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Transmission of Scrapie by Steel-surface-bound Prions

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Abstract

Background: Prions are unusually resistant to conventional disinfection procedures. An electrode used intracerebrally on a Creutzfeldt-Jakob disease (CJD) patient transmitted the disease to two patients in succession and finally to a chimpanzee, despite attempted disinfection. Concerns that surgical instruments may transmit variant CJD have been raised by the finding of PrP^{Sc}, a surrogate marker for infectivity, in various tissues other than brain.
Materials and Methods: Stainless steel wire was exposed to scrapie-infected brain or brain homogenate, washed exhaustively and inserted into the brain of indicator mice to measure infectivity.
Results: A contact time of 5 min with scrapie-infected mouse brain suffices to render steel wire highly infectious and insertion of infectious wire into the brain of an indicator mouse for 30 min suffices to cause disease. Infectivity bound to wires persists far longer in the brain than when injected as homogenate, which can explain the extraordinary efficiency of wire-mediated infection. No detectable amounts of PrP could be eluted with NaOH, however the presence of PrP on infectious wires was demonstrated by chemiluminescence. Several recommended sterilisation procedures inactivated wire-bound mouse prions, but exposure to 10% formaldehyde was insufficient.
Conclusions: Prions are readily and tightly bound to stainless steel surfaces and can transmit scrapie to recipient mice after short exposure times. This system mimics contaminated surgical instruments and will allow an assessment of sterilisation procedures.

Introduction

Prions are more resistant to inactivation than most conventional pathogens (1–4). An electrode used intracerebrally on a patient suffering from sporadic CJD (sCJD) transmitted the disease to two patients in succession and finally to a chimpanzee, despite exposure to benzene, 70% ethanol and formaldehyde vapour after each use (5,6). Concerns that surgical instruments may transmit variant Creutzfeldt-Jakob disease (vCJD) have been raised by the finding of PrP^{Sc} not only in nervous, but also in lymphatic tissue (7–10). We examined the ability of steel surfaces to bind scrapie prions by incubating steel wires overnight with scrapie-infected brain homogenates and inserting them permanently into the brain of indicator mice. This procedure resulted in efficient transmission of disease (11).

However, long-time exposure of steel wires to brain homogenate does not reflect conditions obtaining during surgical interventions. We now show that wires inserted into intact brain for as little as 5 min suffices to render the wires far more infectious than overnight exposure to brain homogenate and as infectious as 0.03 ml of 1% scrapie-infected brain homogenate injected directly into the brain. Furthermore, a contact time of 30 min was sufficient to elicit infection. Our experiments provide a model to assess the effectiveness of sterilisation procedures for steel bound prions and suggest a minimally invasive approach to assess infectivity in organs such as brain and tonsils.

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Materials and Methods

Preparation of Infectious Wire

Stainless steel wire segments (diameter 0.15 mm; 5 mm length) were cut from "Stainless steel suture monofilament wire", Art.Nr. 01614037, USP 4/0, B.Braun Melsungen AG, D-34209 Melsungen, Germany; batch 1/7502 or 1/8452). Gold wire segments (5×0.13 mm, Alfa Aesar Johnson Matthey GmbH Germany) were washed ultrasonically for 15 min in 2% Triton X-100, thoroughly rinsed in distilled water, dried at 37°C for 1 h as described (12). Brains were homogenized in $1 \times$ Dulbecco's phosphate-buffer saline (D-PBS; Gibco BRL, Glasgow, UK) by passing through 21G and 25G needles 8 times each, to give 10% (w/v) homogenates. These were centrifuged at 1,000 rpm (Eppendorf centrifuge 5415c, Hamburg, Germany) for 5 min at room temperature and the supernatants were recovered. We have recently determined that the centrifugation step result

in the precipitation of about 80–90% of the PrP^{Sc} present in the sample (P. Klöhn, unpublished results) so that this step is better avoided. Wires were incubated with centrifuged 10% brain homogenate in PBS for 16 h and washed 5 times 10 min in 50 ml PBS, all at room temperature. The wires were air-dried, stored at room temperature for 1 day and inserted into brain of deeply anaesthetized indicator mice, using a 25-gauge injection needle as a trocar.

Chemiluminescence of Surface-bound PrP

Twenty stainless wire segments (0.15 × 5 mm) were inserted into one brain hemisphere for 5 minutes. The other hemisphere was homogenized and centrifuged as described above. Twenty stainless wire segments were incubated with 0.5 ml 10% centrifuged homogenate for 5 min at room temperature, washed five times for 10 min with 50 ml D-PBS, dried for 24 h and immediately assayed for PrP. Wires were incubated with 0.2 ml of D-PBS containing 5% non-fat dry milk (w/v; Marvel, Premier Brands UK Ltd., Wirral, Merseyside, U.K.) for 1 h with agitation. After removal of the blocking reagent, they were incubated for 1 h with 200 ng/ml of anti-PrP antibody (6H4; Prionics AG, Zürich, Switzerland) in D-PBS containing 1% non-fat dry milk and washed 3 times for 5 min with 0.2 ml of D-PBS, followed by incubation for 1 h with horseradish peroxidase-conjugated rabbit anti-mouse IgG1 (1: 5000 dilution; Zymed, South San Francisco, California, USA). After washing 5 times for 5 min with D-PBS, the wires were exposed to 0.2 ml of SuperSignal ELISA Femto Maximum Sensitivity Substrate (Pierce, Rockford, ILL, USA) according to the manufacturer's instructions. Chemiluminescence was determined by luminometer (AutoLumat LB953; EG&G Berthold GmbH, Bad Wildbad, Germany).

Results

The ability of stainless steel surfaces to bind scrapie infectivity has been previously demonstrated by incubating steel wires (5 × 0.15 mm) for 16 h with 10% w/v brain homogenate of terminally scrapie-sick mice, referred to below as "standard conditions" (11). To model the exposure of surgical instruments to infected tissue more realistically, we inserted wire segments for 5, 30 or 120 min into brains of scrapie-inoculated wild-type mice culled two months before the expected appearance of scrapie symptoms. These "transiently inserted" wires were washed, dried and assayed by permanent implantation into the brain of *Tga20* indicator mice (13). Incubation times of the three groups lay between 65 ± 4 and 69 ± 5 days (Table 1, experiment 1), showing that even the shortest exposure to scrapie-infected brain rendered wires as infectious as intracerebral inoculation with 0.03 ml of 1% homogenate of the same brain homogenate (incubation time of 68 ± 8 days). Gold wires exposed to brain homogenate into brain also acquired infectivity (Table 1, experiment 2).

A second important question regards the length of time an infectious wire must contact brain tissue in order to initiate disease. Infectious wires were

Table 1. Infectivity of steel or gold wires after exposure to intact brain or to brain homogenate of scrapie-infected mice

Inoculation	Sick/Total	Incubation Time ± s.d. (days)
Experiment 1		
Wire transiently inserted for 5 min	5/5	68 ± 2
for 30 min	6/6	65 ± 4
for 120 min	6/6	69 ± 5
Wire exposed to 10% brain homogenate[+]	7/7	75 ± 5
Brain homogenate[+] (1%, 0.03 ml)	4/4	68 ± 8
Experiment 2		
Wires exposed to homogenate		
Steel wire (10%, w/v)	4/4	85 ± 4
Gold wire (10%, w/v)	3/3	74 ± 2
Steel wire (1%, w/v)	4/4	86 ± 8
Gold wire (1%, w/v)	4/4	81 ± 6

For experiment 1, two C57BL/6 mice were culled 87 days after i.c. inoculation with RML, that is, about 2 months before appearance of clinical symptoms. Wires were inserted into brain for the time indicated or exposed to centrifuged 10% brain homogenate for 16 h and processed as described in the Methods section. For experiment 2, wire segments were exposed to centrifuged brain homogenate of RML-infected, terminally sick CD1 mice as described in Methods.

[+]6.8 $logLD_{50}$ units/ml 10% homogenate, as determined by end point titration (23) in *Tga20* mice.

Table 2. Transient insertion of infectious wires into brains of indicator mice

Inoculation	Sick/Total	Incubation Time ± s.d. (days)
Wires infected by exposure to scrapie brain		
(a) Transient insertion into indicator mice		
30 min	4/4[$]	94 ± 10
120 min	2/2[#]	100 ± 18[&]
(b) Permanent insertion into indicator mice		
Wires not previously inserted	3/3	71 ± 2
Wires after transient insertion for:		
30 min	4/4	71 ± 3
120 min	5/5	68 ± 1
(c) Controls		
Wires exposed to brain homogenate	6/6	76 ± 3
Brain homogenate (1%, 0.03 ml)	3/3	69 ± 3

Infectious wires were prepared by insertion for 5 min into the brain of C57Bl6 × 129Sv mice culled 121 days after i.c. inoculation with RML and washed with 50 ml PBS 5 times for 10 min. Infectious wires were inserted into brains of 6 deeply anaesthetised *Tga20* indicator mice for the times indicated. The recovered wires were washed with 1 ml PBS and implanted into *Tga20* indicator mice. As controls, wires incubated with centrifuged 10% homogenate (6.8 log LD50 units/ml) of the same brain and the homogenate itself were introduced into indicator mice.

[$]Two of 6 mice died on the day of the intervention.

[#]Four of 6 mice died within a day of the intervention.

[&]Incubation times were 87 and 113 days.

prepared by insertion for 5 min into the brain of an infected wild-type mouse culled one month before the expected onset of scrapie symptoms. After washing, the wires were inserted transiently into the brains of anaesthetised indicator mice. As shown in Table 2, all mice exposed to a wire for 30 min or 2 h developed symptoms after 94 ± 10 and 100 ± 18 days, respectively. The infectious wires, with or without subsequent exposure to brain tissue, were ultimately assayed in indicator mice and in all cases caused scrapie disease after about 70 days, showing that no detectable amounts of infectivity were lost by exposure to brain.

Earlier experiments had shown that no detectable protein could be eluted with 2 M NaOH (<50 ng protein per wire) from wires exposed to 10% brain homogenate (11). To determine whether wires exposed to brain homogenate or to intact brain had surface-bound PrP, they were incubated with monoclonal PrP antibody 6H4 (14), followed by horseradish peroxidase-conjugated rabbit anti mouse IgG1 and chemiluminescence was measured in the presence of substrate. Fig. 1 shows that chemiluminescence of wires transiently inserted into infected brain of terminally sick indicator mice was about 5.5 times above reagent background. After background subtraction, the values were about 4 times higher than for wires exposed to infected brain homogenate and about 1.8 times higher than for those transiently inserted into uninfected brain. This experiment shows that PrP was bound to the wire surface; the higher

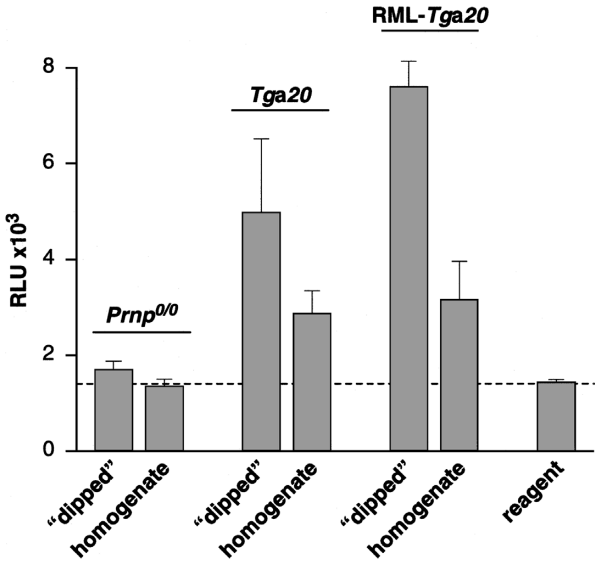

Fig. 1. Chemiluminescence of surface-bound PrP on stainless steel wires exposed to brain or brain homogenates. Stainless steel wire segments were transiently inserted into brains ("dipped") or incubated with 10% brain homogenates (homogenate) from PrP knockout mice (*Prnp*$^{0/0}$), uninfected (*Tga20*) and RML-infected, terminally sick *Tga20* mice (RML-*Tga20*). Wires were washed, treated with anti-PrP antibody 6H4 and horseradish peroxidase-conjugated anti-mouse IgG1 antibody, and chemiluminescence was determined. The dotted line indicates the background level, given by reagents alone (reagent). Averages and standard deviations were calculated from three samples each. RLU, relative light units.

chemiluminescence of the sample from infected brain is in keeping with the finding that total PrP content in terminally infected mouse brain is around 5 times higher than in uninfected controls (13,15), due to accumulation of PrP^{Sc}. We were not able to differentiate between PrP^{C} and PrP^{Sc} on wires because proteinase K treatment abolished immunofluorescence in all cases (data not shown). In an attempt to desorb PrP, we extracted 40 wire segments that had been transiently inserted into scrapie-infected brain with 0.05 ml 2 M NaOH for 1 h, neutralised the eluate with HCl and analysed half the sample by Western blot analysis. No PrP-specific immunoreactivity was detected under conditions where 0.3 ng purified glycosylated murine PrP, dissolved in NaOH and neutralised as described above, was clearly detectable (data not shown). Therefore, one wire released less than 15 pg PrP, that is less than 3×10^8 molecules. Assuming that one $logLD_{50}$ unit of infectivity is associated with 10^5 PrP^{Sc} molecules (16), one wire released less than 3000 LD_{50} units. Yet, the incubation time resulting from one wire is about the same as that following injection of 0.03 ml 1% brain homogenate, which corresponds to about 20′000 LD_{50} units. This somewhat speculative calculation suggests that the amount of PrP that could have been released from the wire surface does not readily account for the wire's infectivity, raising the question whether infectivity is due to irreversibly bound PrP^{Sc} (or PrP*) (17) rather than to desorbed prions.

Why are wire-bound prions as infectious as concentrated homogenates? Upon intracerebral inoculation with brain homogenate, infectivity is rapidly distributed throughout the mouse (18) and after 4 days or less prions are no longer detectable in the brain (19). Perhaps wire-bound prions are more stable and can therefore act over a longer period of time. We assayed infectious wires directly or after leaving them for 1 or 5 days in brains of *Prnp*$^{+/+}$ or *Prnp*$^{0/0}$ mice. Table 3.

Table 3. Infectivity of prion-coated wire after exposure to brain homogenate, PBS or brain of uninfected mice

Inoculation	Sick/Total	Incubation Time ± s.d. (days)
Infectious wire	3/4	62 ± 3
Experiment 1: *In vitro* exposure of infectious wire to:		
(a) Prnp$^{0/0}$ brain homogenate		
Wire	4/4	89 ± 3
Homogenate	1/4*	108
(b) PBS		
Wire	3/3	85 ± 6
PBS	0/4	>260
Experiment 2: *In vivo* exposure of infectious wire to:		
(a) Brain of Prnp$^{+/+}$ mice, 1 day		
Wire	3/3	104 ± 20
Surrounding tissue	0/8†	>260
(b) Brain of Prnp$^{+/+}$ mice, 5 days		
Wire	2/3	86 ± 4
Surrounding tissue	0/8†	>260
(c) Brain of Prnp$^{0/0}$ mice, 1 day		
Wire	3/3	79 ± 4
Surrounding tissue	1/8†,*	101
(d) Brain of Prnp$^{0/0}$ mice, 5 days		
Wire	3/3	91 ± 5
Surrounding tissue	0/8†	>260

Infectious wires were prepared with centrifuged 10% brain homogenate from terminally sick CD1 mice (11). For the *in vitro* assay (expt.1), 20 wires were shaken in Eppendorf tubes for 24 h at 37°C, either with 0.2 ml freshly prepared brain homogenate (10% w/v in PBS) of uninfected *Prnp*$^{0/0}$ mice or with 0.2 ml PBS/0.1% albumin, on a thermomixer (1400 rpm). After washing with 0.2 ml of the cognate solution, wires were assayed for infectivity. Thirty-μl samples of each preparation (0.4 ml) were assayed for infectivity in *Tga20* indicator mice. For the *in vivo* experiment (expt.2), infectious wires were implanted into the brain of uninfected *Prnp*$^{+/+}$ (C57Bl6) or *Prnp*$^{0/0}$ mice. After 1 and 5 days, respectively, the mice were culled and the brain tissue immediately surrounding the wire was dissected out. Wires were washed in 1 ml PBS and assayed. The brain samples (each about 80 mg) were homogenised in PBS to give a 10% homogenate and centrifuged samples were injected i.c. into 3 indicator mice each.

*Scrapie diagnosis was confirmed by histopathology or histoblotting (24)

†One of 9 mice died during or after injection.

Table 4. Infectivity of surface-bound mouse prions after various treatments

Inoculation	Sick/Total	Incubation Time ± s.d. (days)
1. Uninfected wires		
Untreated	0/3	>260
2. Infectious wires		
Untreated	6/6	76 ± 5
Sodium hydroxide (1M, 1 h, 25°C)	0/6	>260
Formaldehyde (10%, 1 h, 25°C)	6/6	92 ± 8
Guanidinium thiocyanate (4M, 16 h, 25°C)	0/6	>260

Infectious wires were prepared with centrifuged brain homogenates and assayed as described (11). End point titration (23) of the homogenate gave a titre of 6.75 log LD50 units/ml 10% homogenate. NaOH and formaldehyde solutions were prepared immediately prior to use; 4 M guanidinium thiocyanate was RNA Lysis buffer (#40082, Applied Biosystems, Foster City, CA, USA). Wires were exposed to 1 ml solution and washed with 1 ml PBS four times prior to implantation.

shows that wires remained infectious even after residing in brain tissue for 5 days, albeit at a lower level, as evidenced by incubation times of about 90 days in indicator mice. Because wire-bound infectivity remains at a locally high concentration for 5 days or longer, it may result in a greater total exposure than injected homogenate.

The wire model will ultimately serve as model for the sterilisation of surgical instruments by recommended (1,3,20) or novel procedures. In a preliminary experiment, infectious wire segments were subjected to different treatments and assayed. Sodium hydroxide (1 M, 1 h) or guanidinium thiocyanate (4 M, 16 h) rendered the wires completely non-infectious to the limits of the bioassay (Table 4), however all 6 indicator mice challenged with formaldehyde-treated, prion-coated wires succumbed to scrapie after 92 ± 8 days.

These preliminary decontamination studies should not be extrapolated to the level of instruments used in surgery. First, we used RML mouse prions, a mouse-adapted scrapie isolate (21) which is less heat stable than mouse-passaged BSE (301V) or the hamster strain 263K (3,22). It is clearly necessary to conduct sterilisation experiments of surface-bound infectivity using CJD, vCJD and BSE prions in an appropriately sensitive host. Second, the area of contact between wire surface and tissue is very small, compared with that of surgical instruments and it will ultimately be necessary to use scaled-up surfaces, such as could be provided by small steel beads introduced into larger indicator animals, to validate results obtained in the mouse.

Finally, it may become possible to use wires "dipped" for short times into brain or tonsils instead of biopsied tissue to determine the presence of PrP^{Sc} by chemiluminescence or infectivity in an appropriate indicator mouse or susceptible cultured cell line.

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References

1. Ernst DR, Race RE. (1993) Comparative analysis of scrapie agent inactivation methods. *J. Virol. Methods* **41(2):** 193–201.
2. Taylor DM, Fraser H, McConnell I, et al. (1994) Decontamination studies with the agents of bovine spongiform encephalopathy and scrapie. *Arch. Virol.* **139(3–4):** 313–326.
3. Taylor DM. (2000) Inactivation of transmissible degenerative encephalopathy agents: A review. *Vet. J.* **159(1):**10–17.
4. Brown P, Gibbs CJ Jr, Amyx HL, et al. (1982) Chemical disinfection of Creutzfeldt-Jakob disease virus. *N. Engl. J. Med.* **306(21):** 1279–1282.
5. Bernoulli C, Siegfried J, Baumgartner G, et al. (1977) Danger of accidental person-to-person transmission of Creutzfeldt-Jakob disease by surgery. *Lancet* **1(8009):** 478–479.
6. Gibbs CJ Jr, Asher DM, Kobrine A, et al. (1994) Transmission of Creutzfeldt-Jakob disease to a chimpanzee by electrodes contaminated during neurosurgery. *J. Neurol. Neurosurg. Psychiatry* **57(6):** 757–758.
7. Hill AF, Zeidler M, Ironside J, Collinge J. (1997) Diagnosis of new variant Creutzfeldt-Jakob disease by tonsil biopsy. *Lancet* **349(9045):** 99–100.
8. Hill AF, Desbruslais M, Joiner S, et al. (1997) The same prion strain causes vCJD and BASE. *Nature* **389(6650):** 448–450.
9. Hill AF, Butterworth RJ, Joiner S. (1999) Investigation of variant Creutzfeldt-Jakob disease and other huma prion diseases with tonsil biopsy samples. *Lancet* **353(9148):** 183–189.
10. Wadsworth JD, Joiner S, Hill AF, et al. (2001) Tissue distribution of protease resistant prion protein in variant Creutzfeldt-Jakob disease using a highly sensitive immunoblotting assay. *Lancet* **358(9277):** 164–165.
11. Zobeley E, Flechsig E, Cozzio A, et al. (1999) Infectivity of scrapie prions bound to a stainless steel surface. *Mol. Med.* **5(4):** 240–243.
12. Williams RL, Williams DF. (1988) Albumin adsorption on metal surfaces. *Biomaterials* **9(3):** 206–212.
13. Fischer M, Rülicke T, Raeber A, et al. (1996) Prion protein (PrP) with aminoproximal deletions restoring susceptibility

of PrP knockout mice to scrapie. *EMBO J.* **15(6):** 1255–1264.
14. Korth C, Stierli B, Streit P, et al. (1997) Prion (PrP^{Sc})-specific epitope defined by a monoclonal antibody. *Nature* **390(6655):** 74–77.
15. Büeler H, Raeber A, Sailer A, Fischer M, Aguzzi A, and Weissmann C. (1994) High prion and PrP^{Sc} levels but delayed onset of disease in scrapie-inoculated mice heterozygous for a disrupted PrP gene *Mol. Med.* **1(1):** 19–30.
16. Bolton DC, Rudelli RD, Currie JR, Bendheim PE. (1991) Copurification of Sp33–37 and scrapie agent from hamster brain prior to detectable histopathology and clinical disease. *J. Gen. Virol.* **72(Pt 12):** 2905–2913.
17. Weissmann C. (1991) Spongiform encephalopathies. The prion's progress. *Nature* **349(6310):** 569–571.
18. Millson GC, Kimberlin RH, Manning EJ, Collis SC. (1979) Early distribution of radioactive liposomes and scrapie infectivity in mouse tissues following administration by different routes. *Vet. Microbiol.* 1979, 89–99.
19. Büeler H, Aguzzi A, Sailer A, et al. (1993) Mice devoid of PrP are resistant to scrapie. *Cell* **73(7):** 1339–1347.
20. Manuelidis L. (1997) Decontamination of Creutzfeldt-Jakob disease and other transmissible agents. *J. Neurovirol.* **3(1):** 62–65.
21. Chandler RL. (1961) Encephalopathy in mice produced by inoculation with scrapie brain material. *Lancet* **1**:1378–1379.
22. Taylor DM, Fernie K, McConnell I, Steele PJ. (1998) Observations on thermostable subpopulations of the unconventional agents that cause transmissible degenerative encephalopathies. *Vet. Microbiol.* **64(1):** 33–38.