Highly sensitive, quantitative cell-based assay for prions adsorbed to solid surfaces

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Prions are comprised principally of aggregates of a misfolded host protein and cause fatal transmissible neurodegenerative disorders of humans and animals, such as variant Creutzfeldt-Jakob disease and bovine spongiform encephalopathy. Prions pose significant public health concerns, including contamination of blood products and surgical instruments; require laborious and often insensitive animal bioassay to detect; and resist conventional hospital sterilization methods. A major experimental advance was the cell culture-based scrapie cell assay, allowing prion titres to be estimated more precisely and an order of magnitude faster than by animal bioassays. Here we describe a bioassay method that exploits the marked binding affinity of prions to steel surfaces. Using steel wires as a concentrating and sensitization tool and combining with an adapted scrapie cell endpoint assay we can achieve, for mouse prions, a sensitivity 100× higher than that achieved in standard mouse bioassays. The rapidity and sensitivity of this assay offers a major advance over small animal bioassay in many aspects of prion research. In addition, its specific application in assay of metal-bound prions allows evaluation of novel prion decontamination methods.

bioassay | decontamination | CJD | PrP | BSE

Prion diseases are a closely related group of fatal neurodegenerative disorders affecting the central nervous system of humans and animals. They include Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker disease (GSS), fatal familial insomnia (FFI), and kuru in humans; bovine spongiform encephalopathy (BSE) in cattle; and scrapie in sheep (1). The identification of variant CJD (vCJD) in the U.K. in 1996 and the subsequent experimental confirmation that BSE in cattle and vCJD in humans are caused by the same prion strain (2–4) has led to a variety of concerns relating to public health.

Iatrogenic transmission of classical (sporadic) CJD by a contaminated neurosurgical instrument has been reported (5), and epidemiological evidence suggests a fraction of apparently sporadic CJD may be caused by unrecognized iatrogenic infection during general surgery (6, 7). The unknown, but potentially substantial (8), prevalence of clinically silent infection with vCJD prions in populations exposed to dietary BSE prions, together with the much wider tissue distribution of infectivity in vCJD (9–11), highlights the concerns of risk of infection through contact with surgical instruments. Secondary vCJD arising from blood transfusion has now been documented, indicating significant prionemia in asymptomatic donors during the incubation period (12). This wide distribution of infectivity makes common surgical and endoscopic procedures, in addition to neurosurgery and eye surgery, a potential risk factor for iatrogenic transmission of vCJD. Further, it is established that tissue prions withstand many forms of sterilization techniques (13) and that the metal-adsorbed agent is even more resistant to both thermal and chemical treatments (14).

According to the widely accepted protein-only hypothesis (15), prions are composed principally, if not entirely, of protein and are devoid of a nucleic acid genome. A normal isoform of host-encoded cellular prion protein (PrP^{C}) is converted to disease-associated forms designated PrP^{Sc} (16, 17). It is proposed that PrP^{Sc} acts as a template that promotes the conversion of PrP^{C} to further PrP^{Sc} and that the difference between these isoforms resides in protein chain conformation and aggregation state. This leads to an autocatalytic propagation of the disease agent, resulting in neuronal dysfunction and death by a pathway yet to be established (18).

In practical terms, quantitative prion assays are laborious and costly. Until recently, prion infectivity in tissue homogenates has been measured by injecting samples intracerebrally into rodents and determining the time to terminal disease or by injecting serial dilutions to determine endpoint (1). Alternatively, a surrogate marker of infection, protease-resistant PrP^{Sc} ($rPrP^{Sc}$) is determined by western blotting, although prion infectivity may accumulate in the absence of detectable levels of this marker (19–23). These methods have been augmented by the scrapie cell assay (SCA) (24), which is based on the ability of murine prions to propagate in selected mouse cell lines (25). SCA is at least as sensitive as rodent bioassay, but more precise and an order of magnitude more rapid and less costly. A major advantage of cell-based assays is also the reduction of the need for animal experimentation. A modification of the standard SCA (SSCA) is the scrapie cell endpoint assay (SCEPA) where cells growing in the wells of 96-well plates are exposed to serial dilutions of a prion isolate and propagated for several successive cell splits. Wells that received only a single infectious unit become extensively infected as evidenced by the accumulation of PrP^{Sc} in many cells in the well as infection spreads between cells. At an appropriate dilution, only a fraction of the wells receive an infectious unit and score positive, and the infectious titer of the starting material can be determined by use of the Poisson distribution and reference to standard curves (24).

It is well documented that prions bind avidly to metal surfaces and that this adsorbed material is highly infectious to mice (26, 27) as well as to cultured cells (21). The approach described herein enhances the sensitivity of prion detection by exploiting their propensity for binding to steel surfaces. Steel wires effectively bind the infectious agent from extremely dilute tissue homogenates, and sensitive cells exposed to the prion-bearing surfaces become infected and can be measured using an adaptation of SCEPA. The possibility of detecting low prion titers on

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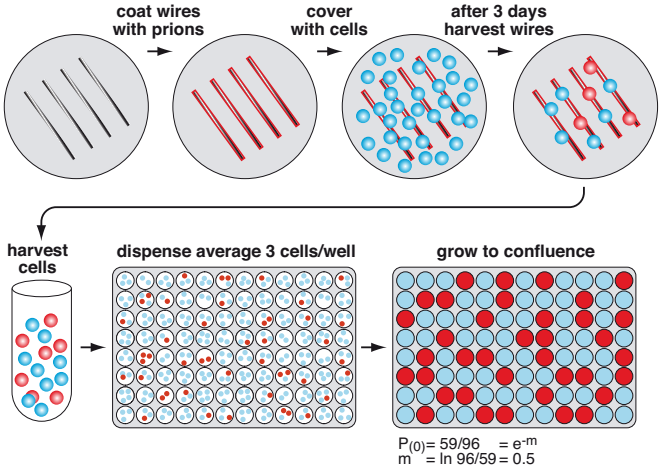

Fig. 1. Schematic of the steel-binding assay. Steel wire segments were exposed to an appropriate dilution of a prion-containing sample, washed to remove loosely adherent material, and covered with susceptible N2aPK1 cells for 3 days. Cells adhering to the wires were then harvested and seeded at set number of cells per well. The cells were grown to confluence and split several times to allow the infection to spread within the well; prion-infected cells were detected by presence of PrP^{Sc} immunoreactivity on Elispot plates. The average number of infectious units delivered to each well (m) was determined from the ratio of uninfected to total wells ($P_{(0)}$) using the Poisson statistical distribution relationship (see *Results* and *Materials and Methods*).

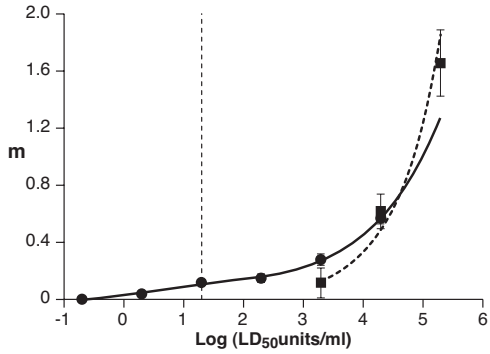

Fig. 2. Correlation of tissue culture infectious units (assayed from wire or tissue homogenate) and LD_{50} units. To determine the correlation between tissue culture infectious units (wire) ($TCIU_w$) per well and LD_{50} units/ml of the solution to which the wires had been exposed, RML prion-infected brain homogenate ($10^{9.3}$ LD_{50} units/g brain) was serially diluted into 10^{-4} *Prnp*$^{0/0}$ brain homogenate in Opti-MEM/10% FCS. Wires were exposed to the dilutions indicated for 3 h, washed briefly in PBS, and processed as described in the legend to Table 1. The m values (for $TCIU_w$) were taken from Table 1 and plotted against log LD_{50} units/ml (black curve). $TCIU_H$ were correlated with LD_{50} units by subjecting the indicated dilutions of brain homogenate to SCEPA using 18,000 cells per well and 24 wells per sample (dashed line). The results represent the average of 3 experiments. Vertical dashed line illustrates the typical limit of detection of titered RML brain homogenate as $10^{1.3}$ intracerebral LD_{50} units/ml by conventional rodent bioassay (47).

metal surfaces by a cell-based assay also provides both a larger dynamic range and more appropriate murine model for assessing methods of surface decontamination.

Results

Development of the Cell-Culture Assay for Surface-Bound Prions. ***Outline of the assay.*** In this study, the level of prion infectivity bound to steel surfaces was measured using an adaptation of SCEPA.

The principle of this assay is shown schematically in Fig. 1. Briefly, an appropriate object—in this case a metal wire—is exposed to prion-infected tissue homogenate, rinsed, transferred to a tissue-culture plate, and then covered with susceptible cells (e.g., N2aPK1). A proportion of the cells become attached to the surface of the object and subsequently become infected. After 3 days, the adherent cells are harvested, and samples containing an average of $N \gg 1$ cell are dispensed into the wells of a multiwell plate. N is chosen such that 10%−90% of the wells receive one or more infected cells. After growing to confluence, the cells are split 3 times 1:3 and 3 times 1:8 to allow the infectivity to spread among the cells in the well. Twenty-five thousand cells from each well are then filtered off onto membranes of a 96-well filtration plate, the samples are treated with proteinase K, and individual cells containing protease-resistant PrP^{Sc}, which is diagnostic of infection, are visualized by an antibody-based assay and counted. Wells with a spot number equal to or greater than the average background value plus 10 standard deviations are scored as positive and therefore initially received one or more infected cells. The average number of tissue culture infectious units (TCIU) per well, m, is calculated using the Poisson distribution according to the equation:

$$P_{(0)} = e^{-m}$$

where $P_{(0)}$ is the number of noninfected wells per total number of wells (see Fig. 1).

The value of m as determined by the wire (w) assay under standard assay conditions ($TCIU_w$ per well) can be translated into LD_{50} units/ml (where one LD_{50} unit represents the dose lethal to 50% of animals inoculated by the intracerebral route) by exposing wires to serial dilutions of a brain homogenate that has previously been titrated by intracerebral injection into wild-type mice, and plotting the resulting m values against the logarithm of the LD_{50} units/ml, as shown in Fig. 2.

Choice and preparation of metal surface. Surgical-grade steel in the form of disks (10 mm diameter × 0.5 mm) and monofilament suture wire (25 × 0.15 mm, USP 4/0) was investigated to determine which format provides the most efficient surface for the binding and transfer of prion infectious particles to susceptible N2aPK1 cells. In these experiments the upper surface of the steel disks was coated with a droplet of homogenate, hence the surface area of the disk exposed was 79 mm^2. Ten wires were completely immersed in homogenate, thus exposing a total surface area of 118 mm^2. The surface area of the disks and wires exposed to infected homogenate was therefore relatively similar. The efficiency of transfer of infectivity from the wires however, was 6- to 7-fold greater than from the steel disks coated with identical infectious samples, so wires were chosen as the preferred carrier [see supporting information (SI) *Results* and Table S1].

Cell culture conditions. To determine the optimal number of wire-exposed N2aPK1 cells to be seeded for SCEPA, a range of cell-seeding densities, from 1 to 4,000 cells per well, was examined (see SI *Results* and Table S2). A seeding density of 1,000 cells per well was optimal for both ease of handling and reproducibility without sacrificing sensitivity of detection in the dilution range of 10^{-4} to 10^{-7}. Greater sensitivity was achieved by increasing the number of cells, with quantifiable detection of infectivity achievable at dilutions of 10^{-10} with 4,000 cells. Interestingly, when wires were coated with a 10^{-2} dilution of Rocky Mountain Laboratory (RML) prion-infected brain homogenate, distribution of a single cell per well could reliably detect the presence of bound infectious material (Table S2).

Capture of Prions and Limits of Sensitivity. To establish which diluent was most appropriate for the capture and assay of low concentrations of RML prions, a dilution series of RML prion-infected CD-1 mouse brain homogenate was compared in 4 different diluents: uninfected CD-1 mouse brain homogenate; modified reduced serum Eagle's Minimum Essential Medium (Opti-

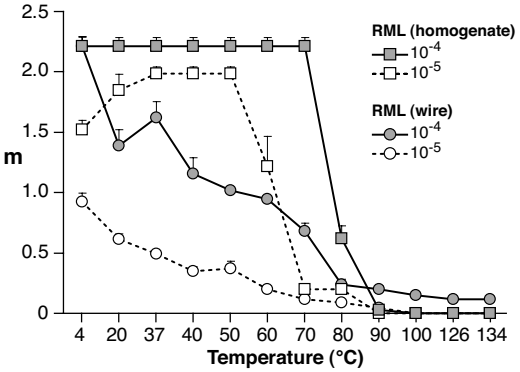

Fig. 3. Thermal stability of prions adsorbed to steel wires or in suspension. Steel wires were exposed to 10^{-4} or 10^{-5} dilutions of RML brain homogenate and rinsed in PBS. The wires and 10^{-4} and 10^{-5} dilutions of RML-infected brain homogenates were incubated across a temperature range of 4 °C to 134 °C for 15 min. Wires were assayed as detailed for the steel-binding assay; homogenate infectivity was assessed by SCEPA. The m values were determined by the Poisson relationship as described in *Materials and Methods*.

MEM) + 10% FCS; PBS; and brain homogenate from $Prnp^{0/0}$ (PrP knockout) mice (28). These serially diluted solutions were exposed to steel wires before incubation with susceptible N2aPK1 cells, which were then assayed using 24 or 48 replica wells for each dilution. Of these 4 diluents, the $Prnp^{0/0}$ homogenate was the only one found suitable for providing a sensitive assay. Reliably, 5 or more of 48 wells of 4,000 cells per well scored positive for wires exposed to a dilution of 10^{-10} RML homogenate (i.e., using this assay, prion infectivity can be detected on steel surfaces exposed to material containing the equivalent of 100 pg of RML prion-infected brain tissue per milliliter). For comparison, in the mouse bioassay of RML, we found that with an inoculum of 30 μl of 10^{-8} diluted brain homogenate, 2 animals were affected of a total of 24. At a 100-fold greater dilution, and thus equivalent to the sensitivity of the wire assay, we would therefore have to use 2,400 animals to have an equivalent chance of detecting 2 positives. Our assay therefore allows us to draw conclusions about the absence of prions on sterilized surfaces with a sensitivity that, for practical reasons, cannot be achieved in animal bioassays.

The unusual stability of infectious prions on metal surfaces has previously been demonstrated using a mouse bioassay (29). The assay we develop here allows us to examine this phenomenon more closely, owing to the ease and convenience of the cell culture method. The stability of RML prion-infected material was examined at a range of temperatures from 4 °C to 134 °C both bound to a metal surface and in suspension as a tissue homogenate (Fig. 3). The infectivity of RML-infected brain homogenate (at 10^{-5} dilution) shows a sharp, single transition, typical of a relatively homogeneous population of particles, with a midpoint temperature of about 60 °C. It is noteworthy that at 90 °C the 10^{-5} dilution of tissue homogenate has no detectable infectivity and, even at 10× the concentration, the infectivity is barely measurable after the 90 °C incubation. By contrast, the steel-bound prion infectivity has a much broader transition, characteristic of multiple populations. The wires exposed to a 10^{-5} or 10^{-4} dilution of RML homogenate show a continuous, slow decline beginning at 10 °C, and in the case of the steel-bound infectivity about 10% of the activity remains after the 90 °C incubation, and slightly more than 5% remains even at 134 °C. This clearly shows that in the case of the surface-adsorbed prions there are subpopulations of varying stabilities. These findings are in agreement with previous reports by Somerville *et al.* (30) where substantial inactivation of various prion strains occurred from 70 °C for 22C or 84 °C for ME7. Our data are also in agreement with earlier findings that a residual level of prion infectivity remains even after autoclaving (14).

Decontamination of surgical steel. Although exposure of metal-bound prions to very high temperatures is capable of reducing the titer substantially, this is not absolute. Treatment of such surfaces with 2 M NaOH or sodium hypochlorite has been shown to eliminate detectable infectivity, although these are not methods suitable for decontaminating delicate and valuable surgical or medical instruments. In view of the prion agent being proteinaceous, it is reasonable to assess the effectiveness of enzymatic methods of degradation. We have previously determined that some combinations of proteases in the presence of ionic detergents can be highly effective at degrading PrP^{Sc} and infectivity (31). Although effective, the published combinations are impractical for commercial use, and an ongoing development program has identified alternative enzyme formulations and detergent/oxidant combinations that are both economical and effective—specifically a mix of peracids, SDS, and a pair of broad-specificity proteases, Alkalase™ and Neutrase™ (Novozymes). Encouragingly, this reagent mix requires as short a contact time as 10 min at either 40 °C or 50 °C to reduce steel-bound RML prions to levels below those that can be detected by our high-sensitivity assay (Table 1). To emphasize the efficacy of this treatment, one may note that the assay is saturated with wires exposed to a 10^{-4} RML dilution, and the lower limit of detection is reached with wires coated with a 10^{-10} dilution. Given the observation that wires exposed to 10^{-4} RML and subjected to the sterilization treatment have no detectable infectivity (i.e., less than that exhibited by wires exposed to 10^{-10} RML), it follows that the reduction in titer is, at the very least, a millionfold. In fact, wires exposed to a 10^{-1} dilution of infected brain homogenate are also completely decontaminated by both the 40 °C and the 50 °C treatments so that the reduction of titer of steel-bound prions by this treatment may be considerably higher than a millionfold.

Discussion

Concern regarding possible iatrogenic transmission of vCJD prion infection via the use of blood, blood products, and surgical instruments has already caused disruption and major costs to public health policy in the U.K. and in other countries. The recent demonstration of subclinical prion infection (carrier states) in animal models (32–36) is also relevant to public health, both with respect to prion zoonoses and iatrogenic transmission of human prions from apparently healthy individuals. Transmission of vCJD via blood and related products has been considered likely since the first 2 case reports in 2004 (37, 38). However, the recent recognition of a further case (12), resulting from red-cell transfusion from amongst this small cohort of known recipients of implicated blood (currently 23 people), emphasizes that transfusion poses a substantial risk and, indeed, suggests that blood transfusion is an efficient route of vCJD transmission. A fourth blood transfusion-associated secondary vCJD case has recently been recognized (unpublished data). Because the clinically silent incubation period of prion infections in humans is known to be long, in some cases exceeding 50 years (39), and the prevalence of asymptomatic infection is unknown (40), the extent of primary infections and iatrogenic transmissions of vCJD cannot be estimated.

There is therefore a pressing need to extend the sensitivity of prion assays to detect the infectivity present at very low concentrations in tissue samples and, particularly, blood derived from humans and animals in early preclinical stages of disease. Until such an assay is available for routine use, effective methods for prion decontamination are required in conjunction with sensitive and relevant assays for their validation.

One possible method by which both of these aims can be achieved is by concentrating the prion agent using its known property of strong adherence to metal surfaces and then devising a sensitive method of detecting this surface-adsorbed material.

Table 1. Evaluation of an enzymatic/detergent method of decontaminating prion-bearing wires

Dilution RML	Treatment	Positive wells/total wells				Cells seeded per well	$TCIU_w$ normalized to 1,000 cells, mean ± SEM
10^{-4}	none	24/24	23/24	23/24	24/24	1,000	>3.1
10^{-5}	none	10/24	9/24	12/24	11/24	1,000	0.57 ± 0.05
10^{-6}	none	10/48	13/48	13/48	11/48	1,000	0.28 ± 0.02
10^{-7}	none	5/48	8/48	8/48	5/48	1,000	0.15 ± 0.02
10^{-8}	none	9/48	12/48	12/48	8/48	2,000	0.12 ± 0.015
10^{-9}	none	2/48	4/48	5/48	2/48	2,000	0.04 ± 0.01
10^{-10}	none	6/48	5/48	6/48	5/48	4,000	0.0029 ± 0.002
U	none	0/48	0/48	0/48	0/48	1,000	<0.017
10^{-1}	10 min at 40°C	0/16	—	—	—	1,000	<0.06
10^{-2}	10 min at 40°C	0/16	—	—	—	1,000	<0.06
10^{-4}	10 min at 40°C	0/16	—	—	—	1,000	<0.06
10^{-1}	10 min at 50°C	0/16	—	—	—	1,000	<0.06
10^{-2}	10 min at 50°C	0/16	—	—	—	1,000	<0.06
10^{-4}	10 min at 50°C	0/19	—	—	—	1,000	<0.06

RML prion-infected brain homogenate ($10^{9.3}$ LD_{50} units/g brain) was serially diluted into 10^{-4} *Prnp*$^{0/0}$ brain homogenate in Opti-MEM/10% FCS. Steelex® monofilament wire segments (60 segments of 2.5 cm length) were exposed to the dilutions indicated for 3 h and washed briefly in PBS. Where indicated, wires were treated with the enzyme/detergent mix described in *Methods* and thoroughly washed in PBS. Wires were covered with 300,000 N2aPK1 cells. After 3 days the cells were harvested and assayed by SCEPA using the number of cells stated per well and the number of wells indicated. The background of the assay was determined in a large-scale experiment by exposing PK1 cells to wires coated with uninfected homogenates and assaying them by SCEPA. Four of 960 wells, or 0.42%, were positive by our criteria; thus, a (negligible) background of 0.0042 × total wells (0.1 wells per 24 wells or 0.2 wells per 48 wells) was subtracted from the positive wells and m values ($TCIU_w$ per well) were calculated as explained in *Methods*. $TCIU_w$ per well was divided by the number of cells seeded per well to give normalized $TCIU_w$ per well.

Using steel wires to concentrate infectivity from RML prion-infected brain homogenate, we have adapted the existing SCA to provide a highly sensitive and quantitative measure of mouse prion infectivity bound to the surface of steel (Fig. 2) that is a 100-fold more sensitive than conventional rodent bioassay.

Thermal inactivation of suspensions of infectious tissue homogenate displays a highly cooperative transition, which suggests prion infectivity is associated with a specific and highly ordered species (Fig. 3). However, this monophasic degradation profile is not observed for steel-bound infectivity, which is characteristic of multiple structures or of proteins in diverse environments and appears to result in the acquisition of heat resistance by populations of prions when they are adsorbed to surfaces, as previously reported (41, 42).

At present it is recommended that surgical equipment be autoclaved at 121 °C or 134 °C for 18 min. However, steel wires exposed to higher titers of infectious material ($10^{5.3}$ LD_{50} units/ml equivalent to 10^{-4} dilution of brain) still harbor significant infectivity after autoclaving at 134 °C for 15 min. This is in agreement with previous studies performed by Taylor *et al.* (42), who observed that autoclaving of surgical steel after exposure to various infectious prion strains is not sufficient to eradicate infectious material bound to the surface. This highlights the present risk that exists for iatrogenic transmission of prion-contaminated surgical equipment even after autoclaving.

A decontamination method applicable to delicate surgical equipment and capable of inactivating prions bound to the surface is a public health priority. There is currently a risk of iatrogenic transmission from asymptomatic prion-infected patients, who are undetectable at present, via the use of contaminated surgical equipment. The decontamination methods used here show destruction of infectivity adsorbed onto steel wires surfaces by more than 10^6-fold by incubating in an enzyme/detergent-based reagent for 10 min at 40 °C (Table 1). This method is neither corrosive nor dependent on high temperatures, therefore making it extremely versatile in its uses.

It has been argued that prion decontamination methods should be evaluated using vCJD prions or a related strain (43). Though use of vCJD prions in animal bioassays may be considered the ideal in principle, in practice it is problematic because available mouse bioassays for vCJD prions have a very limited dynamic range due to transmission barrier effects (35, 40, 44, 45). Though mouse-passaged BSE such as 301C or 301V might be considered a related strain, relatedness is in fact a complex issue, and even biologically cloned strains may be of heterogeneous composition (18). Passage of BSE (or any) strain in a second species is associated with recruitment of host PrP of a different primary structure. Such passaged prions will therefore have different biophysical properties. For example, they have different thermostability and relative protease resistance than the inoculated strain, and so have a different response to decontamination regimens, respectively, involving heat or proteinases (46). It is however important to use a prion strain that has been extensively studied, and the biological and physicochemical properties of which have been well characterized, preferably including its behavior when bound to surgical steel. RML prions have been extensively studied in these regards (26, 27, 31, 47).

In terms of practicality, this assay for murine prion infectivity, unlike the existing rodent bioassay, is able to test multiple samples, in large sample sizes at comparatively very low costs. The assay is completed in 3 weeks compared with several months and is 100-fold more sensitive to infectivity. It has the major advantage of being a cell culture-based assay, hence avoiding the use of live animals and could replace rodent bioassays for many aspects of prion research.

Materials and Methods

Culturing and Storage of N2aPK1 Cells. N2aPK1 cells (24), susceptible to several mouse prion strains, including RML and 22L, were cultured in Opti-MEM 10% FCS and 1% penicillin/streptomycin (OFCS) (Invitrogen).

Preparation and Endpoint Titration of RML Prions. RML prion-infected brain homogenate (designated I6200) was prepared and titrated as previously described (47).

Standard Steel Binding Assay. Monofilament Steelex wires were exposed to appropriately diluted homogenates of prion-infected tissue for 3 h at room temperature, rinsed several times in PBS, and dried. Up to 20 wires were placed into each well of a 6-well cluster plate (Corning) and covered with a suspension

of 3×10^5 N2aPK1 cells in 5 ml OFCS. After 3 days at 37 °C and 5% CO_2, the wires with adherent cells were transferred into fresh wells containing 1 ml OFCS. The adherent cells were dislodged from the wires by vigorous pipetting, collected in a separate tube, and counted. One thousand cells suspended in 300 μl were seeded into wells of 96-well plates (Corning) and after 3 days split at a ratio of 1:3. After reaching confluence, the cells were again split 1:3 twice and 1:8 three times. Once cells were confluent following the second and third 1:8 split, 25,000 cells were seeded onto Elispot plates activated with 70% ethanol (MultiScreen Immobilon-P 96-well filtration plates; Millipore) and washed twice with 160 μl PBS by suction. Plates were dried at 50 °C for 2 h and stored at 4 °C. Elispot plates were developed as described in SI *Materials and Methods*.

Tissue Culture Infectivity Units and LD_{50} Units. SCEPA allows us to calculate m, the average number of infectious units (either in the form of infected cells or prions) delivered into a well, using the Poisson equation $P_{(0)} = e^{-m}$, where $P_{(0)}$ is the probability of a well remaining uninfected (i.e., noninfected wells/total number of wells). Thus, if a certain amount of a sample were to give 9 uninfected wells from a total of 24, then it would contain on average $m = \ln 24/9 = 0.98$ or about one infectious unit which, in the case of the wire-mediated assay under standard conditions, we define as a TCIU wire ($TCIU_w$). We can establish a relationship between LD_{50} units/ml (as established by the mouse bioassay) of the solution to which the wires were exposed, and $TCIU_w$ per well by exposing wires to a serial dilution of mouse-titered brain homogenate and determining m values resulting from the wire assay described previously, as shown in Fig. 2. The SCEPA assay can also be performed directly on appropriate dilutions of brain homogenate, to yield TCIU homogenate ($TCIU_H$) values, which can also be related to LD_{50} units, as shown in Fig. 2.

Decontamination of Wires. To create a reference curve, wires were exposed to a logarithmic dilution series (10^{-4} to 10^{-10}) of RML-infected brain homogenate in 10^{-4} FVB/N-*Prnp*$^{0/0}$ brain homogenate for 3 h. Two batches of wires for decontamination were exposed to 10^{-1}, 10^{-2}, and 10^{-4} dilutions of RML-infected brain homogenate prepared in 10^{-4} FVB/N-*Prnp*$^{0/0}$ brain homogenate as described. Wires were immersed into freshly prepared decontamination solution comprising 1× working solution of PeraSafe™ (DuPont Corp.) + 1% wt/vol SDS + 3.2 mg/ml Alkalase (Novozymes) + 12.6 mg/ml Neutrase (Novozymes). One batch was incubated at 40 °C for 10 min and the other batch at 50 °C for 10 or 20 min. Wires were then rinsed in PBS at room temperature and dried before exposure to N2aPK1 cells as described. Cells were harvested from wires after a 3-day exposure, seeded, and assayed by SCEPA as described, using 16 wells per sample.

Thermal Stability of RML. Wires were exposed to 10^{-4} or 10^{-5} dilution of RML-infected homogenate prepared in 10^{-4} FVB/N-*Prnp*$^{0/0}$ brain homogenate in OFCS for 3 h. Wires were then washed 5×15 min in PBS and incubated at the temperatures indicated for 15 min (Fig. 3) before exposure to N2aPK1 cells. After a 3-day exposure, cells were harvested from the wires, seeded into 12 replica wells, and assayed in the standard way. Homogenates were prepared in an identical manner to those used to coat wires and were subjected to the exact same conditions and incubations. These samples were then applied to susceptible N2aPK1 cells seeded at 18,000 cells per well of a 96-well plate format 18 h before infection. After a 3-day exposure, cells were seeded and assayed by SCEPA as described previously.

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