AD_____

Award Number: DAMD17-03-1-0756

TITLE: Development of an Assay for the Detection of PrPres in Blood and Urine Based on PMCA Assay and ELISA Methods

PRINCIPAL INVESTIGATOR: Robert G. Rohwer, Ph.D.
Luisa Gregori, Ph.D.

CONTRACTING ORGANIZATION: Baltimore Research and Education Foundation, Inc.
Baltimore, MD 21201

REPORT DATE: September 2008

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
1 Sep 2008	Final	1 Sep 2003 – 31 Aug 2008

4. TITLE AND SUBTITLE

Development of an Assay for the Detection of PrPres in Blood and Urine Based on PMCA Assay and ELISA Methods

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0756

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Robert G. Rohwer, Ph.D.
Luisa Gregori, Ph.D.

E-Mail: rrohwer@umaryland.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Baltimore Research and Education Foundation, Inc.
Baltimore, MD 21201

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The focus of this project was the development of a pre-clinical blood-based TSE diagnostic assay. This is the final report for this project. The assay was developed with plasma from hamsters infected with scrapie (263K) and with some modifications the same assay could also be adapted to human plasma. Most of the proposed aims were achieved. We developed and evaluated an immuno-assay for the detection of PrPc and PrPsc from hamster brain and plasma. We conducted a study to determine the PK concentration that preserved plasma infectivity while reducing the level of endogenous PrPc to below the limit of detection of our PrP assay. These studies were designed to assess whether PK can be used as the discrimination method in a PrPsc detection assay. The results indicated that 50-200 µg/ml PK is an appropriate concentration range to discriminate PrPsc in infected plasma. We also found that urine excreted by infected hamsters harbors infectivity with infectivity titers similar to that of hamster plasma. Urine from pre-clinical hamsters did not show detectable levels of infectivity. Lack of infectivity may be due to technical reasons and it should be repeated. Urine could be a useful alternative to blood in a TSE diagnostic assay.

15. SUBJECT TERMS
PrPres, Assay, Diagnostics, ELISA, Blood, Urine

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	37	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Introduction

Transmissible spongiform encephalopathy (TSE) diseases are fatal illnesses for which there is no cure or treatment. Individuals incubating TSE can transmit infectivity by blood transfusion. Four human-to-human transmissions of variant Creutzfeldt-Jakob disease were reported in the United Kingdom[1,2]. TSE infectivity in blood was also demonstrated in natural and experimental animal models such hamster[3,4], mouse[5] and sheep[6]. A TSE diagnostic will greatly improve the safety of the blood supply.

This is the final report for the years 2003 to 2007 with an additional year, 2008, for which we obtained a no-cost extension to complete the plasma PK studies. In the original proposal, we outlined five specific aims. The first specific aim covering the application of the protein misfolding cyclic amplification (PMCA) method to plasma was removed by the review committee and we did not pursue PMCA. The rest of the aims were:
1. To confirm the presence of PrPres in the urine of infected animals and to characterize this PrPres.
2. To develop an ELISA-based format for PrP detection.
3. To optimize the proteinase K digestion conditions for low concentrations of PrPres in the presence of high concentrations of PrPc.
4. To validate all assays developed: any urine-based assay and ELISA format assays.
Over the course of this program, all four specific aims were addressed and the yearly research progresses were reported in the annual reports. Below is a summary of the entire program and it includes an update of the plasma PK study.

Body

Specific aim 1 – Tasks 1 and 2
Prion infectivity in urine has been extensively investigated by us and other groups over the past 4 years. Five studies were reported on TSE infectivity in urine. One study showed trace amounts of urinary infectivity in the 236K hamster strain[11]. The second report indicated that only mice with kidney infection and infected with scrapie excreted infectivity in urine. Mice with scrapie alone did not have infectivity in their urine[12]. The third study was in deer infected with chronic wasting disease and showed no infectivity in urine and feces inoculated together in 3 deer[13]. A second study by the same group suggested detectable level of infectivity in the CWD deer urine assayed with transgenic mice carrying the deer PrP gene (these results will be presented at the Prion2008[10]). Finally, a recent report showed the detection of PrP^{sc} from urine of hamsters

affected by scrapie after PMCA amplification of the endogenous PrPsc[11]. These data all together suggest that low but detectable levels of TSE infectivity is excreted by affected animals. Our laboratory has focused on urinary infectivity in the scrapie hamster model. These studies were recently published and they represent the only quantitative measurements of the titer of infectivity in excreted urine[12].

The details of the study can be found in the publication included in this report (appendix). In summary, we measured 3.8 ± 0.9 ID/ml of urine from clinically infected hamsters. This titer is similar to the titer of infectivity in plasma for the same animal model and reinforces the possibility that urine could be used as the assay material for a TSE diagnostic test. In the same study, we also titered bladder and kidney tissues from infected animals. The titers of the two organs were similar and measured 5 $\log_{10}$ infectious doses_{50} (ID_{50}) per g of tissue. This is the first report showing infectivity in bladder. Kidneys had been investigated before but this is the first quantitative measurement of the kidney infectivity titer. The published studies were completed with immunohistochemistry and histological examinations of the two organs. In collaboration with Dr. Budka at the University of Vienna (Austria) we determined that kidneys and bladders from hamster infected with scrapie do not have signs of infection or other abnormalities aside from the typical changes associated with scrapie. These observations were very important since the Aguzzi lab had reported the opposite findings in mice affected by scrapie[8]. Our results were also confirmed in studies in sheep affected by natural and experimental scrapie[13, 14].

Titration of urine from pre-clinical animals

Following the results of the clinical urine titration, we decided to titer urine collected from hamster at the pre-clinical stage of the scrapie infection. At the time we started this study we did not consider the issue that the infectivity titer in urine might be affected by the volume of urine produced in a given time. The titration with the standard 5 ml of urine inoculated into 100 animals resulted in no infections. This result was unexpected as we anticipated that infectivity in urine, as it is in blood, is present at early incubation times although with lower titers. It is possible that infectivity titers in urine at pre-clinical stage is below the limit of detection of the bioassay or that the same amount of infectivity is excreted but it is diluted in a larger volume of urine compared to the clinical urine (pre-clinical animals are not dehydrated and produce as much as 7 times more urine volume per day than clinically affected animals). Therefore, we are prepared to repeat the study after a method of urine infectivity concentration is developed.

Specific aim 2 – Task 1

In the first year of this project, we tested several combinations antibodies as capture and detector in sandwich ELISA assays. We specifically focused on the Origen Analyzer (BioVeris), the DELFIA (Wallac/PE) and the MPD ELISA (BioTraces). BioTraces had the most sensitive assay in which ^{125}I was used to detect PrP. We worked with the company to implement the assay with biological samples. We confirmed the extremely high sensitivity of the assay with recombinant PrP but we were not able to obtain the same level of sensitivity when biological material such as plasma was used. After extensive investigations we decided to abandon the BioTraces assay and focused on a more practical and also sensitive assay provided by the Origen Analyzer.

Specific aim 2 – Tasks 2 and 3

Using the Origen Analyzer, we developed an assay for TSE infection using scrapie hamsters infected (263K). In our current assay platform, the target test material is plasma and the protein to be detected is PrP^{res}, the biochemical marker for TSE infections. We showed that the assay is capable of detecting abnormal PrP from brain spiked in plasma. In the first attempt to detect PrPres in plasma, no signal was observed after PK. These results highlighted the need to optimize the PK digestion step (see next Task).

Using this detection system we measured PrP in brain and plasma of scrapie infected animals. The details of these studies were reported in a publication (See appendix)and can also be found in previous annual reports. From this analysis we established that the concentration of PrP in normal hamster brain is 7.9±0.9 μg/g and PrPsc in infected brain is 57±9.6 μg/g of tissue. In hamster plasma the concentration of normal PrP was 3 ng/ml and we also calculated the theoretical concentration of PrPsc in infected hamster plasma to be approximately 30 fg/ml of plasma[15]. These measurements represent average values conducted with large pools of brains (50-100 animals) and plasma (10-50 animals).

Specific aim 3 – Tasks 1 and 2

<u>The issue of SDS in the plasma PK assay</u>

In the original proposal we suggested a series of PK digestions using brain homogenates spiked in hamster plasma. These studies using normal and scrapie brain spikes in plasma indicated that 0.5%-1% SDS was necessary to reduce PrP^{c} to below the limit of detection of the assay. This requirement represented a technical challenge for the assay and the bioassay. Following a long

investigation on how to incorporate such high concentration of detergent in the assay, we concluded that the only option was to remove SDS prior to the PrP immunoassay. Several attempts were conducted to find efficient methods of detergent removal from plasma. None of the methods tested was satisfactory because either SDS was not sufficiently removed or together with SDS plasma proteins including PrP were removed as well. The breakthrough came in 2006 when we discovered that SDS in the PK digestion was needed only to digest spiked brain PrP^c but it was not required to remove normal endogenous plasma PrP^c. This observation was critical because it allowed us to apply PK digestion to plasma followed by direct detection of the remaining PrP without further manipulations of the sample.

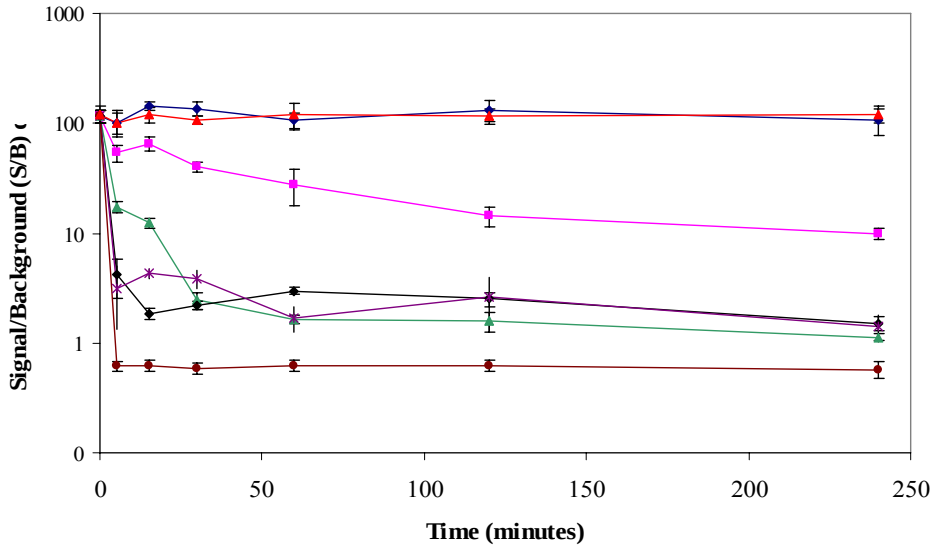
Specific aim 3 – Tasks 1 and 4

PK digestion of PrP from normal hamster plasma

We tried to detect PrP^{res} in hamster infected plasma without success. One of the issues was that the PK digestion was not optimized and therefore it was not possible to know whether PrP^{res} detection failed because of insufficient assay sensitivity or because PK had removed/reduced PrP^{res}. To answer these questions we needed to optimize the PK digestion step using endogenously infected plasma. A series of PK concentration conditions starting from no PK up to 500 μg/ml were tested. The remaining PrP was first captured with an affinity resin (3F4-resin) and then detected with the Origen Analyzer. Since the concentration of normal PrP in plasma is relatively low (3 ng/ml) we expected that low concentrations of PK would be sufficient to digest the protein. On the contrary, we found that relatively high PK concentration (>20 μg/ml) was required to reduce PrP^c below the limit of detection of our assay (4pg/assay).

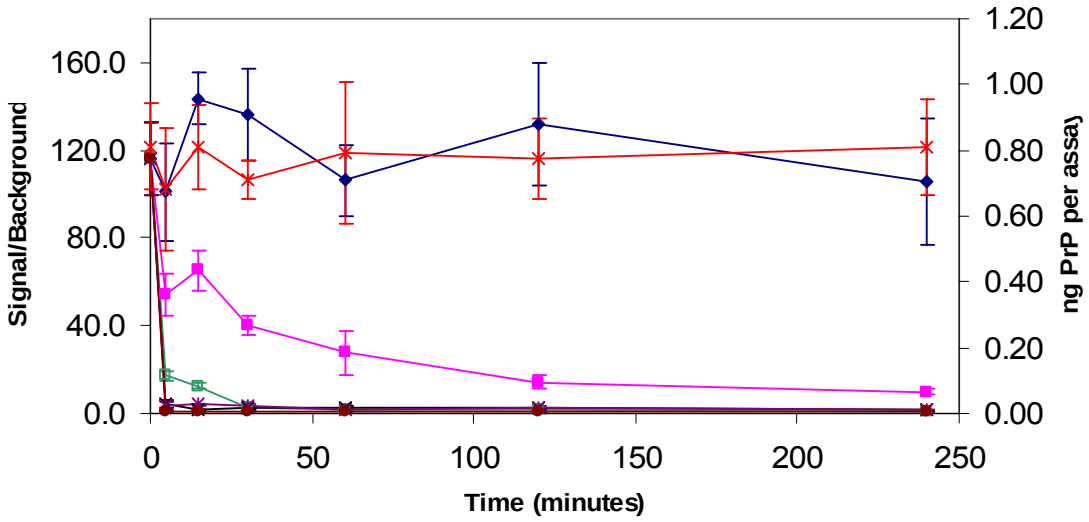
Figure 1 shows the quantitation of PrPc remaining after infected hamster plasma was treated with various concentrations of PK at 37°C and at different times during the incubation. PrPc concentration remained unchanged when PK was not present (lanes blue and green) and the values were the same for normal and infected plasma confirming that PrPsc cannot be detected above the PrPc excess background unless PrPc is fully removed. Low concentration of PK (5 μg/ml, purple lane) did not completely digest PrP while all other PK concentration reduced the level of PrPc to S/B=1.2±0.4 and only 500 μg/ml PK (brown lane) reduced the concentration of PrP below the background level. These data indicate that within the standard deviation all PK concentrations starting with 20 mg/ml removed the PrPc at or below the background level i.e below the limit of detection of the assay.

Figure 1



The data in Figure 1 were plotted together with the measured PrPc mass detected following capture with the 3F4 resin and detection with the Origen Analyzer (Figure 2).

Figure 2



The next step was to determine whether or not the PK condition that reduced PrPc to undetectable levels also digested PrPsc. This point is critical because if PrPsc is also digested it would indicate that this discrimination step could not be incorporated into a diagnostic assay because it would have demonstrated that PK lacked the needed discrimination power to distinguish between the two

proteins in plasma. The difficulty of this demonstration was that PrPsc could not be detected in plasma. To address this issue, we decided to assay TSE infectivity instead of PrPsc thus, using infectivity as a surrogate marker for PrPsc (see below).

Specific aim 3 – Tasks 1 and 4

Plasma PK study

Since the final goal of these studies was to inoculate the PK-treated plasma and measure the infectivity titer, the issue of compatibility of the PK-treated plasma samples with the bioassay needed to be solved. Typically, PK reaction is stopped with PK inhibitors PMSF or pefabloc. Early toxicity studies indicated that both reagents were highly toxic and that the animals tolerated significantly lower concentrations of these inhibitors than was needed to effectively inhibit PK. This was a difficult problem since active PK, we thought, would also be toxic to the animals. To our surprise hamsters tolerated high PK concentrations inoculated intracerebrally much better than the PK inhibitors. After many tests and toxicity experiments, we concluded that the best option was not to add PK inhibitor in the sample prepared for the bioassay.

Based on pilot tests, 3 concentrations of PK, 50 μg/ml, 200 μg/ml and 500 μg/ml were selected (Figures 1 and 2). As the controls, infected plasma with no PK incubated at 37^{o}C for 4 hours and non incubated and normal plasma without PK were also included in the titration for a final 6 titrations (see Table 1). The PK concentrations were chosen because they consistently showed digestion of PrP^{c} to below the detection of the assay. The question asked was under which of PK conditions was infectivity unchanged? Since in this study we assume that reduction of infectivity means degradation of PrP^{res}, those conditions with no change in the infectivity could be selected as TSE assay condition for PrP^{res} detection in infected plasma.

Animal Bioassay

Endogenous plasma infectivity can be measured with the limiting dilution method in which 5 ml of sample is distributed into 100 animals (50 μl per animals) by intracerebral inoculation. This method has been used extensively in our laboratory for those samples with extremely low titers such as blood, blood components and urine. Table 1 shows the number of animals inoculated per condition and the animals so far infected with scrapie. An interim titer is also reported in the Table. The final titer will be calculated after all animals in this study have been euthanized and the brain of each animal has been assayed by Western blot or Elisa to assess the infection status of each animal. The animal study

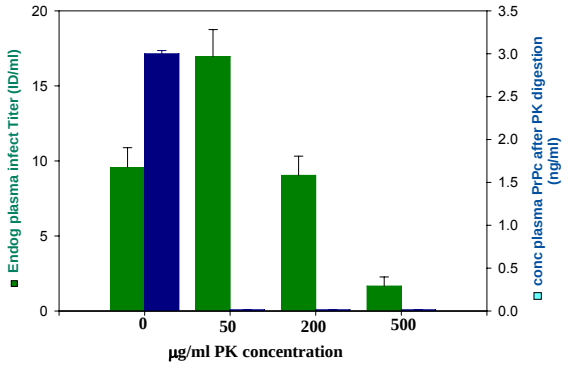
will be terminated at 540 days post inoculation (end of November 2008).

Table 1

Sample	IT	PK (μg/ml)	Inc time (hrs)	# animals inoc (N)	# animals inf (n) (S)	Volume inoc (V)	Titer (ID/ml)	sd
Normal Ha Plasma	471	0	4	106	0	5.3	0.0	0.0
Scrapie Ha Plasma	471	0	4	108	41	5.4	9.5	1.3
Scrapie Ha Plasma	470	50	4	105	60	5.3	16.9	1.8
Scrapie Ha Plasma	470	200	4	110	40	5.5	9.0	1.3
Scrapie Ha Plasma	470	500	4	88	7	4.4	1.7	0.6
Scrapie Ha Plasma	458	0	0	102	30	5.1	7.0	1.2

Figure 4 shows the current titers (green bars) as a function of the PK concentration. Although the exact titer may change by the end of the study, the overall results will not vary significantly. There are two major observations, 500 μg/ml PK caused reduction of the infectivity titer suggesting that this PK concentration is too high and it is not suitable for a PrPres-based assay. Unexpectedly, 50 μg/ml PK showed an increase in the infectivity titer. The significance of this increased titer is not clear at this time. It may be due to possible removal by PK of "inhibitors" of infectivity. Figure 4 also shows the concentration of PrPc measured under those PK conditions (blue bars). The values were calculated for the concentration of PrP in plasma. As indicated in Figure 2, all three PK concentrations chosen for this experiment removed PrPc close or below to the limit of detection.

Figure 4

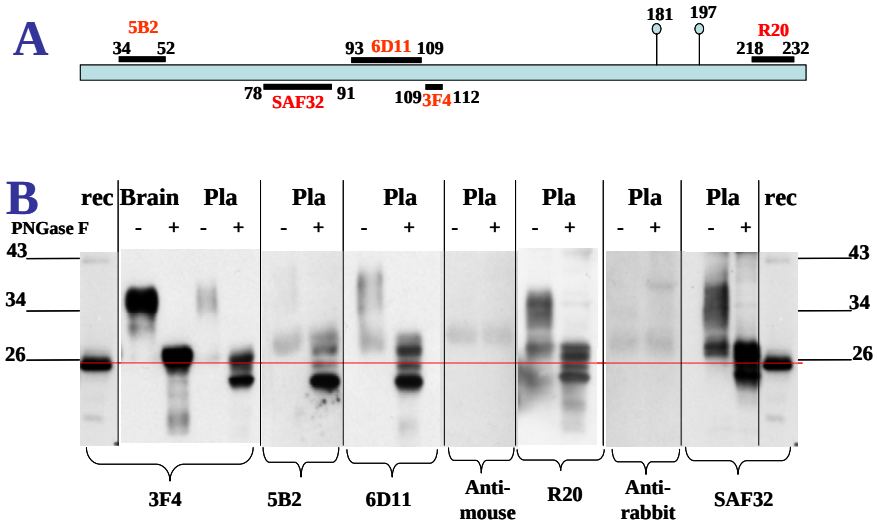


Characterization of plasma PrPc

As part of the characterization of plasma PrPc, the protein was first captured with the 3F4 resin followed by treatment with the deglycosylation enzyme PNGase F. The products were analyzed by Western blot and detected with five different antibodies (Figure

5). As expected PNGase F reduced the apparent molecular weight (MW) of brain PrP. Also plasma PrPc was reduced in MW but its electrophoretic pattern after deglycosylation was more complex than brain PrPc (compare in Figure 5, 3F4 staining). The data also suggest that the protein band with MW below that of recombinant PrP corresponds to the N-terminal proteolytic cleavage.

Figure 5



Specific aim 4
The assay developed was highly reproducible (inter and intra assay variability) and robust. The background was consistently low with all samples tested. Based on the quantitative evaluation, we determined that our assay did not have sufficient sensitivity to detect PrPsc in infected plasma. Our analysis also identified that assay sensitivity and discrimination are the two most critical steps, common to all TSE assays, that are still far from being reliably resolved by the commercially developed TSE assays. We did not evaluate the assay for its predictive power.

As soon the studies are completed, a manuscript for publication will be prepared.

Key research accomplishments

- We developed and characterized the Origen Analyzer assay coupled with an immuno-affinity resin step as an improved and sensitive assay for the detection of PrP. This assay was applied to measure the concentration of PrP in plasma and in brain. These results allowed us to estimate the concentration of PrPsc in infected plasma based on quantitative data. This is valuable information strongly needed to evaluate other TSE assays.

- We have measured the infectivity titer of urine excreted by scrapie infected hamsters. Other groups have also reported infectivity in urine but ours is the first and only quantitative measure of the titer of excreted urine. We have also determined the titers of TSE infectivity in kidneys and bladders from infected hamsters. Until this demonstration, it was not known that hamster kidneys and bladders contained such high levels of infectivity.
- We have generated a valuable PK digestion matrix in which endogenous plasma PrP^{c} digestion is assayed as a function of time and PK concentration.
- The titration of PK-treated infected hamster plasma indicated that PK digestion can be used for discrimination of PrPc and PrPres in plasma. Our results also suggest that endogenous infectivity can be digested if high concentration of PK are used.

Reportable outcomes

Invited Presentations and Posters

Prion2008 meeting in Madrid, Spain. Gregori L, Rose E, Rohwer R. Proteolytic removal of PrPc in plasma with preservation of endogenous infectivity. (Poster)

European Medicinal Agency, TSE Expert meeting, London July 2007. Infectivity in urine of hamster infected with scrapie. (Presentation)

CHI meeting on TSE Diseases Feb 2007. Gregori L and Rohwer R. Infectivity in urine of hamsters infected with scrapie. (Presentation)

International Expert Advisory Group on Risk Modeling Emerging Infectious Diseases Feb 2007. Rohwer R and Gregori L. Quantitative measurement of the titer of TSE infectivity in urine. (Presentation)

Prion2006 Meeting in Turin, Italy. Gregori L and Rohwer R. Infectivity in urine of hamsters infected with scrapie. (Presentation)

CHI meeting on TSE Diseases Feb 2006. Gregori L. Detection of plasma PrP with a sensitive assay. (Presentation)

IPFA International Scientific Workshop on TSEs and the Safety of Blood Components and Plasma Derivatives. April 2006 Paris (France) Gregori L. Highly sensitive assay detection for PrP in hamster plasma. (Presentation)

IBC meeting on Transmissible Spongiform Encephalopathies Nov 2006. Gregori L. Development of a sensitive assay for the detection of PrP in hamster plasma. (Presentation)

Publications

Gregori L, Kovacs GG, Alexeeva I, Budka H, Rohwer RG. (2008) Excretion of transmissible spongiform encephalopathy infectivity in urine. *Emerg Infect Dis* 14:1406-1412.

Gregori L, Gray BN, Rose E, Spinner DS, Kascsak RJ, Rohwer RG. (2008) A sensitive and quantitative assay for normal PrP in plasma. *J Virol Meth* 149:251-259.

Hamir AN, Kunkle RA, Bulgin M, Rohwer RG, Gregori L, Richt JA. (2008) Experimental transmission of scrapie agent to susceptible sheep using intralingual and intracerebral routes of inoculation. *Can J Vet Res*. 72:63-7.

Gregori, L., Rohwer, R.G., 2007. Characterization of scrapie-infected and normal hamster blood as an experimental model for TSE-infected human blood. Dev Biol (Basel) 127, 123-33.

Conclusions

The research program supported by this grant has been very successful. As a consequence, a number of publications and presentations at international meetings have resulted from this work.

Most of the specific aims have been addressed and for the most part the project was on schedule with the exception of a delay in the inoculation of the PK-treated infected plasma due to technical problems. Those problems have been addressed and solved. We did not encounter other problems that required revision of the proposed studies. Because of this delay, the titrations are still on going and we anticipate the completion of the plasma PK study by the end of the year (including the western blot of the brain of 600 hamsters now still alive).

The results of the urine study have been very interesting and had stimulated a discussion among the regulators on whether, based on these data, a new risk assessment for the human urine-derived medicinal product should be conducted. We anticipate that the topic

of infectivity in urine will be a central regulatory issue in the future with more investigations being conducted (we already have seen a flourish of publications on this topic in the past a few months). We did not detect infectivity in urine of pre-clinical animals. This result should be analyzed further because technical issues with urine concentration may have been the cause.

We also anticipate that the PK study will be of equally strong interest in the community as we made the first clear demonstration that PK could be used in the TSE diagnostic assay to remove discriminate between PrPc and PrPsc. We also showed that with excess PK it is possible to destroy infectivity and therefore PrPsc. Those conditions should be avoided and we have indicated those conditions that are appropriate for a TSE assay to detect PrP^{res}.

References

1. Hewitt PE, Llewelyn CA, Mackenzie J, Will RG. Creutzfeldt-Jakob disease and blood transfusion: results of the UK Transfusion Medicine Epidemiological Review study. Vox Sang. 2006 91:221-30.

2. http://www.hpa.org.uk/hpa/news/articles/press_releases/2006/060209_cjd.htm

3. Brown P, Rohwer RG, Dunstan BC, MacAuley C, Gajdusek DC, Drohan WN. (1998). The distribution of infectivity in blood components and plasma derivatives in experimental models of transmissible spongiform encephalopathy. *Transfusion* 38:810-6.

4. Gregori L, McCombie N, Palmer D, Birch P, Sowemimo-Coker SO, Giulivi A, Rohwer RG. (2004) Effectiveness of leucoreduction for removal of infectivity of transmissible spongiform encephalopathies from blood. *Lancet* 364:529-31.

5. Cervenakova L, Yakovleva O, McKenzie C, Kolchinsky S, McShane L, Drohan WN, Brown P. (2003) Similar levels of infectivity in the blood of mice infected with human-derived vCJD and GSS strains of transmissible spongiform encephalopathy. *Transfusion* 43:1687-94.

6. Houston F, Foster JD, Chong A, Hunter N, Bostock CJ. Transmission of BSE by blood transfusion in sheep. (2000) Lancet 356:999-1000.

7. Kariv-Inbal Z, Ben-Hur T, Grigoriadis NC, Engelstein R, Gabizon R. Urine from scrapie-infected hamsters comprises low levels of prion infectivity. Neurodegener Dis. 2006 3:123-8.

8. Seeger H, Heikenwalder M, Zeller N, Kranich J, Schwarz P, Gaspert A, Seifert B, Miele G, Aguzzi A. Coincident scrapie infection and nephritis lead to urinary prion excretion. Science. 2005 310:324-6.

9. Mathiason CK, Powers JG, Dahmes SJ, Osborn DA, Miller KV, Warren RJ, Mason GL, Hays SA, Hayes-Klug J, Seelig DM, Wild MA, Wolfe LL, Spraker TR, Miller MW, Sigurdson CJ, Telling GC, Hoover EA. Infectious prions in the saliva and blood of deer with chronic wasting disease. Science. 2006 314:133-6.

10. Haley N, Hoover E, Telling GC. Detection of CWD prions in urine and saliva of deer by transgenic mouse bioassay. Presentation at the Prion2008 Meeting.

11. Gonzalez-Romero D, Barria MA, Leon P, Morales R, Soto C Detection of infectious prions in urine. FEBS Lett. 2008 582:3161-6.

12. Gregori L, Kovacs GG, Alexeeva I, Budka H, Rohwer RG. (2008) Excretion of transmissible spongiform encephalopathy infectivity in urine. *Emerg Infect Dis* 14:1406-1412.

13. Sisó S, Jeffrey M, Steele P, McGovern G, Martin S, Finlayson J, Chianini F, González L. Occurrence and cellular localization of PrPd in kidneys of scrapie-affected sheep in the absence of inflammation. J Pathol. 2008 215:126-34.

14. Ligios C, Cancedda GM, Margalith I, Santucciu C, Madau L, Maestrale C, Basagni M, Saba M, Heikenwalder M. Intraepithelial and interstitial deposition of pathological prion protein in kidneys of scrapie-affected sheep. PLoS ONE. 2007 2:e859.

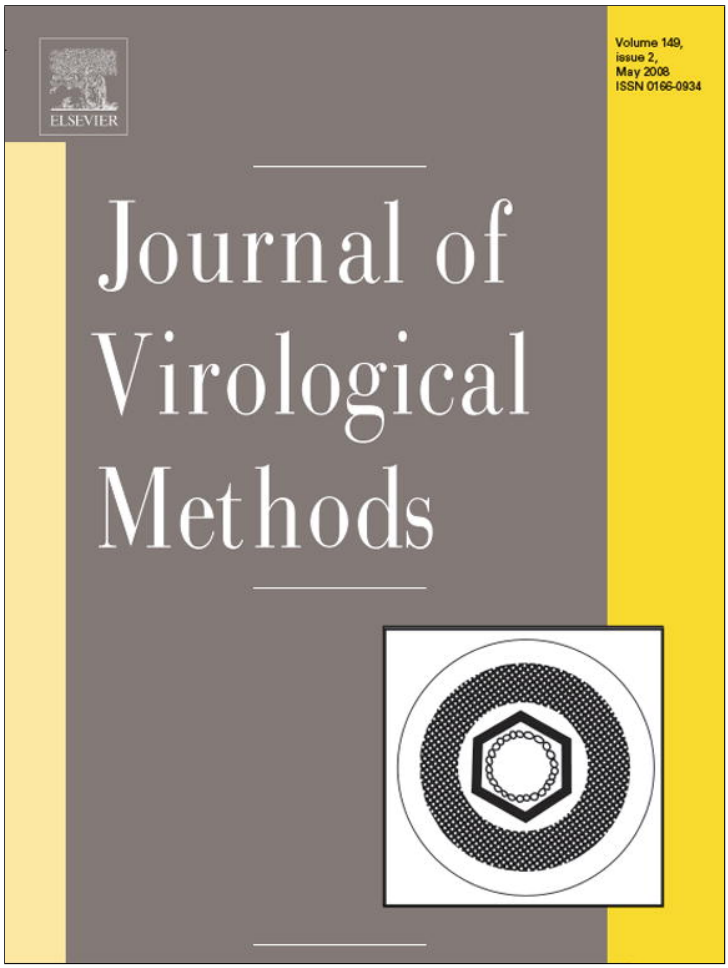
15. Gregori L, Gray BN, Rose E, Spinner DS, Kascsak RJ, Rohwer RG. (2008) A sensitive and quantitative assay for normal PrP in plasma. *J Virol Meth* 149:251-259.

Appendices

Gregori L, Kovacs GG, Alexeeva I, Budka H, Rohwer RG. (2008) Excretion of transmissible spongiform encephalopathy infectivity in urine. *Emerg Infect Dis* 14:1406-1412.

Gregori L, Gray BN, Rose E, Spinner DS, Kascsak RJ, Rohwer RG. (2008) A sensitive and quantitative assay for normal PrP in plasma. *J Virol Meth* 149:251-259.

Hamir AN, Kunkle RA, Bulgin M, Rohwer RG, Gregori L, Richt JA. (2008) Experimental transmission of scrapie agent to susceptible sheep using intralingual and intracerebral routes of inoculation. *Can J Vet Res*. 72:63-7.



Available online at www.sciencedirect.com
ScienceDirect
Journal of Virological Methods 149 (2008) 251–259
Journal of Virological Methods
www.elsevier.com/locate/jviromet

A sensitive and quantitative assay for normal PrP in plasma

Luisa Gregori [a,*], Benjamin N. Gray [a,1], Elaine Rose [a], Daryl S. Spinner [b], Richard J. Kascsak [b], Robert G. Rohwer [a]

[a] *Veterans Affairs Medical Center and University of Maryland, Baltimore, MD 21201, United States*
[b] *New York State Institute for Basic Research in Developmental Disabilities, Staten Island, NY 10314, United States*

Received 10 November 2007; received in revised form 21 January 2008; accepted 28 January 2008
Available online 12 March 2008

Abstract

Transmissible spongiform encephalopathies can be transmitted by blood transfusion. The risk of spreading the disease among the human population could be mitigated with the implementation of a blood screening assay. We developed a two-antibody assay for PrP detection in plasma using the ORIGEN technology with a protocol modification to improve the limit of detection and to increase the sample volume assayed. In the standard 200 μL format, the assay had a detection limit of 7–10 pg of recombinant PrP and 3 pg in 1 mL final volume implementation. PrP concentration measured in normal and scrapie-infected hamster brains was 7.5 ± 0.9 and 57.3 ± 9.6 μg/g, respectively. After a concentration step with an immuno-affinity resin, plasma PrP^c was detected by Western blot and its concentration was measured at 3.5 ± 0.8 ng/mL. From these data and assuming that blood has the same specific infectivity as brain, we estimated the concentration of abnormal PrP in hamster-infected plasma to be 32 fg/mL. The assay also detected abnormal brain PrP spiked into plasma although the limit of detection was affected. This is a novel and sensitive assay for the detection of PrP in plasma that could be developed into a platform for a plasma-based TSE test.

Keywords: TSE; Prion; Brain; Plasma; Immunoassay; Diagnostic

1. Introduction

The successful management of transmissible spongiform encephalopathy (TSE) diseases in humans and animals requires an assay capable of identifying incubating TSE cases during the lengthy preclinical or asymptomatic stages when the animals and humans appear healthy but are incubating infectivity. Diagnoses of TSE diseases are generally conducted post-mortem on tissue from the central nervous system. Almost all TSE diagnostic assays are based on the detection of the abnormal, protease-resistant form of the prion protein PrP generated during the disease from the normal cellular form, PrP^c, by a still unknown mechanism. The abnormal form of PrP is closely associated with TSE infectivity and thus, it is the biochemical marker of TSE infection.

The majority of post-mortem tests rely for specificity on the differential susceptibility of normal and abnormal PrP to proteolytic digestion (Oesch et al., 1985). Most of the pre-mortem blood-based diagnostic assays currently under development do not use proteinase K to distinguish between the two forms of PrP (Lau et al., 2007; Moussa et al., 2006; Pan et al., 2007; Raeber, 2006; Wilson, 2006). Four antibodies with unique specificity to the abnormal form of the protein have been reported (Korth et al., 1997; Moroncini et al., 2004; Paramithiotis et al., 2003; Curin Šerbec et al., 2004). To date, only one of these antibodies has been incorporated into a blood-based diagnostic assay (Raeber, 2006).

Abnormal PrP is present together with and must be detected above a large excess of endogenous blood PrP^c. This requires exquisite assay specificity to ensure genuine abnormal protein signal detection. The sensitivity and limit of detection (LOD) of a TSE PrP-based assay are strongly affected by the high background from the plasma matrix (Safar et al., 1998; Völkel et al., 2001). The LOD of an assay for TSE-infected samples is often reported in terms of infectious doses detected which depends on the starting titer of the brain homogenate used to characterize the assay.

* Corresponding author at: Baltimore Research and Education Foundation, Inc., VA Medical Center, Research Services 151, 10 N. Greene Street, Baltimore, MD 21201, United States. Tel.: +1 410 605 7000x6465; fax: +1 410 605 7959.
E-mail address: lgreg002@umaryland.edu (L. Gregori).
[1] Present address: Cornell University, Department of Biological and Environmental Engineering, Ithaca, NY 14853, United States.

0166-0934/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jviromet.2008.01.028

One means of increasing the assay sensitivity and reducing the background is through enrichment and concentration of the target protein. Approaches that have been described for concentration of abnormal PrP from brain spiked into blood products include selective capture or precipitation mediated by RNA molecules (Zeiler et al., 2003), polymeric ligands (Wilson, 2006), sodium phosphotungstate (Bellon et al., 2003), peptides (Franscini et al., 2006; Lau et al., 2007; Pan et al., 2007). Two affinity ligands have been reported to capture and thereby concentrate brain-derived abnormal PrP spiked in human red blood cell concentrates (Sowemimo-Coker et al., 2005; Gregori et al., 2006a). More recently, one of these ligands was also shown to adsorb endogenous hamster blood scrapie infectivity and presumably abnormal PrP (Gregori et al., 2006b). It has also been reported that brain abnormal PrP may be precipitated by antibodies specific to this form of the protein (Korth et al., 1997; Paramithiotis et al., 2003; Curin Šerbec et al., 2004) and through non-specific interactions with monoclonal antibodies (Morel et al., 2004) or with antibodies to DNA (Zou et al., 2004). A novel method of abnormal PrP amplification known as protein misfolding cyclic amplification (PMCA) was recently reported to detect the amyloid protein in blood (Castilla et al., 2005). Any assay strategy applied to the detection of endogenous plasma abnormal PrP will have to be rigorously validated with blind panels of negative and positive samples from well characterized animal models and with human samples as the ultimate test.

PrP^c in plasma has not been characterized biochemically in terms of glycosylation, solubility, mass and other parameters mostly because the relatively low abundance of PrP^c and the complexity of the plasma matrix have made the isolation of this protein very challenging. We describe here a novel assay for partial purification, detection and quantitation of normal PrP in hamster plasma. We also investigated the assay as a potential platform for a TSE diagnostic test. This investigation highlighted some of the challenges facing a plasma-based TSE assay.

2. Material and methods

2.1. The PrP detection system

PrP was captured with a two-antibody sandwich system and detected with the ORIGEN Analyzer (BioVeris, Gaithersburg, MD). The capture antibody was biotinylated 3F4 (Covance, Berkeley, CA) and the detection antibody was 6D11 labeled with BV-TAG [Ruthenium (II) tris-bipyridine, *N*-hydroxysuccinimide] (BioVeris, Gaithersburg, MD). The 3F4 epitope encompasses residues 109–112 and the 6D11 epitope covers residues 93–109 of the hamster sequence. The immune-complex 3F4:PrP:6D11 was captured by the M-280 streptavidin-coated magnetic beads (Dynal Biotech, Brown Deer, WI) through their interaction with the biotinylated antibody. All reagents were mixed together in solution in a single step. A fixed 200 μL volume of the reaction mixture was drawn into the ORIGEN Analyzer instrument for detection of the electrochemiluminescent (ECL) signal emitted by the tagged 6D11-BV-TAG antibody. Optimization of the reagent concentrations showed that 50 ng 3F4-biotin, 25 ng 6D11-BV-TAG, and 10 μg streptavidin-coated magnetic beads in a 200 μL reaction volume maximized the signal-to-background ratio and these concentrations were used with all experiments. The background value was defined as the ECL signal for a sample containing no recHaPrP. All samples were tested on the ORIGEN Analyzer in duplicate with good reproducibility (%CV $\leq$ 15).

2.2. Preparation of 6D11-BV-TAG

6D11 monoclonal antibodies were grown in the Integra Culture System (Integra Biosciences, AG, Chur, Switzerland) and purified from the culture supernatant using Protein A/G sepharose (Pierce Biotechnology Rockford, IL) according to the manufacturer's instructions. Five-hundred micrograms of purified 6D11 were labeled with BV-TAG according to manufacturer's instructions. A 12:1 molar ratio of BV-TAG:6D11 was used for the labeling procedure, resulting in a final BV-TAG:6D11 molar ratio of 7.15.

2.3. Effect of magnetic wash step on assay sensitivity

Two half-log dilution series of full-length (23–231) recombinant hamster PrP (recHaPrP) were prepared in dilution buffer [PBS with 0.5% (v/v) Tween-20 and 1% (w/v) bovine serum albumin] in duplicate. Purified recHaPrP was provided by Ilia Baskakov (University of Maryland, Baltimore, MD) and was prepared in native conformation according to protocols previously described (Baskakov, 2004) with minor modifications. Two-hundred microliter samples of the dilution series were incubated overnight with 3F4-biotin, 6D11-BV-TAG, and streptavidin-coated magnetic beads. A magnetic rack (Immunometrics Ltd., London, UK) was used to capture the magnetic beads which carried the immune-complex from one series of diluted recHaPrP in duplicate. The second dilution series was not subjected to the magnetic capture step. The magnetic beads were allowed to form a pellet (10 min) while on the magnetic rack and the supernatant was removed along with any unreacted antibodies. Two-hundred microliters of dilution buffer were added and the bead pellet was resuspended to homogeneity. This procedure is referred to as the magnetic wash step. recHaPrP captured on the beads from both dilution series, with and without the magnetic wash step, was detected with the ORIGEN Analyzer. All other experiments in this report were conducted using the magnetic wash step unless otherwise noted.

2.4. Effect of dilution on signal recovery

Samples containing 0, 0.1, 0.03, 0.01, 0.003 ng of recHaPrP were mixed in 200 μL, 500 μL, and 1 mL of dilution buffer. This test was conducted in duplicate. These dilution series were incubated with 3F4-biotin, 6D11-BV-TAG, and streptavidin-coated magnetic beads under constant mixing. The total mass of each reagent was the same at each volume, only the concentration varied. The magnetic beads were subjected to the magnetic wash step (90 min) followed by suspension of the beads in 200 μL of dilution buffer for all samples and processed as described above for ORIGEN Analyzer detection of PrP.

2.5. Detection of PrP from hamster brain homogenates

Ten percent (w/v) brain homogenates were prepared from age-matched normal and scrapie-infected (263K strain) hamsters in PBS (pH 7.2) with extensive sonication. The sonication was conducted on ice with a probe immersed in the sample with four cycles of 30 s on/15 s off for a total of 2 min sonication. The homogenates were visually inspected to confirm complete homogenization of the tissue. Both normal and scrapie brain homogenates represented pools of approximately 100 animals each. The infected animals had been terminated when they showed typical clinical signs of the disease but were still capable of rearing and feeding. The titer of the infected brain pool was 2.3×10^{10} infectious doses$_{50}$ (ID_{50}) per gram of brain as determined by the endpoint dilution titration method (data not shown) (Reed and Muench, 1938).

Brain homogenates were heated at 100 °C in a water bath for 10 min in the presence of 2% SDS, resulting in a final 9% (w/v) brain homogenate. No proteinase K digestion was conducted and the term abnormal PrP is used to indicate the disease-specific form of PrP. The 9% brain homogenates were serially diluted in dilution buffer in half-log steps from 1:10^2 to 1:10^6 in duplicate series. Two-hundred microliters of each brain homogenate dilution were assayed. In parallel, a recHaPrP calibration curve was prepared. All samples were incubated by mixing with 3F4-biotin, 6D11-BV-TAG and streptavidin-coated magnetic beads. This incubation was followed by the standard magnetic wash step and the pellets were treated as indicated above for PrP detection by the ORIGEN Analyzer.

Western blotting of the diluted brain homogenates was used to verify the ECL signals obtained with the ORIGEN Analyzer. To one aliquot (10 μL) of each serially diluted sample was added 5 μL of 3× SDS-PAGE sample buffer to final concentrations of 0.03% dithiothreitol, 2% glycerol, 0.0625 M Tris–HCl (pH 6.8) and 2% SDS. Ten microliters of each sample were loaded on a pre-cast 15% Tris–HCl gel (Bio-Rad, Hercules, CA) and subjected to electrophoresis. The proteins on the gel were transferred to a PVDF membrane (Millipore, Billerica, MA). The membrane was incubated with 5% (w/v) non-fat dry milk (Bio-Rad, Hercules, CA) in Tris-buffered saline containing 0.5% Tween-20 (TBST) before incubation with 3F4 antibodies from ascites fluid diluted 1:10 000 (Kascsak et al., 1987). The membranes were washed in TBST followed by incubation with horseradish peroxidase-conjugated anti-mouse IgG antibody (KPL, Gaithersburg, MD) diluted 1:3000. The ECL + detection kit (Amersham, Piscataway, NJ) was used to visualize the PrP signal on film. Prestained protein markers (SeeBlue 2, Invitrogen, CA) were used to estimate the apparent molecular weight of the protein bands.

2.6. Detection of normal hamster plasma PrP

3F4 immuno-affinity resin was prepared according to the manufacturer's instructions using ImmunoPure® rProtein A IgG Plus Orientation kit (Pierce, Rockford, IL) and 3F4 antibody from ascites fluid. Typically, 0.5 mL of 3F4 ascites fluid containing approximately 5 mg/mL 3F4 antibody concentration was conjugated to 2 mL of resin. The conjugated 3F4 antibodies were cross-linked to protein A with disuccinimidyl suberate (Pierce, Rockford, IL). Extensive washes of the affinity resin were conducted before and after cross-linking to remove uncoupled and uncross-linked antibodies. The 3F4 affinity resin was stored at 4 °C in PBS with 0.02% sodium azide after preparation.

The 3F4 affinity resin was used to capture PrP from a 4 mL aliquot of normal hamster plasma from a pool of 220 mL of citrated blood collected from 55 animals. Plasma was prepared by centrifugation of freshly collected blood at 3300 rpm for 5 min (GS-6KR centrifuge, Beckman Coulter, Fullerton, CA) at ambient temperature. Complete® proteinase inhibitor cocktail (Roche, Indianapolis, IN) and 12 μL of the 3F4 affinity resin were added to 4 mL normal hamster plasma and mixed continuously overnight at ambient temperature. The resin was pelleted by a 30 s centrifugation at 16 100 × g, the pellet was washed three times with 1 mL detergent buffer (0.05 M Tris–HCl, 0.15 M NaCl, 1% Triton X-100, 0.5% sodium deoxycholate) and stored at 4 °C until use. This capture procedure was repeated two more times, each time using the plasma supernatant from the previous incubation with the addition of fresh 3F4 affinity resin. After the third capture, the supernatant was stored at −80 °C until use. We will refer to this plasma supernatant preparation as the PrP-depleted plasma. As a negative control, 12 μL of 3F4 affinity resin was incubated overnight in 1 mL dilution buffer containing proteinase inhibitor cocktail.

PrP was eluted from each of the three 3F4 resin samples by heating the mixture at 100 °C in a water bath for 5 min in 30 μL of SDS-PAGE sample buffer containing final 2% SDS. The samples were briefly centrifuged to pellet the resin and 5 μL in duplicate of the supernatant were diluted 100-fold in dilution buffer and assayed on the ORIGEN Analyzer. This procedure resulted in a final concentration of 0.02% SDS which was established to be compatible with the ORIGEN Analyzer detection system. These samples and a recHaPrP calibration series in duplicate prepared in dilution buffer also containing 0.02% SDS were incubated with mixing overnight with 3F4-biotin, 6D11-BV-TAG and streptavidin-coated magnetic beads. All samples were subjected to the magnetic wash step followed by detection of PrP with the ORIGEN Analyzer. PrP protein concentration was calculated using a standard recHaPrP calibration curve ($r^2 > 0.99$) and using the values from the linear range for both homogenates.

Ten-microliter aliquots of each eluate from the 3F4 affinity resin were also assayed by Western blot along with 10 μL of 0.5% normal hamster brain homogenate included as a standard.

2.7. Capture and detection of denatured brain-derived abnormal PrP spiked into PrP-depleted plasma

Ten percent (w/v) scrapie-infected hamster brain homogenate was denatured by heating the sample in a water bath at 100 °C for 10 min in the presence of 2% SDS to expose the 3F4 antibody epitope. There was no prior digestion by proteinase K. The denatured scrapie-infected brain homogenate was serially diluted in half-log increments in duplicate and each dilution was mixed with 1 mL of the PrP-depleted hamster plasma prepared as described above. A parallel dilution series was also

prepared and mixed with 1 mL of dilution buffer. The final spiked PrP brain masses were: 2, 0.7, 0.2, 0.07 and 0.02 ng based on 57.3 μg/g total PrP concentration (see Section 3). All samples were incubated overnight with 12 μL of the 3F4 affinity resin and continuous mixing. The PrP captured by the 3F4 affinity resin was eluted with 30 μL SDS-PAGE sample buffer; the resin samples were briefly centrifuged and 10 μL in duplicate of each supernatant were diluted 50-fold in dilution buffer. This procedure resulted in 0.04% SDS final concentration which was found to be the highest SDS concentration still allowing the formation of the biotinylated 3F4–PrP complex but was not optimal for 6D11-BV-TAG interaction with PrP. Therefore, we modified the order of addition of the reagents. First, the samples were incubated overnight with 3F4-biotin and streptavidin-coated magnetic beads in parallel with a recHaPrP calibration series in 1 mL dilution buffer with 0.04% SDS as the standard. The beads were pelleted with the magnetic wash step; the supernatant was removed and replaced with 200 μL dilution buffer containing 25 ng of 6D11-BV-TAG. The reaction was mixed for an additional three hours. The unbound 6D11-BV-TAG antibody was removed with a second magnetic wash step and PrP was detected with ORIGEN Analyzer.

3. Results

3.1. Limit of detection of the assay

The limit of detection of the assay was improved by exploiting the key feature of the ORIGEN Analyzer technology to form the 3F4:PrP:6D11 immune-complex in solution. A magnetic wash step conducted on the bench using a magnetic rack was incorporated into the experimental protocol as a method to remove unbound 6D11-BV-TAG that contributed to the background signal. Two recombinant hamster PrP (recHaPrP) dilution series were compared: one incorporating the magnetic wash step and one identical series without this step. Fig. 1 shows the log versus log plot of the signal-to-background ratios and the mass of recHaPrP tested. An average 1.7-fold S/B improvement was observed at all recHaPrP loadings tested as a result of background reduction with the magnetic wash step. The horizontal line on Fig. 1 indicates the ratio S/B = 2.0 which was used as the assay limit of detection. The arrows in the figure indicate that the magnetic wash step reduced the LOD from 0.011 ± 0.001 ng recHaPrP (without magnetic wash) to 0.007 ± 0.001 ng recHaPrP (with magnetic wash).

Table 1
Effect of sample dilution on recHaPrP signal recovery

Volume	recHaPrP (ng)	recHaPrP (ng/mL)	Background (ECL counts)
200 μL	0.006	0.031	2387.0 ± 112
500 μL	0.005	0.009	1486.5 ± 99
1 mL	0.003	0.003	1076.5 ± 58

Nanograms and concentration of recHaPrP detected at the limit of detection and background signals for PrP dilution series in various reaction volumes. Detection limits were interpolated from S/B = 2.0 values observed in half-log dilution series. Background signals were obtained from samples containing only dilution buffer with no recHaPrP.

We explored the added magnetic wash step as a method for concentration of PrP from diluted solutions. The question addressed was whether the same recHaPrP mass detection limit was maintained with the assay performed in 200 μL, 500 μL and 1 mL of dilution buffer. The data in Table 1 and Fig. 2 are representative of three independent repeats of this experiment. Fig. 2 shows the log versus log plot of the signal-to-background ratios of these dilution series plotted against the mass of recHaPrP tested. The arrows point to the mass limit of detection for the three volumes tested. Table 1 shows the background value for each volume tested as well as the corresponding recHaPrP masses at the detection limit (S/B = 2.0) also indicated by the arrows in Fig. 2. The limit of detection was achieved with 0.003 ng PrP in 1 mL (3 pg/mL or 0.13 pM), 0.005 ng PrP in 0.5 mL (9 pg/mL) and 0.006 ng in 200 μL volume (31 pg/mL).

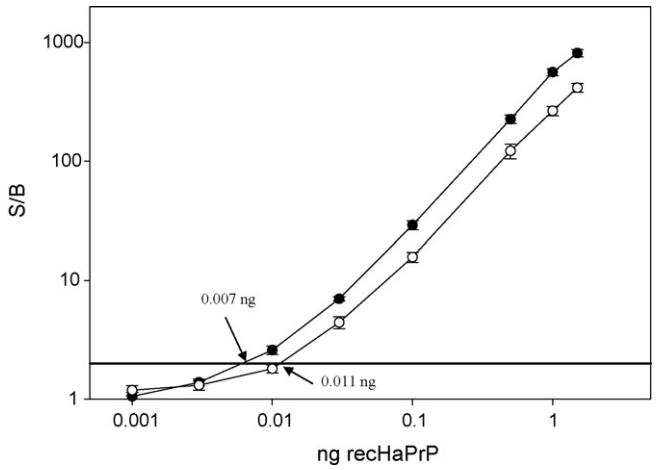


Fig. 1. The effect of the magnetic wash step on ORIGEN Analyzer sensitivity. Half-log dilution series of recHaPrP, from 1.5 to 0.001 ng, with (filled circles) or without (open circles) a magnetic wash step. The line across the graph represents S/B = 2.0 ratio. The arrows point to the mass limit of detection at twice background.

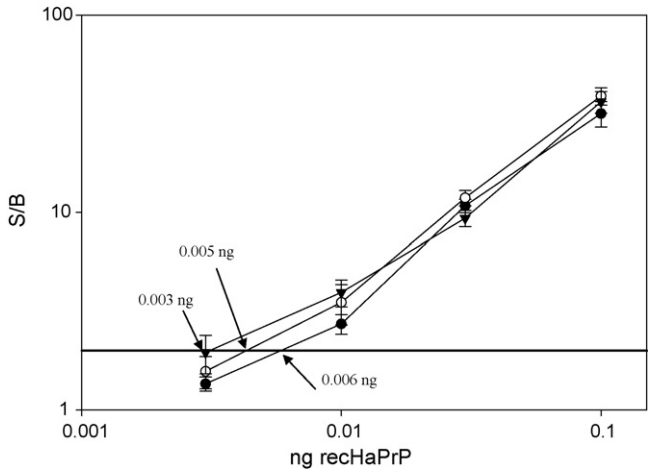


Fig. 2. The effect of dilution on signal-to-background ratio and signal recovery. Half-log dilution series of recHaPrP, from 0.1 to 0.003 ng, diluted in 200 μL (filled circles), 500 μL (open circles), and 1 mL (filled triangles) dilution buffer. The line across the graph represents S/B = 2.0 ratio. The arrows point to the mass limit of detection at twice background.

The assay sensitivity improved in parallel with the increase of the assay volume and this effect was due to the concomitant decrease of the background signals (Table 1).

3.2. Quantitation of normal and abnormal PrP from hamster brains

The assay was used to detect and to quantify PrP from brain homogenate pools of normal and scrapie-infected hamsters after denaturation (see Section 2). PrP denaturation was necessary to solubilize both PrP isoforms and to expose the 3F4 epitope on the abnormal PrP molecule. Fig. 3A shows the dilution curves reported as S/B ratios versus the dilution factor relative to 9% (w/v) brain homogenate. PrP concentrations in normal and scrapie-infected hamster brain pools were calculated to be 7.5 ± 0.9 and 57.3 ± 9.6 μg/g, respectively. Similar results were obtained with three independent replicates of the same pools. These pools were prepared from approximately one hundred brains each. Two-hundred microliter samples of a 10^4 dilution of 9% normal brain homogenate and of a 10^5 dilution of 9% scrapie-infected brain homogenate gave ECL signals at the detection limit at twice background (Fig. 3A). Based on the calculated PrP concentration in brain, these dilutions correspond to 0.014 ± 0.002 ng (0.07 ng/mL) PrP from normal brain and 0.010 ± 0.002 ng (0.05 ng/mL) PrP from scrapie-infected brain equivalent to 2 nL of 9% infected brain. The titer of the scrapie brain homogenate pool was 2.3×10^{10} ID_{50}/g as measured with two independent endpoint titrations. From this titer, the LOD in terms of infectious doses was approximately 4000 ID_{50}.

PrP from serially diluted brain homogenates was also detected by Western blot. In this case, $10^{2.5}$ dilution of 9% normal brain homogenate and $10^{3.5}$ dilution of 9% scrapie-infected brain homogenate represented the limit of detection by Western blot analysis (Fig. 3B). Based on the calculated PrP concentration in normal and scrapie-infected hamster brains, these dilutions correspond to detection limits of 0.016 ng (1.6 ng/mL) and 0.012 ng (1.2 ng/mL) PrP, respectively. The LOD in mass is similar to that calculated for the ORIGEN assay (Fig. 3A) and the increased sensitivity of the ORIGEN assay derived from the ability of this format to accommodate larger sample volumes compared to the Western blot.

3.3. Detection of PrP from normal hamster plasma

The goal of this study was to detect PrP from plasma. However, direct detection from undiluted plasma was unsuccessful due to high protein content in the matrix. To address this issue a PrP concentration step was developed with the aim of reducing the plasma matrix effect while concentrating the target protein. A 3F4 immuno-affinity resin was used to capture endogenous PrP from 4 mL of undiluted normal hamster plasma. Fig. 4A shows that 14.1 ± 2.2 ng of PrP (92% of the total PrP captured)

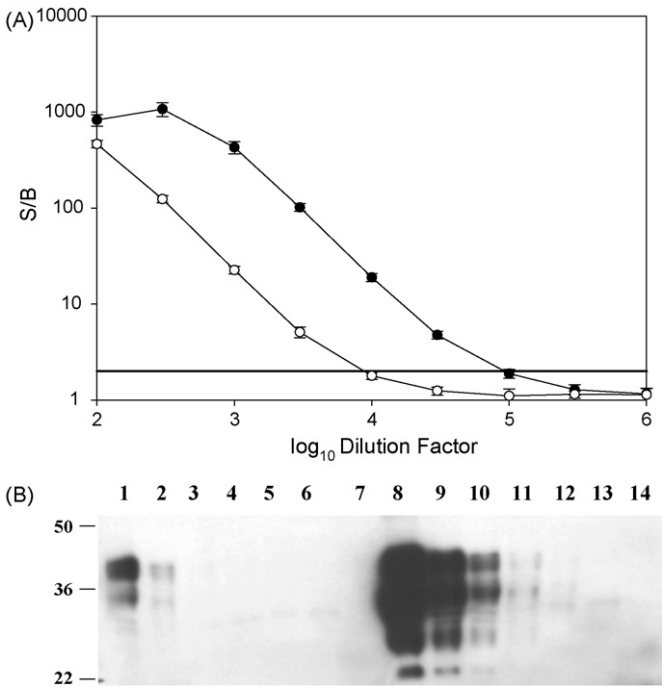


Fig. 3. Normal and scrapie-infected brain dilution series. (A) Half-log dilution series from 10^2 to 10^6 relative to 9% (w/v) normal (open circles) and scrapie-infected (filled circles) hamster brain homogenates detected by ORIGEN Analyzer. The line across the graph represents S/B = 2.0 ratio. (B) Same samples as in (A) detected by Western blot. Lanes 1–7 normal brain and 8–14 scrapie-infected brain. The numbers on the left represent the molecular weights in kDa of the prestained protein standards.

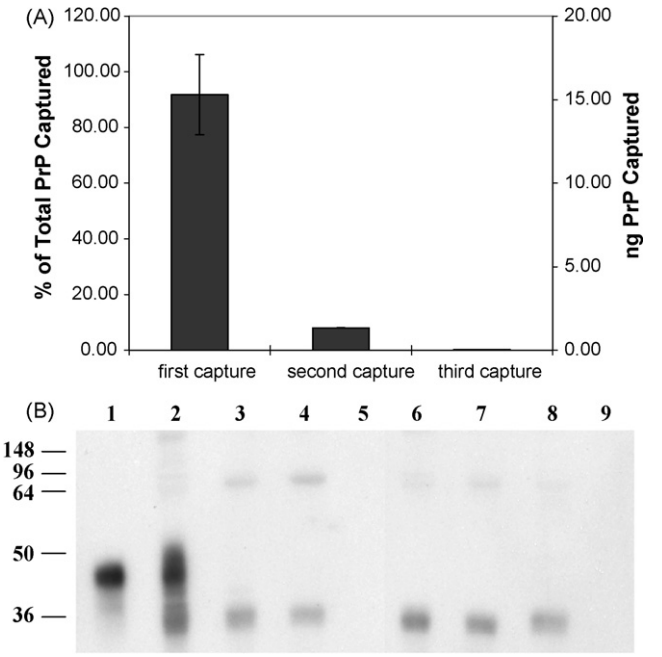


Fig. 4. Endogenous hamster plasma PrP eluted from the 3F4 affinity resin. (A) ORIGEN Analyzer results of plasma PrP signal eluted from each capture passage on 3F4 affinity resin. Quantitation was conducted using a recHaPrP standard curve. The axis on the left refers to the percentage of PrP with the total PrP being the sum of each capture step. (B) Western blot of the same samples tested in (A) with 3F4 primary antibodies (lanes 1–5) and without primary antibodies (lanes 6–9). Lane 1 represents normal brain homogenate. Lanes 2 and 6: first capture; lanes 3 and 7: second capture; lanes 4 and 8: third capture. Lanes 5 and 9 contained negative controls with the resin incubated in buffer without plasma. The numbers on the right represent the molecular weights in kDa of the prestained protein standards.

was bound in the first resin capture and 1.2 ± 0.01 ng of PrP (8% of the total PrP captured) was captured in the second step. The signal captured in the third step was not reduced with a fourth capture step (data not shown) and it was assigned to non-specific interaction between the 3F4 affinity resin and the plasma proteins. The endogenous plasma PrP^c concentration was calculated using a standard recHaPrP calibration curve ($r^2 > 0.99$). In this particular experiment, a total of 15.3 ± 0.3 ng PrP were captured and eluted from 4 mL normal hamster plasma, with a calculated hamster plasma PrP concentration of 3.8 ± 0.1 ng/mL. Similar PrP concentrations were obtained with two large pools of plasma and with a total of six independent experiments. The average of PrP concentration from all experiments was 3.5 ± 0.8 ng/mL. A resin capacity of 1.2 ± 0.3 ng PrP/μL resin was calculated assuming that the resin was saturated in the first PrP capture step.

The ORIGEN Analyzer results were confirmed by Western blot (Fig. 4B). PrP signal was detected in the first capture (lane 2) and it was below the limit of detection of the Western blot in the second capture (lane 3). Western blot of the proteins captured by the 3F4 affinity resin showed a protein band of approximately 40 kDa which was not detected when the primary antibody was omitted (lane 6). We concluded that this band corresponds to endogenous plasma PrP^c. Lane 1 shows normal PrP from hamster brain as the marker. Negative controls containing 3F4 affinity resin incubated in buffer without plasma (lane 5) showed no signal. Protein bands with the apparent molecular weights of approximately 36 and 64 kDa were observed without primary antibody staining (lanes 6–8). These bands were present only in samples that had been exposed to plasma and were tentatively assigned to heavy-chain and light-chain IgG subunits (Hartwell et al., 2005).

3.4. Quantitation and recovery of abnormal brain-derived PrP spiked in PrP-depleted hamster plasma

We evaluated the performance of the 3F4 affinity resin to capture denatured PrP from scrapie-infected brain homogenate spiked into PrP-depleted plasma. For comparison and to assess the effect of plasma proteins on the PrP capture, the test was also conducted in parallel with the same brain homogenate dilution series in dilution buffer.

Fig. 5A compares the results for samples diluted in buffer and in PrP-depleted plasma as log versus log plot of S/B ratios and PrP mass tested (from 2 to 0.02 ng). The signals from the test conducted without brain spikes in buffer and in PrP-depleted plasma were used as the background values for the respective curves. By interpolation to S/B = 2.0 and using a PrP concentration of 57.3 μg/g in scrapie-infected hamster brain, the LOD was 0.04 ng for the sample diluted in buffer and 0.10 ng for the sample diluted in PrP-depleted plasma. The assay was capable of detecting 2.8 pM of abnormal PrP or 20 nL of 9% scrapie brain homogenate spiked in undiluted plasma (abnormal PrP average molecular weight of 35 kDa). The 2.5-fold difference in LOD in Fig. 5A between buffer and PrP-depleted plasma matched the difference in background values for the two conditions. Background–subtracted ECL signals (S–B) were plotted in a log versus log graph against PrP mass for the dilution buffer and PrP-depleted hamster plasma samples (Fig. 5B). The graph shows two overlapping curves indicating that the PrP signals recovered on the resin incubated with buffer and with PrP-depleted plasma were the same. This result suggests that the 3F4 affinity resin captured the same PrP mass under both experimental conditions and that the interaction of PrP with the 3F4 antibody was highly specific and largely unaffected by the presence of plasma proteins. However, plasma increased the background and as a consequence the LOD calculated at twice background increased.

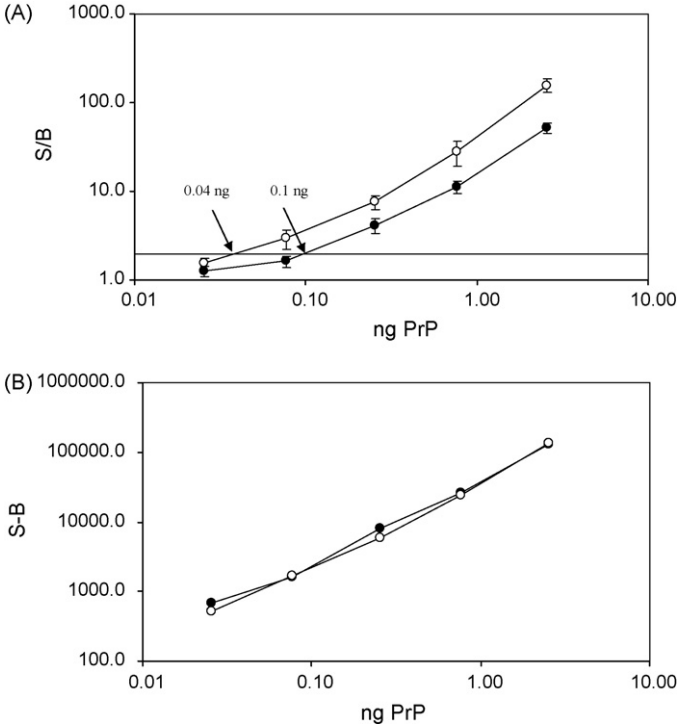


Fig. 5. Detection of denatured PrP^{res} from scrapie-infected brain diluted in buffer and in PrP-depleted plasma. (A) log versus log plot of S/B ratios and amounts of scrapie-infected brain PrP in PrP-depleted plasma (filled circles) and in buffer (open circles) captured by 3F4 affinity resin. The line across the graph represents S/B = 2.0. (B) same samples as in A plotted with background–subtracted ECL signals (S–B) in PrP-depleted plasma and in buffer. The arrows point to the mass limit of detection for twice background.

4. Discussion

We have developed a multistep assay platform to capture, detect and quantitate PrP from hamster plasma using a 3F4 affinity resin. The results were confirmed by Western blot analysis and quantitation was conducted with a sensitive detection technology.

We selected the hamster model because quantitative measurements of infectivity titers in brain and in blood are available for this model and because previous studies have indicated the relevance of the hamster as an experimental animal system to study TSE infectivity in human blood (Gregori and Rohwer, 2007). Our current assay format could also be applicable to human

plasma as both 3F4 and 6D11 antibodies react with human PrP (Kascsak et al., 1987).

PrP concentration in infected and normal hamster brains measured with our assay was in agreement with those previously reported (Beekes et al., 1995; MacGregor and Drummond, 2001; Rubenstein et al., 1994). Assuming that PrP^c concentration in infected brain is the same as in normal brain, the concentration of abnormal PrP in scrapie brains was calculated to be approximately 50 μg/g tissue. Based on the titer of the infected brain homogenate used in these studies, the specific infectivity was 2.2 fg of abnormal PrP per ID_{50}. The measured titer of infected hamster blood is on average 10 ID/mL (Gregori et al., 2006b, 2004). If we use the same specific infectivity calculated for brain and the conversion 1 $ID_{50} = 0.693$ ID (Gregori et al., 2006a), the concentration of abnormal PrP in hamster-infected plasma is estimated to be 32 fg/mL.

The 3F4 affinity resin captured PrP^c from large volumes of undiluted plasma. This is an important feature of our test since in most diagnostic assays plasma needs to be diluted prior to use to reduce the assay background derived from the complex plasma matrix. In our assay, PrP^c was captured from 4 mL of plasma. This step not only concentrated PrP into a small volume of resin and thereby increased the assay sensitivity but it also eliminated most of the interfering plasma proteins and allowed PrP detection by Western blot. Furthermore, it was possible to detect PrP^c using the ORIGEN Analyzer assay which had not been able to detect PrP^c from undiluted plasma. These observations may explain why the highly sensitive DELFIA technology failed to detect PrP^c in hamster plasma (MacGregor and Drummond, 2001). For the detection of PrP^c adsorbed to the affinity resin we exploited the flexibility of the solution-based detection technology and the high affinity of the two monoclonal antibodies, 3F4 and 6D11 for PrP, $K_d^{3F4} = 7.9 \times 10^{-11}$ M (DSS and RJK, unpublished data) and $K_d^{6D11} = 4.0 \times 10^{-11}$ M (DSS and RJK, unpublished data). We reproducibly detected and measured the concentration of endogenous PrP^c from normal hamster plasma with an average value of 3.5 ng/mL. This concentration could be underestimated if not all endogenous PrP was captured by the 3F4 affinity resin or if not all captured PrP was eluted from the resin.

To our knowledge, there are no previous reports on the isolation and detection of PrP^c from hamster plasma and its detection by Western blot. The Western blot signal indicated a predominant single band with the same apparent molecular weight as the diglycosylated species of normal hamster brain PrP. Whether this co-electrophoretic mobility indicates that the two proteins have the same glycosylation patterns remains to be established and we are in the process of further characterizing endogenous plasma PrP^c. Other minor 3F4-specific bands in the same Western blot lane were also detected but they could not be clearly assigned as their signals overlapped with that of a contaminant band. Interestingly, all three typical PrP^c bands were observed in purified PrP^c from human platelets (Jones et al., 2005) suggesting that at least in human all three forms of PrP^c may be found in blood.

Throughout the assay development, a detection limit defined by S/B = 2.0 was adopted to provide a comfortable margin of safety particularly when the assay is applied to complex biological matrix such as plasma. The assay sensitivity was improved by applying a magnetic wash step to remove unreacted 6D11-BV-TAG antibody prior to signal detection thereby reducing the background values. This modification resulted in about 2-fold increase in sensitivity and more importantly, it allowed for the use of sample volumes larger (up to 1 mL) than the conventional 200 μL volume of the 96-well microtiter plate assays. Even under the standard conditions (200 μL assay volume), the detection limit for brain PrP with the ORIGEN Analyzer was 30-fold better than with the Western blot. The assay detection limit for recombinant PrP was more sensitive than that reported by other authors (Kim et al., 2005; Safar et al., 1998) and was at least as sensitive as that of other assays (Biffiger et al., 2002; Dabaghian et al., 2006; Safar et al., 2005; Völkel et al., 2001). Assay formats with extremely low theoretical detection limits have been reported (Barletta et al., 2005; Schmerr et al., 1999), but their applicability to biological samples has not yet been demonstrated. We observed 2-fold increase in LOD between recombinant PrP (0.007 ng) and brain PrP^c (0.014 ng) with the standard assay volume (200 μL). This difference could be due to subtle differences in the antibody interactions with recHaPrP and native PrP. Similar LOD differences were also reported by other authors (Safar et al., 1998; Völkel et al., 2001). Furthermore, direct detection of brain abnormal PrP diluted in buffer was 4-fold more sensitive than the same protein captured with 3F4 affinity resin (compare 0.010 ng from Fig. 3 and 0.04 ng from Fig. 5). It is likely that the different LOD derives from incomplete capture of the abnormal PrP by the 3F4 affinity resin.

The PrP-depleted plasma preparation was used in spiking experiments with brain-derived abnormal PrP to challenge the 3F4 affinity resin capture step in a background of endogenous plasma proteins. The aim of this experiment was to characterize and to test the assay format with known amounts of abnormal PrP and to determine the PrP signal recovery and the limit of detection. We adapted the assay conditions to our current assay format and used denatured abnormal PrP spiked into PrP-depleted plasma. In the presence of plasma, the 3F4 affinity resin successfully captured spiked brain abnormal PrP although the LOD was affected. Comparison of the 3F4 affinity resin capture of brain abnormal PrP diluted in buffer and in plasma showed that the matrix had no influence on the mass of abnormal PrP recovered but it affected the background values and as a consequence caused an increase in the LOD.

The estimated concentration of abnormal PrP in infected plasma was 32 fg/mL and the concentration of normal PrP in hamster plasma was 3.5 ng/mL. The ratio between the two proteins is $1{:}10^5$. Therefore, in a successful TSE diagnostic assay, endogenous PrP^c concentration must be reduced to lower than 32 fg/mL before the abnormal PrP signal can be reliably detected above the remaining PrP^c signal. This corresponds to the removal of more than 99.999% PrP^c molecules without loss of endogenous abnormal PrP. This delicate discrimination step is a necessary requirement for all assays even those that do not

use proteolytic digestion of PrP^c but rely on specific recognition of abnormal PrP.

For a diagnostic application of this assay, the 3F4 antibody in the affinity resin could be substituted with antibody specific to abnormal PrP or with non-conformational dependent antibodies which would remove the requirement for denaturation of the abnormal PrP for epitope recognition. Alternatively, the 3F4 affinity resin could be used to deplete plasma of normal PrP followed by detection of abnormal PrP. Affinity ligands specific for abnormal PrP could also effectively substitute for the immuno-affinity resin. One such a ligand may be already available (Gregori et al., 2006b). The PMCA reaction was shown to be capable of amplifying endogenous abnormal PrP from buffy coat to levels readily detectable by Western blot (Castilla et al., 2005). These results are encouraging although a recent report suggested that under certain conditions PMCA could also generate abnormal PrP from normal brain tissues (Deleault et al., 2007). Thus, the PMCA specificity must be verified, but it is possible that an optimized, more specific PMCA assay or a combination of this amplification technology and an improved capture resin incorporating abnormal PrP specific antibodies or affinity ligands will make possible the practical application of this protocol to a TSE plasma assay.

Acknowledgements

We thank Ilia Baskakov for providing recHaPrP, Regina Kascsak for assisting in the development of 3F4 and 6D11 monoclonal antibodies and the BSL 3 animal facility staff at the VA Medical Center in Baltimore for the excellent hamster care and for the preparation of hamster plasma and brain homogenates.

This work was supported by grants from the Department of Defense #DAMD17-03-1-0756 and the National Health Lung and Blood Institute #HL6390-02 and a contract from the National Institute of Health N01-NS-0-2327.

References

Barletta, J.M., Edelman, D.C., Highsmith, W.E., Constantine, N.T., 2005. Detection of ultra-low levels of pathologic prion protein in scrapie infected hamster brain homogenates using real-time immuno-PCR. J. Virol. Methods 127, 154–164.

Baskakov, I.V., 2004. Autocatalytic conversion of recombinant prion proteins displays a species barrier. J. Biol. Chem. 279, 7671–7677.

Beekes, M., Baldauf, E., Cassens, S., Diringer, H., Keyes, P., Scott, A.C., Wells, G.A., Brown, P., Gibbs Jr., C.J., Gajdusek, D.C., 1995. Western blot mapping of disease-specific amyloid in various animal species and humans with transmissible spongiform encephalopathies using a high-yield purification method. J. Gen. Virol. 76, 2567–2576.

Bellon, A., Seyfert-Brandt, W., Lang, W., Baron, H., Gröner, A., Vey, M., 2003. Improved conformation-dependent immunoassay: suitability for human prion detection with enhanced sensitivity. J. Gen. Virol. 84, 1921–1925.

Biffiger, K., Zwald, D., Kaufmann, L., Briner, A., Nayki, I., Pürro, M., Böttcher, S., Struckmeyer, T., Schaller, O., Meyer, R., Fatzer, R., Zurbriggen, A., Stack, M., Moser, M., Oesch, B., Kübler, E., 2002. Validation of a luminescence immunoassay for the detection of PrP(Sc) in brain homogenate. J. Virol. Methods 101, 79–84.

Castilla, J., Saá, P., Soto, C., 2005. Presymptomatic detection of prions in blood. Nat. Med. 11, 982–985.

Curin Šerbec, V., Bresjanac, M., Popović, M., Pretnar Hartman, K., Galvani, V., Rupreht, R., Černilec, M., Vranac, T., Hafner, I., Jerala, R., 2004. Monoclonal antibody against a peptide of human prion protein discriminates between Creutzfeldt-Jacob's disease-affected and normal brain tissue. J. Biol. Chem. 279, 3694–3698.

Dabaghian, R.H., Barnard, G., McConnell, I., Clewley, J.P., 2006. An immunoassay for the pathological form of the prion protein based on denaturation and time resolved fluorometry. J. Virol. Methods 132, 85–91.

Deleault, N.R., Harris, B.T., Rees, J.R., Supattapone, S., 2007. Formation of native prions from minimal components in vitro. Proc. Natl. Acad. Sci. U.S.A. 104, 9741–9746.

Franscini, N., El Gedaily, A., Matthey, U., Franitza, S., Sy, M.S., Burkle, A., Groschup, M., Braun, U., Zahn, R., 2006. Prion protein in milk. PLoS ONE 20 (1), e71.

Gregori, L., Lambert, B.C., Gurgel, P.V., Gheorghiu, L., Edwardson, P., Lathrop, J.T., MacAuley, C., Carbonell, R.G., Burton, S.J., Hammond, D., Rohwer, R.G., 2006a. Reduction of transmissible spongiform encephalopathy infectivity from human red blood cells with prion protein affinity ligands. Transfusion 46, 1152–1161.

Gregori, L., Gurgel, P.V., Lathrop, J.T., Edwardson, P., Lambert, B.C., Carbonell, R.G., Burton, S.J., Hammond, D.J., Rohwer, R.G., 2006b. Reduction in infectivity of endogenous transmissible spongiform encephalopathies present in blood by adsorption to selective affinity resins. Lancet 368, 2226–2230.

Gregori, L., McCombie, N., Palmer, D., Birch, P., Sowemimo-Coker, S.O., Giulivi, A., Rohwer, R.G., 2004. Effectiveness of leucoreduction for removal of infectivity of transmissible spongiform encephalopathies from blood. Lancet 364, 529–531.

Gregori, L., Rohwer, R.G., 2007. Characterization of scrapie-infected and normal hamster blood as an experimental model for TSE-infected human blood. Dev. Biol. (Basel) 127, 123–133.

Hartwell, R.C., Nelson, M.S., Kislan, M.M., Stenland, C.J., Miller, J.L.C., Pifat, D.Y., Petteway, S.R., Cai, K., 2005. An improved Western blot assay to assess the clearance of prion protein from plasma-derived therapeutic proteins. J. Virol. Methods 125, 187–193.

Jones, M., Head, M.W., Connolly, J.G., Farquhar, C.F., Hornsey, V.S., Pepper, D.S., MacGregor, I.R., 2005. Purification of normal cellular prion protein from human platelets and the formation of a high molecular weight prion protein complex following platelet activation. Biochem. Biophys. Res. Commun. 335, 48–56.

Kascsak, R.J., Rubenstein, R., Merz, P.A., Tonna-DeMasi, M., Fersko, R., Carp, R.I., Wisniewski, H.M., Diringer, H., 1987. Mouse polyclonal and monoclonal antibody to scrapie-associated fibril proteins. J. Virol. 61, 3688–3693.

Kim, J.I., Wang, C., Kuizon, S., Xu, J., Barengolts, D., Gray, P.C., Rubenstein, R., 2005. Simple and specific detection of abnormal prion protein by a magnetic bead-based immunoassay coupled with laser-induced fluorescence spectrofluorometry. J. Neuroimmunol. 158, 112–119.

Korth, C., Stierli, B., Streit, P., Moser, M., Schaller, O., Fischer, R., Schulz-Schaeffer, W., Kretzschmar, H., Raeber, A., Barun, U., Ehrensperger, F., Hornemann, S., Glockshuber, R., Riek, R., Billeter, M., Wüthrich, K., Oesch, B., 1997. Prion (PrPSc)-specific epitope defined by a monoclonal antibody. Nature 390, 74–77.

Lau, A.L., Yam, A.Y., Michelitsch, M.M., Wang, X., Gao, C., Goodson, R.J., Shimizu, R., Timoteo, G., Hall, J., Medina-Selby, A., Coit, D., McCoin, C., Phelps, B., Wu, P., Hu, C., Chien, D., Peretz, D., 2007. Characterization of prion protein (PrP)-derived peptides that discriminate full-length PrPSc from PrPC. Proc. Natl. Acad. Sci. U.S.A. 104, 11551–11556.

MacGregor, I.R., Drummond, O., 2001. Species differences in the blood content of the normal cellular isoform of prion protein, PrP^c, measured by time-resolved fluoroimmunoassay. Vox Sang. 81, 236–240.

Morel, N., Simon, S., Frobert, Y., Volland, H., Mourton-Gilles, C., Negro, A., Sorgato, M.C., Créminon, C., Grassi, J., 2004. Selective and efficient immunoprecipitation of the disease-associated form of the prion protein can be mediated by nonspecific interactions between monoclonal antibodies and scrapie-associated fibrils. J. Biol. Chem. 279, 30143–30149.

Moroncini, G., Kanu, N., Solforosi, L., Abalos, G., Telling, G.C., Head, M., Ironside, J., Brockes, J.P., Burton, D.R., Williamson, R.A., 2004. Motif-

grafted antibodies containing the replicative interface of cellular PrP are specific for PrPSc. Proc. Natl. Acad. Sci. U.S.A. 101, 10404–10409.

Moussa, A., Coleman, A.W., Bencsik, A., Leclere, E., Perret, F., Martin, A., Perron, H., 2006. Use of streptomycin for precipitation and detection of proteinase K resistant prion protein (PrPsc) in biological samples. Chem. Commun. (Camb.) 7, 973–975.

Oesch, B., Westaway, D., Wälchli, M., McKinley, M.P., Kent, S.B.H., Aebersold, R., Barry, R.A., Tempst, P., Teplow, D.B., Hood, L.E., Prusiner, S.B., Weissman, C., 1985. A cellular gene encodes scrapie PrP 27-30 protein. Cell 40, 735–746.

Pan, T., Sethi, J., Nelsen, C., Rudolph, A., Cervenakova, L., Brown, P., Orser, C.S., 2007. Detection of misfolded prion protein in blood with conformationally sensitive peptides. Transfusion 47, 1418–1425.

Paramithiotis, E., Pinard, M., Lawton, T., LaBoissiere, S., Leathers, V.L., Zou, W.Q., Estey, L.A., Lamontagne, J., Lehto, M.T., Kondejewski, L.H., Francoeur, G.P., Papadopoulos, M., Haghighat, A., Spatz, S.J., Head, M., Will, R., Ironside, J., O'Rourke, K., Tonelli, Q., Ledebur, H.C., Chakrabartty, A., Cashman, N.R., 2003. A prion protein epitope selective for the pathologically misfolded conformation. Nat. Med. 9, 893–899.

Raeber, A., 2006. Rapid blood screening test for CJD. Cambridge Healthtech Institute. A Forum on TSE Diagnostic Blood Tests. Baltimore, MD, 7–9 March 2006.

Reed, L.J., Muench, H.A., 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 27, 493–497.

Rubenstein, R., Carp, R.I., Ju, W., Scalici, C., Papini, M., Rubenstein, A., Kascsak, R., 1994. Concentration and distribution of infectivity and PrPSc following partial denaturation of a mouse-adapted and a hamster-adapted scrapie strain. Arch. Virol. 139, 301–311.

Safar, J., Wille, H., Itri, V., Groth, D., Serban, H., Torchia, M., Cohen, F.E., Prusiner, S.B., 1998. Eight prion strains have PrP(Sc) molecules with different conformations. Nat. Med. 4, 1157–1165.

Safar, J.G., Geschwind, M.D., Deering, C., Didorenko, S., Sattavat, M., Sanchez, H., Serban, A., Vey, M., Baron, H., Giles, K., Miller, B.L., DeArmond, S.J., Prusiner, S.B., 2005. Diagnosis of human prion disease. Proc. Natl. Acad. Sci. U.S.A. 102, 3501–3506.

Schmerr, M.J., Jenny, A.L., Bulgin, M.S., Miller, J.M., Hamir, A.N., Cutlip, R.C., Goodwin, K.R., 1999. Use of capillary electrophoresis and fluorescent labeled peptides to detect the abnormal prion protein in the blood of animals that are infected with a transmissible spongiform encephalopathy. J. Chromatogr. A 853, 207–214.

Sowemimo-Coker, S., Kascsak, R., Kim, A., Andrade, F., Pesci, S., Kascsak, R., Meeker, C., Carp, R., Brown, P., 2005. Pall leukotrap affinity prion-reduction filter removes exogenous infectious prions and endogenous infectivity from red cell concentrates. Transfusion 45, 1839–1844.

Völkel, D., Zimmermann, K., Zerr, I., Bodemer, M., Lindner, T., Turecek, P.L., Poser, S., Schwarz, H.P., 2001. Immunochemical determination of cellular prion protein in plasma from healthy subjects and patients with sporadic CJD or other neurologic diseases. Transfusion 41, 441–448.

Wilson, S., 2006. An animal model evaluation and assay protocol for screening blood for vCJD. Cambridge Healthtech Institute. A Forum on TSE Diagnostic Blood Tests. Baltimore, MD, 7–9 March 2006.

Zeiler, B., Adler, V., Kryukov, V., Grossman, A., 2003. Concentration and removal of prion proteins from biological solutions. Biotechnol. Appl. Biochem. 37, 173–182.

Zou, W.Q., Zheng, J., Gray, D.M., Gambetti, P., Chen, S.G., 2004. Antibody to DNA detects scrapie but not normal prion protein. Proc. Natl. Acad. Sci. U.S.A. 101, 1380–1385.

Excretion of Transmissible Spongiform Encephalopathy Infectivity in Urine

Luisa Gregori, Gabor G. Kovacs, Irina Alexeeva, Herbert Budka, and Robert G. Rohwer

The route of transmission of most naturally acquired transmissible spongiform encephalopathy (TSE) infections remains speculative. To investigate urine as a potential source of TSE exposure, we used a sensitive method for detection and quantitation of TSE infectivity. Pooled urine collected from 22 hamsters showing clinical signs of 263K scrapie contained 3.8 ± 0.9 infectious doses/mL of infectivity. Titration of homogenates of kidneys and urinary bladders from the same animals gave concentrations 20,000-fold greater. Histologic and immunohistochemical examination of these same tissues showed no indications of in ammatory or other pathologic changes except for occasional deposits of disease-associated prion protein in kidneys. Although the source of TSE infectivity in urine remains unresolved, these results establish that TSE infectivity is excreted in urine and may thereby play a role in the horizontal transmission of natural TSEs. The results also indicate potential risk for TSE transmission from human urine–derived hormones and other medicines.

Transmissible spongiform encephalopathies (TSEs) are fatal neurologic diseases. In humans, a long asymptomatic incubation period is followed by a progressive clinical course that typically lasts a few months to a year. TSE infectivity and pathologic changes are concentrated in the nervous system; however, much of the transmission risk results from parenteral exposure to the much lower concentrations of infectivity found in tissues outside the nervous system. Thus, despite the very low concentration of TSE infectivity in blood (*1*,*2*), 4 human cases of transmission of variant Creutzfeldt-Jakob disease through blood transfusions have been documented (*3*,*4*). If TSE infectivity were excreted, human urine, which is a source of injectible fertility hormones and other drugs (*5*,*6*), could also pose a risk for transmission. Infected urine might also account for the horizontal transmission of sheep scrapie and might contribute to the natural spread of chronic wasting disease in deer and elk.

Early attempts to transmit Creutzfeldt-Jakob disease by cross-species inoculation of rodents and primates with urine from diseased patients failed (*7*,*8*). More recent attempts in which urine from infected hamsters was injected back into hamsters have produced variable results (*9*,*10*). Two other studies have reported infectivity in urine (*11*) and infectivity with disease-specific prion protein (PrP^{d}) in kidneys of mice with simultaneous scrapie and nephritis but not in those with scrapie alone (*12*). To resolve these discrepancies, we used a highly sensitive and precise method of measuring low concentrations of TSE infectivity, which we have successfully used for quantitation of TSE infectivity in blood (*1*,*2*), to measure the concentration of TSE infectivity in urine of scrapie-infected hamsters.

Materials and Methods

Urine Collection and Processing

Urine was collected from a cohort of 22 Syrian hamsters (Harlan Sprague-Dawley, Haslet, MI, USA) that had been infected by intracranial injection with 10% (wt/vol) scrapie brain homogenate (263K strain) and from a cohort of 8 age-matched, noninoculated control animals. At the time of urine collection, the scrapie-infected hamsters showed clear clinical evidence of disease but were still able to drink and eat (67–74 days postinoculation). Hamsters were placed 2 at a time for 24 hours in metabolism cages in which they had access to water but not food. Food was

Author af liations: Veterans Affairs Medical Center, Baltimore, Maryland, USA (L. Gregori, I. Alexeeva, R.G. Rohwer); University of Maryland, Baltimore (L. Gregori, R.G. Rohwer); and Medical University of Vienna, Vienna, Austria (G.G. Kovacs, H. Budka)

DOI: 10.3201/eid1409.080259

withheld to prevent contamination of the urine. Urine was maintained at 4°C during collection. Separate metabolic cages (Rat metabolic cage no. 2100-R; Lab Products, Seaford, DE, USA) were used for each cohort. The urine produced daily was stored at –80°C. The individual collections were then combined into clinical and control pools of ≈60 mL and ≈125 mL, respectively.

Limiting Dilution Titration of Urine

We used the limiting dilution method of titration developed in our laboratory to measure the concentration of TSE infectivity in urine (*1*,*2*). In this method, a relatively large volume of low-titer sample is injected intracerebrally, 50 μL at a time, into a large cohort of weanling hamsters. Immediately before animal inoculation, aliquots of the clinical and control urine pools were thawed and sonicated on ice with separate sterile ultrasonication probes for each pool. Sonication was for 4 cycles of 15 s on and 10 s off for 1 min of total sonication, using a microtip probe at 40% amplitude (Vibra-Cell 750W; Sonics & Materials, Newtown, CT, USA). Two milliliters of control urine was injected undiluted into 40 hamsters. Clinical urine (urine from hamsters showing clinical signs of disease) was diluted 1:3 with inoculation buffer (phosphate-buffered saline [PBS] supplemented with 1% fetal calf serum and 1× penicillin and streptomycin) to remove concentration-related toxicity. Five milliliters from the clinical urine pool was diluted to 15 mL, and the entire volume was injected into 300 hamsters, 50 μL/animal. Soon after inoculation, 8 animals inoculated with urine from the infected animals died, which left 292 animals in the study. All inoculations were conducted under anesthesia with pentobarbital (40–90 mg/kg). At each step the control urine was processed before the infected urine.

All animals were assessed weekly for early signs of scrapie. At the first signs of disease, animals were separated from their cage mates, observed daily for disease progression, and euthanized after disease was confirmed clinically. After 559 days postinoculation all remaining animals were euthanized. Brains were collected from all animals in the study and assayed for infection-specific, proteinase K–resistant prion protein (PrP^{res}) by Western blot or ELISA, using a dissociation-enhanced lanthanide fluorescent immunoassay (DELFIA) developed in our laboratory (described below). The infection status of each animal was tabulated, and the probabilities of infection and titer were computed as described (*1*,*2*; Table 1).

Tissue Collection and Processing

Kidneys and urinary bladders were harvested from each of 12 infected animals that donated urine either 71 or 76 days postinoculation. Animals were euthanized by asphyxiation with CO_2. The bladder was removed first and immediately frozen in liquid nitrogen. The kidneys were collected next; the renal capsule was removed before freezing the tissue in liquid nitrogen. Both tissues were dissected aseptically with a clean, sterile set of instruments for each animal and each organ; particular care was taken to not touch other organs or tissues. The tissues (12 bladders and 19 kidneys) were pulverized with a cryomill by using separate cryo-capsules for each tissue (Cryogenic Sample Crusher, Model JFC-300; JAI, Tokyo, Japan). The tissue powder was stored at –80°C until use.

End-Point Dilution Titration of Tissues

Pooled bladder powder (1.65 g) and pooled kidney powder (0.64 g) were separately mixed with homogenization buffer (PBS, pH 7.2) to make 10% (wt/vol) tissue suspensions before sonication at 40% amplitude, using separate sterile microtip probes for each homogenate. The kidney homogenate was prepared according to the same schedule of sonication used for the urine pools. The bladder homogenate was sonicated for 10 s, repeated 2 times (20 s total sonication time) at room temperature. Longer sonication times or delays in the injection of the bladder homogenate caused the sample to solidify, which made it impossible to dilute and inject. Immediately after sonication the homogenates were serially diluted 10-fold in inoculation buffer, and each dilution was injected into hamsters in 1 to 5 cages (4 hamsters/cage) for titration by end-point dilution (Table 2).

All dilutions were by weight. The study was terminated at 426 days postinoculation, and the infection status of each animal was confirmed by Western blot of the brain for PrP^{res}. The titers were calculated by the methods of Reed and Muench (*13*), Pizzi (*14*), and Spearman and Karber (*15*).

PrP^{res} Detection Procedures

Immunoblotting

Individual brains were homogenized in PBS, pH 7.2, to 10% (wt/vol) by using a FASTH homogenizer (Consul AR; Villeneueve, Switzerland) according to the manufacturer's instructions. To test for PrP^{res}, brain homogenate

Table 1. Titer of urine from scrapie-infected hamsters

Hamster	Volume assayed, mL	Fold dilution	Volume inoculated, mL	Total no. hamsters	No. infected hamsters	Titer, ID/mL*	SD†
Infected	4.87	3	14.6	292	18	3.8	0.9
Noninoculated	2	None	2	40	1	–	–

*ID, infectious dose. Titer = –ln(P(0)) × (1/v), where P(0) = (noninfected animals)/(total animals inoculated) and v = inoculation volume, 0.05 mL.
†SD = square root (titer/V), where V = 4.87 mL, the total volume of the undiluted urine inoculated (*1*).

Table 2. End-point dilution titration of urinary bladder and kidney from scrapie-infected hamsters

Dilution	Total/no. infected	
	Bladder	Kidney
10^{-1}	19/19	4/4
$10^{-1.3}$	8/8	20/20
$10^{-1.7}$	8/8	8/8
10^{-2}	4/4	8/8
10^{-3}	4/4	4/3
10^{-4}	4/2	4/1
10^{-5}	4/1	4/0
10^{-6}	4/0	4/0
Titer ($\log_{10}$ ID_{50}/g)*	5.5	5.0
Standard error	0.5	0.4

*ID_{50}, 50% infectious dose. Titers calculated by the Reed and Muench method (*13*); standard errors by the Pizzi method (*14*).

was digested with proteinase K at 0.1 mg/mL final concentration as described by Gregori et al. (*1*). Sample buffer containing 2% sodium dodecyl sulfate was added, and the samples were heated at 100°C for 10 min and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Western blots of the samples were developed by using anti-PrP 3F4 monoclonal antibody (Covance, San Diego, CA, USA) for PrP detection (*1*).

ELISA

After proteinase K digestion and heat denaturation as described for immunoblotting, the samples were diluted 100-fold in assay buffer (DELFIA Assay Buffer; PerkinElmer, Waltham, MA, USA). They were then assayed for PrP concentration by DELFIA by using a Wallac Victor V instrument (PelkinElmer) for signal detection, with purified 3F4 monoclonal antibody (Covance) as the capture antibody and purified 7D9 monoclonal antibody (Covance) labeled with Europium according to the manufacturer's instructions (PerkinElmer) as the detection antibody. The molar ratio of Europium:7D9 antibody was 7.4:1 (*2*).

Histologic and Immunologic Tissue Preparation

Formalin-fixed brains were cut and divided on the midline; 1 hemisphere was cut in the sagittal plane; the other was cut coronally at the anterior basal ganglia, the middle of the thalamus, and the brainstem with cerebellum. Spleens, kidneys, and bladders were divided in the middle. All blocks were embedded in paraffin and processed for conventional staining with hematoxylin and eosin and Luxol fast blue/nuclear fast red (for brain) as well as for immunohistochemical detection of PrP with monoclonal anti-PrP antibody 3F4 (1:1,000; Covance). For detection of PrP^{d}, sections were pretreated with 30 min of hydrated autoclaving at 121°C followed by 5 min in 96% formic acid. Immunostained sections were counterstained with hematoxylin.

Animal Husbandry and Decontamination Procedures

Animals were maintained in a Biosafety Level 3 (BSL-3) animal facility at the Veterans Affairs Medical Center in Baltimore, Maryland, USA. Standard operating procedures specifically designed for TSEs, including TSE select agents, were followed. The operation of this facility has been described in detail (*16*). Animal cages were changed once a week, and cages and bedding were decontaminated by autoclaving for 1 h at 134°C. The sonicator probes and dissection instruments were decontaminated by autoclaving for 2 h at 134°C immersed in 2 N NaOH, followed by cleaning, repackaging, and sterilizing. All laboratory surfaces were decontaminated before use with either 2 N NaOH or LpH (Steris Corporation, Mentor, OH, USA) (*16*).

Results

Urine Titration

Urine collections from infected and control animals were combined into separate pools. Pools minimized the possibility of an idiosyncratic measurement from an individual and serve as a resource for future experiments once the titer has been determined. Clinically affected animals consumed lower amounts of water and produced 4–5-fold less urine than control animals. This resulted in slightly elevated specific gravity, proteins, glucose, and ketones as measured with a standard urine dipstick. Elevated urine ketones may also have been caused by fasting. The higher concentration of the urine pooled from infected animals resulted in a toxicity that required a 3-fold dilution in buffer before it could be injected.

TSE developed in 18 of the 292 animals that survived the injection of the 3-fold diluted infected pool. Incubation times are shown in Figure 1. As observed in other studies (*1*,*2*), scrapie incubation times for animals infected with low-titer samples begin at ≈150 days and rarely extend past 500 days. None of the animals from either the infected or noninfected cohorts that survived to the end of the experiment were positive by DELFIA. None of the 24 animals that died during incubation without clinical evidence of scrapie were positive for scrapie infection by Western blot. Only those animals with clinical scrapie had the typical PrP^{res} signal in the brain as assessed by Western blot. The infectivity titer of the urine as calculated from the Poisson distribution was 3.8 ± 0.9 infectious doses (ID)/mL (Table 1).

Scrapie developed (at 425 days postinoculation) in 1 of the 40 hamsters inoculated with control urine. Because none of the control donor animals contracted scrapie and because their brains were negative for PrP^{res}, it is clear that this infection resulted from contamination. However, the contamination was unlikely to have been environmental. Our BSL-3 is managed under a strict regimen of continuous decontami-

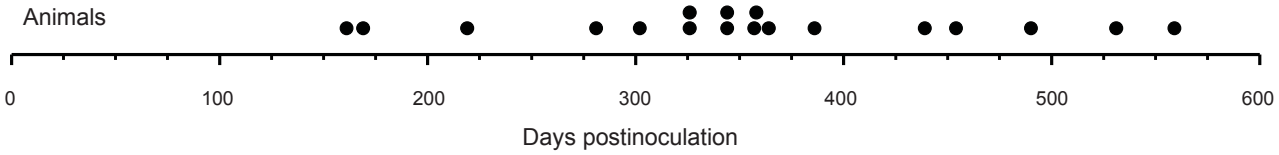


Figure 1. Distribution of incubation times of hamsters infected by injected urine. Each dot represents 1 animal with clinical scrapie that was euthanized at the corresponding day postinoculation. The 22 additional animals that died during the incubation period and the 252 animals that survived to the end of the experiment (559 days) showed no clinical or immunochemical evidence of scrapie and were scored as scrapie negative.

nation and precautionary cleaning (*16*). As evidence of the effectiveness of these measures, we have conducted several titrations, involving hundreds of animals each, in which there were no infections at all during ≥540 days of incubation. One such study was ongoing during the titration of the urine pools reported in this study (*2*). If there are environmental sources of infectivity, the concentration is below the level of detection by the data accumulated in infection-free titrations to date. Instead, after an intensive review of our procedures, we concluded that the most likely source of this contamination was a technical lapse during collection of the urine pools. The level of contamination (1 infection/2 mL of control pool injected vs. 18 infections/4.87 mL of clinical pool injected) is consistent with a pooling error at the time of collection. Nevertheless, had it been an environmental contamination, the associated titer (0.51 ID/mL SD = 0.50 ID/mL) would have had a negligible effect on the value determined for the infected urine.

Tissue Titrations

The concentration of scrapie infectivity in hamster urine is similar to that in plasma of scrapie-infected hamsters at the same stage of disease, which suggests plasma as a possible source of the infectivity. To investigate other possible sources, we also measured the concentration of TSE infectivity in separate pools of kidneys and bladders collected from the same donor animals. The titrations were by the end-point dilution method. The titers calculated by the methods of Reed and Muench (*13*) and Pizzi (*14*) were $10^{5.5 \pm 0.5}$ 50% infectious doses (ID_{50})/g of bladder and $10^{5.0 \pm 0.4}$ ID_{50}/g of kidney. The Spearman and Karber method gave almost identical values (*15*).

Histologic and Immunohistochemical Examination of Tissues

Others have reported TSE infectivity in the urine of scrapie-infected mice with nephritis but not in infected mice without nephritis (*11*,*12*). In contrast, our hamster colony in general, and the animals in this experiment, showed no evidence of inflammation, as indicated by clinical assessments or urine parameters. Nitrates were within normal limits, and no leukocyturia was noted. Proteinuria in the clinical hamsters was likely the consequence of low-volume urine excretion. To further assess whether hamsters infected with scrapie were also affected by kidney inflammation or other abnormalities of the urinary system, we examined the kidneys and the urinary bladders of 8 scrapie-affected hamsters at 84 days postinoculation and 4 preclinically infected hamsters at 49 days postinoculation for PrP^d by immunohistochemical and histologic methods (Figure 2). We also examined control tissues from 10 age-matched uninoculated animals as well as brain and spleen tissues from infected and control animals.

All tissues were evaluated for signs of inflammation and for the pattern of PrP^d immunoreactivity; brains were also examined for spongiform change. No inflammatory changes were found in any tissue examined. In 9 infected animals (clinical and preclinical), we noted nidus formation in the lumina of the bladder with a few neutrophilic granulocytes. However, leukocytes had not invaded the wall of the bladder. Nidus formation is often associated with dehydration.

PrP immunoreactivity was not observed in the bladder wall of scrapie-infected or control animals (data not shown). Spongiform change and deposition of PrP^d was lacking in control animal brains (Figure 2, panel A) and was noted to various extents, according to the stage of the disease, in all scrapie-infected animal brains (Figure 2, panel E). We observed fine synaptic PrP^d immunoreactivity with focal patchy or plaque-like appearance in gray matter structures, but we also noted ependymal, subependymal, perivascular, and white matter PrP^d deposits (data not shown). PrP^d immunoreactivity was observed in the germinal centers of the spleen of all scrapie-infected animals (Figure 2, panel F) but not in those of controls (Figure 2, panel B). None of the control animals exhibited immunoreactivity for PrP^d in the kidneys (Figure 2, panels C, D). PrP^d immunostaining showed fine granular deposits in the collecting tubules of the medulla (Figure 2, panels G, H) in 4 (50%) of 8 animals in the clinical stage of scrapie and in 3 (75%) of 4 animals in the preclinical stage, for a total of 7 (58.3%) of 12 scrapie-infected animals.

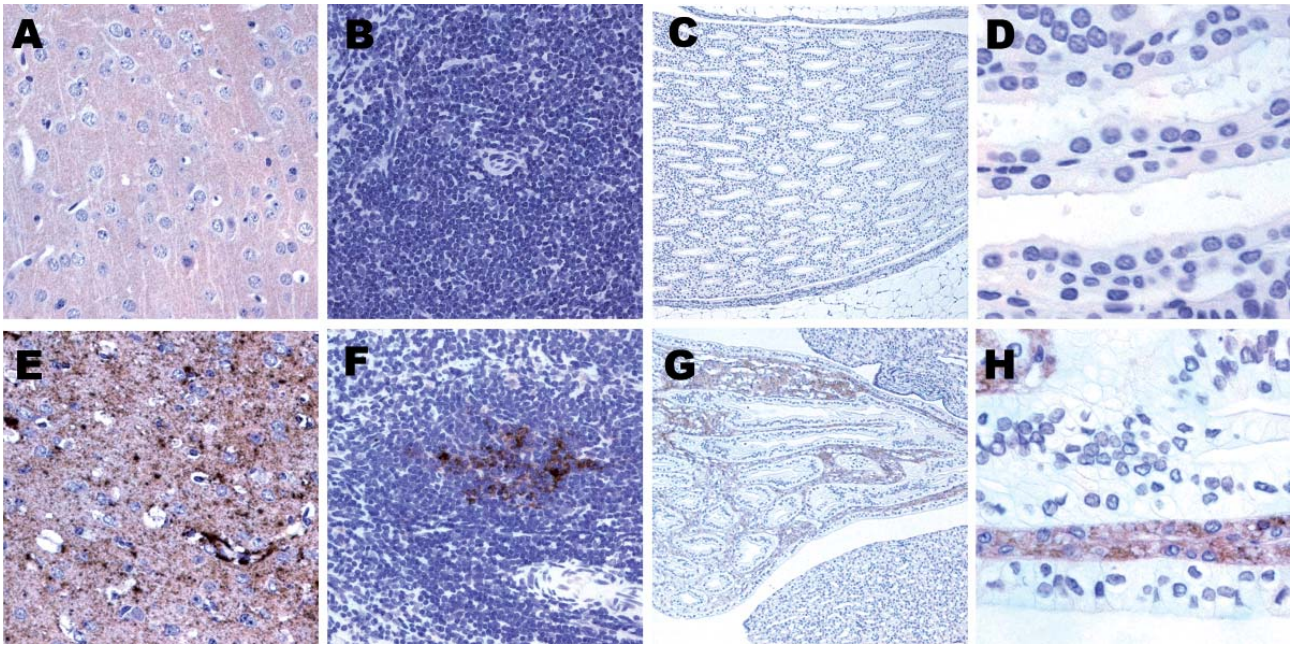


Figure 2. Immunostaining for prion protein (PrP) in control and scrapie-infected hamsters. Deposition of disease-associated PrP is lacking in the brain (A), spleen (B), and kidneys (C,D) of control hamsters. Fine synaptic and plaque-like PrP immunoreactivity in the frontal cortex (E), granular immunoreactivity in the germinal center of spleen (F) and in the collecting tubules of kidneys (G,H) in a representative scrapie-infected animal. Original magni cation ×200 for panels A, B, D, E, F, and H and ×40 for panels C and G.

Discussion

Anticipating that the titer of scrapie infectivity in excreted urine would be low, we measured concentration by using limiting dilution titration, a method with which we have extensive experience quantitating TSE infectivity in blood and blood components. In a limiting dilution titration, all animals in the bioassay are inoculated with the highest concentration of inoculum that is tolerated by the intracranial (most efficient) route. Infectivity assorts randomly into the inoculated animals; provided that at least some, but not all, of the animals are infected, the concentration can be calculated from the Poisson distribution of the infections (*1*). The method is highly sensitive and far more precise than other methods of TSE titration. We considered concentrating the urine before bioassay, but to circumvent uncertainties about the recovery of endogenous infectivity, we decided to inject the urine as collected.

We found TSE infectivity in the urine of hamsters that had no evidence of kidney or bladder inflammation. In contrast, Seeger et al. did not detect infectivity in the urine of scrapie-infected mice (*11*) unless the mice were also affected by nephritis, in which case they found low levels of infectivity. Whether the bioassay they used was capable of detecting infectivity at the concentration we observed for hamsters is not clear. If it was not capable, then detection of infectivity in mice with nephritis implies a higher concentration of infectivity in urine excreted by a nephritic kidney. In another study, urine and feces from deer with chronic wasting disease failed to demonstrate infectivity when orally given to the same susceptible species (*17*). Although usually an inefficient route of inoculation, the oral route did successfully transmit chronic wasting disease infectivity in saliva. The authors identified several possible reasons for the unsuccessful transmission by excreta, including incubation time, genotype, or sample size.

In our experiments, cross-contamination by feces can not be excluded as a source of infectivity. Although the metabolism cage effectively separated urine and feces, some contact is possible because of the anatomy of the hamster.

Protein misfolding cyclic amplification uses sonication to generate PrP^{res} and infectivity in vitro. Although we routinely disperse all samples by ultrasonication before injection, our conditions are much harsher than those used to generate PrP^{res} de novo (*18*) and do not support protein misfolding cyclic amplification of PrP^{res}, or presumably infectivity (L. Gregori and R.G. Rohwer, unpub. data).

The kidney and bladder titers were far greater than expected compared with findings of historical studies in which, with only rare exceptions (*19–21*), most attempts at transmission have been unsuccessful. These titers cannot be explained by the infectivity in residual blood (10 ID/mL) (*1*,*2*). In addition, we observed PrP^{d} in the kidneys of scrapie-infected animals that had no indications of tissue inflammation. Heikenwalder et al. found PrP^{d} staining within

follicular infiltrates only in kidneys of mice affected by nephritis and not in control mice with noncomplicated scrapie (*12*). These data together with those by Seeger et al. (*11*) suggested that renal inflammation might be a prerequisite for TSE infectivity in renal tissue and its excretion in urine. In contrast, our results indicate that renal inflammation is not necessary for the deposition of PrP^d in kidneys or for excretion of infectivity. One interpretation is that nephritis enhances the accumulation of PrP^d at sites of inflammation, consistent with the excretion of higher levels of infectivity inferred above for this same condition (*11*).

Two studies of scrapie in naturally and experimentally infected sheep reported PrP^d depositions in the renal papillae (*22*) and in the intraepithelial cortex, medulla, and papillae (*23*). Similar to our findings, both studies indicated that not all scrapie tissues examined were positive for PrP^d. In chronic wasting disease, PrP^d staining was uniquely localized in the ectopic lymphoid follicle of the kidney of a whitetail deer (*24*). All studies indicated either no changes (*22*,*24*) or mild to no inflammatory changes of the kidney (*23*). Thus, our histologic and immunohistochemical results for scrapie-infected hamsters are consistent with results found for sheep and deer and suggest that under normal conditions TSE diseases do not have concomitant inflammatory changes in the kidney.

That urine titer is similar to that of plasma suggests that urine infectivity may originate from blood (*25*), but how the infectivity would be excreted is not clear. In general, proteins >40 kDa are not excreted and smaller proteins crossing the glomeruli are reabsorbed in the renal tubule and returned to the blood. If TSE infectivity is particulate (>40 kDa), its presence in urine might indicate abnormalities in renal filtration, perhaps related to the accumulation of PrP^d in the collecting tubules of the medulla. The accumulation of immunoglobulins in the urine of TSE-infected hamsters and humans may also indicate malfunction of the urinary system (*9*,*26*). Excretion of a small C-terminal fragment of the normal cellular form of the prion protein in urine of infected and noninfected animals has been reported (*27*), but PrP^{res} or PrP^d forms can only be inferred from the presence of infectivity. Nevertheless, excretion of proteins similar to PrP^{res} or PrP^d forms has been documented. Follicle-stimulating hormone is a glycosylated protein of 203 amino acids organized mostly as a β-sheet, which bears some remarkable similarities to β-rich forms of the prion protein. Follicle-stimulating and several similar hormones are excreted in urine at great enough concentration to be extracted commercially. Alternatively, TSE infectivity may be excreted by processes analogous to those responsible for the low-level virurias that occur during infections of the nervous system by mumps, measles, and West Nile virus (*28*–*30*).

To the extent that results from the hamster model can be generalized to other TSE infections (and it has so far proven highly predictive), then even the very low concentrations of infectivity measured here could result in substantial environmental contamination. Several liters of urine and several thousand doses of TSE infectivity may be excreted daily over the course of the illness; even higher titers might be excreted by an animal with nephritis. The high stability of TSE infectivity would account for its persistence in pasture years after infected animals are removed (*31*). Recent studies have shown that infectivity that is adsorbed and immobilized by soil minerals (*32*) can still infect hamsters by oral exposure 29 months later (*33*). Our study also warns of a possible risk from TSE contamination to fertility hormones and other medicinal products extracted from human urine.

Acknowledgments

We thank the BSL-3 animal staff at the Veterans Affairs Medical Center in Baltimore for their excellent animal care.

This work was funded by Department of Defense grant 17-03-1-0756 to R.G.R. and by a National Institutes of Health contract N01-NS-0-2327 for R.G.R. The pathologic investigations were performed within the PrioGen subproject of the European Union Network of Excellence NeuroPrion.

Dr Gregori is deputy director of the Molecular Neurovirology Laboratory in the Veterans Affairs Medical Center in Baltimore and a faculty member of the Department of Neurology at the University of Maryland in Baltimore. Her primary research interest is TSEs, with particular focus on TSE transmission by secondary exposure such as blood transfusion.

References

1. Gregori L, McCombie N, Palmer D, Birch P, Sowemimo-Coker SO, Giulivi A, et al. Effectiveness of leucoreduction for removal of infectivity of transmissible spongiform encephalopathies from blood. Lancet. 2004;364:529–31. DOI: 10.1016/S0140-6736(04)16812-8
2. Gregori L, Gurgel PV, Lathrop JT, Edwardson P, Lambert BC, Carbonell RG, et al. Reduction in infectivity of endogenous transmissible spongiform encephalopathies present in blood by adsorption to selective affinity resins. Lancet. 2006;368:2226–30. DOI: 10.1016/S0140-6736(06)69897-8
3. Hewitt PE, Llewelyn CA, Mackenzie J, Will RG. Creutzfeldt-Jakob disease and blood transfusion: results of the UK Transfusion Medicine Epidemiological Review study. Vox Sang. 2006;91:221–30. DOI: 10.1111/j.1423-0410.2006.00833.x
4. Health Protection Agency. A new case of variant CJD associated with blood transfusion [cited 2008 Jan 2]. Available from http://www.hpa.org.uk/hpa/news/articles/press_releases/2007/070118_vCJD.htm
5. Matorras R, Rodriguez-Escudero FJ. Prions, urinary gonadotrophins and recombinant gonadotrophins. Hum Reprod. 2003;18:1129–30. DOI: 10.1093/humrep/deg176
6. Reichl H, Balen A, Jansen CA. Prion transmission in blood and urine: what are the implications for recombinant and urinary-derived gonadotrophins? Hum Reprod. 2002;17:2501–8. DOI: 10.1093/humrep/17.10.2501
7. Kuroda Y, Gibbs CJ Jr, Amyx HL, Gajdusek DC. Creutzfeldt-Jakob disease in mice: persistent viremia and preferential replication of virus in low-density lymphocytes. Infect Immun. 1983;41:154–61.

8. Gajdusek DC, Gibbs CJ Jr, Alpers M. Transmission and passage of experimenal "kuru" to chimpanzees. Science. 1967;155:212–4.
9. Shaked GM, Shaked Y, Kariv-Inbal Z, Halimi M, Avraham I, Gabizon R. A protease-resistant prion protein isoform is present in urine of animals and humans affected with prion diseases. J Biol Chem. 2001;276:31479–82. DOI: 10.1074/jbc.C100278200
10. Kariv-Inbal Z, Ben-Hur T, Grigoriadis NC, Engelstein R, Gabizon R. Urine from scrapie-infected hamsters comprises low levels of prion infectivity. Neurodegener Dis. 2006;3:123–8. DOI: 10.1159/000094770
11. Seeger H, Heikenwalder M, Zeller N, Kranich J, Schwarz P, Gaspert A, et al. Coincident scrapie infection and nephritis lead to urinary prion excretion. Science. 2005;310:324–6. DOI: 10.1126/science.1118829
12. Heikenwalder M, Zeller N, Seeger H, Prinz M, Klöhn PC, Schwarz P, et al. Chronic lymphocytic inflammation specifies the organ tropism of prions. Science. 2005;307:1107–10. DOI: 10.1126/science.1106460
13. Reed LJ, Muench H. A simple method of estimating fifty per cent endpoints. American Journal of Hygiene. 1938;27:493–7.
14. Pizzi M. Sampling variation of the fifty percent end-point, determined by the Reed-Muench (Behrens) method. Hum Biol. 1950;22:151–90.
15. Spearman C. The method of "right and wrong cases" ("constant stimuli") without Gauss's formulae. British Journal of Psychology 1908;2:227–42.
16. Elliott EJ, MacAuley C, Robins D, Rohwer RG. Working safely with transmissible spongiform encephalopathies. In: Richmond JY, Editor. Anthology of biosafety, vol. 7. Mundelein (IL): American Biological Safety Association; 2005.
17. Mathiason CK, Powers JG, Dahmes SJ, Osborn DA, Miller KV, Warren RJ, et al. Infectious prions in the saliva and blood of deer with chronic wasting disease. Science. 2006;314:133–6. DOI: 10.1126/science.1132661
18. Deleault NR, Harris BT, Rees JR, Supattapone S. Formation of native prions from minimal components in vitro. Proc Natl Acad Sci U S A. 2007;104:9741–6. DOI: 10.1073/pnas.0702662104
19. Hadlow WJ, Race RE, Kennedy RC. Temporal distribution of transmissible mink encephalopathy virus in mink inoculated subcutaneously. J Virol. 1987;61:3235–40.
20. Marsh RF, Burger D, Hanson RP. Transmissible mink encephalopathy: behaviour of the agent in mink. J Infect Dis. 1969;120:713–9.
21. Brown P, Gibbs CJ Jr, Rodgers-Johnson P, Asher DM, Sulima MP, Bacote A, et al. Human spongiform encephalopathy: the National Institutes of Health series of 300 cases of experimentally transmitted disease. Ann Neurol. 1994;35:513–29. DOI: 10.1002/ana.410350504
22. Sisó S, González L, Jeffrey M, Martin S, Chianini F, Steele P. Prion protein in kidneys of scrapie-infected sheep. Vet Rec. 2006;159:327–8.
23. Ligios C, Cancedda GM, Margalith I, Santucciu C, Madau L, Maestrale C, et al. Intraepithelial and interstitial deposition of pathological prion protein in kidneys of scrapie-affected sheep. PLoS One. 2007;2:e859. DOI: 10.1371/journal.pone.0000859
24. Hamir AN, Kunkle RA, Miller J. M, Hall SM. Abnormal prion protein in ectopic lymphoid tissue in a kidney of an asymptomatic white-tailed deer experimentally inoculated with the agent of chronic wasting disease. Vet Pathol. 2006;43:367–9. DOI: 10.1354/vp.43-3-367
25. Brown P, Rohwer RG, Dunstan BC, MacAuley C, Gajdusek DC, Drohan WN. The distribution of infectivity in blood components and plasma derivatives in experimental models of transmissible spongiform encephalopathy. Transfusion. 1998;38:810–6. DOI: 10.1046/j.1537-2995.1998.38998408999.x
26. Head MW, Kouverianou E, Taylor L, Green A, Knight R. Evaluation of urinary PrPSc as a diagnostic test for sporadic, variant, and familial CJD. Neurology. 2005;64:1794–6. DOI: 10.1212/01.WNL.0000161842.68793.8A
27. Narang HK, Dagdanova A, Xie Z, Yang Q, Chen SG. Sensitive detection of prion protein in human urine. Exp Biol Med (Maywood). 2005;230:343–9.
28. Krause CH, Eastick K, Ogilvie MM. Real-time PCR for mumps diagnosis on clinical specimens—comparison with results of conventional methods of virus detection and nested PCR. J Clin Virol. 2006;37:184–9. DOI: 10.1016/j.jcv.2006.07.009
29. Rota PA, Khan AS, Durigon E, Yuran T, Villamarzo YS, Bellini WJ. Detection of measles virus RNA in urine specimens from vaccine recipients. J Clin Microbiol. 1995;33:2485–8.
30. Tonry JH, Brown CB, Cropp CB, Co JK, Bennett SN, Nerurkar VR, et al. West Nile virus detection in urine. Emerg Infect Dis. 2005;11:1294–6.
31. Georgsson G, Sigurdarson S, Brown P. Infectious agent of sheep scrapie may persist in the environment for at least 16 years. J Gen Virol. 2006;87:3737–40. DOI: 10.1099/vir.0.82011-0
32. Johnson CJ, Phillips KE, Schramm PT, McKenzie D, Aiken JM, Pedersen JA. Prions adhere to soil minerals and remain infectious. PLoS Pathog. 2006;2:e32. DOI: 10.1371/journal.ppat.0020032
33. Seidel B, Thomzig A, Buschmann A, Groschup MH, Peters R, Beekes M, et al. Scrapie agent (strain 263K) can transmit disease via the oral route after persistence in soil over years. PLoS One. 2007;2:e435. DOI: 10.1371/journal.pone.0000435

Address for correspondence: Robert G. Rohwer, Veterans Affairs Medical Center, Research Service, 10 North Greene St, Mailstop 151, Baltimore, MD 21201, USA; email: rrohwer@umaryland.edu

Experimental transmission of scrapie agent to susceptible sheep by intralingual or intracerebral inoculation

Amir N. Hamir, Robert A. Kunkle, Marie S. Bulgin, Robert G. Rohwer, Luisa Gregori, Juergen A. Richt

Abstract

Scrapie, a transmissible spongiform encephalopathy (TSE), is a naturally occurring fatal neurodegenerative disease of sheep and goats. This study documents survival periods, pathological findings, and the presence of abnormal prion protein (PrP^{Sc}) in genetically susceptible sheep inoculated with scrapie agent. Suffolk lambs (AA/RR/QQ at codons 136, 154, and 171, respectively) aged 4 mo were injected by the intralingual (IL) or intracerebral (IC) route with an inoculum prepared from a pool of scrapie-affected US sheep brains. The animals were euthanized when advanced clinical signs of scrapie were observed. Spongiform lesions in the brain and PrP^{Sc} deposits in the central nervous system (CNS) and lymphoid tissues were detected by immunohistochemical and Western blot (WB) testing in all the sheep with clinical prion disease. The mean survival period was 18.3 mo for the sheep inoculated by the IL route and 17.6 mo for those inoculated by the IC route. Since the IC method is occasionally associated with anesthesia-induced complications, intracranial hematoma, and CNS infections, and the IL method is very efficient, it may be more humane to use the latter. However, before this method can be recommended for inoculation of TSE agents, research needs to show that other TSE agents can also transmit disease via the tongue.

Résumé

La tremblante fait partie des encéphalopathies spongiformes transmissibles (TSE) et est une maladie neuro-dégénérative fatale naturelle rencontrée chez les moutons et chèvres. La présente étude fait état des résultats obtenus quant aux périodes de survie, aux trouvailles pathologiques et à la présence de protéines prions anormales (PrP^{Sc}) chez des moutons génétiquement susceptibles inoculés avec l'agent de la tremblante. Des agneaux Suffolk (AA/RR/QQ aux codons 136, 154 et 171, respectivement) âgés de 4 mo ont été injectés par voie intra-linguale (IL) ou intracérébrale (IC) avec un inoculum préparé à partir d'un pool de cerveaux de moutons souffrant de tremblante provenant des États-Unis. Les animaux ont été euthanasiés lorsque des signes cliniques avancés de tremblante étaient observés. Les lésions spongiformes dans le cerveau et des dépôts de PrP^{Sc} dans le système nerveux central (CNS) et les tissus lymphoïdes ont été détectés par immunohistochimie et immuno-buvardage (WB) chez tous les moutons présentant une maladie à prion clinique. La période moyenne de survie était de 18,3 mois pour les moutons inoculés par la voie IL et de 17,6 mois pour ceux inoculés par la voie IC. Comme la méthode d'inoculation IC est parfois associée avec des complications dues à l'anesthésie, des hématomes intracrâniens et des infections du CNS, et que la méthode IL est très efficace, il serait plus éthique d'utiliser cette dernière. Toutefois, avant que cette méthode ne soit recommandée pour l'inoculation d'agents de TSE, les recherches doivent démontrer que d'autres agents de TSE peuvent également être transmis via la langue.

(Traduit par Docteur Serge Messier)

Scrapie belongs to a group of diseases known as transmissible spongiform encephalopathies (TSEs). It is a naturally occurring, genetically influenced, fatal neurodegenerative disease of sheep and goats. Infection by the causative agent, considered to be the post-translationally modified form of the host-encoded membrane-bound prion protein (PrP^{c}), leads to spongiform encephalopathy associated with accumulation of the abnormal form of prion protein (PrP^{Sc}) in tissues of the nervous and lymphoid systems, as well as in the placenta (1).

The most likely portal of entry in natural scrapie has been suggested to be the alimentary tract; other potential portals, such as scarified skin or the conjunctiva, have been effective experimentally (1). There is a paucity of information on experimental studies with scrapie in Suffolk sheep, the dominant sheep breed in the United States. In particular, the various routes of infection, other than oral and intracerebral (IC) with the US scrapie agent (2), have not been documented previously. This study attempted to partially fill this void by comparing intralingual (IL) and IC administration of the US scrapie agent to genetically susceptible Suffolk sheep.

Nine 4-mo-old Suffolk lambs (4 females and 5 castrated males) were obtained from a scrapie-free sheep flock at the National Animal Disease Center (NADC), Ames, Iowa. All were AA/RR/QQ at codons 136, 154, and 171, respectively, of the *PRNP* gene. The animals were divided into 2 groups: 4 lambs received the scrapie inoculum by the IL route, and 5 lambs received it by the IC route.

The inoculum (X124) was prepared from a pool of 7 scrapie-affected sheep brains from a single flock (3). All 7 sheep were QQ at codon 171 of the *PRNP* gene, and their brains were positive by

National Animal Disease Center, Agricultural Research Service, US Department of Agriculture, 2300 Dayton Avenue, PO Box 70, Ames, Iowa 50010, USA (Hamir, Kunkle, Richt); Department of Animal and Veterinary Science, University of Idaho, Caldwell, Idaho, USA (Bulgin); Laboratory of Molecular Neurovirology, VA Medical Center, University of Maryland, Baltimore, Maryland, USA (Rohwer, Gregori).

Address all correspondence and reprint requests to Dr. Amir N. Hamir; telephone: (515) 663-7544; fax: (515) 663-7458; e-mail: ahamir@nadc.ars.usda.gov

Received February 13, 2007. Accepted June 25, 2007.

Table I. Findings consistent with scrapie in tissues of susceptible Suffolk sheep injected with US scrapie agent X124 by the intralingual (IL) or intracerebral (IC) route

Sheep ear-tag no., sex	Survival period[a]	Histologic lesions of SE/PrP^{Sc} by IHC					PrP^{Sc} by IHC			PrP^{Sc} by WB; BS
		O	C	SC	H	RC	R	LN	T	
IL route										
3511, F[b]	9 d	−/−	−/NE	−/NE	−/NE	−/NE	NE	NE	NE	−
3513, M	18 mo	+/+	+/+	+/+	−/+	+/+	+	+	+	+
3514, F	19 mo	+/+	+/+	+/+	−/+	+/+	+	+	+	+
3502, M	18 mo	+/+	+/+	+/+	−/+	−/+	+	+	+	+
IC route										
3501, F	15 mo	+/+	+/+	+/+	+/+	+/+	+	+	+	+
3510, M	16 mo	+/+	+/+	+/+	+/+	+/+	+	+	+	+
3512, M	15 mo	+/+	+/+	+/+	+/+	+/+	+	+	+	+
3509, F	15 mo	+/+	+/+	+/+	−/+	+/+	+	+	+	+
3504, M	26 mo	+/+	+/+	+/+	+/+	+/+	NE	−	+[c]	+

SE — spongiform encephalopathy; PrP^{Sc} — abnormal prion protein; IHC — immunohistochemical testing; O — obex; C — cerebellum; SC — superior colliculi; H — hippocampus; RC — rostral cerebrum; R — retina; LN — retropharyngeal lymph node; T — tonsil (palatine and pharyngeal); WB — Western blot testing; BS — brainstem; NE — not examined.

[a] Time between inoculation and euthanasia.

[b] This lamb did not have signs of scrapie; it was euthanized at 9 d for detection of inoculated material in the tongue.

[c] Only the palatine tissue was positive.

Western blot (WB) analysis. The brains were sonicated, and a final concentration of 10% (w/v) was prepared with phosphate-buffered saline. The animals were each injected with a standard 1 mL of the inoculum.

For the IL route, the lambs were not given any sedation. The inoculum was injected with a 20-gauge needle into the ventral aspect of the tongue, approximately 2.5 cm from the tip. The IC method has been described previously (4). Briefly, the lambs were sedated with xylazine, a midline incision was made in the skin at the junction of the parietal and frontal bones, and a 1-mm hole was trephined through the calvarium. The inoculum was injected into the midbrain with a 22-gauge, 9-cm-long needle as the needle was withdrawn from the brain. The skin incision was closed with a single suture.

The inoculated animals were housed separately in a biosafety level 2 containment facility for 2 wk and then moved to 2 outside pens at NADC. They were fed pelleted growth and maintenance rations that contained no ruminant protein, and clean water was freely available.

One lamb, injected by the IL route, was euthanized 9 d after inoculation for immunohistochemical (IHC) detection of inoculated material in the tongue tissue; at necropsy, only the brain (for negative-control samples) and the tongue were obtained. The other 8 animals were euthanized when advanced clinical signs of scrapie developed. In these 8, a detailed gross examination did not show any lesions. Two sets of representative tissue samples were collected. One set of tissues included representative sections of liver, kidney, spleen, skin, striated muscles (heart, tongue, diaphragm, masseter), tonsils (pharyngeal, palatine), thyroid gland, turbinates, lung, tonsils, intestines (ileum), adrenal gland, lymph nodes (retropharyngeal, mesenteric), pituitary gland, Gasserian ganglion, brain (hemisections of cerebral cortex, cerebellum, superior colliculi, and brainstem, including obex), and eye (retina). For the tongue, a minimum of 8 cross-sections from the site of inoculation of each animal that received an IL injection were obtained. These tissues were fixed in 10% buffered formalin, embedded in paraffin wax, sectioned at 5 μm, and stained with hematoxylin and eosin (H–E) for light microscopy. The 2nd set of tissues was frozen.

All paraffin-embedded tissues and negative-control sections were labeled by an automated IHC method for detection of PrP^{Sc} as described previously (2). For WB detection of PrP^{Sc} in the brainstem, a commercial kit (Bio-Rad, Marnes-La-Coquette, France) with monoclonal antibody P4 was used as described previously (5).

Except for the lamb euthanized 9 d after inoculation, the animals showed clinical signs of scrapie before euthanasia. Initial signs were a progressive decrease in appetite and associated weight loss. Later signs were fine head tremors, listlessness, progressive problems with locomotion, and terminal sternal recumbency. None of the sheep exhibited obvious pruritus or loss of wool.

Table I shows the survival period after inoculation for the individual animals. The mean was 18.3 mo for the group inoculated by the IL route and 17.6 mo for the group inoculated by the IC route; analysis of variance did not show a significant difference ($P = 0.76$). Table I also shows the distribution of histologic, IHC, and WB findings. In all the sheep except the lamb euthanized 9 d after inoculation (sheep 3511), microscopic spongiform lesions characteristic of prion disease (Figure 1) were observed, and characteristic PrP^{Sc} deposition was present in the CNS samples. In addition, PrP^{Sc} deposition was present in the retropharyngeal lymph node and the tonsils (Figure 2), as well as all the tested retinas (Figure 3), of the sheep with clinical scrapie. A single submucosal lymphoid aggregate was detectable in the 3rd-eyelid samples from 1 of the sheep with an IHC-positive brain; however, this follicle contained no detectable PrP^{Sc}. Submucosal lymphoid follicles were not present in the other examined 3rd eyelids.

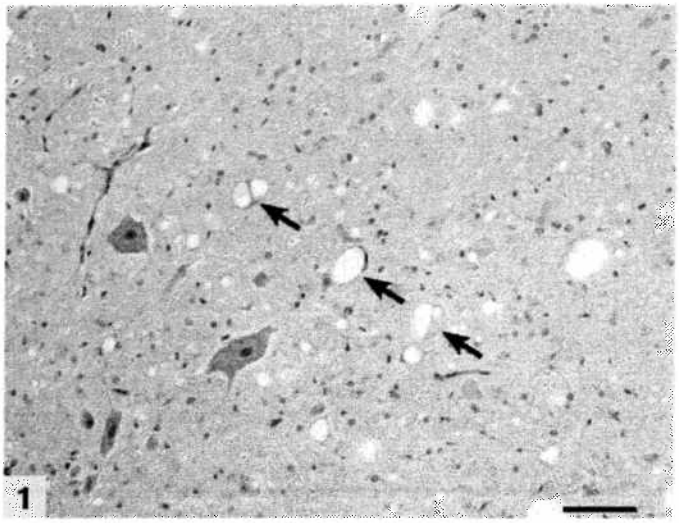


Figure 1. Medulla oblongata at the obex level of sheep 3502, with clinical scrapie, showing extensive vacuolation of the neuropil and multiple vacuoles in the neuronal perikaryon (arrows). Note the paucity of normal-appearing neurons. Hematoxylin and eosin (H–E). Bar — 30 μm.

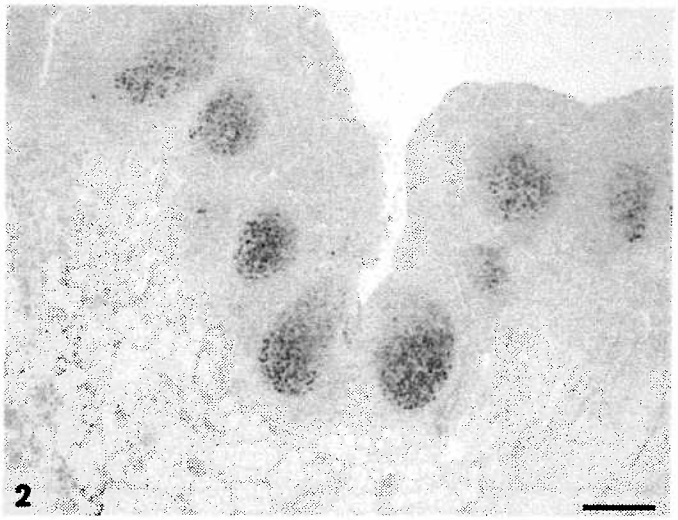


Figure 2. Pharyngeal tonsil of sheep 3502, showing immunohistochemical (IHC) staining (red) of the abnormal form of prion protein (PrP^{Sc}), which is mainly in the germinal centers of follicles. Immunoalkaline phosphatase and (counterstain) hematoxylin. Bar — 250 μm.

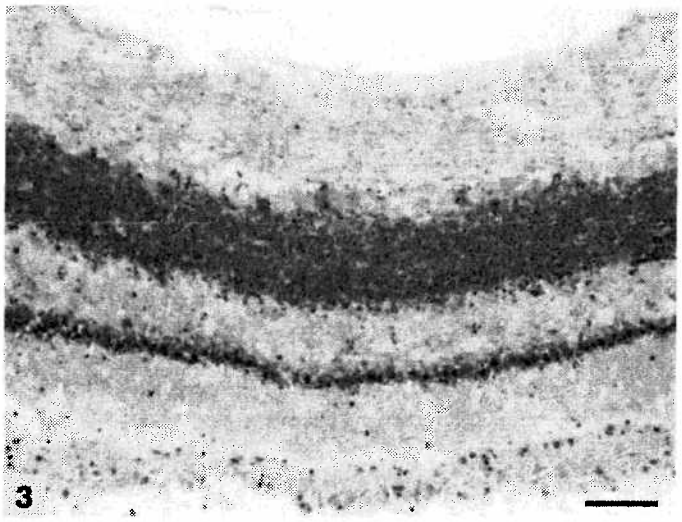


Figure 3. Retina of sheep 3502, showing extensive diffuse PrP^{Sc} labeling in both inner and outer plexiform layers. Bar — 20 μm.

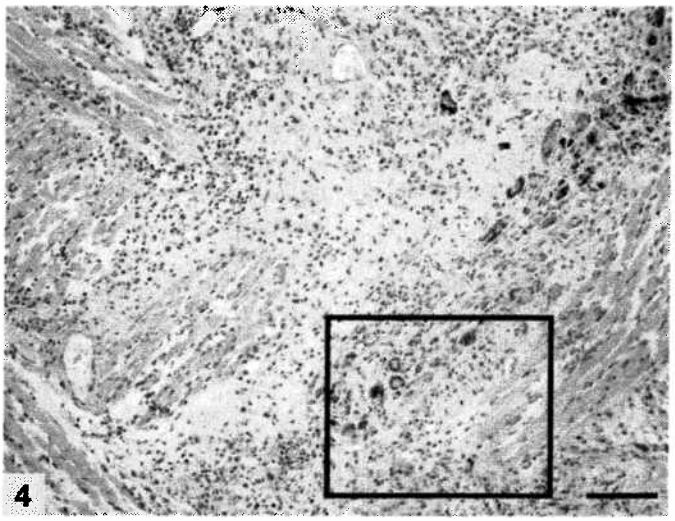


Figure 4. Tongue of sheep 3511, euthanized 9 d after inoculation with scrapie agent X124, showing extensive infiltration of epithelioid macrophages and lesser numbers of multinucleated giant cells between muscle fibers. H–E. Bar — 100 μm.

In sheep 3511, a locally extensive cellular infiltrate was noted at the site of inoculation in the tongue. The inflammatory cells were predominantly epithelioid macrophages arranged in broad sheets (Figure 4). At scattered sites within the infiltrate there were small numbers of multinucleated giant cells and multiple small areas of mineralization (Figure 5). In some areas away from the main focus of inflammation, muscle fibers were separated by edema fluid and lesser numbers of macrophages. The IHC-stained section of this area revealed foci of PrP^{Sc} within the macrophages (Figure 6).

Except for the tongue of sheep 3511, the IHC-stained sections of non-CNS and nonlymphoid tissues, including striated muscle (heart, diaphragm, and masseter muscle), did not reveal PrP^{Sc}. The adrenal medulla of sheep 3514 revealed PrP^{Sc} in the cytoplasm of multiple endocrine cells in a focally restricted area.

In the WB analysis with monoclonal antibody P4, brainstem samples from all the sheep except the negative-control lamb were positive for PrP^{Sc}, showing the typical profile of 3 bands of proteinase-K-resistant isoforms of PrP^{Sc} (Figure 7), which represent the diglycosylated, monoglycosylated, and unglycosylated polypeptides. The molecular pattern of the 3 isoforms in the samples from the animals inoculated by the IC route was similar to the pattern for the animals inoculated by the IL route.

In this study, the localization of PrP^{Sc} deposits in brain, lymphoid tissues, and retina in the scrapie-positive sheep was similar to that previously seen in sheep with scrapie after inoculation by the oral or IC route (2). Labeling of variable intensity was seen in various sections of the brain. The labeling was predominantly either particulate and diffuse or multifocal and extensive but also appeared as scattered small aggregates of consolidated plaques. Labeling

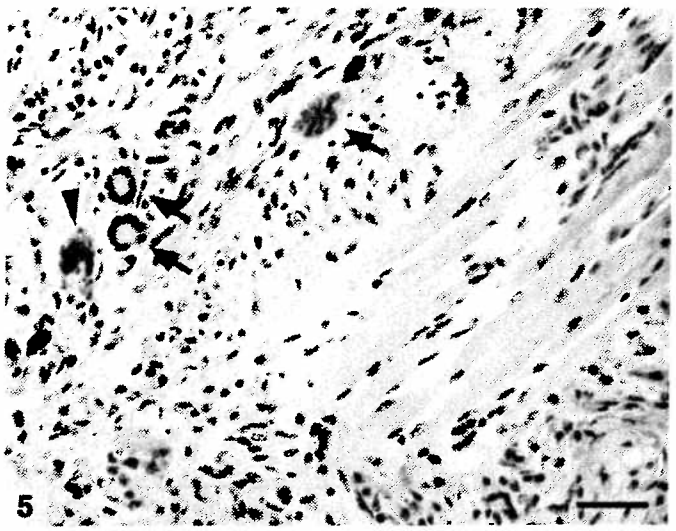


Figure 5. Higher magnification of area demarcated in Figure 4, showing multinucleated giant cells (arrows) and isolated mineralized muscle fibers (arrowhead). H–E. Bar — 40 μm.

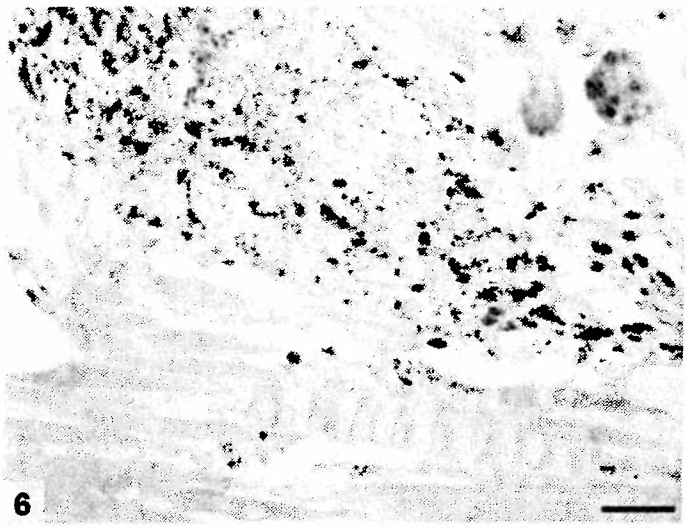


Figure 6. Tongue of sheep 3511, showing focal area of dense PrP^{Sc}-positive material (red) in the area of inflammation and, in the inset, within the epithelioid macrophages. Immunoalkaline phosphatase and hematoxylin. Bar — 40 μm.

predominated in the grey matter neuropil as perineuronal accumulations and in the perikaryon. Staining in the white matter was markedly less intense and appeared as scattered aggregates of particles. Labeling of the inner and outer plexiform layers of the retina was characterized by diffuse coalescing particles forming roughly uniform sheets. In IHC-positive lymphoid organs, PrP^{Sc} labeling was principally confined to the germinal centers of follicles and was either particulate or appeared as small aggregates of particles. Sparse punctate labeling of cells in the paracortex was noted in some sections.

Labeling of the tongue with PrP^{Sc} was documented in 7 of 10 sheep with naturally occurring scrapie in European breeds of sheep (6). However, in the present study, except for the sheep euthanized 9 d after inoculation, which had IHC staining of the tongue, PrP^{Sc} labeling was not observed in striated muscles of the scrapie-affected animals. This observation is in accordance with previous findings in 20 animals (cattle, sheep, elk, and raccoons) that had a TSE after experimental inoculation: PrP^{Sc} was found by IHC examination in the CNS but not in striated muscle tissues (7). Recent investigations with an enriched WB technique (8,9) have enabled the detection of PrP^{Sc} in the tongues of some sheep and elk experimentally infected with the agents of scrapie and chronic wasting disease (CWD), respectively (Richard Bessen, Montana State University, Bozeman, Montana: personal communication, 2006). This technique, however, failed to detect PrP^{Sc} in tongues of cattle in which a TSE developed after inoculation with the agent of CWD or that of transmissible mink encephalopathy (TME) (Richard Bessen, Montana State University: personal communication, 2006). The contrast in IHC results between the present study and the European study (6) could be due to differences between scrapie strains, breeds of sheep, natural and experimental inoculation, or the IHC procedure.

Inoculation route apparently did not influence the molecular phenotype of the PrP^{Sc} observed in the CNS tissues in our study. Alternative routes of infection and spread of prion disease have been suggested as being responsible for different disease phenotypes, as in cattle with typical and atypical bovine spongiform encephalopathy (9).

In general, the IC route of inoculation is considered to be the fastest means of transmitting TSE agents to susceptible hosts. In a study of sheep injected by the IC route with a different US scrapie inoculum (no. 13-7), the average incubation time was 18.8 mo (2); in the present study, with injection by this route of a different US scrapie inoculum (no. X124), the mean time to development of advanced clinical signs of scrapie was 17.6 mo. The sheep in both studies had clinical scrapie, lesions of spongiform encephalopathy, and PrP^{Sc} deposits in their tissues. Although sheep 3504 in our study had a significantly longer survival than the other sheep inoculated by the IC route, no difference in survival time was noted between the IL and IC groups. Recently, hamsters in which TME agent was inoculated into the tongue and 4 other non-neuronal anatomic sites were found to have PrP^{Sc} in submandibular lymph nodes and the hypoglossal nucleus in the brain within 2 wk after inoculation (10).

The IC route of inoculation is an invasive surgical procedure that involves the use of light anesthesia and carries the risks of intracranial hematoma, CNS infection, and complications of anesthesia. Aside from being safer, the IL method may mimic the natural route of infection for prion diseases. Development of an inoculation method that has an incubation time comparable to that of the IC method and is safer for the recipient animals is desirable. Although the numbers of animals used in this study were small, the results of the IL method were comparable to those of the IC method, and the former can be performed without general anesthesia. However, before the IL method of inoculation can be recommended for general use in TSE studies, similar studies need to be done with other TSE agents and need to involve larger numbers of susceptible hosts to validate the efficacy of the IL inoculation method.

Results of this study show that the transmission of scrapie via the IL route is possible. In comparison, oral inoculation required an average survival time of 32 mo and resulted in a lower attack rate (2). In another study (9), the IC route required a smaller volume of

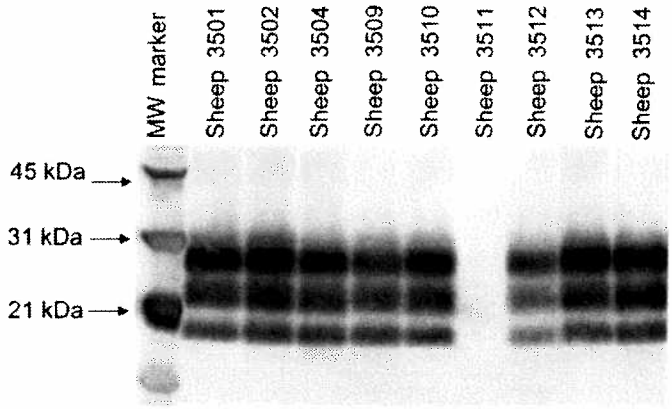


Figure 7. Western blot analysis of brainstem material from sheep inoculated with scrapie agent X124 by the intralingual or the intracerebral route. The immunoblot was developed with the use of monoclonal antibody P4. MW — molecular weight.

inoculum than the oral route (1 mL versus 30 mL of a 10% brain suspension), yet had a higher attack rate (100% versus 56%). It therefore appears that under field conditions the oral cavity in general, and the tongue in particular, may serve as a portal of entry for prions to gain access to the host's PrP^{Sc}-susceptible cell population.

Acknowledgments

We thank Dr. Marcus Kehrli for statistical analysis, Dr. Katherine O'Rourke for the prion antibodies for IHC testing, Drs. Lawayne Nusz and Jean Laufer for clinical assistance, and James Fosse for the photomicrographs. Expert technical assistance was provided by Martha Church, Dennis Orcutt, Joseph Lesan, Deb Clouser, Trudy Tatum, Sharla Van Roekel, Ginny Montgomery, Micky Fenneman, and the TSE animal caretakers.

A grant from the US Department of Defence and a contract from the National Institutes of Health (NO1-NS-0-2327) supported the laboratories of Drs. Bulgin, Rowher, and Gregori.

This study was carried out under the guidelines of the NADC Institutional Animal Care and Use Committee. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

References

1. Detwiler LA. Scrapie. Rev Sci Tech Off Int Epizoot 1992;11: 491–537.
2. Hamir AN, Kunkle RA, Richt JA, et al. Experimental transmission of sheep scrapie by intracerebral and oral routes to genetically susceptible Suffolk sheep in the United States. J Vet Diagn Invest 2005;17:3–9.
3. Bulgin MS, Sorensen SJ, Matlock ME. Association between incubation time and genotype in sheep experimentally inoculated with scrapie-positive brain homogenate. Am J Vet Res 2006;67:498–504.
4. Hamir AN, Miller JM, Stack MJ, et al. Failure to detect abnormal prion protein and scrapie-associated fibrils 6 wk after intracerebral inoculation of genetically susceptible sheep with scrapie agent. Can J Vet Res 2002;66:289–294.
5. Richt JA, Kunkle RA, Alt D, et al. Identification and characterization of two bovine spongiform encephalopathy cases diagnosed in the United States. J Vet Diagn Invest 2007;19:142–154.
6. Casalone C, Corona C, Crescio MI, et al. Pathological prion protein in the tongues of sheep infected with naturally occurring scrapie. J Virol 2004;79:5847–5849.
7. Hamir AN, Miller JM, Cutlip RC. Failure to detect prion protein by immunohistochemistry in striated muscle tissues of animals experimentally inoculated with agents of transmissible spongiform encephalopathy. Vet Pathol 2004;41:78–81.
8. Mulcahy ER, Bartz JC, Kincaid AE, et al. Prion infection of skeletal muscle cells and papillae in the tongue. J Virol 2004;78: 6792–6798.
9. Casalone C, Zanusso G, Acutis P, et al. Identification of a second bovine amyloidotic spongiform encephalopathy: molecular similarities with sporadic Creutzfeldt–Jakob disease. Proc Natl Acad Sci U S A 2004;101:3065–3070.
10. Bartz JC, Kincaid AE, Bessen RA. Rapid prion neuroinvasion following tongue infection. J Virol 2003;77:583–591.