Enzymatic detergent treatment protocol that reduces protease-resistant prion protein load and infectivity from surgical-steel monofilaments contaminated with a human-derived prion strain

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The unconventional nature of the infectious agent of prion diseases poses a challenge to conventional infection control methodologies. The extraneural tissue distribution of variant and sporadic Creutzfeldt–Jakob disease has increased concern regarding the risk of prion disease transmission via general surgical procedures and highlighted the need for decontamination procedures that can be incorporated into routine processing. In this study, the ability of preparations of enzymatic medical instrument cleaners to reduce the infectivity associated with a rodent-adapted strain of human prion disease, previously reported to be resistant to decontamination, was tested. Efficient degradation of the disease-associated prion protein by enzymatic cleaning preparations required high treatment temperatures (50–60 °C). Standard decontamination methods (1 M NaOH for 1 h or autoclaving at 134 °C for 18 min) reduced infectivity associated with the human-derived prion strain by less than 3 log10 LD50. In contrast, a 30 min treatment with the optimized enzymatic cleaning preparation protocols reduced infectivity by more than 3 log10 LD50 and when used in conjunction with autoclave cycles eliminated detectable levels of infectivity. The development of prion decontamination procedures that are compatible with routine cleaning and sterilization of medical and surgical instruments may reduce the risk of the transmission of prion disease in general surgery.

INTRODUCTION

The transmissible spongiform encephalopathy or prion diseases are a group of transmissible neurodegenerative disorders affecting both humans and animals. In humans, the disease manifests in sporadic, familial and acquired forms, with sporadic Creutzfeldt–Jakob disease (spCJD) accounting for over 80% of cases reported worldwide. Regardless of its initiating aetiology, all forms of the disease are transmissible by exposure to infected tissue. Transmission is highest from tissue of the central nervous system where accumulation of the disease-associated form of the prion protein is associated with spongiform degeneration.

Concern regarding the transmission of variant Creutzfeldt–Jakob disease (vCJD), a prion disease associated with the consumption of beef products contaminated with bovine spongiform encephalopathy, has led to increased regulation of blood products. These measures appear to have been well founded, with four instances of transfusion-acquired vCJD now reported (Editorial team, 2007; Llewelyn et al., 2004; Peden et al., 2004) and a predicted incidence of vCJD within the British population of 237 cases per million head of population (Hilton et al., 2004). This prediction included individuals homozygous for valine at polymorphic residue 129 of the prion protein (Ironside et al., 2006), a genotype not previously reported to be susceptible to vCJD infection. This is significantly higher than the 161 clinical cases reported to date and suggests that a significant subclinical population of vCJD may exist. In addition to concern regarding the neurological and haematogenous transmission of vCJD is the risk of disease transmission through tissue transplantation and general surgery, as the disease-associated form of the prion protein has been detected in lymphoid organs (Wadsworth et al., 2001) and skeletal muscle of vCJD patients (Peden et al., 2006). The disease-associated isoform of the prion protein has also been reported in extraneural tissue of spCJD (Glatzel et al., 2003), highlighting the risk of disease acquisition through general surgical procedures in the wider population (Collins et al., 1999).

A principal component of the infectivity associated with prion diseases is an abnormal protease-resistant isoform (PrPSc) of the host-encoded prion protein (PrPC). The unconventional nature of the infectious agent or prion poses a challenge to conventional infection control methodologies due to the resistance of the prion to general
methods of cleaning and disinfection, and the incompatibility of recommended prion disinfection methods with the materials used in the construction of medical instruments. This, in conjunction with the prolonged pre-symptomatic period of prion diseases and the absence of suitable diagnostic test methods, make the application of universal infection control strategies problematic.

Recent studies have suggested that detergent preparations with and without the addition of enzymes may be effective in the decontamination of prions from surgical steel wires (Fichet et al., 2004; Jackson et al., 2005; Peretz et al., 2006; Yan et al., 2004). Prions bind tightly to surgical steel surfaces and effectively transmit infectivity to recipient animals (Flechsig et al., 2001; Zobeley et al., 1999). These systems have therefore been developed to assess methods for the decontamination of prions from surgical instruments, which have been shown to be more resistant to decontamination than prions present in a suspension of infected tissue homogenate (Peretz et al., 2006). These studies also support differences in the sensitivity of different prion strains to decontamination. The latest report suggests that human sPCJD prions are 10^5 times more resistant to decontamination by acidic SDS when compared with prions derived from scrapie-infected hamster tissue (Peretz et al., 2006).

In this report, we demonstrate that cleaning prion-contaminated steel wires with an enzymatic medical instrument cleaning solution followed by autoclave treatment significantly reduces infectivity associated with a strain of mouse-adapted human prions. Only partial inactivation of this strain was observed using current World Health Organization (WHO)-recommended protocols of 1 M NaOH for 1 h or an autoclave cycle of 134 °C for 18 min.

**METHODS**

**Source of chemical decontaminants.** The enzymatic medical instrument cleaning preparations used in this study were Rapid Multi Enzyme Cleaner trial formulation A (RMEC A) and trial formulation B (RMEC B) and trial variations prepared by Novapharm Research (Australia) Pty Ltd, Endozyme AW Plus (RuboIn) and Klenzyme (Steris). Preparations of RMEC A and B were prepared without enzymes (Novapharm Research) and are referred to as Cleaner A and Cleaner B, respectively. NaOH (Pronalysis) was freshly prepared in distilled water for each experiment.

**Infectious material.** The M1000 prion strain used in this study is derived from the Fukuoka-1 (or FU-1) strain of mouse-adapted human prions and was originally derived from a patient who died from a familial form of prion disease (Tateishi et al., 1979). This strain was originally isolated by passage in mice and has been maintained by passage in mice (Brazier et al., 2006). A 10% (w/v) infectious brain homogenate (IBH) was prepared in PBS from brain tissue derived from a pool of BALB/c mice at the terminal stage of disease. This pool was used in all experiments. The mean lethal dose (LD50) of this pool was determined by end-point titration in Tg(a2/20) mice as −10^8 LD50 per g wet-tissue equivalents. A 10% (w/v) uninfected brain homogenate (UBH), prepared from the brain tissue of healthy, uninfected BALB/c mice was used as a negative control. Purified PrPSc was prepared by the method of Bolton et al. (1987).

**In vitro analysis of PrPSc degradation.** The PrPSc-degrading efficacy of decontamination methods and formulations was assessed by Western blot analysis of treated IBH. Briefly, IBH was treated with benzoxane (1.2 mM, 5 min at 37 °C) to reduce the viscosity of the sample and then mixed with an appropriately diluted volume of product prepared in distilled water. Samples were incubated for the specified period of time and at the specified temperature. At the conclusion of the incubation period, the action of proteases present in the trial formulation was stopped by reducing the pH of the solution to 3 by the addition of HCl. Alkaline treatment methods (NaOH) were neutralized by the addition of HEPES-buffered HCl before protease degradation, as described previously (Kasermann & Kempf, 2003). The ability of NaOH and cleaner preparations to form protease-sensitive PrP was determined by incubation of treated IBH with proteinase K (PK; 100 μg mL−1; Invitrogen) for 30 min at 37 °C. The action of PK was subsequently stopped by the addition of Pefabloc SC (4 mM; Roche). For samples that were not further concentrated by centrifugation, an equal volume of 2 × sample buffer [containing 6% (v/v) β-mercaptoethanol] was then added and the sample was heated at 80 °C for 10 min. Samples were otherwise concentrated by centrifugation at 20 000 g for 1 h (Lee et al., 2000) (unless otherwise stated) and the resulting pellet resuspended in 1 × sample buffer [containing 3% (v/v) β-mercaptoethanol] and heated at 80 °C for 10 min. Samples were then either snap frozen in liquid nitrogen and stored at −80 °C or immediately subjected to SDS-PAGE using Novex 12% Tris/glycine gels (Invitrogen) in Tris/glycine running buffer (200 V for 60 min) or NuPAGE Novex 12% Bis/Tris gels (Invitrogen) in MES running buffer (200 V for 50 min). For quantitative assessment of PrPSc degradation, half-log serial dilutions were prepared in the appropriate sample buffer and electrophoresed on NuPAGE Novex 12% Bis/Tris gels as described above or 6% E-PAGE gels following the manufacturer’s directions (Invitrogen).

All gels were transferred in a Tris/glycine transfer buffer (25 mM Tris, 200 mM glycine, 20% methanol) to a PVDF membrane (Millipore) at 0.38 A for 45 min and developed with the PrP-specific monoclonal antibody (mAb) ICSM18 (raised against aa 143–153 of human PrP; D-Gen; White et al., 2003) and secondary antibody (horseradish peroxidase-conjugated anti-mouse Ig; GE Healthcare). Membranes were developed with ECL-Advance chemiluminescent reagent (GE Healthcare) and imaged using ECL Hyperfilm (GE Healthcare).

**Preparation of prion-contaminated steel wires.** Steel wires were contaminated with prion infectivity as previously described (Fichet et al., 2004), with minor modifications. Briefly, 30 mm lengths of stainless steel monofilament (B. Braun) were cleaned by sonication in 2% (v/v) Triton X-100 for 15 min, rinsed clean in sterile distilled water and air dried. The tip of each wire was then immersed to a depth of approximately 10 mm in a tube containing IBH. For dose titration experiments, the IBH was diluted tenfold from 10−1 to 10−9 in UBH. A batch of wires was also contaminated with UBH as a negative control. Wires were incubated in UBH or IBH for 1 h at room temperature and air dried overnight before unbound contaminant was removed by washing the wires once in PBS (Gibco) for 5 min. Wires were then air dried for at least 30 min before decontamination procedures were performed. Chemical and heat decontamination procedures were performed on two to four batches of five wires. Chemical decontamination procedures were performed in 1 ml volumes of freshly prepared chemical decontaminant in 1.5 ml screw-cap tubes with agitation at 300 r.p.m. Following treatment, wires were rinsed in distilled water and air dried for at least 30 min before being stored at −80 °C.
Autoclave procedures were performed by placing batches of wires in paper autoclave bags, which were then placed in an open container. Autoclave cycles were performed in a Getinge GE 660/920.2 autoclave using the gravity displacement cycle. Wires were cooled to room temperature before being stored at −80 °C. Where a chemical treatment protocol was followed by a physical treatment protocol, the wires were rinsed in distilled water and air dried before being subjected to autoclave cycles as described above. Untreated M1000-contaminated wires were left in a sealed Petri dish at room temperature while the decontamination procedures were being performed.

Wires were implanted into the left cerebral hemisphere of 6–8-week-old Tga/20 transgenic (PrPC overexpressing) mice (Fischer et al., 1996). Mice were anaesthetized using ketamine/xylazine. A 0.5 cm length was cut from the contaminated end of each wire and inserted into the end of a 26G needle. The needle was then inserted through the skull of the anaesthetized animal into the left parietal region, where the inserted wire was displaced from the needle.

Inoculated Tga/20 mice were observed daily for signs of disease. Animals were sacrificed under methoxyflurane anaesthesia when persistent signs consistent with prion disease were evident, such as reduced motor activity, weight loss, hunched posture, hind limb paresis and ataxia. Mice were given food and water ad libitum, with all handling according to prescribed national guidelines and ethical approval from the University of Melbourne Animal Ethics Committee (AECC Register No. 03237).

**Confirmation of infectivity in symptomatic animals.** Prion disease was confirmed in all clinical mice by Western blot analysis (Collins et al., 2005) using the anti-PrP rabbit polyclonal antibody 03R19 (raised against aa 89–103 of mouse PrP; Vella et al., 2007). Mice negative for PrPSc by conventional Western blot methods and healthy mice euthanized at the end of the experimental period were tested for asymptomatic prion disease using high-sensitivity Western blot methods (Wadsworth et al., 2001), as described previously (Collins et al., 2005). With the exception of groups of asymptomatic mice in which no disease was recorded (low-dose titration groups 10−2 to 10−3 and mice implanted with wires decontaminated with RMEC B and autoclave cycles), all animals negative for PrPSc by Western blot approaches were also examined by immunohistochemistry (IHC) (Collins et al., 2005).

Using these methods, only one asymptomatic mouse, implanted with a wire contaminated with a 10−2 dilution of IBH, was identified using conventional Western blot methods.

**Statistical analysis.** The significance of delays in incubation time was determined using one-way analysis of variance with Tukey’s post-test (GRAPHPAD PRISM).

**RESULTS**

In this study, the M1000 strain of mouse-adapted human prions was used to investigate the ability of enzymatic medical instrument cleaners to inactivate prion infectivity. The M1000 strain is derived from the Fukuoka prion strain, a strain previously reported for its resistance to inactivation with standard protocols using NaOH (Tateishi et al., 1988). In support of this, we observed that a protease-resistant subpopulation of M1000 PrPSc remained after a 15 min exposure to 25 mM NaOH (Fig. 1a). This same treatment was previously reported to eliminate PrPSc associated with the 263K scrapie prion strain (Kasermann & Kempf, 2003). Furthermore, a weak protease-resistant signal was detected in homogenates treated with 1 M NaOH for 60 min at room temperature (data not shown). This strain was also highly resistant to degradation with PK (Fig. 1b) with an enzyme decay curve yielding a half-life of 12–14 h (Fig. 1c). This suggests that the M1000 strain has an intermediate level of resistance to PK digestion relative to the RML strain, which is reported to be relatively sensitive to PK digestion, and the 263K strain, which is highly resistant to PK digestion (Kuczius & Groschup, 1999).

Combinations of enzymes and detergents have been reported to inactivate the infectivity associated with prions (Fichet et al., 2004; Jackson et al., 2005). To investigate the
efficacy of enzymatic cleaning preparations to degrade M1000 PrPSc, IBH and UBH were treated in a 0.8% (v/v) solution of four different enzymatic medical instrument cleaning solutions prepared in distilled water, and incubated for 5 min at 43 °C. Wet-tissue equivalents (10 μg) were electrophoresed and Western blotted using mAb ICSM18. Lanes: 1, PrPSc present in UBH (5 μg wet-brain equivalents loaded); 2, PrPSc present in IBH after PK treatment (100 μg ml⁻¹, 30 min, 37 °C); 3–10, digestion with RMEC A (lanes 3 and 4), RMEC B (lanes 5 and 6), Steris Klenzyme (KLENZ) (lanes 7 and 8) or Ruhof Endozime AW plus (ENDO) (lanes 9 and 10). Molecular mass markers (kDa) are indicated.

PrPSc present in M1000 IBH was not completely degraded by exposure to these enzymatic medical instrument cleaning solutions. RMEC B cleared all detectable PrP from the UBH and reduced the amount of PrPSc detected in M1000 IBH relative to the PK-treated control. Treatment of 263K prions with Klenzyme under these conditions was reported previously to reduce prion infectivity by 3.5 log LD50 units (Fichet et al., 2004). However, this treatment did not reduce the amount of PrPSc present in M1000 IBH.

We considered that the poor performance of enzymatic medical instrument cleaning preparations against the M1000 prion strain might reflect the previously reported resistance of this strain to decontamination (Tateishi et al., 1988). To identify conditions that were suitable for the inactivation of this human-derived prion strain, we compared the effect of time and temperature of treatment for RMEC A and B.

A quantitative assessment of time versus temperature indicated that both solutions were more effective at degrading PrPSc after long (30 min) exposure times and at higher treatment temperatures (Table 1). A more detailed titration of the effect of temperature indicated that the enzymatic medical instrument cleaning preparations did not reduce PrPSc at temperatures below 40 °C and that their efficacy was reduced at temperatures above 60 °C (Fig. 3). In contrast, increasing temperature did not affect the sensitivity of M1000 PrPSc to degradation with PK in PBS. Both enzymatic cleaning preparations reduced the amount of detectable PrPSc at temperatures of 50–60 °C, with RMEC A most effective at 50 °C and RMEC B most effective at 60 °C. A quantitative assessment of PrPSc degradation indicated that treatment of M1000 IBH with RMEC A under the optimized conditions of 30 min at 50 °C reduced PrPSc by approximately 2 log10 and treatment with RMEC B under optimized conditions reduced PrPSc by approximately 2.5 log10 (Fig. 4).

The protease present in the RMEC solutions is a subtilisin derived from Bacillus subtilis. The activity of this enzyme is greatest between 50 and 65 °C (>80% relative activity) and has less than 50% relative activity at temperatures below 40 and above 70 °C (Novozymes). The activity profile of subtilisin was therefore similar to that observed for PrPSc degradation by the RMEC solutions. This suggested that the cleaning solution may effectively unfold PrPSc into a protease-sensitive form at all temperatures tested, but that the limiting step in the degradation of PrPSc is the inability of the protease to degrade all of the protease-sensitive material at temperatures where its activity is reduced. We therefore investigated the resistance of M1000 PrPSc to PK degradation in the presence of the cleaning solution without the subtilisin enzyme. The degradation profile for PrPSc treated with PK in the presence of the RMEC B cleaning solution (cleaner B) was identical to the degradation of PrPSc by RMEC B (Fig. 3c), thus suggesting that the subtilisin enzyme present in the cleaning solution had degraded all of the protease-resistant material present in the IBH within the treatment time. In contrast, more PrPSc was digested by PK in the presence of the RMEC A cleaning solution (cleaner A) than was degraded by RMEC A alone (Fig. 3b). Of particular note was the efficacy of cleaner A plus PK at temperatures of 20–40 °C. However, the overall inactivation profile of cleaner A plus PK was the same as RMEC A and B, with temperatures between 50 and 60 °C resulting in the most PrPSc degradation.

To understand further the mechanism of PrPSc degradation, formulations of RMEC A were prepared in which the

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**Table 1. Effect of treatment time and temperature on PrPSc degradation**

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<tr>
<th>Treatment</th>
<th>5 min</th>
<th>30 min</th>
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<tr>
<td>RMEC A</td>
<td>&lt;3</td>
<td>&lt;2</td>
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<td>RMEC B</td>
<td>&lt;2.5</td>
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Results are shown as the amount of M1000 PrPSc (log10) detected by serial dilution and Western blot analysis of IBH treated with RMEC A or B at low (30 °C) and high (65 °C) treatment temperatures and with increasing treatment times. Total M1000 PrPSc present in an equivalent IBH after treatment with PK (100 μg ml⁻¹, 30 min, 37 °C) was 3 log10.
concentration of protease, solvent, anionic surfactant and amylase were varied. The ability of these formulations to degrade M1000 PrPSc present in IBH was examined in a 1 % (v/v) solution with a treatment time of 15 min at 45°C (Fig. 5). Little improvement in PrPSc degradation was achieved using protease concentrations greater than 50 % of that present in RMEC A, even at concentrations four times that present in the RMEC solution (Fig. 5a). The protease resistance of the PrPSc population was then compared at a low (30°C) and high (60°C) temperature in the presence of a low (16%), intermediate (100%) and high (400%) protease concentration. At 30°C, all three protease concentrations showed similar levels of PrPSc degradation and only at high temperatures (60°C) was a difference observed between low and high protease concentrations. We therefore concluded that heat is required to unfold the PrPSc structure and make it accessible to digestion by the proteases present in the RMEC preparations.

Solvent and amylase did not contribute to PrPSc degradation and the presence of the anionic surfactant appeared to adversely affect PrPSc degradation under the conditions tested (Fig. 5b). Agitation of the IBH during digestion had no effect on PrPSc degradation.

To investigate the ability of the optimized treatment protocols for RMEC A and B to reduce M1000 infectivity, surgical steel wires were contaminated with M1000 IBH and treated with the RMEC protocols or the WHO-recommended protocols before bioassay in Tga/20 mice. In order to quantify the reduction of infectivity, a dose titration was performed on wires exposed to serial dilutions

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**Fig. 3.** Effect of temperature on the protease resistance of M1000 PrPSc in the presence of RMEC or cleaning solutions. Western blot analysis of PrP present in M1000 IBH after treatment with PK, (100 μg ml⁻¹) (a), a 1 % (v/v) solution of RMEC A or cleaner A (b) or a 0.3 % (v/v) solution RMEC B or cleaner B (c). Treatment with cleaning solutions was performed in the presence of PK (100 μg ml⁻¹). Treatments were performed for 30 min at the specified temperature. Proteolysis by RMEC formulations was stopped by the addition of HCl to pH 3. PK activity was stopped by the addition of Pefabloc (4 mM). The samples were concentrated by centrifugation (20 000 g for 60 min), and 200 μg wet-tissue equivalent was analysed by SDS-PAGE and Western blotting with mAb ICSM18. Molecular mass markers (kDa) are indicated.

**Fig. 4.** Western blot analysis of M1000 PrPSc after treatment under optimized RMEC conditions. Wet-tissue equivalents (μg) of M1000 IBH were treated with PK (100 μg ml⁻¹, 30 min at 37°C) (a), RMEC A [1 % (v/v) solution, 30 min at 50°C] (b) and RMEC B [0.3 % (v/v) solution, 30 min at 60°C] (c). Proteolysis was stopped by the addition of HCl to pH 3 or Pefabloc (4 mM) and samples were concentrated by ultracentrifugation (250 000 g, 60 min at 4°C). Half-log serial dilutions of the homogenate were analysed by SDS-PAGE and Western blotting with mAb ICSM18. Wet-tissue equivalents (μg) per lane and molecular mass markers (kDa) are indicated.
of M1000 IBH diluted in UBH. The method of Reed & Muench (1938) was subsequently used to calculate the infectivity associated with each 5 mm length of steel wire. This gave a log10 dose of 5.5 LD50 units per wire contaminated with 10% (w/v) IBH. Linear regression analysis was performed to fit a dose–response curve to the titration and subsequently used to calculate the load remaining on treated wires based on incubation times (Fig. 6a).

A calculation of infectivity, based on incubation time, for untreated wires contaminated with the 10% (w/v) IBH used as the positive control in the treatment protocol resulted in a log10 dose of 5 LD50 units, thus validating the use of linear regression analysis of incubation time to calculate the infectious dose of prions bound to surgical steel surfaces, as the error associated with this method used to calculate infectivity from prions in suspension has been reported previously as 0.5 log10 LD50 units (Prusiner et al., 1982).

In agreement with the inability of NaOH to inactivate infectivity associated with the Fukuoka prion strain, M1000 infectivity was poorly inactivated by treatment with 1 M NaOH for 1 h at room temperature, with all mice succumbing to disease within the period of the bioassay without a significant increase in incubation time relative to untreated wires (Fig. 6b).

All autoclave treatments were also poorly efficacious (Fig. 6b), including treatment of wires at 134 °C for 18 min, which did not significantly improve the incubation period or mortality rates relative to the conventional sterilization techniques of 121 °C for 20 min and 134 °C for 3 min.

Both RMEC treatments resulted in decreased mortality and prolonged incubation periods relative to untreated wires (RMEC A, P < 0.001; RMEC B, P < 0.05). The efficacy of the RMEC B protocol was further improved when combined with a standard autoclave cycle of 134 °C for 3 min, where only one mouse succumbed to disease, and 121 °C for 20 min, in which no mortality was recorded (Fig. 6c).

Tga/20 mice were also implanted with wires contaminated with UBH. One mouse from this group with neurological symptoms of kyphosis and ataxia was culled during the incubation period. However, Western blot and IHC examination did not detect PrPSc in this mouse and it was concluded that the neurological symptoms in this mouse were due to the implantation of the steel wire. Prion disease was confirmed in all clinical mice implanted with M1000-contaminated steel wires by Western blot analysis, and all non-clinical mice culled at the end of the experiment were confirmed as negative for PrPSc by sodium phosphotungstic acid Western blotting. Asymptomatic infection was not detected in mice implanted with wires from the treatment groups. Further IHC examination confirmed this result (data not shown).

**DISCUSSION**

Biochemical and in vivo experiments using the M1000 strain of prions confirmed earlier reports of the resistance of this strain, derived from the mouse-adapted human prion strain Fukuoka, to decontamination with NaOH or autoclave cycles. This report supports the finding of Peretz et al. (2006) that human prion strains are more resistant to inactivation than rodent-adapted sheep scrapie strains. However, this study further suggests that human prion strains retain their resistance to inactivation, despite passage in a rodent species.

Enzymatic detergent preparations have been shown to have a range of efficacies against prions derived from rodent-adapted scrapie strains (Fichet et al., 2004; Jackson et al., 2005; Yan et al., 2004). Four enzymatic medical instrument cleaning preparations were tested for their efficacy against M1000 prions present in an IBH. All four preparations tested were unable to degrade all of the PrPSc present within the M1000 IBH and showed variable efficacy against PrPSc present in UBH after brief exposure times at moderate temperatures. These results support the variable protein-degrading capacity of enzymatic medical
instrument cleaning preparations (Cheetham & Berentsveig, 2002) and confirmed the variable efficacy of such preparations in degrading PrP\textsuperscript{Sc}.

Optimization of RMEC A and B preparations indicated that temperatures of 40 °C or greater were required for efficient degradation of PrP\textsuperscript{Sc}. Increasing the incubation time at lower temperatures did not completely overcome the limitations of temperatures below 40 °C, indicating that the effect of temperature was not directly related to the temperature-dependent activity of the enzyme.

PK was able to digest PrP\textsuperscript{Sc} in the presence of cleaner A more efficiently and over a greater temperature range than the protease (subtilisin) present in RMEC A. This was not simply related to differences in enzyme concentrations, as a fourfold increase in subtilisin concentration in RMEC A did not improve PrP\textsuperscript{Sc} degradation. Heat alone did not render the PrP\textsuperscript{Sc} protease sensitive, as PK digestion performed on a homogenate diluted in PBS had little effect on the protease resistance of PrP\textsuperscript{Sc}. Therefore, it would appear that, although subtilisin and PK are both serine endopeptidases, they differ in their activity towards PrP\textsuperscript{Sc} in the presence of the components of cleaner A. As PrP\textsuperscript{Sc} degradation was found to be optimal at temperatures between 40 and 60 °C, we concluded that higher temperatures assist the unfolding of PrP\textsuperscript{Sc} into a protease-sensitive form that can be digested efficiently by proteolytic enzymes in the enzymatic medical instrument cleaning solutions.

Our results with cleaner A in the presence of PK would suggest that cleaner A alone is able to unfold most PrP\textsuperscript{Sc} into a protease-sensitive form. However, we caution against prionocidal products that destabilize PrP\textsuperscript{Sc} without the benefit of degradation (Lemmer et al., 2004). PrP\textsuperscript{Sc} has been reported to be reversibly denatured by concentrations of guanidine, reforming PrP\textsuperscript{Sc} with converting activity after the denaturant has been diluted (Kocisko et al., 1996). Detachment and destabilization of PrP\textsuperscript{Sc} without the added step of degradation may enable PrP\textsuperscript{Sc} to refold into an infectious form under less stringent conditions, thus introducing an environmental contamination risk.

Interestingly, WHO-recommended prionocidal protocols using NaOH or sodium hypochlorite were the only methods shown by Lemmer et al. (2004) to degrade PrP\textsuperscript{Sc} after removal from a prion-contaminated steel surface.

Formulations of RMEC A containing various concentrations of an anionic surfactant, solvent, protease or amylase were tested against M1000 IBH. At 45 °C, amylase and

![Fig. 6. Effect of infectious dose of surgical-steel-bound M1000 prions on time to disease development in Tga/20 mice. (a) The relationship between the inoculation dose and incubation period for surgical steel wires contaminated with M1000 prions was assayed in Tga/20 mice. Regression analysis using the method of least-squares was used to construct the fitted line with the regression equation \( y = 180.5 - 22.05 \log(x) \), where \( y \) equals mean incubation (days) and \( x \) equals dose (LD\textsubscript{50} units). The strength of the association was tested. \( R^2 = 0.5587, P < 0.0001 \). (b, c) The time to disease (incubation period) for Tga/20 mice implanted with M1000-contaminated steel wires following treatment with standard sterilization and prion decontamination methods (b) and decontamination using the optimized RMEC protocols with and without autoclave sterilization (c) are shown. Incubation times that were significantly extended relative to untreated controls are indicated: *, \( P < 0.05 \), **, \( P < 0.001 \).]
solvent did not contribute to PrPSc degradation. Interestingly, at 45 °C, the absence of anionic surfactant improved PrPSc degradation. The surfactant present in RMEC A preparations is sodium dodecyl benzene sulphonic acid. This molecule contains an extra benzene group that significantly increases its ionic power relative to SDS, a detergent that has been reported to contribute to prion inactivation (Jackson et al., 2005; Kimberlin et al., 1983; Peretz et al., 2006; Tateishi et al., 1991). In previous studies, successful decontamination of prions by SDS has required high temperatures. Therefore, the adverse effect of sodium dodecyl benzene sulphonic acid at the relatively low temperature of 45 °C may further support the importance of temperature as a factor contributing to the PrPSc-degrading efficacy of RMEC A.

In vivo studies using M1000 prion-contaminated steel wires supported biochemical studies with treatment using RMEC A or B. Biochemical studies indicated that the optimized conditions reduced PrPSc by at least 2 log10. In vivo infectivity was reduced by more than 4 log10 LD50 units by RMEC A and more than 3 log10 LD50 units by RMEC B. The improved performance of each product when directed against surface-bound prions, which are more resistant to decontamination than suspensions, may reflect the reduced contaminant-to-product ratio that can be assayed in the surface-bound system and the ability of the tested products both to clean efficiently and to inactivate prions from the contaminated surface. This is an important benefit of using surface-bound prions to develop inactivation strategies, as it considers the load of the contaminant and the importance of cleaning in the sterilization process.

It was recently reported that polyanionic molecules, in particular RNA, can stimulate the formation of protease-resistant PrP in a cell-free assay of prion propagation (Deleault et al., 2003) and that the incubation and sonication of PrPsc in the presence of polyRNA can generate de novo infectivity (Deleault et al., 2007). In our study, the IBH used in biochemical analysis was treated with benzonase prior to treatment with enzymatic medical instrument cleaning preparations to reduce the viscosity of the homogenate. In contrast, homogenates used to contaminate surgical steel wires were not treated with benzonase and as such may have represented a more challenging target for decontamination.

This study confirms the earlier work of Fichet et al. (2004) demonstrating that the infectivity of surface-bound prions can be titrated directly from the solid support to which it is tightly bound, thus bypassing the need to remove the infectivity from the wires for titration (Weissmann et al., 2002). We have further shown that the incubation time interval assay reproduced the titre of infectivity associated with surface-bound prions, thus validating the use of this assay to calculate surface-bound prion titre. The assay further enables the chemical decontaminant to be washed from the surface, thus eliminating potential problems associated with toxicity and the effect of the chemical on prion incubation time (Taylor & Fernie, 1996). Finally, in conjunction with the PrPsc-overexpressing transgenic mouse line used here, this assay represents a rapid assay that suitably represents prion contamination, cleaning and sterilization of surgical steel instruments in a medical setting.

A significant drawback to protocols currently available is the length of time of treatment required to reduce infectivity effectively. Traditional methods using NaOH and sodium hypochlorite require a minimum treatment period of 1 h. Recently reported protocols have included 15 min treatments with an alkaline detergent (Fichet et al., 2004), a 1 h three-step treatment in a detergent/enzyme solution (Jackson et al., 2005) and treatments of 2 h or more in acidified SDS (Peretz et al., 2006). In the current study, treatment times of 30 min were tested at temperatures of 50–60 °C. At higher temperatures, PrPSc degrad-ation was limited, most probably due to inactivation of the proteolytic enzyme responsible for prion degradation at higher temperatures. Chemical modification to increase enzyme stability at higher temperatures holds the promise of shorter treatment times at higher temperatures for routine decontamination of heat-resistant surgical instruments.

The M1000 strain of mouse-adapted human prions is highly resistant to decontamination using the WHO-recommended method of treatment with 1 M NaOH for 1 h at room temperature. In the current study, the incubation period of mice implanted with M1000-contaminated steel wires treated with 1 M NaOH was not significantly delayed relative to untreated M1000-contaminated steel wires and reduced the infectious prion load by only 2.7 log10 LD50 units. In contrast, optimization of the RMEC A product at 50 °C inactivated more than 4 log10 LD50 units of prion infectivity (Table 2) and significantly delayed incubation time relative to untreated (P<0.001) and NaOH-treated wires (P<0.001). This cycle is compatible with heat-sensitive instruments such as endoscopes, which may come into contact with peripheral sources of prion infectivity. Combining the RMEC B protocol with an autoclave cycle of 121 °C for 20 min eliminated detectable levels of infectivity and therefore reduced the infectious prion load by more than 5 log10 LD50 units. Consistent with the slightly reduced efficacy of an autoclave cycle of 134 °C for 3 min compared with an autoclave cycle of 121 °C for 20 min, one mouse succumbed to disease when the former cycle was performed in conjunction with the RMEC B protocol. The RMEC B studies indicated that the RMEC B and autoclaving protocols targeted different prion populations, as inactivation using both methods approximated the sum of the individual methods (Taylor, 2000). Thus, it can be predicted that combining the RMEC B protocol (3.5 log10 LD50 unit reduction in infectivity) with the most effective autoclave cycle of 134 °C for 18 min (2.2 log10 LD50 unit reduction in infectivity) might result in prion inactivation approaching 6 log10 LD50 units and that better results may be obtained by combining the more effective RMEC A protocol with an autoclave cycle.
The RMEC formulations are non-corrosive, neutral pH detergents that have been developed for cleaning of endoscopes and surgical equipment. Therefore, the protocols described here can be used for routine cleaning of surgical instruments without the corrosive effect associated with NaOH methods (McDonnell & Burke, 2003) and may minimize the risk of prion disease transmission through general surgical procedures (Collins et al., 1999). It will be of importance to validate further the efficacy of these protocols against other human prion strains.

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REFERENCES


Table 2. Effect of chemical and physical treatment methods on the infectivity associated with M1000 prion-contaminated surgical steel wires

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean incubation period in days (sd)</th>
<th>Total death/total number</th>
<th>log10 reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>71 (2)</td>
<td>4/4</td>
<td>0</td>
</tr>
<tr>
<td>1 M NaOH</td>
<td>130 (19)</td>
<td>9/9</td>
<td>2.7</td>
</tr>
<tr>
<td>121 °C 20 min</td>
<td>106 (2)</td>
<td>10/10</td>
<td>1.6</td>
</tr>
<tr>
<td>134 °C 3 min</td>
<td>104 (3)</td>
<td>9/9</td>
<td>1.5</td>
</tr>
<tr>
<td>134 °C 18 min</td>
<td>120 (5)</td>
<td>10/10</td>
<td>2.2</td>
</tr>
<tr>
<td>RMEC A 1 %, 50 °C, 30 min</td>
<td>204 (18)*</td>
<td>8/10†</td>
<td>≥4.5§</td>
</tr>
<tr>
<td>RMEC B 0.3 %, 60 °C, 30 min</td>
<td>147 (13)†</td>
<td>6/10</td>
<td>≥3.5§</td>
</tr>
<tr>
<td>RMEC B protocol +134 °C 3 min</td>
<td>166</td>
<td>1/10†</td>
<td>≥4.3§</td>
</tr>
<tr>
<td>RMEC B protocol +120 °C</td>
<td>–</td>
<td>0/10</td>
<td>≥5</td>
</tr>
</tbody>
</table>

†Incubation time was significantly extended beyond that of mice implanted with untreated wires (*P < 0.001, †P < 0.05).

§One mouse in each of these groups was culled with an intercurrent illness 300+ days post-implantation. These mice were negative for PrPSc and have been included in the analysis as non-clinical mice.

§log reduction is shown as ≥ value to indicate the survival of some mice in these groups.


