Immunoglobulins in urine of hamsters with scrapie

Ana Serban¹, Giuseppe Legname¹,², Kirk Hansen⁴, Nadia Kovaleva¹ and Stanley B. Prusiner¹,²,³*

¹Institute for Neurodegenerative Diseases and Departments of ²Neurology, ³Biochemistry and Biophysics, and ⁴Pharmaceutical Chemistry, University of California, San Francisco

*Corresponding author. Please address all correspondence to: Institute for Neurodegenerative Diseases, Box 0518, University of California, San Francisco, CA 94143-0518; Tel: (415) 476-4482; Fax: (415) 476-8386.
ABSTRACT

In the prion diseases, a prolonged, asymptomatic incubation period precedes the onset of neurologic dysfunction. At present, a noninvasive test is not available for the presymptomatic diagnosis of prion disease and thus the report of a test for prions using urine has been of great interest (1). Using Western immunoblots with the anti-prion protein (PrP) 3F4 monoclonal antibody (mAb) and an anti-mouse IgG secondary antibody, a protease-resistant PrP was reported in the urine of Syrian hamsters and humans with prion disease. Here we demonstrate that this purportedly “protease-resistant PrP” band in the urine of diseased hamsters is detectable using the anti-mouse IgG secondary antibody in the absence of the 3F4 mAb. Mass spectrometric analysis identified immunoglobulin light chain in the band but found no PrP peptides. No similar band was found in the urine of uninfected hamsters or in brain homogenates from normal or prion-infected hamsters. Moreover, the band in the urine of infected hamsters was not detected using two chimeric human–mouse (HuM) recombinant anti-PrP antibody fragments followed by an anti-human IgG secondary antibody. Our results indicate that the band detected under previously published conditions is due to the cross-reactivity of the anti-mouse IgG antibody with IgG light chains and possibly heavy chain fragments in urine, and is not PrP.
INTRODUCTION

Prions cause neurodegeneration in humans and animals (2). The human prion diseases can manifest as hereditary, sporadic and infectious disorders. Much evidence argues that prions causing bovine spongiform encephalopathy (BSE) have been transferred to humans by ingestion of tainted beef products (3,4). The resulting disease in humans has been designated variant Creutzfeldt-Jakob disease (vCJD), which occurs primarily in teenagers and young adults. Compared to sporadic (s) CJD that accounts for ~85% of all prion disease in humans, vCJD seems to be caused by a strain of prions that is much more lymphotrophic. Evidence for the lymphotropism of vCJD prions comes from studies of the tonsils and appendix, both of which contain readily measurable levels of the disease-causing isoform (PrP\textsuperscript{Sc}) of the prion protein (PrP) (5). The lymphotropism of vCJD prions has raised considerable concern about the safety of the blood supply in Great Britain and led the United States to prohibit blood donations from individuals who have lived in Europe for prolonged periods of time (6). Findings that both scrapie and BSE prions in sheep can transmit disease after blood transfusions in sheep are also of importance (7). Recently, a 69-year-old man, who had received a blood transfusion 6.5 years earlier, died of vCJD (8). The blood donor died of vCJD three years ago. Because of the advanced age of the transfusion recipient, which contrasts with the young age of all other vCJD cases, it has been suggested that this may be the first case of human prion disease transmitted by blood transfusion.

The foregoing observations argue for the need for a test for prion infection in both humans and animals when they are still asymptomatic. The report of a protease-resistant PrP molecule in the urine of Syrian hamsters, cattle, and humans with prion disease was met with enthusiasm since it seem to herald a means of detecting prions in asymptomatic livestock and humans, perhaps years before they manifest illness (1). It is noteworthy that prion infectivity was not detected in urine. Other approaches to developing antemortem tests include measuring
PrP<sub>Sc</sub> in muscle (9-11) and detecting the protease-sensitive (s) form of PrP<sub>Sc</sub> in serum of hamsters (12).

We report here that the Western blot signal of a protease-resistant molecule of ~33 kDa is present as previously described in the urine of prion-infected hamsters and is absent from the urine of uninfected controls (1). Unexpectedly, we found that this ~33-kDa molecule is not PrP but seems to be composed of IgG light chains.

**EXPERIMENTAL PROCEDURES**

**Animals and inocula**

Syrian hamsters (LVG:Lak) were inoculated intracerebrally with Sc237 prions. They were sacrificed after 60 to 65 days, their brains were collected and immediately frozen in liquid N<sub>2</sub>.

**Urine collection**

Urine from normal and diseased hamsters was collected overnight in metabolic cages (Rodent Metabolic Cage, Nalgene, Naperville, IL). In the morning, this urine was centrifuged at 500 × g for 5 min and then dialyzed against 0.85% NaCl with three changes as described (1). The dialysis bags were 6000 to 8000 molecular weight cut-off (MWCO), either CelluSep T2 (Membrane Filtration Products, Seguin, TX) or Spectra/Por (Spectrum Chemical). After this stage, the samples were aliquotted and kept at −80°C.

**Sample preparation**

Each urine sample was started with 2 ml of dialyzed urine from normal or ill Syrian hamsters. The urine sample was concentrated by methanol precipitation at a ratio of 1:10 (v/v), for 2 h at −20°C and centrifuged at 2500 × g for 30 min. The pellet was resuspended in 500 µl STE buffer (10 mM Tris-HCl, pH 7.5; 10 mM NaCl; 1 mM EDTA; 2% Sarkosyl) (1). Each sample was divided in two equal parts. One half was digested with proteinase K (PK; Gibco BRL, Carlsbad,
CA) at a final concentration of 40 µg/ml for 60 min in a water bath, at 37°C. The reaction was stopped with 2 mM phenylmethylsulfonyl fluoride (PMSF). The other sample was kept as an undigested control.

Ten percent (w/v) brain homogenates were prepared from hamsters killed in the terminal stages of disease. The brains were homogenized in phosphate buffered saline (PBS) without calcium and magnesium chloride (GIBCO, Invitrogen) with 2% Sarkosyl, by three, 15-sec strokes of a PowerGen homogenizer (Fisher Scientific International, Tustin, CA). The homogenate was centrifuged at 500 × g for 5 min and the supernatant was used for the preparation of the sample.

**SDS-PAGE/Western blotting**

The urine was analyzed on a 12% SDS-PAGE. We added an equal volume (250 µl) of 2× sodium dodecyl sulfate (SDS) sample buffer with 1.5 M urea to each sample. The samples were boiled for 7 min, then cleared by centrifugation for 1 min in the tabletop microfuge and 60 µl was applied to the gel. SDS-PAGE was performed in a 1.5-mm-thick, 12% polyacrylamide gel and then electrotransferred to a cellulose nitrate membrane (Hoefer/Pharmacia Biotech, San Francisco, CA). The membrane was blocked for 30 min with 5% nonfat milk in Tris-buffered saline with 5% Tween-20 (TBST) at room temperature. As suggested to reduce the background due to nonspecific IgG in the urine (1), we performed a second blocking with a cocktail of goat anti-rabbit IgG heavy and light chain (H&L) and rabbit anti-mouse IgG (H&L; Pierce, Rockford, IL), both diluted 1:3000 in TBST. We developed the blot with the primary monoclonal antibody (mAb) 3F4 (13), diluted 1:5000, followed by the secondary antibody goat anti-mouse IgG (H&L) conjugated to alkaline phosphatase (AP; Promega, Madison, WI), diluted 1:7500 in TBST. The substrate was the Lumi-Phos WB Chemiluminescent Substrate (Pierce). The blots were exposed to CL-Xposure Film (Pierce).
Electrospray ionization mass spectrometry (ESI-MS)

In-gel digestion was carried out by excising bands from the unstained half of the gel. After reduction and alkylation, gel slices were resuspended in approximately 25 µl of 5 ng/µl trypsin (Promega) and digested for 12 h at 37 °C. Peptides were extracted from gel slices using 50% acetonitrile, 2% formic acid; sample volumes were concentrated and resuspended in 0.1% formic acid. The extracted peptides were injected onto a reverse-phase column using a nanoflow (~350 nL/min) HPLC system (Eldex, Napa, CA). The column effluent was coupled directly via a fused silica capillary transfer line to a LTQ linear ion trap mass spectrometer (Thermo Finnegan, San Jose, CA) equipped with a nanospray ion source. LC runs of 50-min duration were monitored by sequentially recording the precursor scan (MS), a zoom-scan to allow for correct charge state assignment (narrow window MS) followed by collision-induced dissociation (CID) acquisitions (MS/MS). Automatic gain control was used to define acquisition times with the average for all three scan types taking approximately 1.5 seconds. Singly charged ions were excluded from CID selection. Normalized collision energies were employed using nitrogen as the collision gas.

For database searches, the Mascot Distiller Program (Matrix Science, Boston, MA) was used to create centroided peak lists from the raw spectra. These peak lists were submitted for database searching using an in-house Mascot server (Version 2.0, Matrix Science). In these searches, we allowed mass tolerances of ±2.0 Da for MS peaks and ±0.5 Da for MS/MS fragment ions. Samples were searched against all species entries in the UniProt (www.pir.uniprot.org, release date May 10, 2004) and nrNCBI (www.ncbi.nlm.nih.gov, release date May 10, 2004) databases. The modifications allowed in the searches included: oxidation of methionine residues, N-acetylation of proteins, pyroglutamic acid formation at the peptide N-termini, and up to two trypsin missed cleavages. Homology searches were carried out using the program MS-Homology (proteinprospector.ucsf.edu, in-house version 4.7).
RESULTS

Protease-resistant proteins in urine of hamsters

Urine samples were collected from normal and symptomatic Syrian hamsters. For comparison, we prepared brain homogenates from Syrian hamsters dying of prion disease. We evaluated these samples in parallel on a Western blot, which was developed with the 3F4 mAb (13), followed by immunostaining with a goat anti-mouse IgG-AP secondary antibody (Fig. 1A). A control gel was probed with only the secondary antibody (Fig. 1B).

At 29-kDa, a protease-sensitive band was present in the normal urine (Fig. 1A, lane 1) while urine from ill hamsters had two strong bands, at 29 kDa and 33 kDa, with the latter band resistant to limited PK digestion (Fig. 1A, lane 2). Interestingly, there was down shift in the molecular weight of the 33-kDa protein after proteolysis as noted previously (1). Brain homogenates from ill hamsters presented the usual pattern with the characteristic shift in molecular weight after limited proteolysis (Fig. 1A, lane 3).

Surprisingly, the same signals were detected on the control blot that stained with only the secondary antibody (Fig. 1B). The protease-sensitive, 29-kDa band, as well as the protease-resistant 33-kDa band, was found in the urine of both normal and diseased hamsters with the secondary antibody alone. In contrast, we detected neither full length PrPSc nor N-terminally truncated PrPSc (PrP 27–30) in diseased brain homogenates subjected to limited PK digestion using the secondary antibody alone (Fig. 1B, lane 6).

When we developed the Western blot with the goat anti-mouse IgG conjugated to horseradish peroxidase (HRP), the same pattern was observed, with a weaker signal corresponding to the 33 kDa band (Fig. 1C). This was likely due to the low concentration of antigen in the urine sample and the lower sensitivity of the HRP system.
**PrP signals were not detected using recombinant Fabs that bind PrP**

To characterize the 33-kDa band detected in the urine of ill hamsters, a Western blot was developed with two antibody fragments (Fab) known to react well with Syrian hamster PrP. These mouse Fabs were isolated by phage display (14) and then engineered into an expression vector that created a human–mouse (HuM) chimeras denoted HuM-D18 and HuM-P ((15,16), respectively). HuM-P binds to an epitope comprised of PrP residues 96–105 (16), which is adjacent to the 3F4 mAb epitope at residues 104–113 (17,18). HuM-D18 binds to an epitope in the region of PrP between residues 133–157 (15). Both Fabs were detected with an anti-human IgG secondary antibody.

HuM-P recognized PrP\textsuperscript{Sc} in hamster brain homogenates, but did not detect PrP in any of the urine samples (Fig. 2A, left panel). Similarly, the Fab HuM-D18 did not detect any PrP in the urine samples but clearly recognized PrP\textsuperscript{Sc} in brain homogenates (Fig. 2B, left panel). In contrast to the results presented in Fig. 1B, the goat anti-human IgG secondary antibody alone did not detect any PrP in the urine of hamsters (Fig. 2A and 2B, right panels).

**Mass spectrometric identification of kappa light chain peptides**

An SDS-PAGE gel was prepared as described above with urine from infected hamsters in parallel lanes. One lane was analyzed by Western blotting to establish approximate molecular weight. At approximately 33 kDa in an unstained parallel lane, a 20-mm segment was excised. From this segment, 10 equal bands were cut and numbered from 1 to 10 from lowest to highest molecular weight (bottom to top of the gel segment). These 10 samples were placed in individual tubes and subjected to in-gel reduction, alkylation, and digestion with trypsin. The 10 samples were sequentially analyzed by tandem mass spectrometry, resulting in approximately 5000 CID sequence spectra that were submitted for protein database searching. At this time, the databases used for these searches contain no hamster immunoglobulin sequences;
therefore, any matches are from completely homologous peptide sequences of other species. Four peptides extracted from band 4 were identified as belonging to immunoglobulin light chain from various species. Two of the spectra that gave unambiguous identifications for the peptides LLIYWASTR and DSTYSLSSTLTLTK are shown in Fig. 3.

To confirm that the peptide sequence is unique to immunoglobulin kappa chain, these peptides were submitted to a protein homology database search. With no species specified and allowing for isoleucine/leucine substitutions, 501 (I→L) and 1 (L→I) peptides from the nrNCBI database were found match the two sequences. All peptides appeared to be derived from immunoglobulin light chains from various species. None of the database searches or manual data analysis resulted in the identification of any PrP peptides even when using very low statistical significance threshold criteria ($p < 0.5$).
DISCUSSION

The development of antemortem tests for prion diseases is of utmost importance. Such tests would have immediate application in the routine testing of human blood and the testing of livestock, such as cattle and sheep, as well as free-ranging animals, including deer and elk. Thus, the report of a protease-resistant form of PrP in the urine of Syrian hamsters, cattle and humans with prion disease was met with considerable enthusiasm (1). Unlike PrP<sup>Sc</sup>, which is N-terminally truncated upon limited proteolysis, the protease-resistant PrP in urine was unaltered in size after limited PK digestion. Moreover, no prion infectivity could be recovered from the urine of Syrian hamsters infected with Sc237 prions.

Multiple attempts to identify the protease-resistant form of PrP reported to be present in urine of animals and humans with prion disease (1) were initially judged to be reproducible in our studies of urine collected from prion-infected Syrian hamsters. Western blots developed with the anti-PrP 3F4 mAb showed the presence of a PK-resistant protein with unconventional behavior in the urine of ill hamsters. However, when we analyzed the Western blot with the anti-mouse IgG secondary antibody alone, we detected the same signal. This indicates that the band detected on Western blot was not PrP as initially thought but is a protein or protein fragment that shares epitopes with mouse IgG. That the band is likely to be mouse IgG is supported by additional studies in which an anti-human IgG secondary antibody was used and no signal was detected. We also found no immunoreactivity with the anti-human IgG secondary antibody, which we used for the detection of two different anti-PrP recombinant HuM chimeric antibodies.

Among all antibodies used in our studies, only the anti-mouse IgG secondary antibody detected a protein band in the urine of prion-infected hamsters. This finding argues for cross-reactivity with a non-PrP hamster protein. Furthermore, we found by means of ESI-MS that this
protein band contains immunoglobulin light chain fragments. We suggest that this protein band might be Bence Jones protein. The Bence Jones protein is a mixture of aggregated kappa and lambda light chains from immunoglobulins (19). The molecular weight of the Bence Jones protein varies from low molecular weight fragments to 22-kDa monomers or 44-kDa dimers (20). It has been documented that the Bence Jones proteins or light chain immunoglobulins form amyloid structures, which can be stained with Congo red (21). Polymerization of such proteins in amyloid might explain the apparent protease-resistance of these urinary proteins.

It is unclear why Bence Jones proteins should accumulate in the urine of humans, cattle, and rodents infected with prions. Whether prions cause a nephrotic syndrome or CNS damage leading to inactivity that in turn results in a nephrotic syndrome remains uncertain.

The report of a protease-resistant PrP in urine prompted speculation that urine may carry infectious prions and that horizontal transmission of scrapie in flocks or CWD in herds of deer may have occurred through prion contamination of pastures via urine (22,23). To date, no one has convincingly identified prions in urine but a much more likely source of prions is fecal matter.

Miyazawa and co-workers reported finding protease-resistant PrP in urine of seven of nine CJD patients examined (24). Possibly, these investigators did not perform control studies in which they omitted the primary anti-PrP antibody. Furukawa and colleagues reported their experience with patients suffering from CJD, Alzheimer’s disease (AD) and non-dementing disorders as well as healthy controls. Twenty-nine of 38 CJD patients had protease-resistant IgG bands in urine, similar to results reported here for urine of Syrian hamsters inoculated with prions; two of 19 non-demented patients were also positive (25). None of the AD patients (n=20) and healthy controls (n=19) exhibited protease-resistant IgG bands in urine. Those findings and these reported here provide a compelling argument that if urine contains any protease-resistant
PrP, then the levels must be quite low. Moreover, it seems doubtful that measuring protease-resistant IgG bands in urine will form the basis for a useful diagnostic test for prion disease. Such measurements will be fraught with all the difficulties encountered by medical investigators who have attempted to use elevated levels of the stress protein 14-3-3 in cerebrospinal fluid as a diagnostic marker for prion disease (5,26,27).

In summary, we report the presence of protease-resistant immunoglobulin molecules in the urine of prion-infected Syrian hamsters. It remains to be investigated whether or not immunoglobulin fragments might serve as a surrogate marker for prion disease or other neurodegenerative diseases. Whether such a marker can be used as a diagnostic test that reliably reports the replication of prions long before clinical signs of neurologic dysfunction appear remains to be established.

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FIGURE LEGENDS

**Fig. 1.** Western immunoblot of PrP in urine of normal and prion-diseased Syrian hamsters, compared with brain homogenates of ill hamsters. (A) Western blot probed with mAb 3F4 followed by the goat anti-mouse IgG-AP secondary antibody. (B) Western blot probed only with the goat anti-mouse IgG-AP secondary antibody, used as a control. (C) Western blot probed with 3F4 mAb followed by the goat anti-mouse IgG-HRP secondary antibody. The minus (−) symbol designates undigested samples and the plus (+) symbol represents samples after limited PK digestion. Paired lanes are: (1, 4) urine from normal hamsters; (2, 5) urine from ill hamsters; (3, 6) 10% brain homogenates from hamsters with prion disease. Molecular weight markers based on migration of protein standards are shown in kDa.

**Fig. 2.** Western immunoblot of PrP from urine of normal and prion-diseased Syrian hamsters, compared to brain homogenates of ill hamsters. (A) Western blot probed with recombinant Fab HuM-P followed by the goat anti-human IgG (Fab´)2-AP secondary antibody. The right panel was blotted with the secondary antibody alone. (B) Western blot probed with recombinant Fab HuM-D18 followed by the goat anti-human IgG (Fab´)2-AP secondary antibody. The right panel was developed with the secondary antibody alone. The minus (−) symbol designates undigested samples and the plus (+) symbol represents samples after limited PK digestion. Paired lanes are: (1, 4) urine from normal hamsters; (2, 5) urine from ill hamsters; (3, 6) 10% brain homogenates from hamsters with prion disease. Molecular weight markers based on migration of protein standards are shown in kDa.

**Fig. 3.** Mass spectra of kappa light chain peptides. (A) Product ion spectra (MS/MS) used to identify the sequence LLIYWASTR, which matches the Uniprot entry for human Ig kappa chain. The probability that the matched sequence is a random event is 4.5e⁻⁰⁶. (B) Spectra used to identify the sequence DSTYSLSTLTLTK matching the nrNCBI database entry for mouse Ig
kappa chain. The peptide DSTYSSSTLTTLTK appears to be the most common homologue of this peptide, with 281 entries in the nrNCBI database almost exclusively part of light chain sequences. The probability that the matched sequence is a random event is $3.5 \times 10^{-5}$. Only selected ions are labeled for simplicity. The superscript $^\circ$ on b ions represents the loss of H$_2$O.
Figure 3

A
LLYWASTR

B
DSTYLSSTLTTLK

Relative abundance vs. m/z for LLYWASTR and DSTYLSSTLTTLK.