

Decontamination of surgical instruments from prion proteins: *in vitro* studies on the detachment, destabilization and degradation of PrP^{Sc} bound to steel surfaces

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Effective reprocessing of surgical instruments ensuring elimination of inadvertent contamination with infectious agents causing transmissible spongiform encephalopathies (TSEs) is essential for the prevention of iatrogenic transmission of Creutzfeldt–Jakob disease (CJD) or its new variant (vCJD) from asymptomatic carriers. In a search for effective yet instrument-friendly and routinely applicable reprocessing procedures, we used an *in vitro* carrier assay to assess the decontamination activity exerted by different reagents on pathological prion protein (PrP^{Sc}), the biochemical marker for TSE infectivity, attached to steel surfaces. In this assay, steel wires were contaminated with 263K scrapie brain homogenate and reprocessed for decontamination by exposure to several different test reagents. Residual contamination with PrP^{Sc} and its protease-resistant core PrP27-30, still present after reprocessing on the wire surface or in the cleaning solution, was monitored by sensitive Western blot detection without or after proteinase K digestion. Using this approach, various reagents and processing conditions were screened for both their efficacy of decontamination and their active principles, such as detachment, destabilization or degradation of surface-bound prion protein. This revealed that, under appropriate conditions, relatively mild reagents such as 0.2% SDS/0.3% NaOH (pH 12.8), a commercially available alkaline cleaner (pH 11.9–12.2), a disinfectant containing 0.2% peracetic acid and low concentrations of NaOH (pH 8.9) or 5% SDS (pH 7.1) exert potent decontaminating activities on PrP^{Sc}/PrP27-30 attached to steel surfaces. For *in vivo* validation, wires reprocessed in these reagents have been implanted into reporter animals in ongoing experiments.

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INTRODUCTION

Transmissible spongiform encephalopathies (TSEs) such as Creutzfeldt–Jakob disease (CJD) and its variant form (vCJD) in humans, bovine spongiform encephalopathy (BSE) in cattle and scrapie in sheep are invariably fatal neurodegenerative diseases of the central nervous system. The agents that cause TSEs are widely believed to represent a unique biological principle of infection. According to the prion hypothesis (Prusiner, 1982, 1998), TSE agents (so-called proteinaceous infectious particles or prions) consist essentially – if not entirely – of a misfolded form of the prion protein (PrP), which is known as PrP^{Sc} and derived from a host-encoded cellular precursor (PrP^C). Although the exact molecular nature of TSE agents remains to be determined, there is substantial evidence that PrP^{Sc} (or its protease-resistant core, PrP27-30) provides a practical biochemical marker for these pathogens (McKinley *et al.*, 1983; Jendroska *et al.*, 1991; Beekes *et al.*, 1996; Baldauf *et al.*, 1997; Ironside, 2000; Wadsworth *et al.*, 2001).

Following the emergence of BSE (Wells *et al.*, 1987) and vCJD (Will *et al.*, 1996), substantial evidence has accumulated that the latter can most likely be attributed to transmission, presumably via contaminated food, of BSE from cattle to man (Bruce *et al.*, 1997; Hill *et al.*, 1997; Cousens *et al.*, 1999; Scott *et al.*, 1999). The countermeasures implemented in response to the BSE epidemic are expected effectively to prevent further spread of this disease to humans, thereby minimizing the risk of new primary vCJD infections. However, additional challenges for public health in the context of TSEs arise from the hypothetical as well as the established risks of human-to-human transmission of vCJD and classical CJD, respectively (Brown *et al.*, 2001; Taylor, 2003; Beekes *et al.*, 2004; Llewelyn *et al.*, 2004). The experience with iatrogenic CJD, of which 267 cases were reported until July 2000 (Brown *et al.*, 2000), and the detection of infectivity or PrP^{Sc} in a variety of tissues from vCJD patients in addition to the brain and spinal cord (e.g. lymphatic system and peripheral nervous system; Bruce *et al.*, 2001; Wadsworth *et al.*, 2001; Hilton

et al., 2002; Haik *et al.*, 2003; Ramasamy *et al.*, 2003) have led to the formulation of national and international recommendations and guidelines aiming at the prevention of iatrogenic transmission of these diseases (Simon & Pauli, 1998; World Health Organization, 1999; Abschlussbericht der Task Force vCJK, 2002).

In hospitals, it is of the utmost importance to avoid the spread of TSE infectivity via surgical instruments by effective and safe decontamination (e.g. cleaning, chemical disinfection, sterilization; Beekes *et al.*, 2004; Schulster, 2004). This is highlighted by the fact that PrP^{Sc} has also been detected in skeletal muscles of CJD patients (Glatzel *et al.*, 2003) and is present in lymphatic tissues, and possibly blood, during preclinical phases of vCJK incubation (Hilton *et al.*, 1998, 2002; Llewelyn *et al.*, 2004).

The high resistance of TSE agents to conventional methods of chemical or thermal inactivation and to UV or ionizing radiation (Alper *et al.*, 1966, 1967; Brown *et al.*, 1982, 1986; Kimberlin *et al.*, 1983; Taguchi *et al.*, 1991; Tateishi *et al.*, 1991; Ernst & Race, 1993; Taylor *et al.*, 1994; Manuelidis, 1997; Taylor, 1999; for review see Taylor, 2000), as well as their high binding affinity to and tenacity on steel surfaces (Zobeley *et al.*, 1999; Flechsig *et al.*, 2001), warrant specific decontamination procedures in the reprocessing of surgical instruments (Rutala & Weber, 2001). Treatments that are considered appropriate for decontamination include use of 1–2 M NaOH solution (for 24 h), 2.5–5 % NaOCl solution (for 24 h) as well as 3, 4 or 6 M GdnSCN solution (for 24 h, 1 h or 15 min, respectively), followed by steam sterilization at 134 °C for 18 min to 1 h (Simon & Pauli, 1998; World Health Organization, 1999; Hörnlimann *et al.*, 2001). Such stringent conditions, which are mandatory for the reprocessing of non-disposable instruments used in patients with CJD (or those with a recognizable risk of it), are hazardous to both equipment and operators, therefore they do not offer an option for the routine maintenance of surgical instruments used on patients without a recognizable risk of human TSE. Here, generally applicable decontamination strategies that take into account the theoretical risk of CJD and vCJK transmission from asymptomatic carriers on the one hand, without compromising the conventional processes for cleaning, disinfection and sterilization on the other, are required.

In the search for effective yet instrument-friendly and routinely applicable procedures complying with these requirements, we used an *in vitro* carrier assay to assess the decontamination efficacy exerted by various reagents on PrP^{Sc} and its proteinase K (PK)-resistant core PrP^{Sc}/PrP^{Sc}-30 (referred to here as PrP^{Sc}/PrP^{Sc}-30), attached to steel surfaces. The rationale of our experiments is based on the paradigm of a steel wire assay originally described by Zobeley *et al.* (1999) and Flechsig *et al.* (2001), as well as on the notion that a reduction of the amount of PK-resistant prion protein achieved by decontaminating reagents should, in principle, correlate with a decrease of infectivity (Beekes *et al.*, 2004). However, this correlation

needs to be carefully validated for each individual setup of inactivation experiments, as highlighted by McLeod *et al.* (2004). Comprehensive studies in which PrP^{Sc} was visualized by Western blotting after immunolabelling with the monoclonal anti-PrP antibody 3F4 (mAb 3F4) have previously shown a close quantitative correlation between PrP^{Sc}/PrP^{Sc}-30 and infectivity in the brains of hamsters infected with 263K scrapie agent (Beekes *et al.*, 1996; Baldauf *et al.*, 1997). In addition, studies have demonstrated an inactivation of 263K scrapie agent concomitant with the disappearance of PK-resistant PrP in hamster brain homogenates following incubation with an alkaline cleaning reagent (Baier *et al.*, 2004). We simulated contamination of surgical instruments using stainless steel wires incubated in 263K scrapie brain homogenate containing PrP^{Sc}/PrP^{Sc}-30 (as well as a minor proportion of normal PrP^C). Other than intact brain tissue, which is known to contain varying infectivity titres and concentrations of PrP^{Sc} in different cerebral areas, brain homogenate allows defined amounts of material to be dried onto carriers and facilitates the standardization and reproducibility of decontamination experiments within the same or in different laboratories. Scrapie brain homogenates have been used as the substrate of choice in a variety of inactivation studies (Taylor, 2000, 2004). Hard data formally proving that homogenized brain tissue dried onto wire surfaces provides an appropriate equivalent to intact brain tissue contaminating surgical instruments are limited, but there are reported findings that suggest this. First, inactivation of murine ME7 scrapie agent by formic acid was found to be similarly effective if applied to brain homogenate or intact, even fixed brain tissue (Taylor *et al.*, 1997). Second, under certain experimental conditions, homogenates were actually found to provide a more challenging substrate for inactivation than intact tissue: scrapie infectivity was inactivated in intact scrapie-infected mouse brain, but not in 10 % homogenates of brain tissue, by 2 % peracetic acid (Taylor, 1991). 'One explanation for the presence of heat-or-chemical resistant subpopulations of scrapie agent might be the protective effect of aggregation which could occur in homogenates of infected tissue but not in undiluted tissue' (Taylor, 2004). Third, it has been observed that scrapie agent in partially smeared tissue dried onto glass or metal surfaces is more resistant to inactivation by autoclaving than in intact undisrupted tissue, which is thought to result from rapid PrP^{Sc}-fixation in the dried film (Taylor, 2000, 2004). Brain tissue, which provides one of the most challenging contaminations in the routine reprocessing of surgical instruments (Köhnlein *et al.*, 2004) is rich in lipids, compounds that are known to further increase the resistance to inactivation of TSE agents and the stability of PrP^{Sc} (Appel *et al.*, 2001). Thus, particularly when additionally fixed by air-drying as in our studies, scrapie brain homogenate attached to steel wires can be considered a relevant model for a worst-case scenario in nosocomial instrument decontamination.

With this paradigm, we reprocessed batches of steel wires for decontamination by exposure, under various conditions,

to several different test reagents. Residual contamination of wires still present after reprocessing was then monitored by sensitive Western blot detection (Beekes *et al.*, 1995; Thomzig *et al.*, 2003, 2004) of full-length PrP (PrP^{Sc} and PrP^C) and PrP27-30 which could be eluted from the carriers without or after PK digestion, respectively. Finally, the cleaning solutions in which the wires had been incubated were also scrutinized for their content of total PrP and PrP27-30.

A broad spectrum of reagents and processing conditions were screened for both efficacy of decontamination and the underlying active principles such as detachment, destabilization or degradation of surface-bound prion protein. This revealed several different, relatively mild candidate reagents which appear to be potentially suitable for the thorough decontamination of steel surfaces from PrP^{Sc}/PrP27-30.

METHODS

Reagents for decontamination. The following reagents were tested in various concentrations and incubation conditions, either individually or in combination, for their ability to detach, destabilize or degrade PrP^{Sc}/PrP27-30 bound to steel surfaces: sodium hydroxide, guanidine thiocyanate and urea (Merck); sodium hypochlorite (2.5% stock solution with >20 000 p.p.m. available chlorine) and SDS (Roth); peracetic acid (Degussa) titrated before use; an alkaline cleaner (Baier *et al.*, 2004) and a disinfectant containing 0.2% peracetic acid and sodium hydroxide in the range 0.075–0.225% (the stock solution of this disinfectant contains 5–15% NaOH according to the manufacturer's specification), both of which (alkaline cleaner and disinfectant) are used in the routine maintenance of certain medical devices. Distilled water served as a control.

In vitro carrier assay. A stock of 10% 263K scrapie brain homogenate, containing $\sim 10^8$ 50% intracerebral lethal doses (LD_{50i.c.}) of 263K agent and ~ 10 µg pathological prion protein (PrP^{Sc}/PrP27-30) ml⁻¹ was prepared from brains of Syrian hamsters in the terminal stage of disease (Beekes *et al.*, 1995, 1996) and stored at -70 °C in aliquots. Stainless steel wire (DIN-No. 1.4301, Forestadent, Pforzheim, Germany; diameter 0.25 mm) was cut into small pieces 5 mm long. These test bodies (here called wires) with a surface area of ~ 4.0 mm² ($2\pi rh + 2\pi r^2$), were washed in 2% Triton X-100 for 15 min under constant ultrasonication (Sonorex RK 102 P; Bandelin Electronics), rinsed in distilled water, dried and sterilized in a steam autoclave at 121 °C for 20 min. In order to contaminate the carriers *in vitro* with PrP^{Sc}/PrP27-30, batches of 30 wires were incubated in 150 µl 10% scrapie brain homogenate for 2 h under constant shaking at 37 °C and 700 r.p.m. in a thermomixer (Amersham Biosciences). Control batches of wires were similarly incubated in 10% normal hamster brain homogenate. Following removal of the homogenate, wires were transferred to and placed separately from each other in Petri dishes, air-dried for 1 h, and stored overnight (~ 16 h) at room temperature. Subsequently, batches of 30 contaminated wires were incubated, in a volume of 1.5 ml, in the reagents to be tested for decontaminating activity (Table 1). These incubations were performed in a thermomixer (400 r.p.m.) at the temperatures and for the times specified in Table 1. Finally, the wire batches were rinsed under constant shaking five times, each time in 45 ml distilled water for 10 min at room temperature. To assess the influence of drying on the fixation of PrP^{Sc}/PrP27-30 to steel surfaces, a subset of wire batches was rinsed five times in 45 ml distilled water for 10 min at room temperature immediately after incubation in scrapie brain homogenate. After rinsing in distilled water, processing

of wires was finished by air-drying in Petri dishes for 1 h, storing overnight (~ 16 h) at room temperature, and recollection of batches in test tubes. For each reagent tested four batches of 30 wires were processed independently. Residual prion protein contaminations of wires, which were still present after processing as outlined above, were eluted from the carrier surface as follows. Of the four batches independently processed per test reagent, two were boiled for 5 min in 52 µl electrophoresis sample loading buffer (62.5 mM Tris pH 6.8, 10% glycerol, 5% mercaptoethanol, 2% SDS, 0.025% bromophenol blue) containing 4 M urea; the remaining two batches were treated with 150 µg PK ml⁻¹ in a volume of 52 µl TBS-Sarkosyl (50 mM Tris/HCl, 150 mM NaCl pH 7.5, 1% Sarkosyl) for 1 h at 37 °C, subsequently mixed with an equal volume of 2 × sample loading buffer containing 8 M urea, and boiled for 5 min. Aliquots of wire eluates (20 µl) were removed (leaving the wires on the bottom of the tube) and analysed by SDS-PAGE and Western blotting for the presence of prion protein.

Additionally, the release of total PrP and PrP27-30 into the cleaning solutions in which the wires had been incubated was monitored by SDS-PAGE and Western blotting. For this purpose, batches of 30 wires were incubated in 500 µl portions of the various reagents in a thermomixer (400 r.p.m.) at the temperatures and for the times specified in Table 1. Subsequently, the cleaning solutions were either directly mixed 1:1 with 2 × sample loading buffer and boiled for 5 min, or subjected to PK digestion. For the latter, 10 µl of the cleaning solutions were diluted in 42 µl TBS-Sarkosyl containing 150 µg PK ml⁻¹, incubated for 1 h at 37 °C, subsequently mixed 1:1 with 2 × sample loading buffer and finally boiled for 5 min. SDS-PAGE and Western blotting for prion protein were performed with 20 µl aliquots of these samples. It should be noted that PrP^C, which is present at similar levels in normal (see Fig. 2a, lane 1) and scrapie brain homogenates, may contribute partially to PrP immunostaining in samples not subjected to PK digestion.

SDS-PAGE and Western blotting. SDS-PAGE and Western blot analyses using the monoclonal anti-PrP antibody 3F4 (Kasczak *et al.*, 1987) were performed as described elsewhere (Beekes *et al.*, 1995, 1996), with recently published modifications (Thomzig *et al.*, 2003). PrP signals were visualized on X-OMAT AR film (Kodak; Sigma-Aldrich) after various exposure times individually adjusted to optimize the signal-to-noise ratio of each blot. Molecular mass (MW) marker proteins of 14.4, 20.1, 30.0, 45.0, 66.0 and 97.0 kDa were used (Amersham Biosciences). PK-digested homogenate from scrapie hamster brains, used as an internal PrP27-30 standard in the Western blot analyses, was prepared as outlined previously (Beekes *et al.*, 1995; Thomzig *et al.*, 2003). Based on the infectivity titre and content of PrP^{Sc} determined previously in brain homogenates from our 263K scrapie hamsters (Beekes *et al.*, 1995, 1996), 1×10^{-6} g scrapie-infected hamster brain homogenate contains $\sim 1-3 \times 10^3$ LD_{50i.c.} and, after digestion with PK, ~ 100 pg PrP27-30. The processed batches consisting of 30 wires represented a total steel surface of 120 mm² each. To facilitate quantification and comparison of PrP immunostaining, the amount of sample material blotted in Figs 1–4 is specified by the wire area (mm²) it corresponded to.

RESULTS

Binding of PrP^{Sc}/PrP27-30 to steel surfaces

The amount of PrP^{Sc} bound to the surface of steel wires after incubation in scrapie brain homogenate can be assessed by comparing the intensity of PrP immunostaining displayed by eluates from contaminated wires (Fig. 1a, lanes 1 and 4; lanes 2 and 5, representing 4.6 and 0.46 mm²

Table 1. Efficacy of PrP decontamination by various reagents in the *in vitro* carrier assay

Residual contamination of steel wires and cleaning solutions following processing was monitored by Western blotting for PrP without and after PK digestion. The intensity of detected PrP Western blot signals is indicated by + + +, very strong; + +, strong; +, weak but clearly discernible; (+), faint shades which may potentially, but not necessarily, indicate the presence of minute traces of residual protein (ND, not determined). Mechanisms underlying the observed decontamination activity of the test reagents such as detachment, destabilization and degradation of PrP were only deduced if they were unambiguously demonstrated (and indicated by +) or at least conclusively suggested [indicated by (+)] by the experimental findings according to the criteria outlined in the results section. In all other cases, no conclusions on the active principles of the test reagents were attempted.

Chemical	Concentration	pH*	Time (min)	Temperature (°C)	Detectable residual PrP on wires		Detectable residual PrP in cleaning solution		Observed mechanism of decontamination			
					-PK	+PK	-PK	+PK	Detach-ment	Destabi-lization	Degrada-tion	
Distilled water		6.0	0.1	23	+++	+++	+++	++	+			
			60	23	++	++	+++	++	+			
			10	55	++	++	+++	++	+			
			10	90	+++	+++	ND	ND				
Sodium hydroxide	1.0 M	13.5	5	55	++	++	++	+	+	(+)		
			60	90	++	++	++	+				
			10	55	-	-	ND	ND				+
			30	23	-	-	-	-				+
	0.5 M	13.4	5	55	-	-	ND	ND				
			10	55	-	-	-	-				+
			30	23	(+)	-	++	-	+	+		
			60	23	-	-	++	-	+	+		
	0.1 M	13.0	5	55	(+)	-	ND	ND				
			10	55	(+)	-	++	-	+	+		
			30	23	++	-	++	-	+	+		
			60	23	+	-	++	-	+	+		
Sodium hypochlorite	2.5 %	12.1	5	23	-	-	-	-				+
			10	23	-	-	-	-				+
			60	23	-	-	ND	ND				
	1.0 %	11.9	5	23	-	-	-	-				+
			10	23	-	-	-	-				+
			60	23	-	-	ND	ND				
Guanidine thiocyanate	4.0 M	5.3	10	23	+++	-	ND	ND				
			60	23	+++	-	+	-	+	+		
Urea	4.0 M	7.4	5	55	++	++	ND	ND				
			10	55	++	++	+++	++	+			
Alkaline cleaner	1.0 %	12.2	5	55	+	-	ND	ND				
			10	55	+	-	+++	-	+	+		
			60	23	+	-	ND	ND				
SDS	0.5 %	11.9	5	55	+	-	+++	-	+	+		
			10	55	+	-	+++	-	+	+		
			60	23	+	-	+++	-	+	+		
	5.0 %	7.1	10	90	(+)	-	+++	-	+	+		
			60	90	-	-	+++	-	+	+		
			60	55	+	-	ND	ND				
2.5 %	7.1	960	90	-	-	ND	ND					
		960	23	+	+	ND	ND					
		10	90	(+)	-	ND	ND					
			60	90	-	-	+++	-	+	+		

Table 1. cont.

Chemical	Concentration	pH*	Time (min)	Temperature (°C)	Detectable residual PrP on wires		Detectable residual PrP in cleaning solution		Observed mechanism of decontamination		
					-PK	+PK	-PK	+PK	Detach-ment	Destabi-lization	Degrada-tion
SDS/NaOH	0.2%/0.3%	12.8	2.5	23	(+)	-	+++	-	+	+	
				5	(+)	-	+++	-	+	+	
				10	(+)	-	+++	-	+	+	
Peracetic acid	0.25%	2.8	60	23	+++	+++	(+)	-	(+)		
				23	+++	+++	ND	ND			
Disinfectant with peracetic acid/NaOH	0.2%/≥0.075%†	8.9	120	23	(+)	-	++	(+)	+	+	
				23	-	-	++	(+)	+	+	

*As determined using a pH meter with glass electrode.

†Concentration of NaOH is in the range 0.075–0.225% according to the manufacturer's specification.

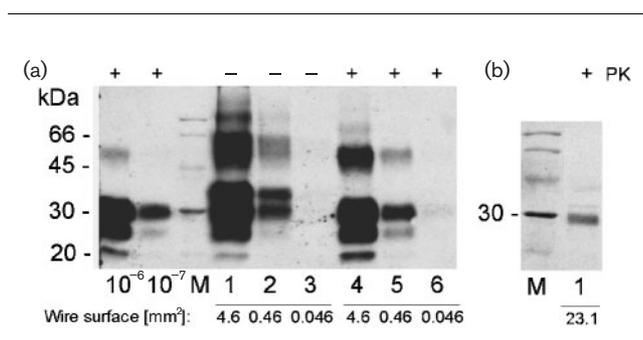


Fig. 1. Binding of PrP^{Sc} to steel surfaces. Western blot detection of PrP^{Sc} and PrP27-30 attached to steel wires after contamination with 263K scrapie brain homogenate. (a) Lanes 10⁻⁶ and 10⁻⁷, internal standards: PK-digested brain homogenate from scrapie hamsters corresponding to 1 × 10⁻⁶ and 1 × 10⁻⁷ g brain tissue. Lane M, molecular mass marker. Lanes 1–3, serial dilutions (1:10, 1:100 and 1:1000) of protein eluate from 30 contaminated wires: 20 µl out of a total sample volume of 52 µl per 30 wires was used as starting material for PrP^{Sc} detection in the dilution series; samples correspond to 4.620, 0.462 and 0.046 mm² of wire surface. Lanes 4–6, serial dilutions (1:5, 1:50 and 1:500) of protein eluate from 30 contaminated wires which were incubated with proteinase K prior to subsequent processing to visualize PrP27-30: 20 µl out of a total sample volume of 104 µl per 30 wires was used as starting material for the dilution series; samples correspond to 4.620, 0.462 and 0.046 mm² of wire surface. Wires analysed in lanes 1–6 were air-dried after contamination, then rinsed in distilled water before eluting proteins. (b) Lane M, molecular mass marker. Lane 1, undiluted protein extract from 30 contaminated wires which were rinsed without prior drying immediately after incubation in brain homogenate with distilled water: 20 µl out of a total sample volume of 104 µl (from 30 wires) was used for PrP27-30 detection following incubation of wires with PK; sample corresponds to 23.1 mm² of wire surface.

of wire surface, respectively) with Western blot signals from internal PrP27-30 standards (Fig. 1a, lanes 10⁻⁶ and 10⁻⁷). This revealed that the amount of PrP^{Sc}/PrP27-30 bound per mm² of wire surface approximately corresponded to that present in approximately 2–3 × 10⁻⁷ g scrapie brain tissue. The sensitivity of our assay allowed the detection of PrP in eluates from batches of 30 wires down to a dilution of 1:1000 (Fig. 1a, no detectable signal in lane 3 but weak immunostaining in lane 6; samples represent 0.046 mm² of wire surface). It should be noted that the amount of surface-bound PrP^{Sc}/PrP27-30 was substantially (at least 50-fold) reduced when wires were not air-dried after incubation in scrapie brain homogenate, but were immediately rinsed with water [Fig. 1b, compare lane 1 and lane 5 (1a)].

Efficacy and active principles of test reagents for decontamination of steel surfaces from PrP^{Sc}/PrP27-30

The efficacy of the decontamination of steel wires by various test reagents was assessed by comparing the initial load of contamination with the amount of total PrP and PrP27-30 residually attached to the carriers or released into the cleaning solution after processing.

This analytical approach also shed light on the active principles underlying the effects of the different reagents (degradation, detachment or destabilization of PrP^{Sc}). (i) If – without PK treatment – PrP could be detected only in a substantially reduced amount, or not at all, on the steel wires and in the cleaning solution, this indicated degradation of the protein; (ii) if – without or after PK treatment – PrP was found in the cleaning solution, the protein was at least in part detached from the wire surface; (iii) if prion protein visible in the Western blot prior to PK treatment was markedly reduced in its amount or

completely disappeared upon digestion with PK, this showed that the reagent destabilized the protease-resistant core of PrP^{Sc} molecules in that it made this core more susceptible to enzymic degradation.

Table 1 summarizes the decontamination efficacy and active principles observed in our *in vitro* carrier assay for the test reagents examined.

Distilled water. The mildest processing conditions were simulated by incubating wires in distilled water at different temperatures up to 90 °C. This treatment left massive contamination attached to the carriers. Compared with incubation at 23 and 90 °C for 60 min, or 55 °C for 10 min, exposure of wires to water at 90 °C for 10 min appears to exert a fixing effect (Fig. 2a and b, compare lanes 3, 4 and 6 against lane 5). However, even by simple processing in water, considerable proportions of PrP were detached from the wire surface and released into the aqueous phase (see Fig. 4a and b, lanes 10, 11, 17 and 18), and after 60 min at 90 °C a proportion of the released PrP^{Sc}/PrP27-30 appeared to be destabilized and rendered susceptible to PK digestion (Fig. 4a and b, lane 18). Such an effect was not observed with the fraction of PrP^{Sc}/PrP27-30 that remained attached to the wire surface under these processing conditions (Fig. 2b, lane 6).

Sodium hydroxide and sodium hypochlorite. In accordance with a wealth of previous findings on these reagents (Kimberlin *et al.*, 1983; Brown *et al.*, 1986; Taylor *et al.*, 1994; Taylor, 2000; Flechsig *et al.*, 2001; Rutala & Weber, 2001), incubation of wires in 1 M NaOH or 2.5 and 1% NaOCl (containing at least 20 000 and 8000 p.p.m. available chlorine, respectively) under the conditions specified in Table 1 led to apparently complete degradation of PrP^{Sc}/PrP27-30, as revealed by Western blotting (Figs 3a, lane 1; 3b, lanes 1–2; 4a, lanes 1, 4 and 5). [The cross-reactive band visible after PK digestion in several samples (Fig. 3a, lane 2; 3b, lane 3; 4b, lane 5) resulted from the protease; Korth *et al.*, 2000.] NaOH was similarly effective at a concentration of 0.5 M if applied at 55 °C. However, after incubation for 30 min at 23 °C with 0.5 M NaOH, some residual PrP remained detectable in several runs prior to PK digestion (Fig. 3a, lane 3). Following treatment with 0.1 M NaOH, PK-sensitive residual PrP could be found attached to the wire surface as well as released into the cleaning solution (Fig. 3a, lanes 5 and 6; Fig. 4a and b, lane 3). This shows that 0.1 M NaOH exerts a detaching and destabilizing effect on the protein.

Guanidine thiocyanate. After incubation in 4 M GdnSCN, strong Western blot signals for residual PrP contaminations of wires were observed (Fig. 3c, lanes 1 and 2). In wire eluates, PrP immunostaining could be detected down to a dilution of 1:100; these dilution samples represented 0.462 mm² of wire surface and showed weak but still clearly discernible signals (not shown). Only a small proportion of the protein was detectable in the cleaning solution (Fig. 4a,

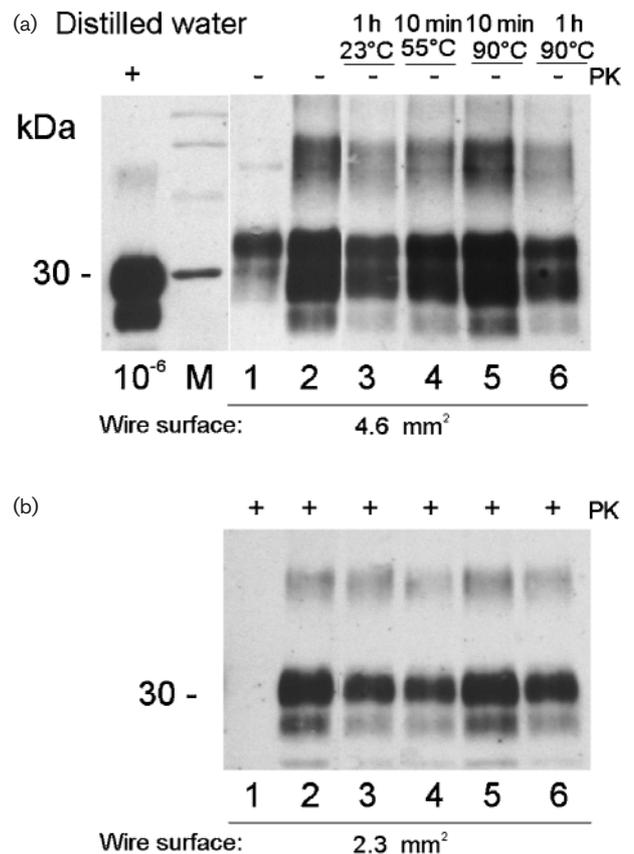


Fig. 2. Effect of distilled water on PrP^{Sc} bound to steel surfaces. Western blot detection of total PrP and PrP27-30 in eluates from contaminated steel wires after incubation in distilled water under varying conditions without (a) or after (b) PK digestion. Lane 10⁻⁶, internal standard: PK-digested brain homogenate from scrapie hamsters corresponding to 1 × 10⁻⁶ g brain tissue. Lane M, molecular mass marker. Lane 1, wires contaminated with normal brain homogenate from uninfected donors rinsed with water immediately after drying. Lanes 2–6, wires contaminated with scrapie brain homogenate rinsed with water (2) or incubated in water for 60 min at 23 °C (3), 10 min at 55 °C (4), 10 min at 90 °C (5) and 60 min at 90 °C (6). Samples in lanes 1–6 correspond to 4.620 and 2.31 mm² of wire surface, respectively.

lane 12). Thus, there was no evidence for substantial degrading or detaching activity exerted by this reagent. However, complete disappearance of PrP^{Sc}/PrP27-30 after incubation with PK (Figs 3c, lane 3; 4b, lane 12) indicated that the protease-resistant core of PrP^{Sc} was destabilized and rendered susceptible to enzymic degradation by this chaotropic reagent.

Urea. Following treatment with 4 M urea, substantial amounts of PrP were retained on the wire surface (Fig. 3d, lanes 1 and 2) as well as being released into the cleaning solution (Fig. 4a, lanes 15 and 16). Large proportions of the residual PrP^{Sc}/PrP27-30 on the wires and in

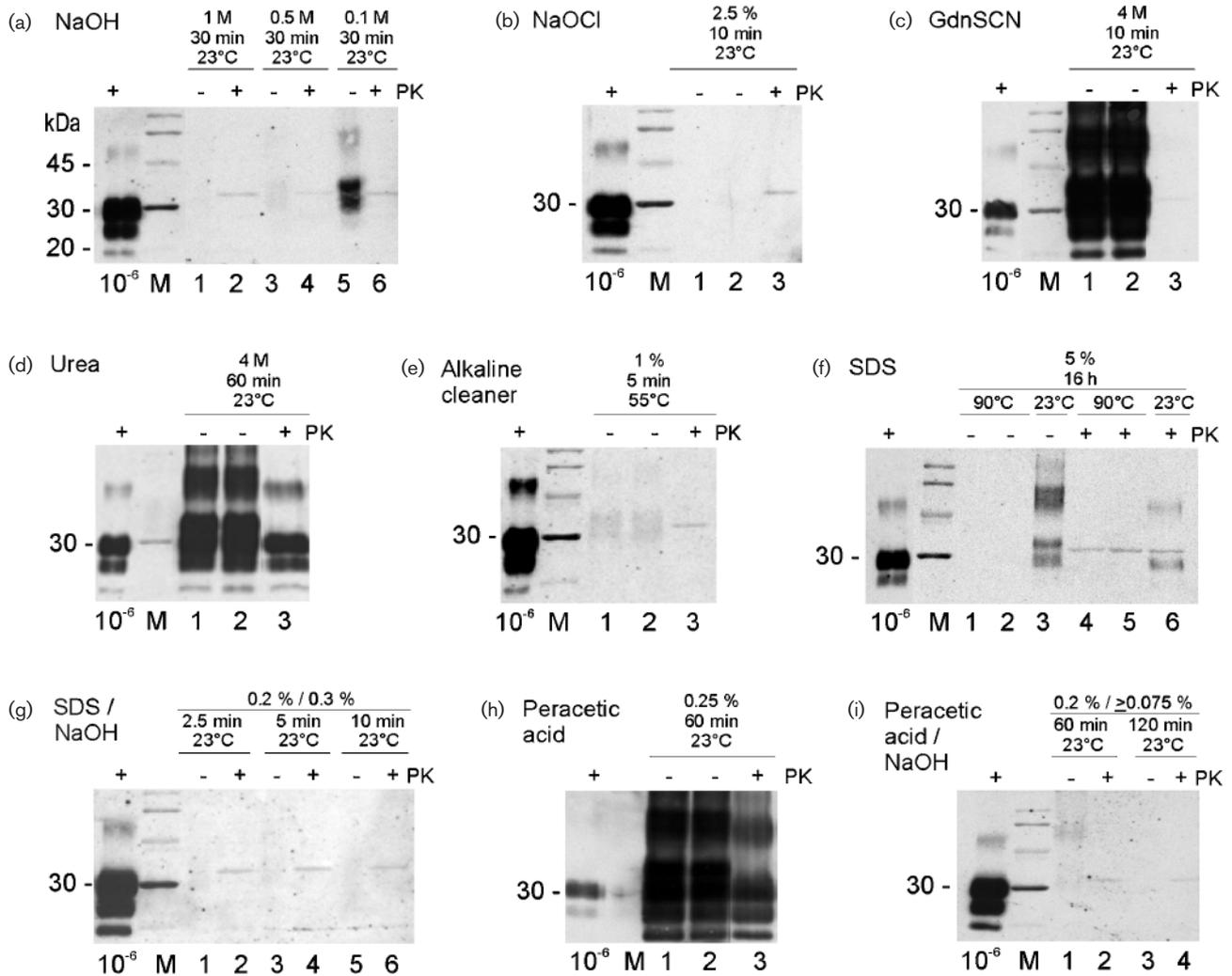


Fig. 3. Efficacy of reagents for the decontamination of steel surfaces from PrP^{Sc}. Detection of full-length PrP and PrP27-30 in eluates from contaminated steel wires after incubation in various reagents without and after PK digestion, Western blots providing a representative selection of findings. (a–i) Lanes 10⁻⁶, internal standard: PK-digested brain homogenate from scrapie hamsters corresponding to 1 × 10⁻⁶ g brain tissue. Lane M, molecular mass marker. Numbered lanes represent protein eluates from 30 contaminated wires incubated under various conditions in the following solutions containing reagents to be tested for decontaminating activity: (a) 1 M (lanes 1, 2), 0.5 M (lanes 3, 4) or 0.1 M NaOH (lanes 5, 6) for 30 min at 23 °C; (b) 2.5 % NaOCl for 10 min at 23 °C (lanes 1–3); (c) 4 M guanidine thiocyanate for 10 min at 23 °C (lanes 1–3); (d) 4 M urea for 60 min at 23 °C (lanes 1–3); (e) 1.0 % solution of an alkaline cleaner used in the routine maintenance of certain medical devices for 5 min at 55 °C (lanes 1–3); (f) 5 % SDS for 16 h at 90 °C (lanes 1, 2, 4, 5) or at 23 °C (lanes 3, 6); (g) mixture of 0.2 % SDS and 0.3 % (0.075 M) NaOH for 2.5 min (lanes 1, 2), 5 min (lanes 3, 4) and 10 min (lanes 5, 6) at 23 °C; (h) 0.25 % peracetic acid for 60 min at 23 °C (lanes 1–3); (i) a disinfectant used in the routine maintenance of certain medical devices containing 0.2 % peracetic acid and NaOH in the range 0.075–0.225 % (0.019–0.057 M) for 60 min (lanes 1, 2) and 120 min (lanes 3, 4) at 23 °C. PK-treated samples correspond to 23.1 mm², samples not subjected to PK digestion to 46.2 mm² of wire surface.

the cleaning solution were resistant to digestion with PK (Figs 3d, lane 3; 4b, lanes 15 and 16). PrP immunostaining in wire eluates examined after or without PK digestion could be detected down to a dilution of 1:100, with very weak signals in the samples representing 0.462 mm² of wire surface (not shown). These findings demonstrate that 4 M urea, under the conditions used, exerted a detaching

but no prominent destabilizing or degrading activity on PrP^{Sc}/PrP27-30.

Alkaline cleaner. The commercially available alkaline cleaner considerably reduced the load of prion protein attached to the wires (Fig. 3e, lanes 1 and 2), apparently at least in part by mediating substantial release of PrP

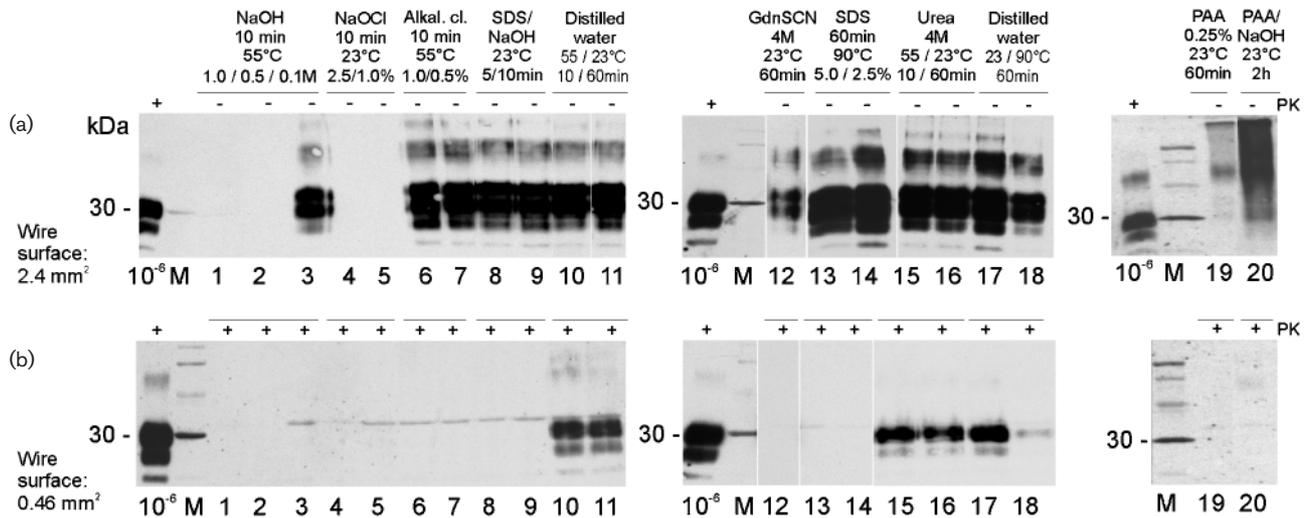


Fig. 4. Release of prion protein from steel surfaces into the cleaning solution. Detection of full-length PrP and PrP27-30 in solutions of decontamination reagents in which the wires had been incubated, Western blots providing a representative selection of findings. Findings without (a) and after (b) PK digestion of the cleaning solutions. Lanes 10⁻⁶, internal standard: PK-digested brain homogenate from scrapie hamsters corresponding to 1 × 10⁻⁶ g brain tissue. Lane M, molecular mass marker. Numbered lanes in (a) and (b) represent 1 M (lane 1), 0.5 M (lane 2) or 0.1 M NaOH (lane 3) after incubation for 10 min at 55 °C; 2.5% (lane 4) or 1% NaOCl (lane 5) after incubation for 10 min at 23 °C; 1.0% (lane 6) or 0.5% (lane 7) solution of an alkaline cleaner used in the routine maintenance of certain medical devices after incubation for 10 min at 55 °C; mixture of 0.2% SDS and 0.3% (0.075 M) NaOH after incubation at 23 °C for 5 min (lane 8) or 10 min (lane 9); distilled water after incubation for 10 min at 55 °C (lane 10) or 60 min at 23 °C (lane 11); 4 M guanidine thiocyanate after incubation for 60 min at 23 °C in (lane 12); 5% (lane 13) or 2.5% SDS (lane 14) after incubation for 60 min at 90 °C; 4 M urea after incubation for 10 min at 55 °C (lane 15) or 60 min at 23 °C (lane 16); distilled water after incubation for 60 min at 23 °C (lane 17) or 90 °C (lane 18); 0.25% peracetic acid after incubation for 60 min at 23 °C (lane 19); disinfectant used in the routine maintenance of certain medical devices containing 0.2% peracetic acid and NaOH in the range of 0.075–0.225% (0.019–0.057 M) after incubation for 120 min at 23 °C (lane 20). PK-treated samples correspond to 0.46 mm², samples not subjected to PK digestion to 2.4 mm² of wire surface.

into the cleaning solution (Fig. 4a, lanes 6 and 7). Digestion with PK led to a complete disappearance of visible residual PrP^{Sc}/PrP27-30 contamination (Figs 3e, lane 3; 4b, lanes 6 and 7). Taken together, these observations show that the alkaline cleaner exerted a detaching and strong destabilizing effect.

SDS. SDS at 5 and 2.5% achieved marked decontamination of the wires when applied at 90 °C for 1 h or longer, with no residual PrP detectable on the carrier surface without or after PK digestion (Fig. 3f, lanes 1 and 2, 4 and 5). These processing regimes caused release of a massive proportion of wire-attached PrP into the SDS solution (Fig. 4a, lanes 13 and 14). On digestion with PK, all residual PrP immunoreactivity disappeared completely (Fig. 4b, lanes 13 and 14). Thus processing of contaminated wires in SDS, as specified above, exerted a strong detaching and destabilizing effect on PrP^{Sc}/PrP27-30. However, exposure of wires to 5% SDS at 23 °C, even for 16 h, resulted in incomplete decontamination (Fig. 3f, lanes 3 and 6).

SDS/sodium hydroxide. When contaminated steel wires were processed in a mixture of 0.2% SDS and 0.3%

NaOH (previously used for decontamination purposes in the laboratory of Professor H. Diringler at the Robert Koch-Institut) for 2.5, 5 or 10 min at 23 °C, only very weak shadows in the molecular mass range of PrP immunoreactivity (Fig. 3g, lanes 1 and 3), or no PrP at all (Fig. 3g, lane 5), could be detected by Western blotting of carrier eluates. However, substantial amounts of PrP were released into the cleaning solution (Fig. 4a, lanes 8 and 9). After digestion with PK, no residual PrP^{Sc} could be found on the wires or in the cleaning solution (Figs 3g, lanes 2, 4 and 6; 4b, lanes 8 and 9). These observations indicate a marked detaching and destabilizing activity exerted by this reagent mixture on PrP^{Sc}/PrP27-30.

Peracetic acid. Following treatment of contaminated wires in 0.25% peracetic acid for 1 h at 23 °C, very strong Western blot signals (Fig. 3h, lanes 1 and 2) indicated massive residual contamination of carriers. On exposure to PK, the residual surface-bound PrP showed strong resistance to enzymic degradation (Fig. 3h, lane 3). In the eluates of wires treated with peracetic acid, it was possible to detect PrP both before and after PK digestion down to a dilution of 1:100, with prominent Western blot signals observed in samples representing 0.462 mm² of wire

surface. Only minute traces of immunoreactive material were released into the peracetic acid solution, displaying an apparent molecular mass between that of the 45 and 66 kDa MW marker proteins (Fig. 4a, lane 19). This material was apparently sensitive to digestion with PK (although the lower amount of material represented in Fig. 4b, lane 19 compared with that in 4a, lane 19, has to be taken into consideration). In any case, peracetic acid, a potent oxidizing biocide, had no visible decontaminating effect and did not exert significant detaching, destabilizing or degrading effects on PrP^{Sc}/PrP27-30 if applied at a concentration of 0.25 %.

Peracetic acid/sodium hydroxide. The disinfectant containing peracetic acid and NaOH reduced the load of PrP to the threshold of detection if applied for 2 h at 23 °C (Fig. 3i, lane 3). The reagent mixture detached substantial amounts of immunoreactive material from the carriers, displayed predominantly as a broad band from the top of the gel down to 45 kDa (Fig. 4, lane 20). This smear possibly resulted from reagents present in the sample that severely interfered with the electrophoretic separation of proteins. After incubation with PK a weak immunostaining signal, possibly indicating PrP dimers, could still be detected in the cleaning solution at a molecular mass between ~60 and 66 kDa (Fig. 4b, lane 20). Taken together, these findings suggest that the protease-resistant core of PrP^{Sc} was substantially destabilized by the disinfectant used, which also appeared to exert a strong degrading activity.

Candidate reagents for thorough decontamination of steel surfaces from PrP^{Sc}/PrP27-30

In the test assay used, failure to detect residual prion protein in wire eluates which were not subjected to prior PK digestion indicated an efficacy of surface decontamination in the range of at least 500- to 1000-fold compared with the initial load of PrP bound to the carriers. Such thorough decontamination efficacy – the highest possible measurable in our assay – or at least a reduction of contamination to the threshold of detection (as indicated by faint shadows in the Western blot which may potentially, but not necessarily, point to the presence of minute traces of residual PrP) could be achieved with the following reagents and minimum incubation times/temperatures: 1 M NaOH (30 min/23 °C; 5 min/55 °C); 0.5 M NaOH (60 min/23 °C; 5 min/55 °C); 2.5 and 1 % NaOCl (5 min/23 °C); 5 and 2.5 % SDS (60 min/90 °C); 0.2 % SDS/0.3 % NaOH (2.5 min/23 °C); and the disinfectant containing peracetic acid and NaOH (120 min/23 °C).

Of these treatments, only those based on highly concentrated NaOH and NaOCl did not cause any detectable contamination of the cleaning solution. With the disinfectant containing peracetic acid and NaOH, substantial amounts of immunoreactive material extending from the top of the gel down to 45 kDa were found in the cleaning solution,

and on treatment with 0.2 % SDS/0.3 % NaOH, or highly concentrated SDS alone, strong PrP signals occurred in this analyte. However, with the possible exception of the disinfectant containing peracetic acid and NaOH, any PrP^{Sc}/PrP27-30 detached by these reagents under the specified conditions from the surface of contaminated wires was substantially destabilized, as revealed by apparently complete disappearance after digestion with PK.

The alkaline cleaner routinely used cleared the carriers to a very large extent, although not completely, from PrP at concentrations of 1 % (60 min/23 °C; 5 min/55 °C) and 0.5 % (60 min/23 °C; 10 min/55 °C), and rendered the residual protein, which remained attached to the wires, susceptible to degradation by PK. The latter was also observed for 4 M GdnSCN (10 min/23 °C), although this chaotropic reagent did not show prominent detaching or degrading activity. PrP^{Sc}/PrP27-30 released into the cleaning solution during incubation of wires in the alkaline cleaner and 4 M GdnSCN showed marked sensitivity to PK due to the strong destabilizing effects exerted by these reagents.

DISCUSSION

***In vitro* and *in vivo* carrier assays in testing reprocessing procedures for decontamination of surgical instruments from TSE agents**

The development of effective methods for decontamination of surgical instruments from TSE agents that can be used routinely without damaging the equipment would considerably facilitate the risk management of iatrogenic transmission of classical and variant CJD.

Assuming that a specific misfolded state of PrP^{Sc} or its protease-resistant core PrP27-30 is more or less essential for maintaining TSE infectivity, virtually all strategies to inactivate TSE agents aim at modifying the conformation, folding or aggregation of PrP^{Sc} so that it becomes accessible to complete enzymic degradation by PK (Beekes *et al.*, 2004). Therefore, and in the light of the experimentally established close correlation between TSE infectivity and PK-resistant PrP^{Sc}/PrP27-30, testing for the latter in an *in vitro* carrier assay such as that used in our study appears to be a suitable rapid screening method for identification of candidate decontamination reagents. Probing the detaching, destabilizing and degrading activity of test reagents on PrP^{Sc} in the wire assay is easily accomplished by Western blotting with and without PK digestion of samples eluted from the wire surface and taken from the cleaning solution. Precise knowledge of how much PrP^{Sc} remains ‘invisibly’ attached to the wire surface after boiling in sample loading buffer containing urea would further specify the sensitivity of our *in vitro* carrier assay. However, as crude scrapie brain homogenate was used for the contamination of wires with PrP^{Sc} (and for a variety of other experimental/technical reasons) this information is

difficult to obtain by conventional protein and amino acid analyses.

In any case, bioassays still provide the most sensitive and accurate method for detection and titration of TSE agents, and constitute the ultimate touchstone for measuring the efficiency of decontamination procedures for TSEs. Therefore, any loss of infectivity suggested by the reduction of PK-resistant prion protein in the *in vitro* carrier assay needs to be confirmed by validating the decontamination of carriers in reporter animals. As recent reports have consistently shown, monitoring the efficacy of wire reprocessing *in vivo* is feasible (Flechsig *et al.*, 2001; Yan *et al.*, 2004). If carried out sequentially in two steps (first in an *in vitro* then in an *in vivo* format) the steel wire assay can contribute considerably to the reduction of animal experiments in TSE research, which – in addition to ethical considerations – are time-consuming and cost-intensive. Apart from these aspects, the carrier assay provides further advantages: (i) it models specific problems of instrument reprocessing more realistically than inactivation studies on liquid tissue homogenates; and (ii) it allows the examination of substances that are not suitable for bioassays using tissue homogenates because they are toxic and cannot be adequately removed or neutralized.

Candidate reagents for decontamination identified in the *in vitro* carrier assay

Using the approach described here, several different reagents were found to exert a potent decontaminating activity on PrP^{Sc}/PrP27-30 attached to steel surfaces. The strong decontaminating activities of NaOH and NaOCl previously established by comprehensive infectivity studies (Kimberlin *et al.*, 1983; Brown *et al.*, 1986; Taylor *et al.*, 1994; Taylor, 2000; Flechsig *et al.*, 2001; Rutala & Weber, 2001) and their underlying active principles were mirrored in the *in vitro* carrier assay, which confirmed the suitability of the assay for pre-assessment of candidate reprocessing procedures. The alkaline cleaner biochemically identified in the *in vitro* carrier assay as a promising candidate reagent for surface decontamination has recently been shown in tissue homogenates to achieve efficient destabilization of PrP^{Sc} and inactivation of 263K scrapie agent (Baier *et al.*, 2004). Furthermore, 4 M GdnSCN, which displayed a strong destabilizing activity in our *in vitro* assay, has already been shown completely to inactivate the infectivity of murine Rocky Mountain laboratory scrapie agent bound to steel wires (Flechsig *et al.*, 2001). Finally, again consistent with our findings, a high decontaminating efficiency has been reported for 5% SDS used at higher temperatures (Taylor *et al.*, 1999; Taylor, 2004). On the other hand, water and 4 M urea (applied for 1 h), both of which are suggested by our assay to exert no significant effect of decontamination, are well known for not substantially inactivating TSE infectivity (Brown *et al.*, 1986; Prusiner *et al.*, 1993). Thus for the range of reference reagents examined here, our biochemical screening assay has provided reliable information for the preselection of candidate decontaminants as

well as insights into their active principles. The results observed after reprocessing wires with 0.2% SDS/0.3% NaOH or the disinfectant containing peracetic acid and NaOH suggest that further testing of these reagents under various conditions in the *in vivo* format of the carrier assay would be of interest. Such *in vivo* validations are currently under way after implantation of reprocessed wires into reporter animals.

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