinfectants investigated were Endoyme Plus (●), LpH (▼) and LpHse (△), which prolonged the incubation period but had little effect on overall survival. Autoclaving at both 121 and 134 °C prevented the manifestation of a clinical syndrome within the experimental observation period of 273 days, as did a combined SDS/Pronase/papain treatment. (b) Wires coated with RML prions and not subjected to any decontamination procedure (○) and i.c. inoculation with 30 μl of a 1% (w/v) RML-infected brain homogenate (●) produced short incubation periods in 100% of the animals. In this experiment, a three-stage enzymic decontamination using SDS, PK and Pronase ('enzyme mix') was tested, which resulted in only one animal showing clinical signs of infection at 101 days post-inoculation (□). (c) Untreated wires coated with RML prions produced clinical disease in 5/7 of the animals (○), with shorter incubation periods than direct inoculation with 30 μl of a 1% (w/v) RML-infected brain homogenate (●). All the methods tested were completely effective at decontaminating the surgical steel wires.
Table 3. Bioassay of infected wires in large groups of Tg20 mice

The corresponding survival curve is displayed in Fig. 4(b). See Table 2 for other details.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Clinically affected</th>
<th>Incubation time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected wire</td>
<td>0/20</td>
<td>–</td>
</tr>
<tr>
<td>1% RML i.c.</td>
<td>20/20</td>
<td>63 (0-5)</td>
</tr>
<tr>
<td>Infected wire, no sterilization</td>
<td>17/17</td>
<td>62 (0-7)</td>
</tr>
<tr>
<td>Autoclaved (134 °C, 20 min)</td>
<td>13/13*</td>
<td>108 (12-4)</td>
</tr>
<tr>
<td>Enzyme-treated (40 °C, 60 min)</td>
<td>1/18</td>
<td>101</td>
</tr>
</tbody>
</table>

*A additional mouse was culled for animal care reasons at 91 days. This mouse was also infected as assessed by subsequent neuro-pathological examination, giving a total of 14/14 animals infected.

Disturbingly, autoclaving at 134 °C, while clearly reducing the infectious titre of wire-bound material, as evidenced from the increased incubation period, still allowed 100% transmission of disease (Table 3). In this experiment, for local biosafety reasons, wires were transferred to the autoclave inside microfuge tubes partially sealed with loose caps. A subsequent experiment showed that, despite being loosely applied, the caps restrict the entry of steam to the tubes and therefore would have presumably impaired the efficiency of autoclaving (data not shown). In other experiments (Tables 2 and 4; Fig. 4a, c), where wires were fully accessible to steam, autoclaving was more effective.

Decontamination methods were also assessed using a second mouse model: wild-type CD-1 mice. When exposed to a 20% (w/v) homogenate of RML-infected brain, implanted wires produced clinical prion disease in 5 of 7 CD-1 mice with a mean incubation period of 152 days (Table 4; Fig. 4c). As with Tg20 mice, this was a shorter incubation period than that produced by direct i.c. inoculation of 30 μl of a 1% (w/v) homogenate of RML. The decontamination methods tested (high-temperature autoclaving, SDS–PK–Pronase treatment, NaOH and red heat) were effective at preventing transmission of disease (Table 4; Fig. 4c). The extreme method of heating the wires until glowing red was included as a secure positive control of prion decontamination.

**DISCUSSION**

Defining effective and practical regimes for the removal of prion infectivity from surgical and medical instruments is an important public health issue in the UK and other countries with significant population exposure to BSE prions, where unknown numbers of individuals may silently harbour prion infection in a range of tissues for many years. No suitable test to detect asymptomatic prion infection exists to date, a situation which precludes the introduction of measures that could limit the spread of infection by this route.

While the three-stage detergent/enzymic procedure we have developed will not ensure absolute sterility of prion-contaminated instruments, its application would be expected to degrade any infectivity risk by several orders of magnitude and very substantially reduce risks of iatrogenic transmission of human prion infection during routine surgery. Importantly, the method has been shown to be effective against native vCJD PrPSc in homogenized tissue and against RML prions adsorbed onto surgical steel surfaces rather than simply with tissue homogenates. As there is no sensitive indicator animal in which we could assay vCJD infectivity on the wires, the experimental approach we describe here is the most rigorous available at the moment. The use of stainless steel wires as a model of surgical instruments is an essential element of validating potential decontamination regimes, as metal-bound prions appear to have a higher specific infectivity than prions in tissue homogenates and may be more resistant to degradation (Flechsig et al., 2001).

The application of extreme heat or incubation in NaOH prior to autoclaving (as recommended by the WHO) confirmed that these methods can be effective against prion-contaminated steel. An independent study using a wire implantation model in hamsters indicated that 134 °C autoclaving was partially effective, with only 1 of 10 implanted animals succumbing to prion disease (Yan et al., 2004), with a combination treatment of soaking in NaOH followed by autoclaving of a comparable efficiency, with 2 of 10 animals still becoming infected. The application of NaOH is of limited use in a hospital environment where it would be damaging to instruments and a risk to the operator. High-temperature autoclaving at 134 °C is the mainstay of current UK procedures. It may be effective, but appears sensitive to steam penetration in our experimental model. The application of loose tops to the tubes containing the prion-contaminated wires led to 100% transmission in subsequent bioassay of the wires. In hospital situations, such full steam penetration and uniform
heating of a large load of instruments may not always be achieved, even when the autoclave is functioning optimally. Hence, relying on autoclaving at 134 °C for prion decontamination may be unsafe. Recent studies of instruments have reported persistence of macroscopic contamination in complex areas of surgical instruments (such as hinges), despite washing prior to autoclaving (Dinakaran & Kayarkar, 2002). It is possible that during routine autoclaving some instruments would be partially protected from the ingress of steam, thereby compromising the effectiveness of autoclaving as a prion decontamination method.

The use of safe water-soluble reagents such as ionic detergents and proteases in conjunction with autoclaving appears to offer the best approach to improve the effectiveness of existing procedures at decontaminating prion infectivity. It ought to be possible to produce effective formulations of these reagents that can be readily and reproducibly applied in existing hospital washing and sterilization equipment at modest additional expense. While these methods have been experimentally verified on metal surfaces, it will be important to determine whether they are as effective on other surfaces such as neoprene. This type of polymer is a component of sensitive diagnostic instruments such as endoscopes, which will not withstand autoclaving and might also be decontaminated by this detergent–enzyme method.

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