

Reduction of transmissible spongiform encephalopathy infectivity from human red blood cells with prion protein affinity ligands

Luisa Gregori, Brian C. Lambert, Patrick V. Gurgel, Liliana Gheorghiu, Peter Edwardson, Julia T. Lathrop, Claudia MacAuley, Ruben G. Carbonell, Steven J. Burton, David Hammond, and Robert G. Rohwer

BACKGROUND: There is a demonstrated risk of infection by transmissible spongiform encephalopathies (TSEs) through transfusion from asymptomatic donors. Currently, blood-borne TSE infectivity cannot be detected with a diagnostic test, nor is it likely to be amenable to inactivation; however, its depletion with specific adsorptive ligand resins is possible.

STUDY DESIGN AND METHODS: Six ligands that bind the prion protein, PrP, were selected by screening large solid-phase combinatorial chemical libraries. The selected resins were placed in columns and challenged with a unit of leukoreduced human red blood cells (RBCs) spiked with hamster brain-derived scrapie infectivity. The performance of each ligand was assessed by comparing the TSE infectivity titer in the RBCs before and after passage through each of five resin columns in series.

RESULTS: Four resins were able to reduce infectivity titer by 3 to more than 4 log ID₅₀ per mL. The reduction was not due to nonspecific matrix interactions since a chemical modification of the most effective ligand completely abolished its ability to bind infectivity (negative control). A small subfraction of the infectivity, 0.01 percent, could not be removed, even upon repeated passage through successive columns.

CONCLUSION: If endogenous TSE infectivity in RBCs binds to the ligands in the same proportion as brain-derived infectivity spiked into RBCs, the four most effective ligands would remove 3 to 4 log ID₅₀ per mL. A follow-up experiment is in progress to test whether endogenous blood-borne infectivity is also reduced.

ABBREVIATIONS: PK = proteinase K; PrP = prion protein; PrP^c = normal prion protein; PrP^{res} = protease-resistant prion protein; PrP^{TSE} = infectious specific forms of PrP; TSE(s) = transmissible spongiform encephalopathy(-ies); vCJD = variant Creutzfeldt-Jakob disease.

From the Veterans Affairs Maryland Health Care System, VA Medical Center, University of Maryland, Baltimore, Maryland; ProMetic BioSciences Ltd, Cambridge, United Kingdom; the Plasma Derivatives Department, American Red Cross Biomedical R&D, Rockville, Maryland; and the Department of Chemical and Biomolecular Engineering, North Carolina State University, Raleigh, North Carolina.

Address reprint requests to: Robert G. Rohwer, PhD, 10 North Greene Street, Medical Research Services 151, VA Medical Center, University of Maryland, Baltimore, MD 21201; e-mail: rrohwer@umaryland.edu.

Financial support for this work was provided by Pathogen Removal and Diagnostic Technologies (a joint venture between the American Red Cross and ProMetic BioSciences) with a contract to the Baltimore Research and Education Foundation. LGr, BCL, CM, and RGR were in part funded by this contract. RC and PVG were in part funded by a contract between the American Red Cross and the NCSU. LGH, JL, and DH are employees of the American Red Cross. PE and SB are employees of ProMetic BioSciences.

Declaration of potential conflicts of interest: Pathogen Removal and Diagnostic Technologies is a joint venture of the American Red Cross and ProMetic BioSciences established in part to develop reduction devices for TSE infectivity. DH, RGC, and RGR are founders of PRDT, have equity in the company, and sit on its scientific and executive boards with SJB. Thus, American Red Cross and ProMetic BioSciences have direct financial interest in the work presented. LGr is PI on a contract from PRDT to conduct the infectivity studies required for the device development. All other authors are employees either of the American Red Cross or ProMetic Biosciences or members of the Rohwer or Carbonell laboratories.

Received for publication October 4, 2005; revision received November 23, 2005, and accepted November 28, 2005.

doi: 10.1111/j.1537-2995.2006.00865.x

TRANSFUSION 2006;46:1152-1161.

The original cases of variant Creutzfeldt-Jakob disease (vCJD), a transmissible spongiform encephalopathy [TSE] of humans, were almost certainly transmissions from bovine spongiform encephalopathy (BSE) infected cattle.¹ More recently, there have been three cases of transfusion transmitted vCJD among 50 recipients of blood donated by persons who later died of vCJD.^{2,3,4} One of these was a subclinical case discovered 5 years after transfusion in a person who died from an unrelated cause.³ Only 19 recipients have lived long enough to present evidence of vCJD. Three cases of transfusion transmitted vCJD, diagnosed either at autopsy or clinically, are consistent with a transmission rate of at least 3 per 19 or approximately 15 percent. The infection status of the remaining 16 recipients, including those who have died, has not been determined.⁴ Additional cases in this cohort would increase the estimated transmission rate.

To date, there have been more than 170 confirmed or probable cases of vCJD worldwide but the incidence of UK cases has waned during the last two years suggesting that the disease might have reached its peak. In contrast, a prevalence study based on the detection of vCJD infection-specific forms of the prion protein (PrP^{TSE}) in surgically removed tonsils and appendixes indicated approximately 4000 incubating cases presuming detection of 100 percent of cases by this method.⁵ The ascertainment rate is unknown but almost certainly less than 100 percent as there was no PrP^{TSE} in the tonsils or appendix of the incubating transfusion-transmitted case where infection was instead detected in the spleen and in the cervical lymph node.

The subclinical case of transfusion transmission was the first to be recognized in a person heterozygous for methionine-valine at codon 129 of the prion gene.² All previous vCJD cases were homozygous for methionine even though that genotype is found in only 39 percent of the UK Caucasian population.⁵ The presence of infection in a codon 129 heterozygote extends the risk of blood-borne vCJD infection to at least 89 percent of the human population.⁵ The lack of codon 129 M/V heterozygotes among persons clinically affected by vCJD suggests a potential asymptomatic carrier state from which the disease could be spread by transfusion.

Infected individuals might donate for decades before developing clinically recognizable disease. An unknown proportion would die from unrelated causes so that a carrier state might never be detectable. Secondary, same-species transmissions almost invariably shorten the incubation time and increase the efficiency of transmission of TSE infections in new hosts. This combination of undetectable transmissions between humans and increased efficiency of transmission harbors a dangerous potential for silently expanding the vCJD epidemic.

The traditional defenses against blood-borne pathogens are deferral, screening, inactivation, and removal.

The recent implementation in the United Kingdom of a deferral from blood donation by previous recipients of blood components will prevent secondary transmissions from persons that acquire their infections by this route; however, it cannot protect the blood supply against donors who have acquired the infection by means other than blood donation. Geographical deferrals from donation by persons who have spent significant periods of time in the United Kingdom have been implemented in many countries to minimize exposure of their blood supplies to risk from vCJD.⁶ These deferrals, however, do not guarantee safety; a recent case of vCJD in a Japanese citizen who had only spent approximately 1 month in the United Kingdom demonstrates the limitations of this strategy.⁷

Although preclinical diagnosis of TSEs in asymptomatic individuals is highly desirable both as a public health measure and as a necessary prerequisite for prophylactic therapies should they be developed, a test for PrP^{TSE} in blood presents formidable challenges due to the very low concentration of PrP^{TSE} that is expected from the very low levels of infectivity found there. The concentration of blood-borne TSE infectivity in symptomatic rodents, where such measurement is feasible, is approximately 10 ID per mL⁸ (R.G. Rohwer laboratory, ongoing studies). Infectivity is detectable throughout the last two-thirds of the incubation period, but at even lower concentration (R.G. Rohwer laboratory, manuscript in preparation). As yet there has been no validated demonstration of the biochemical marker of the disease, PrP^{TSE}, in blood. That does not mean that it is not present, but only that a method for detecting it has not yet been developed.

Inactivation is problematical for the notoriously refractory TSE pathogens. In contrast, removal is technically feasible and may prove to be both more effective and more economical than mass screening even if diagnostics are developed.

Blood-borne TSE infectivity is concentrated in buffy coat; thus, the most obvious TSE removal strategy is leukoreduction. It was hoped that universal leukoreduction would, in addition to its other benefits, also rid blood of TSE infectivity. A leukofiltration conducted at full scale with 450 mL of endogenously infected hamster blood and a commercial leukofilter, however, removed less than one-half of the scrapie infectivity while reducing the number of white blood cells (WBCs) by 99.9 percent.⁸ The remaining infectivity is not associated with platelets (PLTs)⁹ and is unlikely to be intrinsically associated with red blood cells (RBCs; L. Gregori et al., work in progress). We presume that it is in the plasma compartment. Thus, while leukoreduction is necessary to remove the TSE infectivity associated with WBCs, the plasma-associated infectivity must be removed by other means. We report here on the development of an affinity-based technology to remove non-cell-associated TSE infectivity from blood.

MATERIALS AND METHODS

Immobilized ligands

Six ligands selected by screening large solid-phase combinatorial libraries were synthesized at similar ligand densities on a proprietary matrix. Resin 91 was a chemical modification of resin 13, which rendered it nonfunctional. It served as a negative control.

Blood compatibility studies

Each resin was packed in a 10-mL column and tested for hemocompatibility by passing 1 unit of leukoreduced RBCs through the column at room temperature. Percent hemolysis was measured by the ratio of plasma hemoglobin as determined by plasma hemoglobin analyzer (HemoCue, Lake Forest, CA) to total hemoglobin as determined by hemocounter (Cell-Dyne 3700). Activation of Factor VII was measured by coagulation time with a coagulometer and reagents (BCS coagulometer, Dade-Behring, Deerfield, IL). Activation of PLTs was measured by CD61/CD62 ratio (BD Biosciences, San Jose, CA) with a flow cytometer (FACSCalibur, BD Biosciences, San Jose, CA). Activation of complement was measured by C3a-desArg levels (Progen Biotechnik, Archerfield, Australia). All tests followed the manufacturers' protocols.

Preparation of the RBC pool

Each resin was challenged with 1 unit of leukoreduced human RBCs. Because seven resins were to be tested, 7 RBC units of type O+ blood in additive solution (Adsol, Baxter, Deerfield, IL) were obtained from the American Red Cross (Rockville, MD). Each unit was passed through a commercial leukoreduction filter (Pall Leukotrap-SC RC filter, Pall, Inc., Port Washington, NY) following the manufacturer's instructions. The WBC counts in the leukoreduced RBC filtrates were below the detection limit of a Cell-Dyn 3700 cell counter (Abbott Diagnostics). All seven leukofiltered units were combined in a single 5-L media bag (Stedim, Concord, CA) and gently blended for 10 minutes at room temperature by rocking by hand.

Preparation of the infectivity spike and addition to the RBC pool

Brains harvested from hamsters showing clinical signs of infection with the 263K strain of scrapie were homogenized in phosphate-buffered saline (PBS), pH 7.2, to give a 10 percent (w/v) suspension. To prevent mechanical trapping by the resin bed, the suspension was extensively sonicated and, just before mixing with the RBCs, was treated with 0.5 percent Sarkosyl for 30 minutes on ice with constant mixing. The solution was centrifuged at $12,000 \times g$ for 15 minutes at room temperature, and the

clarified supernatant was used as the spike. The spike was diluted 100-fold into the RBC pool (final concentration, 0.1% with respect to whole brain) by slow addition with a peristaltic pump at 2.2 mL per minute with continuous, gentle mixing by hand. The spiked RBC pool was thoroughly mixed for an additional 30 minutes to ensure homogeneity before challenge of the resins. The final concentration of Sarkosyl was 0.005 percent, which had no effect on RBC integrity or on PrP binding to the resins.

The spiked RBC pool was redistributed into seven blood bags to reconstitute the original 7 units of blood. Each bag was weighed to ensure that each immobilized ligand was challenged with equivalent amounts of blood and infectivity. Weights ranged from 303 to 327 g.

Configuration of reduction devices and adsorption runs

Slurries of each resin (10 mL) were packed into custom column chromatographic housings (the removal devices). For each ligand, 1 unit of RBCs was passed through five of these devices in series (Fig. 1). The effluent from each device was collected in a downstream blood bag from which a 20-mL aliquot was removed for infectivity assay before passage through the next device in series. Resin L03 was tested with only four devices in series due to a technical problem with the fifth device. The flow rates, which were controlled by increasing or decreasing the height between the bags, varied from 8 to 21 mL per minute. We had previously determined that flow rates of 3, 10, and 22 mL per minute had no impact on the binding of the PrP to the resins (data not shown). All work was conducted at room temperature.

Analysis of the proteins bound to the immobilized ligands

After each run, the resin contained in each device was extensively washed in the housing with 20 mmol per L Na-citrate-140 mmol per L NaCl, pH 7.0, and then removed. The amount of protease-resistant PrP, PrP^{res}, bound to the resins was assessed by Western blot analysis. Proteinase K (PK) digestion was conducted directly on the resin-bound proteins. One 50- μ L aliquot of the resin was digested by addition of 7.5 μ L of PK (1 mg/mL in 50 mmol/L Tris, 10 mmol/L NaCl, and 2 mmol/L CaCl₂, pH 8.0) and 17.5 μ L of 2 percent SDS, while a second 50- μ L aliquot of resin was treated with 50 μ L of 2 \times sample buffer with reducing agent (NuPAGE, Invitrogen, Carlsbad, CA). The samples were vortexed thoroughly to mix the resin and incubated for 1 hour at 37°C with agitation. After incubation, the samples containing PK were treated with 3 μ L of phenylmethylsulfonyl fluoride (44 mg/mL in methanol), and 25 μ L of 4 \times NuPAGE sample buffer with reducing agent (Invitrogen) was added. All samples were then

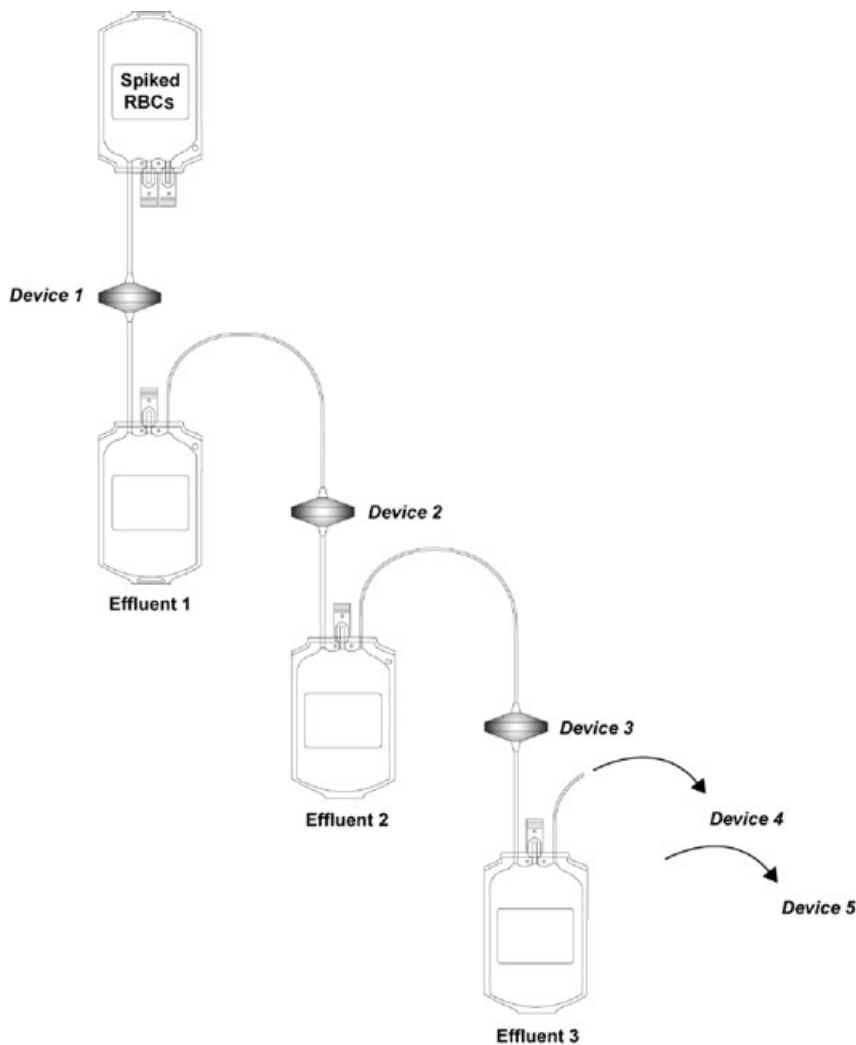


Fig. 1. Schematic representation of the removal scheme for a single ligand where each device in series contains the same immobilized ligand. This scheme was repeated seven times, once for each ligand tested (L03, L13, L21, L46, L51, L70, and L91).

heated for 10 minutes at 90°C to denature and release undigested protein. Ten microliters of each supernatant was loaded on NuPAGE precast 14 percent gels with 2-(*N*-morpholino)ethanesulfonic acid running buffer. After electrophoretic transfer of the separated proteins to polyvinylidene fluoride membranes (Invitrogen), the blot was developed with mouse anti-PrP monoclonal antibody 3F4 (Signet Laboratories, Dedham, MA) with a Western blot immunodetection kit (Western Breeze, Invitrogen) according to the manufacturer's instructions. The chemiluminescent signal was visualized on X-ray film (Amersham Biosciences, Little Chalfont, UK).

Infectivity titers

The animal studies in this report were conducted in accordance with NIH guidelines after approval by the Institu-

tional Animal Care and Use Committee of the University of Maryland at Baltimore.

Dose-response curves. Two independent 10-fold serial dilutions of the spiked RBCs were prepared from 10^{-3} to 10^{-10} (relative to whole brain at 10^0) with RBCs as diluent. Each dilution was confirmed gravimetrically by converting weights to volumes with 1.06 g per mL as the density of RBCs in Adsol solution. Fifty microliters of each dilution of each series was inoculated intracranially into each of eight weanling hamsters anesthetized before inoculation with pentobarbital (40-60 mg/kg body weight) administered intraperitoneally.¹⁰ The incubation time endpoints were determined empirically as the day an animal's weight declined to 20 percent of its maximum body weight. Normal hamsters gain weight continuously throughout the first year of their lives. Infected animals started losing weight when they were showing clear signs of hyperreactivity. The endpoints corresponded roughly to when they no longer reared spontaneously. The mean values of the incubation times at each dilution were fit by an arbitrary equation,

$$\log(\text{dilution factor}) = -8.05 + 83.06e^{(-\text{day after inoculation}/34.41)},$$

selected for its high correlation coefficient, 0.986, with computer software (TableCurve 2D v4, Jandel Scientific, San Rafael, CA). This equation was used to calculate the dilution intercepts of

the average incubation times of all device effluents tested.

Incubation time titrations. Each effluent that was tested was inoculated undiluted into eight hamsters, 50 μ L per animal, by the intracranial route, and the animals were scored as for the dose-response curves. All animals inoculated with effluents died of scrapie within 239 days after inoculation with the exception of one animal inoculated with effluent 4 of resin 13 that was terminated 506 days after inoculation without evidence of infection. This animal was not included in the calculation of the mean incubation time. This is a conservative treatment to avoid overestimation of the reduction achieved. The titer of an inoculum that does not infect every animal in a cohort that has been inoculated is at limiting dilution and can therefore be estimated more precisely from the Poisson distribution by the limiting dilution method.⁸

Endpoint dilution titration. All remaining animals from the dose-response curve determinations were terminated 365 days after inoculation, and the endpoint dilution titers were computed separately for each determination by the interpolation method of Reed and Muench,¹¹ estimating the standard error by the method of Pizzi,¹² and by the method of Spearman and Karber. The two methods gave nearly identical results. Endpoint dilution titrations of the untreated, and Sarkosyl-treated and clarified, 10 percent hamster brain homogenate that comprised the spike were also conducted. Serial 10-fold dilutions from 10^{-1} to 10^{-11} relative to brain were made into inoculation buffer, PBS containing 1× penicillin-streptomycin (Biofluids, Rockville, MD) and 1 percent fetal bovine serum (USDA-tested FBS, HyClone, Logan, UT). Each dilution was inoculated intracranially into four animals, except for the dilutions at 10^{-8} and 10^{-9} , which were inoculated into eight animals to increase precision at the expected dilution endpoint.

RESULTS

By screening millions of chemical ligand structures from combinatorial libraries of peptides and chemical structures immobilized on chromatography resin beads,¹³⁻¹⁵ Pathogen Removal and Diagnostic Technologies, Inc., identified approximately 200 compounds that, in immobilized form on inert supports, adsorbed prion proteins in the presence of RBCs, plasma, and whole blood.¹⁶ More than 100 of these compounds were further evaluated for their ability to bind PrP from a variety of sources with a Western blot assay. Compounds were evaluated for their ability to bind normal PrP, PrP^c, from humans and hamsters, and PrP^{res}, the PK-specific form associated with the disease, from sporadic CJD vCJD, and hamster scrapie, without adversely affecting other constituents, that is, plasma proteins, PLTs, and RBCs. Six ligands were selected for further development into TSE removal devices for RBCs. The activity of one of the resins (L13) was neutralized by a chemical modification of the ligand functional groups to create a negative control for the activity of the others.

Each of the selected resins was also screened for hemocompatibility. After passage through the resin in column format, total hemolysis after 42 days of storage was less than 0.8 percent, and no significant activation of plasma coagulation factors above background was

observed. Visual inspection of RBCs before and after treatment with the devices indicated no gross differences.

Each resin was challenged with 1 unit of leukodepleted RBCs drawn from a common pool that had been spiked (to a final concentration of 0.1% relative to whole brain) with a highly dispersed and clarified homogenate of scrapie-infected hamster brain. In each experiment five identical devices containing 10 mL of resin each were tested in series as depicted in Fig. 1. After passage through each column, a 20-mL aliquot was removed for infectivity assay, and the column itself was retained for Western blot assay. The experiments were configured in this way with two objectives: the first was to determine the resin volume required for maximum removal of infectivity; the second was to investigate what proportion of the spiked infectivity was accessible to removal by any ligand. For example, if infectivity escaping the first device was readily captured by the second device, it would indicate that the immobilized ligand performed appropriately, but the device configuration or operating conditions were suboptimal, or that the capacity of the resin for the targeted infectivity was exceeded in the first device. Alternatively, if the second device removed no additional infectivity, it would indicate that the unbound infectivity was in a physical state that was not accessible to the ligand and could not be removed under these conditions regardless of how much ligand was present.

The removal of PrP^{res} by each device in the series was assessed by Western blot assay of the proteins bound to the resin. Each panel in Fig. 2 shows the PrP^{res} bound to

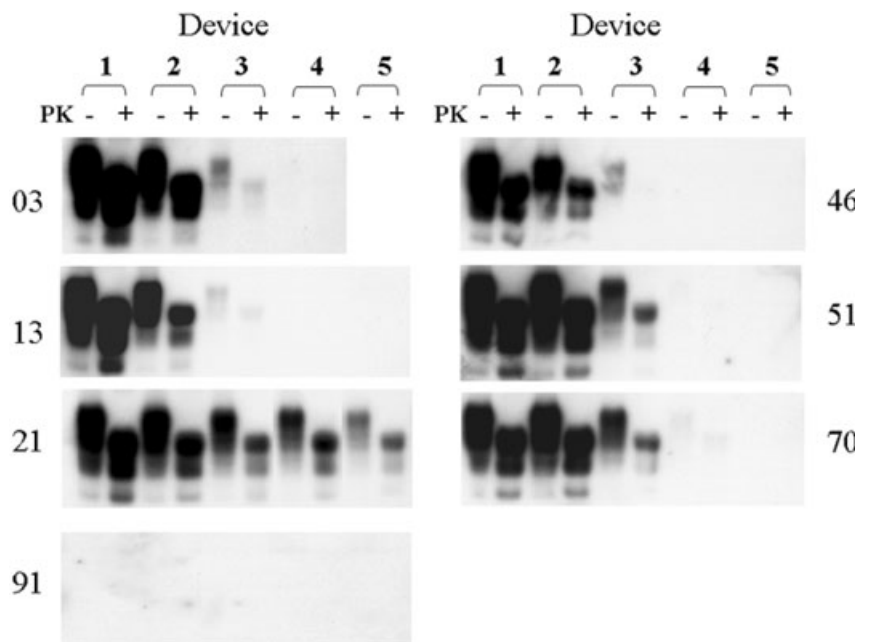


Fig. 2. Western blot detection of the PrP bound to the ligands in each device in series. The PrP eluted from each device was analyzed with and without PK digestion. Ligand L03 was tested with only four devices.

device one through five for each resin. A strong Western blot signal indicates that the device captured large amounts of PrP^{res}. Lack of signal could indicate either failure of the device to capture PrP^{res} or lack of PrP protein in the challenge to that device due to depletion by the previous device. The identity of PrP^{res} was confirmed by its characteristic electrophoretic mobility shift after PK digestion.

No detectable PrP^{res} was captured by the negative control, resin L91. In contrast, all six of the functional resins captured large amounts of PrP^{res} as evidenced by strong Western blot signals. Four resins, L03, L13, L46, and L51, showed no Western blot-detectable PrP^{res} signal after device 3, whereas L70 had a weak but detectable signal captured by device 4. L21 behaved differently from all other resins. It removed significant but decreasing amounts of PrP^{res} at each passage in the series including the last, suggesting a lower capacity, a lower affinity, or less efficient binding than the other resins. It may be that the association rate was too slow to achieve maximum binding under the conditions of the experiment compared to those used for its original selection.

Based on the Western blot results, effluents 2, 3, 4, and 5 from all resins that showed PrP^{res} signal reduction after device 3 (L03, L13, L46, and L51) were selected for TSE infectivity assay. Effluent 1 was not assayed for any sample because it was estimated from the Western blot results that its infectivity titer would be indistinguishable from that of the challenge. In the case of the resins that showed inefficient capture of PrP^{res} (L21 and L70), and the negative control (L91), only effluents from devices 2 and 5 were assayed.

The incubation time bioassay requires fewer animals and is more economical than endpoint dilution titration, at the expense of precision and sensitivity. Because we were interested only in identifying those resins that produced dramatic reductions in infectivity, however, this weakness was inconsequential. To evaluate the reproducibility of the incubation time data, we prepared the dose-response curve in duplicate. To eliminate any possibility of matrix effects on the dose response, we constructed both curves by serial dilution of the spiked RBC pool into RBCs reserved before the spike was added. The diagnosis of scrapie symptoms is subject to large interobserver and even intraobserver variation. To eliminate any possibility of bias in the determination of endpoints, we used loss of body weight as an empiric endpoint. These measures resulted in tightly clustered data at the higher concentrations on the dose-response curves and a high level of concordance between the two independent curves.

The data are plotted in Fig. 3B, and the mean incubation time of the infections at each dilution and for both dilution series are reported in Table 1. The dilution series starts at 10⁻³ because whole brain (dilution 10⁰) was diluted 1000-fold to prepare the RBC challenge. At low concentrations of infectivity, the incubation time is no longer strongly coupled to the infectivity titer and varies randomly from 150 to 540 days (R.G. Rohwer laboratory, unpublished). Nevertheless, a sample of unknown titer producing a similar pattern of long, highly scattered incubation times can still be compared to the curve and should be presumed to have a similarly low titer.

Both determinations of the dose-response curve are presented in Fig. 3B. The graph emphasizes incubation times up to 250 days after inoculation because all but six animals died within this interval. The incubation times and mean for the eight animals inoculated with the final effluent in each ligand series are plotted in Fig. 3A. All inocula in Fig. 3A are undiluted effluents at the same 10⁻³ dilution, relative to whole brain, as the original undiluted, spiked RBC pool from which they were derived. Increases in the incubation times of the infections relative to those at the 10⁻³ dilution of the dose-response curve are due to removal of infectivity by the devices. The magnitude of the transposition required to place the data for each ligand at its intercept on the dose-response curve is a measure of the reduction of infectivity by the resin.

The cumulative log reduction for each effluent is the absolute value of the difference between the log dilution calculated for the effluent from the fitted curve and that of the log dilution of the challenge, -3, where both dilutions are relative to whole brain. The results are summarized in Table 3. The fluctuations in cumulative reduction seen between successive devices in some of the series reflects the low titer of the residual infectivity in the effluents from those devices as the incubation times of individual animals become longer and more scattered for low-dose inoculum.

There was no retention of either infectivity (Table 2) or PrP^{res} (Fig. 2) by the negative control, resin L91, thereby

TABLE 1. Mean incubation times and animal survival as a function of inoculation dose

Dilution	Determination I			Determination II			Mean I +II	
	S/N*	IT†	SD	S/N	IT	SD	IT	SD
-3	8/0	94	4	8/0	99	5	96	5
-4	8/0	106	8	8/0	105	7	106	7
-5	8/0	114	5	8/0	111	4	113	5
-6	8/0	129	9	8/0	118	5	123	9
-7	8/0	164	76	8/0	160	59	162	65
-8	6/2	227	49	5/3	182	73	207	62
-9	1/7	147		2/6	206	104		
-10	1/7	161		0/8				

* S/N = number of scrapie infected animals versus number of uninfected animals 365 days after inoculation.

† IT = mean incubation time in days after inoculation to 80 percent of maximum weight.

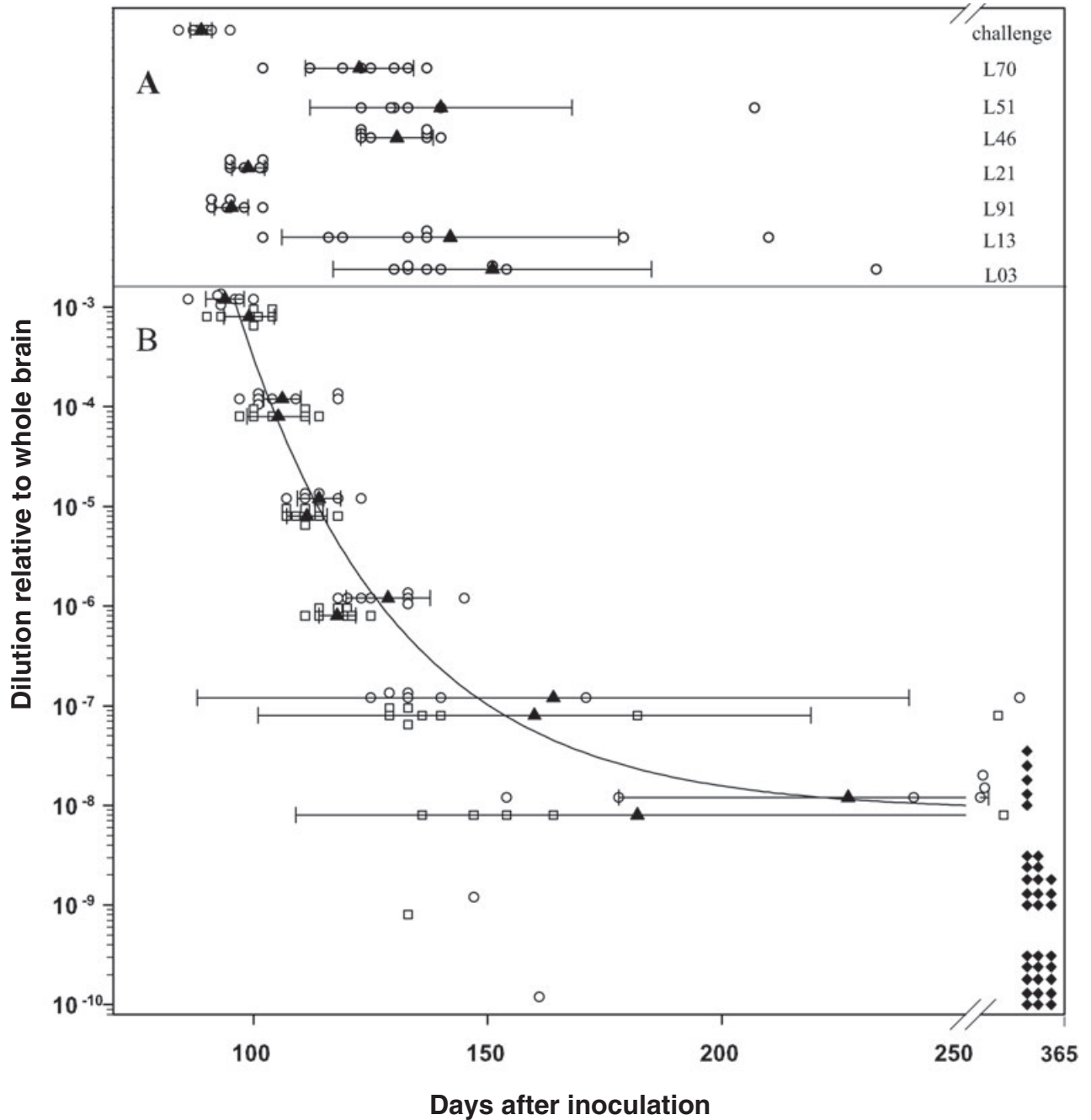


Fig. 3. Incubation time assay of residual infectivity. (A) The incubation times and mean for the eight animals inoculated with the final effluent in each ligand series. (○) Incubation times for individual animals; (▲) mean value at each dilution. Standard deviations (SDs) of the mean are plotted as horizontal bars. All inocula in A are undiluted effluents at the same 10^{-3} dilution relative to whole brain as the original spiked RBC pool from which they were derived. Increases in the incubation times of the infections relative to those at the 10^{-3} dilution of the dose-response curve are due to removal of infectivity by the devices. The magnitude of the transposition required to place the data for each ligand on its intercept on the dose-response curve is a measure of the reduction of infectivity by the resin. (B) Dose-response curves. (○) Incubation times for individual animals; (▲) mean value at each dilution. SDs of the means are plotted as horizontal bars. The two independent determinations are displaced slightly above and below each inoculated dilution, and the incubation times of individual animals are displaced vertically as needed to separate the data visually. The graph emphasizes incubation times up to 250 days after inoculation because all but six animals died within this interval. The remaining six animals are plotted in compressed form after the axes break point. Inoculated animals that were not infected are plotted as closed diamonds on the extreme right axis of the chart.

TABLE 2. Mean incubation times and dose-response curve intercepts for each effluent titered

Ligand	Effluent											
	2			3			4			4		
	IT*	SD	Dilution intercepts†	IT	SD	Dilution intercepts	IT	SD	Dilution intercepts	IT	SD	Dilution intercepts
L03	103	8	-3.9	131	20	-6.2	151	34	-7.0			
L13	104	4	-4.0	164	42	-7.3	158	39	-7.2	142	36	-6.7
L21	95	7	-3.0							99	3	-3.4
L46	102	3	-3.7	127	5	-6.0	134	12	-6.3	131	8	-6.2
L51	101	3	-3.7	119	9	-5.4	150	55	-7.0	140	28	-6.6
L70	100	5	-3.5							123	12	-5.7
L91	92	4	-3.0							95	4	-3.0

* IT = mean incubation time in days after inoculation to 80 percent of maximum weight.

† The dilution intercepts of the mean incubation times for each effluent calculated from an equation fit to the dose-response curves (see Materials and methods).

establishing that removal by the functional resins was mediated by the ligands themselves rather than nonspecific interactions or mechanical trapping. Unexpectedly, resin L21 performed nearly as poorly as the control, reducing the titer of the challenge by only 0.4 log ID per mL, or 60 percent, after five columns. In contrast to the control, however, each column retained a strong, if gradually diminishing, Western blot signal for PrP^{res}. This result highlights an important distinction that must be made between the mass of PrP^{res} bound by the columns and the proportion bound. For example, the masses of PrP^{res}, represented by 99 percent removal and 99.99 percent or greater removal, are virtually identical and thereby indistinguishable by Western blot. They represent, however, vastly different levels of risk reduction. The proportional removal can only be determined from the titer of the residual infectivity in the effluents. The Western blot is not sensitive enough to detect the very low levels of residual PrP^{res} in the effluents, especially in the presence of plasma proteins.

Without the benefit of the infectivity measurements for L21, the diminishing effectiveness of each column in the series might have been interpreted as indicating complete removal of PrP^{res}. In fact, because the effluent concentrations of PrP^{res} and infectivity remained high, it suggests instead that this resin was preferentially binding to, and depleting only, a distinct subfraction of the total PrP^{res} protein. The pattern of removal exhibited by resin L21 warns against the overinterpretation of binding data for the evaluation of infectivity removal and confirms the need for infectivity studies.

Resin L70, while more effective than L21, nevertheless gave a weak but definite PrP^{res} signal on the Western blot from device 4 consistent with only a 2.7 log ID₅₀ per mL reduction in infectivity over all five columns. The poor performances of L21 and L70 were unexpected and may result from kinetic factors that were not present in the earlier screening. Resins L03 and L51 reduced the titer of

the challenge by approximately 4 log ID₅₀ per mL with four devices.

The best performing resin was L13, which achieved a 4 log ID₅₀ per mL reduction in titer with three devices. One of the animals inoculated with effluent 4 of resin L13 did not develop disease, indicating that the inoculum was, by definition, at limiting dilution. A much more precise estimate of residual titer can be calculated from limiting dilution data.⁸ In the case of L13, it is likely that the effluents from columns 3, 4, and 5 were essentially at the same concentration such that there was 1 uninfected animal of 24 inoculated. The probability, P(0), of escaping infection with this inoculum was therefore 1 in 24 and the titer calculated from the Poisson distribution was

$$-\ln(1/24) \text{ ID}/50 \mu\text{L} = 3.18 \text{ ID}/50 \mu\text{L} \text{ or } 63.6 \pm 36 \text{ ID}/\text{mL}$$

equivalent to 91.7 ID₅₀ per mL. Comparison with the titer of the challenge, 6.74 log ID₅₀ per mL, gives a reduction of 6.74 - 1.96 = 4.78 log ID₅₀. If, because of their long and scattered incubation times (Table 2), we consider that the residual infectivity in effluent 4 of resin L03; effluents 3, 4, and 5 of resin L13; and effluents 4 and 5 of resin L51 are all equivalent, then the limiting dilution titer of the residual infectivity in all maximally depleted samples would be 2.05 log ID₅₀ and the reduction by the three best resins would be 4.69 log ID₅₀.

The reduction in infectivity estimated from the dose response was approximately 4 log ID₅₀ for the best resins and somewhat greater when calculated by the more precise limiting dilution method; however, for every resin a point was reached where subsequent devices failed to remove further infectivity. This demonstrates that a fraction of brain-derived infectivity was not captured by any of the immobilized ligands tested in this format.

The number of scrapie-infected versus uninfected animals at each dilution of the dose-response curves was used to compute the endpoint dilution titer of the scrapie-infected RBC pool used to challenge the resins. The values

from the Reed and Muench calculation were $10^{6.79 \pm 0.25}$ ID₅₀ per mL and $10^{6.69 \pm 0.32}$ ID₅₀ per mL with a geometric mean of $10^{6.74}$ ID₅₀ per mL. Endpoint dilution titration was also used to titer the 10 percent scrapie brain homogenate from which the spike was prepared and the clarified spike after Sarkosyl treatment. The titer of the 10 percent brain homogenate was $10^{9.2}$ ID₅₀ per mL. After Sarkosyl treatment, it dropped to $10^{8.1}$ ID₅₀ per mL indicating a loss of 90 percent of the infectivity to the Sarkosyl pellet. The Sarkosyl conditioning removed large particles and aggregated materials that if trapped mechanically would have given false indications of removal, but at a high cost in total infectivity. Fortunately, the high brain titers of 263K-infected hamsters, typically 1×10^{10} to 2×10^{10} ID₅₀ per g, make it possible to sustain the conditioning losses and still retain sufficient infectivity to conduct meaningful removal experiments. The lack of retention of infectivity, or PrP^{res}, by the control resin, L91, validated the effectiveness of the Sarkosyl treatment.

DISCUSSION

The mean and median concentrations of TSE infectivity in blood pooled from hamsters clinically affected with 263K scrapie has consistently been approximately 10 ID per mL^{8,9} (R.G. Rohwer laboratory, ongoing projects). Similar values have been obtained for bovine spongiform encephalopathy-infected mice (R.G. Rohwer laboratory, unpublished). Approximately one-half of the infectivity in blood is removed with the WBCs during leukofiltration.⁸ Elsewhere we have shown that TSE infectivity is not associated with purified PLTs⁹, and we have preliminary evidence that this is also true of RBCs (L. Gregori, in progress); therefore, the remaining infectivity must be associated with plasma. For a hematocrit level of 50 percent, the concentration of infectivity in the plasma would be similar to that in whole blood, approximately 10 ID per mL (since approximately one-half of the volume of the leukodepleted whole blood is occupied by un-infected RBCs). If a 350-mL unit of RBCs contained 20 percent plasma (a high estimate), the expected concentration of TSE infectivity would be approximately 2 ID per mL. In comparison, the concentration of TSE infectivity in the spiked RBCs used to challenge the resins was 6.54 log ID per mL (obtained by conversion of the endpoint dilution titer of 6.63 log ID₅₀/mL to log ID/mL, where the conversion factor is 0.693 ID/ID₅₀). Thus, the spiked RBCs contained nearly 2,000,000-fold greater TSE infectivity than would ever be expected from a TSE-infected unit of RBCs, and the most efficacious ligands demonstrated a vast excess capacity for removing those infectious forms that they were capable of removing. The most effective ligand, L13, captured approximately 10^9 ID total with the first two devices in series, leaving 1.3×10^8 ID, 99.95 percent of which was captured by device 3 (Table 3). The only infec-

TABLE 3. Cumulative log reduction of TSE infectivity*

Ligand	Effluent			
	2	3	4	5
L03	0.9	3.2	4.0	
L13	1.0	4.3	4.2	3.7
L21	0			0.4
L46	0.7	3.0	3.3	3.2
L51	0.7	2.4	4.0	3.6
L70	0.5			2.7
L91	0			0

* The cumulative log reduction for each effluent is the absolute value of the difference between the log dilution calculated for the effluent from the curve fitted to the dose-response data and that of the log dilution of the challenge, -3, where both dilutions are relative to whole brain.

tivity remaining after device 3 was a small subfraction that was not recognized by the resin. The infectivity captured in a single step by device 3 (1.3×10^8 ID) was substantially greater than that present in an equivalent volume of blood (approx. 5000 ID) or RBCs (approx. 600 ID). Thus, a single device provided a large margin of safety for removal of those infectious forms that it was capable of removing. It is possible that other solid support matrices, other physical conformations of the matrix (particles vs. membranes), or configurations of the final device could increase the efficiency of binding even further.

If the distribution of TSE infectivity in leukoreduced blood is the same as in the Sarkosyl-conditioned brain spike, the unadsorbed fraction would be less than 1 part per 10,000 of the total infectivity for the best resins. In a 500-mL unit of blood, at 5 ID per mL after leukoreduction, the total unadsorbed infectivity would be 0.25 ID total. This is identical to the predicted level of residual WBC-associated infectivity after leukoreduction. In a 350-mL unit of RBCs containing 20 percent plasma, the residual plasma associated infectivity would be approximately 0.07 ID total, far less than that remaining from leukoreduction. In actual practice, the residual infectivity titers may be far lower. Few donations are likely during symptomatic disease when blood infectivity titers are highest (at least in the hamster 263K scrapie model [R.G. Rohwer laboratory, manuscript in preparation]). The infectivity removal device may also trap and remove additional WBCs. Most importantly, the efficiency of transfusion transmission of TSE infections is uncertain. In rodents, the efficiency of intravascular inoculation of brain-derived infectivity has been reported to be 0.1- to 0.2-fold that of an intracranial inoculation, thus making the effective titer lower in proportion. In the hamster, the transmission efficiency for transfusion of endogenous infectivity in whole blood is at least 50-fold lower still (R.G. Rohwer laboratory, manuscript in preparation). It is also possible that the unadsorbed fraction in the brain spike may not be present

in blood. If, on the other hand, this fraction has a greater representation in blood than in brain, the effectiveness of the device will be diminished in proportion.

The ability of immobilized ligands to bind the endogenous TSE infectivity in blood, versus brain-derived infectivity spiked into blood, can only be determined by direct challenge of the ligands with endogenously infected blood. The total reduction in infectivity that can be demonstrated in such an experiment is limited by the titer and volume of the sample that is inoculated. The potential reduction will be far less than for a brain spike, but such reduction as can be demonstrated will be of the relevant form of infectivity. Low infectivity samples like blood can be accurately titered by limiting dilution titration. If 5 mL of leukoreduced blood with a titer of 5 ID per mL is inoculated 50 µL per hamster into 100 hamsters, and there are no infections after passage through the resin, the study could demonstrate approximately 1.4 log of endogenous TSE infectivity reduction. Such a study is currently ongoing in our laboratory.

In the case of TSE infectivity, removal is one of the few options available for reducing the risk from blood infectivity. It also may have unique advantages for other blood pathogens as well. At high ligand concentrations, there is no lower limit for removal, whereas there is for detection. Thus removal has the potential to eliminate the risk from window-period infections more comprehensively, more sensitively, and less expensively than screening.

ACKNOWLEDGMENT

The authors are grateful to the BSL3 animal facility staff for the excellent animal care.

REFERENCES

1. Will RG. Acquired prion disease: iatrogenic CJD, variant CJD, kuru. *Br Med Bull* 2003;66:255-65.
2. Llewelyn CA, Hewitt PE, Knight RS, et al. Possible transmission of variant Creutzfeldt-Jakob disease by blood transfusion. *Lancet* 2004;363:417-21.
3. Peden AH, Head MW, Ritchie DL, Bell JE, Ironside JW. Preclinical vCJD after blood transfusion in a PRNP codon 129 heterozygous patient. *Lancet* 2004;364:527-9.
4. Transfusion Medicine Epidemiology Review (TMER) [homepage on the Internet]. Edinburgh: The National Creutzfeldt-Jakob Disease Surveillance Unit; 2005. Available from: <http://www.cjd.ed.ac.uk/TMER/TMER.htm>
5. Hilton DA, Ghani AC, Conyers L, et al. Prevalence of lymphoreticular prion protein accumulation in UK tissue samples. *J Pathol* 2004;203:733-9.
6. Guidance for industry: questions and answers on FDA guidance entitled "Revised preventive measures to reduce the possible risk of transmission of Creutzfeldt-Jakob (CJD) disease and variant Creutzfeldt-Jakob disease (vCJD) by blood and blood products" [monograph on the Internet]. Rockville (MD): U.S. Food and Drug Administration, Center for Biologics Evaluation and Research (CBER), Food and Drug Administration; 2004. Available from: <http://www.fda.gov/cber/gdlns/cjdvcjdq&a.htm>
7. Kokunai ni okeru henikei Creutzfeldt-Jakob-byo (vCJD) no hassei ni tsuite [monograph on the Internet]. Press release. Tokyo: Japanese Ministry of Health, Labour and Welfare; 2005 Feb 4. Available from: <http://www.mhlw.go.jp/houdou/2005/02/h0204-3.html>
8. Gregori L, McCombie N, Palmer D, et al. Effectiveness of leucoreduction for removal of infectivity of transmissible spongiform encephalopathies from blood. *Lancet* 2004;364:529-31.
9. Holada K, Vostal JG, Theisen PW, et al. Scrapie infectivity in hamster blood is not associated with platelets. *J Virol* 2002;76:4649-50.
10. Elliott EJ, MacAuley C, D'Addio V, Rohwer RG. Carotid artery transfusion in the hamster. *Contemp Top Lab Anim Sci* 2005;44:28-30.
11. Reed LJ, Muench H. A simple method of estimating fifty per cent end points. *Am J Hygiene* 1938;27:493-7.
12. Pizzi M. Sampling variation of the fifty percent end point, determined by the Reed-Muench (Behrens) method. *Hum Biol* 1950;22:151-19.
13. Baumbach GA, Hammond DJ. Protein purification using affinity ligands deduced from peptide libraries. *BioPharm* 1992;May:24-35.
14. Furka A, Sebestyen F, Asgedom M, Dibo G. General method for rapid synthesis of multicomponent peptide mixtures. *Int J Pept Protein Res* 1991;37:487-93.
15. Lam KS, Salmon SE, Hersh EM, et al. A new type of synthetic peptide library for identifying ligand-binding activity. *Nature* 1991;354:82-4.
16. Hammond D, Lathrop J, Cervenakova L, Carbonell R, inventors. Prion protein ligands and methods of use. U.S. patent WO 2004/050851A2. 2003 Dec 3. 