Prion Disease Detection, PMCA Kinetics, and IgG in Urine from Sheep Naturally/Experimentally Infected with Scrapie and Deer with Preclinical/Clinical Chronic Wasting Disease

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Prion diseases, also known as transmissible spongiform encephalopathies, are fatal neurodegenerative disorders. Low levels of infectious agent and limited, infrequent success of disease transmissibility and PrPSc detection have been reported with urine from experimentally infected clinical cervids and rodents. We report the detection of prion disease-associated seeding activity (PASA) in urine from naturally and orally infected sheep with clinical scrapie agent and orally infected preclinical and infected white-tailed deer with clinical chronic wasting disease (CWD). This is the first report on PASA detection of PrPSc from the urine of naturally or preclinical prion-diseased ovine or cervids. Detection was achieved by using the surrounding optical fiber immunoassay (SOFIA) to measure the products of limited serial protein misfolding cyclic amplification (sPMCA). Conversion of PrPC to PrPSc was not influenced by the presence of poly(A) during sPMCA or by the homogeneity of the PrP genotypes between the PrPSc source and urine donor animals. Analysis of the sPMCA-SOFIA data resembled a linear, rather than an exponential, course. Compared to uninfected animals, there was a 2- to 4-log increase of proteinase K-sensitive, light chain immunoglobulin G (IgG) fragments in scrapie-infected sheep but not in infected CWD-infected deer. The higher-than-normal range of IgG levels found in the naturally and experimentally infected clinical scrapie-infected sheep were independent of their genotypes. Although analysis of urine samples throughout the course of infection would be necessary to determine the usefulness of altered IgG levels as a disease biomarker, detection of PrPSc from PASA in urine points to its potential value for antemortem diagnosis of prion diseases.

Transmissible spongiform encephalopathies (TSEs), or prion diseases, are fatal neurodegenerative diseases associated with unconventional agents containing an aberrant isoform (PrPSc) of the cellular prion protein (PrPC). They include scrapie agent in sheep, bovine spongiform encephalopathy (BSE) in cattle, and Creutzfeldt-Jakob disease (CJD) in humans. A line of evidence suggests that BSE has been transmitted to humans; this disease is designated a new variant of CJD (vCJD). Various reports of experimental and natural transmission have shown that blood carries infectivity (1, 3, 19, 22). Secondary transmission of vCJD by blood transfusion raised a public concern about the safety of blood transfusion and blood-derived products (23, 25). Therefore, the development of a method for early and sensitive diagnosis is essential to control, and possibly prevent, disease transmission.

Detection of PrPSc is commonly used for the conclusive diagnosis of prion diseases. Although the highest concentration of PrPSc is present in the central nervous system, its presence has been reported with a variable degree of success in peripheral tissues, such as lymphoid organs, peripheral nerves, skeletal muscle, kidney, mammary glands, olfactory mucosa, and cerebrospinal fluid (1, 18, 31). Blood and urine represent the ideal biological fluids for routine diagnosis.

There have been several attempts to transmit TSEs from human and animal urine samples. Some of these have not been successful (2, 12), presumably due to the species barrier between the host source and test animals inoculated. More recent studies with urine from rodents and cervids have been more successful, albeit still limited and variable (14, 15, 17, 20, 28, 30). Two of these have reported infectivity in urine (28) and infectivity with PrPSc in kidneys of mice with simultaneous scrapie infection and nephritis but not in those with scrapie infection alone (17). Gregori et al. (14) reported that urine from clinically 263K-infected hamsters contained almost 4 infectious doses/ml of infectivity, and titration of kidneys and urinary bladders from the same animals gave concentrations 20,000-fold greater. However, histologic and immunohistochemical examinations of these same tissues showed no indications of inflammatory or other pathological changes except for occasional deposits of disease-associated prion protein in kidneys. To date, all of the urine studies involved urine collection from and examination of experimentally infected animals at the time of clinical disease.

There have been several studies on the detection and char-
acterization of the PrP isoforms in urine. Identification and characterization of human urine PrP\textsuperscript{C} using immunoprecipitation, electrophoresis, and mass spectrometry (8) demonstrated that urinary PrP\textsuperscript{C} (uPrP\textsuperscript{C}) is truncated mainly at residue 112 but also at other residues up to 122. Further, uPrP\textsuperscript{C} is glyco-sylated and carries an anchor which lacks the inositol-associated phospholipid moiety, indicating that uPrP\textsuperscript{C} is probably shed from the cell surface.

The detection of uPrP\textsuperscript{Sc} by Western blotting of prion-affected Syrian hamsters and human subjects was first reported by Shaked et al. (30). However, subsequent studies failed to detect PrP\textsuperscript{Sc} in urine from prion disease-affected individuals and demonstrated that the false-positive results arose from the cross-reaction of anti-mouse IgG with either contaminating bacterial proteins (11) or urinary IgG fragments (29). PrP\textsuperscript{Sc} has been identified by serial protein misfolding cyclic amplification (PMCA) in the urine of scrapie-infected sheep, hamsters, and mice and infrequently in both chronic wasting disease (CWD)-infected deer and transgenic mice (2, 13, 14, 20, 24). Further, it has been shown that a major protease-resistant protein in urine from prion disease-affected humans and animals is light chain IgG (15, 29). Following target amplification by PMCA or prion amplification–aggregation–fluorescent amplification catalyzed by T7RNA polymerase technique (Am-A-FACIT), PrP\textsuperscript{Sc} has been detected in blood of 263K scrapie strain-infected hamsters, scrapie-infected sheep, and deer with CWD (5, 6, 32).

Recently, PrP\textsuperscript{Sc} was detected in blood from scrapie-infected sheep and CWD-infected deer by using PMCA combined with the rapid and highly sensitive immunooassay known as suround optical fiber immunoassay (SOFIA) (27). In this report we extend those studies by demonstrating the detection of prion disease-associated seeding activity (PASA) in urine of symptomatic scrapie-infected sheep as well as CWD-infected preclinical or clinical white-tailed deer. To our knowledge, this is the first successful report on the detection of PASA in urine from both naturally infected clinical sheep and cervids at the preclinical stage of CWD. Interestingly, we also show that the levels of IgG are significantly increased in the urine of prion disease-infected sheep but not CWD-infected deer and that these proteins are, in contrast to previous reports, protease sensitive.

**MATERIALS AND METHODS**

**Animals, sample collection, and preparation.** All animal care and sampling protocols followed Institutional Animal Care and Use Committee (IACUC)-approved established protocols. In the case of the sheep studies, the facilities, animal care, and procedures used were approved by the University of Idaho IACUC (protocol 2003-53) and met federal recommendations found in the *Guide for Care and Use of Agricultural Animals in Agricultural Research and Teaching* (10). The white-tailed deer work was reviewed and approved by the Colorado Division of Wildlife IACUC (file number 07-2004) in compliance with U.S. Department of Agriculture regulations.

All scrapie-infected sheep were at the late stage of clinical disease at the time of urine collection. The clinical signs in sheep infected with scrapie collectively included pruritis, wasting, ataxia, bunny hopping, and recumbency (4), although each of the symptoms was not observed in every animal. Scrapie infection was confirmed by postmortem immunohistochemical staining of PrP\textsuperscript{Sc} from lymph nodes and brain. Unsedated sheep were placed in a sheep metabolism crate with access to water for up to 2 h. The urine was collected into a beaker and then strained through 4 layers of cheesecloth, placed in plastic bottles, and stored frozen. Separate crates were used for each animal, with the control sheep having their own crate which the scrapie-infected sheep never entered. Crates were cleaned between sheep.

White-tailed deer care and sampling protocols have been published previously (33, 34). Food, water, and supplements were provided ad libitum in all paddocks. At about 6 months of age, fawns were orally inoculated with about 0.5 g con-specific, pooled, infected brain material containing 6 μg PrP\textsuperscript{CWD} brain tissue per gram (26, 36). Urine samples were collected by holding deer in individual metabolic cages (1.5 m by 2.5 m) for two consecutive days; feed and water were provided ad libitum. During this period, about 100 ml of voided urine was collected daily, composited, and stored at −20°C for later analysis. Clinical signs of CWD included behavioral changes, loss of body condition, ataxia, and salivation or polydipsia. The five CWD-infected deer used for this study had PrP\textsuperscript{Sc} accumulation in tonsil biopsies by 253 or 343 days postinfection (p.i.) (36) and were confirmed to be prion infected at postmortem examination at 891 to 1,774 days p.i. Urine samples were collected from the five inoculated white-tailed deer at approximately 891 days p.i. At the time of sampling, one animal had end-stage clinical CWD, two were showing some loss of body condition, and the other two were clinically normal.

Table 1 describes and characterizes the animals used in the study as a source of urine and brain-derived PrP\textsuperscript{C}. All urine samples were first centrifuged at 400 x g for 2 min, and 500 μl of supernatant was used for initiating PMCA. Normal sheep and deer brain tissues were used in PMCA as the source of PrP\textsuperscript{C}. A 10% normal brain homogenate (NBH) from sheep and deer was prepared in phosphate-buffered saline (PBS) containing 150 mM NaCl, 1.0% Triton X-100, 4 mM EDTA, and Complete protease inhibitor cocktail (Calbiochem). The homogenates were then centrifuged at 400 x g for 2 min, and the supernatants were aliquoted and stored at −80°C.

**PMCA and SOFIA.** PMCA and SOFIA were carried out using a similar procedure as previously described (25). In brief, urine samples (500 μl) were mixed with 100 μl of 10% NBH (prepared in 1% Triton X-100, 4 mM EDTA, 1× protease inhibitor cocktail) and transferred into microcentrifuge tubes. For experiments utilizing poly(A), 300 μg poly(A) (poly(A) potassium salt; Sigma Chemical Co.) was added to the NBH prior to initiating PMCA. A single cycle of PMCA consisted of incubation (1 h at 37°C) with shaking followed by 36 pulses of sonication at 2.5 s each (90 s total; Sonics/VCT750 at 28-W power output). Prior to the start of each cycle, the reaction mixture was supplemented with an additional 100 μl of the NBH [with or without poly(A)]. After 10 cycles, 500 μl of the sample was transferred to a new tube and PMCA continued. At the completion of serial PMCA (40 cycles [PMCA\textsubscript{40}], 80 cycles [PMCA\textsubscript{80}], and 120 cycles [PMCA\textsubscript{120}]), 500 μl of the amplified product was immunoprecipitated (IP) with the MagnaBind protein G (Pierce)-monoclonal antibody (MAB) SE9 complex. Following IP, the sample was resuspended in 500 μl PBS, boiled, centrifuged, and analyzed by SOFIA using MAB 11F12 (5 μg/ml) as the capture reagent with biotinylated MAB SD6 (4 μg/ml) and streptavidin–rhodamine red X conjugate (Life Technologies) for detection.

**Western blotting.** Twenty-microliter urine samples or PMCA products were treated with proteinase K (PK; 100 μg/ml final concentration; 30 min at 50°C), followed by the addition of 1× protease inhibitor cocktail (Calbiochem). Sample buffer was added to a 1× final concentration (60 mM Tris- HCl [pH 6.8], 0.1% SDS, 100 mM dithiothreitol, 5% glycerol, and 0.01% bromophenol blue; pH 6.8). Samples were heated at 100°C for 10 min, microcentrifuged at 400 x g for 2 min, and electrophoresed (12% acrylamide gels) at 130 V. Following electrophoresis, the nitrocellulose membranes were blocked (3% bovine serum albumin in PBS; 1 h at room temperature), washed in PBS containing 0.2% Tween 20 (PBST), and incubated with MAB SE9 (1:5,000 in PBST; 1 h at room temperature). After washing three times in PBST, the blots were incubated with goat anti-mouse IgG Fab fragments conjugated to alkaline phosphatase (1:2,000 in PBST; 1 h at room temperature) and washed in PBST, and proteins were visualized following the addition of substrate (nitroblue tetrazolium/bromo-chloro-indolyl phosphate [NBT/BCIP]). The color reaction was allowed to continue until maximum signal intensity was achieved for the control samples (usually 5 to 15 min).

For the Western blot analysis of Ig in normal and infected deer and urine, 20-ml aliquots of pooled samples were dialyzed overnight at 4°C against normal saline. Following Speed-Vac (Savant) concentration, 10% Sarkanoy (in Tris-buffered saline [TBS], pH 7.4) was added to a 5-μg aliquot of each sample for a final Sarkanoy concentration of 2%. One-half of each sample was digested with 50 μg/ml PK (Sigma, St. Louis, MO) for 60 min at 50°C. The digestion was stopped by adding 1 mM phenylmethylsulfonyl fluoride. Following the addition of SDS-PAGE loading buffer and electrophoresis, immunostaining was performed using biotinylated rabbit anti-sheep or anti-deer IgG (KPL, Gaithersburg, MD), streptavidin conjugated to alkaline phosphatase, and with NBT/BCIP as the substrate.
Ig analysis. The isolation of deer IgG was carried out using 500 μl of the MagnaBind protein G beads (Pierce) and 100 μl of deer serum as per the manufacturer’s instructions. Elution of IgG was carried out with 0.2 M glycine-HCl, pH 2.5, followed by neutralization in 1 M Tris-HCl, pH 9.0.

For capture enzyme-linked immunosorbent assay (ELISA), the plates were coated with affinity-purified chicken anti-sheep IgG antibody or affinity purified chicken anti-deer IgG antibody (Gallus Immunotech, Fergus, Ontario) at a concentration of 0.5 μg/ml in PBS. After overnight incubation at 4°C wells were washed 2x with PBST and blocked by adding 200 μl of 1% casein in TBS, pH 7.4.

A 100 μl aliquot of dialyzed (24 h at 4°C with two changes of normal saline [0.9% NaCl]) urine diluted to 1:10 and 1:100 was untreated or PK treated (50 μg/ml PK, 60 min at 50°C), added to each well, and incubated for 1 h at room temperature. Wells were then washed five times with PBST followed by the addition of 100 μl of 0.1 μg/ml detection antibody (alkaline phosphatase-labeled rabbit anti-sheep IgG or anti-deer IgG; KPL, Gaithersburg, MD) for 1 h at room temperature. Wells were washed five times with PBST and, following the addition of 100 μl of p-nitrophenyl-phosphate (PNPP) solution, incubated at room temperature for 30 min. Optical density at 405 nm readings were compared to a standard curve prepared using serial dilutions of purified sheep IgG (Lampire Biological Labs, Pipersville, PA) or deer IgG ranging from 0.005 μg/ml to 5 μg/ml.

RESULTS

PrP analysis. Western blot analysis for PrP was performed on untreated and PK-treated urine samples from normal sheep and deer as well as clinically affected scrapie sheep in addition to preclinical and clinical CWD-infected deer. Western blot immunostaining using PrP-specific Mabs 11F12 or 8E9 revealed no detectable PrP isoforms in any of the urine samples from normal or scrapie-infected sheep urine and normal or CWD-infected deer (data not shown). PMCA was performed on the urine samples from both uninfected and infected sheep and deer. Western blot analysis of the PK-treated sPMCA products at the completion of both sPMCA80 and sPMCA120 did not reveal PrPSc immunostaining (data not shown).

In addition, PrPSc could not be detected in urine samples from infected sheep or deer by direct analysis using SOFIA. However, PASA in urine from scrapie-infected sheep could be measured by SOFIA of sPMCA products. As can be seen in Fig. 1, SOFIA was used to measure the levels of PrPSc at the

FIG. 1. Detection of PrPSc by SOFIA after increasing cycle numbers of sPMCA, using PASA from scrapie-infected sheep urine. Urine from normal uninfected (N1 to N4) and scrapie-infected (S5 to S8) was subjected to sPMCA for 40, 80, or 120 cycles. At the completion of sPMCA, the samples were analyzed for PrPSc as described in Materials and Methods. By comparison, the influence of poly(A) on the extent of PMCA was analyzed by also conducting sPMCA40 in the presence of poly(A) in urine from two normal (N3p and N4p) and two infected (S7p and S8p) animals. PrPSc detection by SOFIA was expressed as signal intensity of the sample-to-background ratio.

**TABLE 1. Description of samples**

<table>
<thead>
<tr>
<th>Animal group and sample no.</th>
<th>PrP genotype</th>
<th>Infection</th>
<th>Disease status*</th>
<th>Specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal sheep</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 AA136RR154RR171</td>
<td>NA*</td>
<td>NA</td>
<td>Brain**</td>
<td>Brain**</td>
</tr>
<tr>
<td>2 AA136RR154QQ171</td>
<td>NA</td>
<td>NA</td>
<td>Bone</td>
<td>Bone</td>
</tr>
<tr>
<td>3 AV136RR154OR171</td>
<td>NA</td>
<td>NA</td>
<td>Bone</td>
<td>Bone</td>
</tr>
<tr>
<td>Normal deer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 QQ95GG96AA116ψ</td>
<td>NA</td>
<td>NA</td>
<td>Brain**</td>
<td>Brain**</td>
</tr>
<tr>
<td>2 QQ95GS96AA116ψ</td>
<td>NA</td>
<td>NA</td>
<td>Bone</td>
<td>Bone</td>
</tr>
<tr>
<td>CWD-infected deer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 QQ95GS96AA116ψ</td>
<td>Exptl</td>
<td>Clinical (late stage)</td>
<td>Urine</td>
<td></td>
</tr>
<tr>
<td>4 QQ95GS96AA116ψ</td>
<td>Exptl</td>
<td>Clinical (early stage)</td>
<td>Urine</td>
<td></td>
</tr>
<tr>
<td>5 QQ95GS96ND116ND</td>
<td>Exptl</td>
<td>Clinical (late stage)</td>
<td>Urine</td>
<td></td>
</tr>
<tr>
<td>6 QQ95GS96AA116ψ</td>
<td>Exptl</td>
<td>Preclinical</td>
<td>Urine</td>
<td></td>
</tr>
<tr>
<td>7 QQ95GS96AA116ψ</td>
<td>Exptl</td>
<td>Preclinical</td>
<td>Urine</td>
<td></td>
</tr>
</tbody>
</table>

*Animal condition at the time of sample collection.  
b NA, not applicable.  
c Brain homogenate was used as the source of PrPSc for PMCA.  
d ND, not determined.  
e Experimentally infected via the oral route.  
ψ, pseudogene absent.  
ψ′, pseudogene present.
end of each 40-cycle interval up to a maximum of 120 cycles. Relative to the dilution of the original sample through the 120 PMCA cycles, the signal intensity obtained by SOFIA from scrapie-infected sheep urine increased by approximately 2 logs at the completion of each 40-cycle interval (sPMCA40, $10^2$; sPMCA 80, $10^3$; sPMCA 120, $10^4$). These values were unexpectedly consistent between samples from different clinical animals, suggesting that factors related to disease progression and independent of the individual urine samples play a role in PrPSc amplification. There was no appreciable increase in the SOFIA signal intensity from the control samples throughout the 120 PMCA cycles (Fig. 1), indicating there were no false-positive results and no generation of spontaneous PrPSc (32).

Studies using urine from normal and CWD-infected preclinical and clinical deer (Fig. 2) resulted in a pattern similar to the sheep studies. That is, increases in SOFIA signal intensity with urine samples from CWD-infected animals could be demonstrated as the number of PMCA cycles increased from 40 to 120. This pattern was consistent qualitatively and similar quantitatively for all urine samples regardless of whether or not there were matching genotypes between the source of PrPSc (AA136RR154RR171 versus AA136RR154QR171 for sheep PrPSc and QQ95GG96AA116 versus QQ95GG96AA116 for deer PrPSc) and the infected animals (Table 1; Fig. 1 and 2).

Interestingly, for both sheep and deer, a direct comparison of SOFIA results (without adjusting for the dilution effect relative to the original sample) from the PMCA products of the three amplified groups (1 to 40, 41 to 80, and 81 to 120 cycles) demonstrated that the PrPSc levels measured by SOFIA were relatively constant (Table 2). That is, the levels of PrPSc amplified as a result of the PMCA were offset by the sample volume dilution carried out by the completion of each 40-cycle interval (1:81 dilution for each of the amplified groups: 1 to 40 cycles, 41 to 80 cycles, and 81 to 120 cycles).

**TABLE 2. Direct SOFIA measurements of PrPSc from sheep and deer urine following PMCA**

<table>
<thead>
<tr>
<th>Animals</th>
<th>Health status</th>
<th>SOFIA signal intensity$^a$ after indicated no. of PMCA cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>40</td>
</tr>
<tr>
<td>Sheep</td>
<td>Normal</td>
<td>1.02 ± 0.39</td>
</tr>
<tr>
<td></td>
<td>Scrapie infected</td>
<td>3.12 ± 0.34</td>
</tr>
<tr>
<td>Deer</td>
<td>Normal</td>
<td>0.92 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>CWD infected</td>
<td>2.91 ± 0.2</td>
</tr>
</tbody>
</table>

$^a$ PrPSc generated from PASA in individual urine samples from the four groups (normal and infected sheep and deer) was measured by SOFIA after 40, 80, and 120 PMCA cycles. The direct SOFIA measurements from the individual samples (without accounting for the dilution effect relative to the original sample) in each of the four groups were pooled and are expressed as means ± standard deviations. Background SOFIA values after 40, 80, and 120 PMCA cycles with diluent alone were 1.01 ± 0.21, 1.04 ± 0.10, and 1.03 ± 0.09, respectively.

**FIG. 2.** Detection of PrPSc by SOFIA after increasing cycle numbers of sPMCA using PASA from urine of CWD-infected white-tailed deer. Urine from normal uninfected (N1 and N2) and CWD-infected sheep (CWD3 to CWD7) were subjected to sPMCA for 40, 80, or 120 cycles. At the completion of sPMCA, the samples were analyzed for PrPSc as described in Materials and Methods. By comparison, the influence of poly(A) on the extent of PMCA was carried out by also conducting sPMCA in the presence of poly(A) on two normal (N1p and N2p) and two infected (CWD6p and CWD7p) urine samples. PrPSc detection by SOFIA was expressed as the signal intensity of the sample-to-background ratio.

**FIG. 3.** Regression fit analysis of PrPSc detection in urine by SOFIA with increasing PMCA. Regression fits show the SOFIA data adjusted for dilution of sheep urine samples (solid line) and exponential fit [dashed line]) and deer urine samples (linear regression fit [solid line] and exponential fit [dashed line]) for 40, 80, and 120 PMCA cycles. The slopes for the linear fits are 0.0123 ± 0.0004 for sheep and 0.0164 ± 0.0009 for deer, while the growth constants are 0.0035 (sheep) and 0.0046 (deer), with $R^2$ values of 0.9948 (sheep) and 0.9876 (deer).
signal intensities that were measured after sPMCA_{40}, sPMCA_{60}, and sPMCA_{120} with respect to the actual measurements at the completion of each 40-cycle set as well as the amplification with respect to the dilution relative to the original sample. As shown in Fig. 3, the original sample did not have a sufficient concentration of PrP^Sc to produce a measurable SOFIA signal. Following sPMCA, the signal was measurable by SOFIA and the signal data plots of the sheep and deer samples were, respectively, fitted to linear as well as exponential functions. A measure of the appropriateness and accuracy of the representation is provided by the $R^2$ value that accompanies each function for each of the data sets. The sheep signal is represented by the linear function $y = 0.0126x + 2.6488$, with an $R^2$ value equal to 0.9989. The sheep signal is also accurately represented by the exponential function $y = 2.751 \exp(0.0035)$, with an $R^2$ value of 0.9948. In the case of the deer data, it is represented by the linear function $y = 0.0164x + 2.3163$, with an $R^2$ value of 0.9964, as well as by the exponential function $y = 2.4825 \exp(0.0046)$, with an $R^2$ value of 0.9876. As $R^2$ approaches a value of 1, the ideal situation for accuracy, we can see that the data are well represented and accurately summarized by the linear as well as by the exponential functions. Based on our data, it can be deduced that the amplification factor per PMCA cycle must have a low value, since for small arguments (the value of the exponent), an exponential function is virtually indistinguishable from a linear function.

**Immunoglobulins in normal and scrapie-infected sheep urine and normal and CWD-infected deer urine.** Histopathological examination of the kidneys was performed on formalin-fixed sheep tissue. No inflammatory or pathological changes of clinical significance could be found from either uninfected or infected clinical sheep (data not shown). All the urine specimens for this Ig analysis were the same original samples used for PMCA and SOFIA as described above. Western blot analysis using anti-sheep IgG antibody (Fig. 4) showed strong staining of heavy and light chain IgG bands that migrated at 55 kDa and 25 kDa in samples of pooled normal and pooled scrapie-infected sheep urine, respectively. The ratio of heavy versus light chain appeared to favor the light chain fragment. In addition, the immunostaining of IgG from scrapie-infected sheep urine appeared to be more intense than from normal sheep urine. Further, the IgG levels from both scrapie-infected and normal sheep urine were sensitive to PK digestion. Similar results were observed for the IgG obtained from normal and infected white-tailed deer, with significantly less intense immunostaining from the deer samples than for the sheep. These differences in IgG concentration were confirmed and quantified by ELISA of the same individual urine samples used for SOFIA. Within the scrapie-infected sheep urine group, the concentration of IgG varied by several orders of magnitude (40 to 2,000 μg/ml) but was consistently and significantly greater than the IgG levels in normal sheep urine (0.3 to 0.7 μg/ml) (Fig. 5). On the other hand, compared to sheep IgG, Western blot analysis of deer urine showed significantly less immunostaining of IgG, although the staining intensity in the CWD-infected sample was slightly greater than in the uninfected samples. The levels of IgG were quantitated by ELISA and supported the Western blotting data. The IgG levels from uninfected deer were 0.2 to 0.3 μg/ml, while in CWD-infected deer, the IgG concentrations ranged from 0.3 to 0.6 μg/ml (Fig. 5). Furthermore, similar to the sheep samples, the IgG from both infected and uninfected deer were PK sensitive (Fig. 4 and 5).

**DISCUSSION**

Attempts to demonstrate the TSE infectious agent in urine through transmission studies have been unrewarding as well as inconsistently and variably successful (2, 12, 14, 15, 20, 28). Similarly, reports on the detection of PrP^Sc in urine were initially met with controversy (20, 29, 30) but have become more, but not universally, convincing with the advent of PMCA.
Using the hamster model for prion disease and Western blotting for PrPSc detection, one group (24) reported the need for a minimum of 120 h (120 cycles) of PMCA while, using a modified protocol, a minimum of 480 h (240 cycles) of PMCA has also been reported (13).

Detection of PrPSc using PK-untreated sheep and deer blood samples has previously been reported with sPMCA followed by SOFIA (27). Chang et al. (7) demonstrated that in PK-untreated clinical sheep brain, PrPSc was detectable in the femtomolar range. Urine-derived PrPSc could not be detected by ELISA or Western blotting analysis of the IP products following sPMCA40 through sPMCA120. However, PrPSc could be readily measured by the highly sensitive SOFIA method.

Undoubtedly, the detection of PrPSc in urine from infected animals by ELISA or Western blotting would require many more rounds of PMCA, which would also increase the possibility of false-positive results (32).

Although implied, PrPSc has not yet been directly detected in urine from infected animals and, as such, cannot be assumed to provide the initial seed for PMCA. It should be noted that PASA may or may not be synonymous with urine containing PrPSc. Nevertheless, based on the limits of detection (LOD) previously reported for scrapie-infected sheep and CWD-infected deer brain PrPSc levels (7) and the SOFIA measurements of PrPSc following sPMCA40 in plasma from infected sheep and deer (27), we estimate that, compared to plasma, urine contains approximately 5- to 10-fold less PASA. This is in agreement with findings reported by Gonzalez-Romero et al. (13), who reported that PrPSc concentrations in urine from infected hamsters were approximately 10-fold lower than in blood.

By utilizing the PMCA protocol described in this report, dilution of the reaction mixture (which occurs with the addition of NBH at each cycle of PMCA) approximately matches the amplification from the PMCA process. This can be seen in Table 2, which shows the SOFIA signal strength as a function of cycles of PMCA for 40, 80, and 120 cycles. The underlying assumption of PMCA is that of the autocatalytic process captured in the prion hypothesis. Although the data are insufficient to determine the validity of this assumption, a numerical fit to the data is informative as to PMCA performance. Our results of linear and exponential regression analyses (following data correction based on the dilution due to the addition of the NBH at each cycle) showed that both regression fits match the data to a very good extent, as can be seen by the coefficient of determination, $R^2$, which in each case was close to 1. This indicates that these model fits are both good predictors of signal for a given number of cycles, within the range of data given. That exponential and linear models both fit the data this well indicates that the exponential model is a fit in the linear part of the exponential curve (i.e., during early times of exponential growth). As previously reported (7), the difference between the LOD for SOFIA and ELISA in the hamster model is a factor of $10^6$. The use of PMCA in our study required 40 cycles (a factor of 200 based on the linear and exponential fits shown in Fig. 3) to reach the LOD for SOFIA. If the underlying assumption is justified to the extent that these model fits are valid at longer times, then we can estimate the number of cycles it would take to amplify the PrPSc in the sample to the LOD for ELISA. If the amplification were linear, to achieve a $10^6$ increase would require $10^6 \times 40/200$, or 200,000 cycles of PMCA (or about 22 years, assuming our definition of a single cycle of 1 h). However, if the amplification were exponential, as per the prion hypothesis, then by the model fit the number of cycles required to achieve an amplification of $10^6$ would be about $\ln(10^6)/0.132$, or 104.6 cycles, or about 4.5 days. These numbers are optimistic, since we are measuring the exponential behavior under ideal conditions where the normal protein is always in excess. However, while this gives the optimal estimates for the growth constant, it does not take into account the saturation of growth one expects at higher concentrations of the resulting PrPSc product. While the linear fit is clearly not valid for estimates at long times or high concentrations, it is valid at the low concentrations at which the data were obtained. Thus, it yields a rough estimate of the concentration of PrPSc in the original sample. Since it took 40 cycles to reach the threshold for detection by SOFIA, which is in the attomolar range, we can make the approximation that these urine sample likely had initial concentrations of 0.005 attomol of PrPSc/ml.

Further, our studies at 120 cycles indicated that amplification does not follow an exponential increase as one would predict from the prion hypothesis.

By hypothesis there are competing processes occurring in PMCA. The amplification of PrPSc occurs through the autocatalysis of PrPSc, while the number of nucleation sites where this can happen is diminished by aggregation of the protein and hindered by competitive binding. These latter processes will only be important if the relative concentration of PrPSc is sufficiently high. These competitive effects would cause significant deviation of the growth curve from the ideal case for PMCA, the pure exponential growth of PrPSc, or $[\text{PrPSc}]_t = [\text{PrPSc}]_0 \exp(\alpha t)$, where $t$ = time and $\alpha$ is a constant to be determined experimentally.

Table 2 shows the signal from SOFIA of samples taken at 40, 80, and 120 cycles of PMCA as performed in this study. The data, which were not corrected for the dilution of the sample by the normal protein added at each cycle, show a small linear growth in signal. This indicates that the amplification in signal (PrPSc) approximately offset this dilution. A linear least squares fit of the data yielded slopes of 0.016 and 0.012 per cycle for deer and sheep, respectively, and correlation $R^2$ values of 0.9964 and 0.9989 (or well over 90% reliability).

That the growth and dilution nearly balanced each other indicates that the reaction is approximately occurring at a fixed concentration, which is ideal for determining the growth constant, $\alpha$, from the assumed exponential form. The data, adjusted for the sample dilution at each cycle, are shown in Fig. 1 and 2. The dilution factor for each set of 10 cycles is just the ratio of the initial sample to the total volume at which the sample was serially reduced after 10 cycles (see Materials and Methods), that is, 400 µl/1,500 µl, or 0.266. The growth rate to match this requires a gain of 3.75 every 10 cycles. From this, and assuming the ideal exponential growth form as given above, we estimate the growth constant $\alpha$ as 0.132 (where we are expressing time in cycles). It should also be noted that depending on the details of the aggregation process, the form of the interference to the amplification could also follow an exponential increase resulting in an apparent overall exponential growth curve with a smaller value of $\alpha$ than that for ideal, unobstructed amplification.
Gregori (14) reported PrPSc in the kidneys of scrapie-infected animals that had no evidence of tissue inflammation. This is in contrast to findings reported by Heikenwalder et al. (17) but supports our findings of no inflammatory or pathological changes in the infected sheep kidneys. Further, it was reported (30) that the proteins identified as uPrP are not a form of PrP but rather are Igs that have cross-reacted with the secondary antibody utilized to identify PrP. It has previously been reported (21, 29) that PrPSc cannot be detected in urine of symptomatic scrapie-infected hamsters, and instead Ig was identified that was specific to the diseased animals. This Ig was further characterized as PK-resistant IgG light chain fragments. In addition, Halimi et al. (16) reported that urine from CJD patients comprises an amyloidotic aggregate of PK-resistant light chain IgG and glycosaminoglycans, mostly chondroitin sulfate. This PK-resistant property seems to be related to the presence of glycosaminoglycans in the urine of these patients, since digestion of the samples with chondroitinase rendered light chain IgG PK sensitive. It is possible that the PK-resistant IgG previously reported and the PK sensitivity of the IgG stated in this report are due to factors including species specificity, sample processing, and aggregation or PK-protective proteins bound to IgG.

The ELISA developed in the present study demonstrated a quantitative analysis of IgG present in urine of scrapie-infected sheep and CWD-infected deer. With IgG levels as high as 2 mg/ml in scrapie-infected sheep urine, compared to 0.7 µg/ml in normal sheep urine, this approach distinguished normal from scrapie-infected sheep but could not differentiate normal and CWD-infected deer, in which much lower levels of IgG were detected. Williams and Young (35) reported that a significant feature of mule deer infected with CWD is a low urine specific gravity. Thus, this highly dilute urine may account for the low IgG values in the deer. However, the normal unaffected deer as well as infected preclinical animals also showed similar low urine IgG levels. The increased levels of Igs in urine of scrapie-infected sheep, as reported in this study for sheep and by Serban et al. (29) for hamsters, may be a consequence of metabolic and/or protein processing alterations resulting from the prion replication process. The accumulation of IgG in the urine of TSE-infected hosts may also indicate malfunction of the urinary system. Our results demonstrating a difference in Ig concentration that can approach 3 logs clearly indicate that a variation in Ig concentration between urine of scrapie-infected versus normal sheep, rather than unique protease resistance Ig properties, distinguish the urine of scrapie-infected sheep. The increase in light chain IgG deserves further investigation to determine why it is not a generalized prion disease phenomenon and whether it plays a role in the pathogenesis and/or is a dynamic biomarker for select TSE infections.

The findings in this paper point to the urine as a noninvasive sampling source for analysis of PASA and the detection of prion diseases. The presence of PASA in the urine from the natural sheep disease indicates what has already been implied from experimental infections reported in our manuscript and by others, that urine can serve as a natural route of infectious agent transmission. Exposure of surfaces to animal secretions and/or excretions can undoubtedly act as reservoirs for the infectious agent. Further, the detection of PASA in the urine of preclinical CWD-infected deer indicates that the spread of the infectious agent can begin in nonsymptomatic animals and therefore increases the risk for the continuous and unrealized spread of disease. Our studies may help in understanding the dynamics of horizontal spread of prion diseases in farmed and free-ranging animals.

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