diluted and subjected to SDS-PAGE and immunoblotting to semiquantitate the amount of sorbed protein. The binding capacity of a mineral was attained when subsequent PrP^{Sc} additions did not further increase the dilution factor required to reach the limit of immunoblotting detection (Table 1). Of the minerals examined, Mte exhibited the highest PrP^{Sc} adsorption capacity (~100 $\mu g_{protein}$ mg_{Mte}^{-1}). The adsorption capacity of the quartz microparticles was nearly 10-fold less (~15.6 $\mu g_{protein}$ $mg_{microparticle}^{-1}$), and that of Kte was nearly 100-fold less than Mte (~2 $\mu g_{protein}$ mg_{Kte}^{-1}). When expressed on a surface-area basis (Table 1), the adsorption capacities of Mte and quartz microparticles were indistinguishable by our measurement method; that of Kte was 25 times less. These data demonstrate that mineral surface properties contribute to differences in the amount of PrP^{Sc} bound.

PrP^{Sc} Desorbed from Mte Surfaces Is Cleaved

Unexpectedly, PrP^{Sc} desorbed from Mte surfaces exhibited a lower molecular mass (~27–31 kDa) than the starting material (~33–35 kDa) (Figure 1A). Neither contaminant proteases nor metal oxide coatings on Mte particles appeared responsible for PrP^{Sc} cleavage, as treatments to counteract each did not prevent cleavage (unpublished data). Prior to sorption experiments, Mte was boiled in a solution of 10 mM NaCl for 10 min to denature contaminant proteases, or binding experiments were performed in the presence of a cocktail of protease inhibitors to inactivate them. Neither treatment prevented PrP^{Sc} cleavage. Amorphous metal oxide coatings on clay mineral particles can alter their surface reactivities and could potentially be responsible for PrP^{Sc} cleavage. The size-fractionated Mte used in this study has been reported to not contain such impurities at levels detectable by X-ray diffraction analysis [23], and precautionary pretreatment of the clay with a buffered neutral citrate-bicarbonate-dithionate solution to remove metal oxide coatings [24] failed to prevent cleavage.

Prion protein desorbed from Kte and quartz did not exhibit a change in molecular mass (Figure 1A), suggesting that surface properties specific to Mte were responsible for the cleavage. Previous studies on protein interaction with Mte have not noted reductions in molecular mass upon desorption [25,26]. We incubated PrP^{Sc} with Mte for short time periods (1–15 min) to qualitatively investigate initial adsorption and cleavage kinetics. Adsorption of PrP^{Sc} to Mte was apparent within 1 min, and reduction in protein molecular mass was discernable (Figure 2A). Prion protein cleavage consistently occurred early within the first 15 min of contact with Mte and appeared maximal by 60 min. Cleavage of PrP^{Sc} caused by sorption to or desorption from Mte seemed to be a phenomenon specific to this protein. We examined sorption and desorption of scrapie-infected hamster brain homogenate (BH) to Mte. Desorption of brain proteins from Mte produced no changes in the overall molecular mass distribution as visualized by Coomassie blue staining (unpublished data). Subunit C2 of the 20S proteasome (~29 kDa), an unrelated protein similar in size to PrP likewise did not appear cleaved upon desorption from Mte (Figure 2B). In contrast, PrP^{Sc} in BH was cleaved (Figure 2C).

Cleavage of PrP^{Sc} involved loss of the N-terminal portion of the protein, which is not necessary for infectivity [3]. Prion protein desorbed from Mte lost immunoreactivity with an antibody directed against amino acids 23–37 on the protein N terminus, indicating that all or part of the epitope of this antibody was missing from the desorbed protein (Figure 2D).

Table 1. PrP^{Sc} Adsorption Capacities for the Minerals Examined[a]

Mineral	Binding Capacity (Sorbent Mass Basis) ($\mu g_{protein}$ $mg_{mineral}^{-1}$)	Binding Capacity (Sorbent Surface Area Basis) ($mg_{protein}$ $m_{mineral}^{-2}$)
Mte	87–174	2.8–5.7
Kte	1.7–2.6	0.15–0.22
Quartz microparticles	13.6–27.1	2.7–5.4

[a]Protein concentration determined by Bradford assay; PrP^{Sc} concentration was taken as 87% of total protein [45]. Reported adsorption capacities represent upper estimates, as the fraction of PrP^{Sc} in clarified preparations may have been lower.

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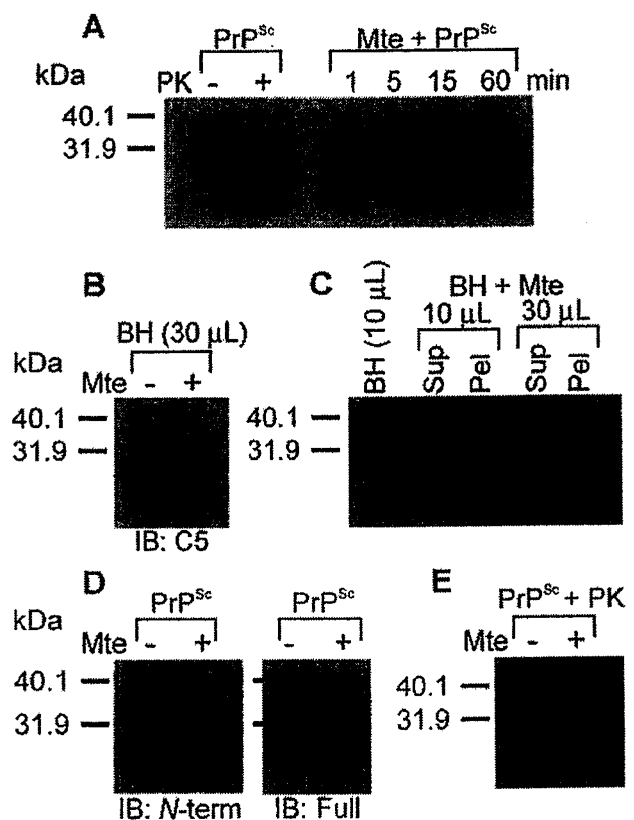

Figure 2. PrP^{Sc} Desorbed from Mte Is Cleaved

(A) PrP^{Sc} cleavage occurs after short contact times with Mte surfaces.
(B) The molecular mass protein C2 of the 20S proteasome subunit from BH was unaltered following desorption from Mte.
(C) Cleavage of PrP^{Sc} present in infected BH was apparent after desorption from Mte.
(D) PrP^{Sc} desorbed from Mte lost immunoreactivity against an antibody recognizing the N-terminal portion of the mature protein.
(E) PrP^{Sc} pretreated with PK bound to Mte and did not exhibit further reduction in molecular mass when desorbed.
Immunoblots (A, B, and E) used mAb 3F4. Immunoblots (C and D) employed anti-C2 and R20 polyclonal antibodies, respectively. Pel, PrP^{Sc} associated with pelleted mineral particles; Sup, unbound PrP^{Sc} in supernatant.

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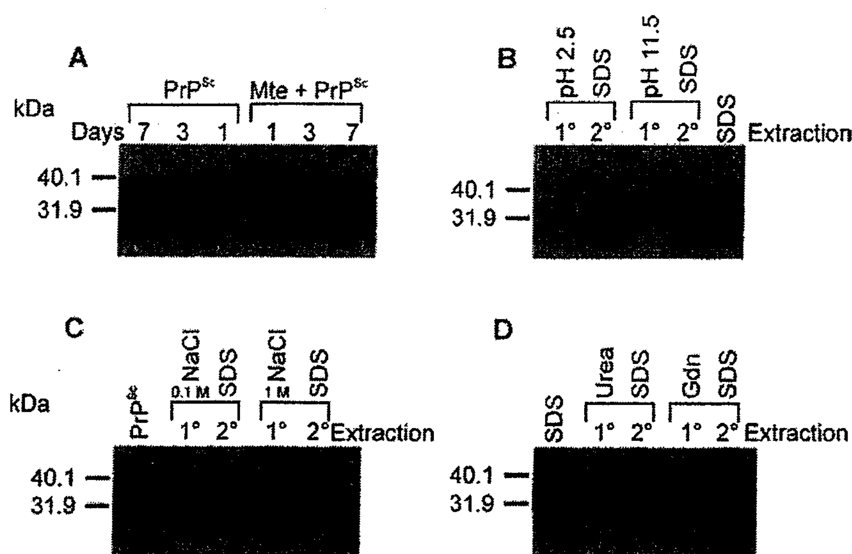

Figure 3. PrP^{Sc} Adsorbed to Mte Avidly and Remained Stable

(A) PrP^{Sc} was stable when adsorbed to Mte for at least 7 d. (B) Extremes in pH (100 mM phosphate at pH 2.5 or 11.5), (C) sodium chloride (100 mM or 1 M), and (D) chaotropic agents (8 M urea or 8 M guanidine [Gdn]) did not desorb detectable amounts of PrP^{Sc} from Mte. Primary extractions (1°) were followed by secondary extractions (2°) extractions with a 10% SDS solution at 100°C. Immunoblots (A–D) employed mAb 3F4. Pel, PrP^{Sc} associated with pelleted mineral particles; Sup, unbound PrP^{Sc} in supernatant.

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In contrast, probing identical samples with a polyclonal antibody against full-length PrP demonstrated that PrP^{Sc} was desorbed from the Mte. Although the precise cleavage site was not determined, these data suggest that the N terminus of PrP^{Sc} was removed; the fate of the cleaved amino acid residues is not known, as they may have remained bound to the clay or may have been extracted but not detected. When the N-terminal ~70 amino acids were removed from PrP^{Sc} by pretreatment with proteinase K (PK) prior to adsorption to Mte, we observed sorption to the Mte, but no further reduction in molecular mass upon desorption, evidence that other regions of the protein remain intact when associated with Mte (Figure 2E). These results also indicate that the N terminus of PrP^{Sc} is not necessary for adsorption to Mte.

Strength of PrP^{Sc} Binding to Mte

PrP^{Sc} attachment to Mte was avid, and sorbed PrP^{Sc} was stable. Washing Mte-PrP^{Sc} with the background solution used in sorption experiments did not induce detachment of detectable amounts of PrP^{Sc} from Mte (unpublished data). Contact of PrP^{Sc} with Mte for up to 1 wk did not result in additional degradation, indicating that the protein was not rendered more susceptible to cleavage by further structural rearrangements on the clay surface (Figure 3A). The strength of PrP^{Sc} attachment to Mte was surprising, even in light of reports of protein sorption-desorption hysteresis on mineral surfaces [26]. Conditions previously employed to desorb other proteins from soil minerals were largely ineffective in detaching PrP^{Sc} from Mte surfaces [26,27]. In our experiments, described above, a solution containing 10% SDS at 100 °C was used to remove the PrP^{Sc} from mineral surfaces. Changes in pH often alter interactions between clay surfaces and sorbed proteins [27,28]. Incubation of Mte-bound PrP^{Sc} in 100 mM phosphate buffer at pH 2.5 or 11.5, proton activities substantially higher and lower than the reported isoelectric points for PrP^{Sc} [29], failed to release the protein (Figure 3B). Likewise, increases in ionic strength (0.1 M or 1 M NaCl) failed to remove detectable PrP^{Sc} from Mte (Figure 3C). Strong chaotropic agents can be effective in desorbing proteins from soil minerals by disrupting hydrogen bonds [26]; however, neither 8 M urea nor 8 M guanidine released detectable amounts of PrP^{Sc} from Mte (Figure 3D). Our data indicate the interaction between PrP^{Sc} and Mte is strong and of high affinity.

PrP^{Sc} Bound to Mte Remains Infectious

Sorption of proteins to soil particles often results in structural rearrangements that cause loss or diminution of function [25,27,30]. If binding to Mte surfaces results in (partial) unfolding of PrP^{Sc}, a reduction or loss of infectivity would be expected, as denaturation renders the protein non-infectious [31]. We therefore tested whether PrP^{Sc} adsorbed to Mte remained infectious by intracerebrally inoculating hamsters with Mte-PrP^{Sc} complexes (Table 2). The time to onset of clinical symptoms after inoculation provides a measure of infectivity [32]. Hamsters inoculated with Mte-PrP^{Sc} exhibited clinical symptoms of scrapie 93 dpi. To control for any unbound prion protein that may have cosedimented with Mte particles, mineral-free PrP^{Sc} suspensions were processed in the same manner as in sorption experiments. The sedimented fraction of these control samples (mock pellets) showed substantially less infectivity than Mte-PrP^{Sc} pellets with a mean incubation period of 178 d, 105 d longer than Mte-PrP^{Sc} pellets. Hamsters inoculated with supernatants from these control samples (mock supernatants) showed clinical symptoms 103 dpi. Animals intracerebrally inoculated with Mte alone and uninoculated animals did not exhibit TSE symptoms during the course of the experiment (200 d).

Table 2. Prions Adsorbed to Montmorillonite Clay Retain Infectivity

Inoculum	Positive Animals/ Total Animals	Onset of Clinical Symptoms (dpi)[a]
None	0/8	>200[b]
Mte (no PrP^{Sc})	0/8	>200[b]
Mte-PrP^{Sc} complex	10/10[c]	93 ± 4[d]
Mock supernatant[e] (no Mte)	8/8	103 ± 0[d]
Mock pellet[e] (no Mte)	8/8	178 ± 21[d]

[a]Mean dpi ± SD to the onset of clinical symptoms of TSE infection.
[b]None of the animals showed clinical symptoms of TSE infection or had protease-resistant PrP accumulation at the termination of the experiment at 200 dpi.
[c]Although 12 animals were inoculated, two non-TSE intercurrent deaths occurred at 8 dpi.
[d]Brains of infected animals were positive for protease-resistant PrP.
[e]Mock supernatant and mock pellet samples were generated by adding clarified PrP^{Sc} (~0.2 μg) to buffer in the absence of soil minerals and processing identically to samples containing Mte.
DOI: 10.1371/journal.ppat.0020032.t002

Whole Soils Bind PrP^{Sc}

To examine the extent of prion protein binding by whole soils, we conducted PrP^{Sc} sorption experiments with four soils differing in texture and mineralogy (Table S2). When equal masses of soil (0.5 μg) were used, all soils bound PrP^{Sc} to a similar extent (Figure 4); no detectable PrP^{Sc} remained in the supernatant at the level of protein used in this experiment. Prion protein desorbed from the soils did not appear cleaved. Several nonmutually exclusive factors may have contributed to this finding, including (1) relatively small amounts of Mte in some samples, (2) occlusion of Mte cleavage sites by metal oxide and/or natural organic matter coatings, and (3) competition among the various sorption domains (both inorganic and organic) for PrP^{Sc}, limiting interaction with Mte. The amount of immunoreactive PrP^{Sc} recovered from each soil differed slightly; for example, the immunoreactive protein desorbed from the Elliot soil was less than that from the Boardman soil. This may have been due to stronger interaction of PrP^{Sc} with the Elliot soil than with the Boardman soil, leading to incomplete extraction, consistent with the larger fraction of clay-sized particles in the Elliot soil (Table S2).

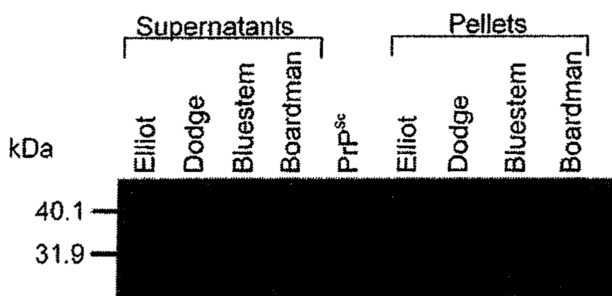

Figure 4. Whole Soils Bind PrP^{Sc}

Elliot, Dodge, Bluestem, and Boardman soils bound PrP^{Sc} (pelleted soils). No immunoreactivity (i.e., no unbound PrP^{Sc}) was detected in the supernatants. Immunoblot employed mAb 3F4.
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Discussion

Environmental transmission of prion diseases has been noted for decades [7,8,14]. In this study, we provide evidence indicating that soil and soil minerals serve as a reservoir of TSE infectivity. While extrapolation of in vitro studies to the environment must be made with caution, our findings suggest that PrP^{Sc} released from diseased animals may be sequestered near the soil surface, maintaining the TSE agent in an environmental medium with which livestock and wildlife come in contact. Our experiments demonstrate that Mte-bound PrP^{Sc} remains infectious and suggest that soil may harbor more TSE agent than previously assumed on the basis of water extraction of prions from garden soil [13].

Our results demonstrate that all soil mineral surfaces examined bound PrP^{Sc} and that Mte and quartz have larger specific binding capacities for PrP^{Sc} than does Kte (Figure 1). Although not relevant to TSE transmission, nonglycosylated, recombinant PrP^{C} has been shown to bind to Mte [33]. Interestingly, the N terminus of PrP^{Sc} desorbed from Mte was truncated (Figures 1A and 2). While Mte is known to catalyze several reactions, including the deamination of free glutamine and aspartic acid [34] and the polymerization of RNA into oligomers [35], protease activity has not been noted previously. The interaction between Mte and PrP^{Sc} is remarkably avid, as the only extractant used in this study that effected desorption was a solution containing 10% SDS at 100 °C (Figure 3B–3D). Prion protein appears unlikely to readily desorb from Mte in the environment. The propensity for PrP^{Sc} to tenaciously bind to Mte could be exploited in landfills to isolate prion-infected materials and prevent migration of the infectious agent.

The observation that prions remained infectious when bound to Mte is intriguing in light of the results of the desorption experiments; PrP^{Sc} adsorbed to Mte was extremely difficult to remove. Current mechanistic models for conversion of PrP^{C} to the pathological form require direct PrP^{C}–PrP^{Sc} interaction [36]. The brain is unlikely to possess microenvironments capable of extracting significant amounts of PrP^{Sc} from clay surfaces. The 10-d increase in incubation period for Mte-adsorbed PrP^{Sc} relative to clay-free controls (mock supernatant) was statistically significant ($p < 0.05$) and would correspond to approximately a 1-log increase in infectivity [32]. This result suggests that PrP^{Sc}-Mte complexes are inherently more infectious than the unbound protein and/or adsorption to Mte reduces clearance from the brain. We consider it likely that PrP^{Sc} adsorbed to Mte surfaces was available to convert PrP^{C} in the brain to the pathological isoform. Our findings are reminiscent of reports in which metal wires exposed to scrapie agent harbored significant infectious agent despite attempts to remove attached PrP^{Sc} [37,38].

The infectivity of soil- and soil mineral-sorbed PrP^{Sc} following oral exposure warrants investigation. The binding of PrP^{Sc} to soil particles could reduce oral bioavailability such that soil serves as a sink rather than a reservoir for infectivity. Conversely, association with mineral particles may protect the agent from degradation in the gastrointestinal tract, possibly enhancing transmission [39]. For example, bovine rotaviruses and coronaviruses retain infectivity via the oral route when bound to clay minerals [40]. While desorption of the protein from soil particles is more likely to occur in the

gut than in the brain, removal of PrP^{Sc} from mineral particles may not be necessary to initiate infection.

In conclusion, soil and soil minerals have the potential to bind PrP^{Sc} and maintain infectivity. These findings will serve as the basis for further study on the interaction of PrP^{Sc} with other soil components (humic substances, quartz, and other minerals), the stability of soil-bound PrP^{Sc} under typical environmental conditions (UV light, freeze-thaw cycles) and the effect of soil microorganisms and extracellular enzymes on protein integrity. Our current results suggest that sorption of PrP^{Sc} to clay minerals may limit its migration through the soil column. Maintenance of prion infectivity at the soil surface may contribute to the propagation of CWD and scrapie epizootics and enhance the likelihood of interspecies transmission of these diseases.

Materials and Methods

Preparation of soil minerals and soils. Montmorillonite (SWy-2) and kaolinite (KGa-1b) clays, obtained from the Clay Minerals Society Source Clays Repository (West Lafayette, Indiana, United States), were size-fractionated by wet sedimentation to obtain particles with d_h = 0.5–2 μm and saturated with sodium. These reference clay samples were extensively characterized previously [23,41]. Fine quartz sand (d_h = 125–250 μm) and SiO_2 microparticles (d_h = 1–5 μm; 99% purity) were obtained from Sigma (St. Louis, Missouri, United States). The fine quartz sand was soaked for 24 h in 12 N HCl to remove impurities. X-ray diffraction analysis and infrared photoacoustic spectroscopy indicated that the SiO_2 microparticles were composed of quartz.

We examined PrP^{Sc} sorption to four soils (Table S2). The Elliot soil was a silty clay loam purchased from the International Humic Substances Society (St. Paul, Minnesota, United States). Organically amended Dodge soil (sandy clay loam) was obtained from a glaciated upland area in Madison, Wisconsin. The Bluestem soil was a sandy clay loam collected from a fluvial deposit in Cedar Rapids, Iowa. The Boardman soil was a silt loam taken from an eolian deposit in Boardman, Oregon. Characteristics of these soils are presented in Table S2.

Source of PrP^{Sc}. Syrian hamsters (cared for according to all institutional animal care and handling protocols of the University of Wisconsin, Madison) were experimentally infected with the Hyper strain of hamster-adapted transmissible mink encephalopathy agent. PrP^{Sc} was purified to a P_4 pellet from brains of infected hamsters by a modification of the procedure described by Bolton et al. [42,43]. The P_4 pellet prepared from four brains was resuspended in 1 ml of 10 mM Tris (pH 7.4) with 130 mM NaCl. For experiments employing PK-treated PrP^{Sc}, 20% brain homogenate was treated with 50 μg ml^{-1} of proteinase K for 30 min at 37 °C. After blocking PK activity with 5 mM phenylmethylsulfonyl fluoride, purification was performed as above.

Batch sorption experiments. Larger prion aggregates were removed from purified PrP^{Sc} by collecting supernatants from two sequential 5-min centrifugations at 800 *g* (clarification step). Clarified PrP^{Sc} (~0.2 μg) was added to 500 μg of Mte or fine quartz sand, 1,500 μg of Kte, or 3.2 mg of quartz microparticles in 10 mM NaCl buffered to pH 7.0 with 10 mM 3-*N*-morpholinopropanesulfonic acid (MOPS) (500 μl final volume). In some cases, Mte experiments were conducted in unbuffered 10 mM NaCl. Sorption experiments with Mte performed in buffered and unbuffered 10 mM NaCl yielded comparable results. Experiments with Mte, Kte, and quartz microparticles each employed equivalent (external) mineral surface areas. In sorption experiments with whole soil samples, ~2 μg of clarified PrP^{Sc} was added to 5-ml suspensions of each soil (5 mg) in 5 mM $CaCl_2$. Samples were rotated at ambient temperature for 2 h or an indicated time period. Sorption appeared complete within 2 h, as longer incubation times did not result in changes in levels of bound protein.

Each PrP^{Sc}-mineral suspension and a 500-μl aliquot of each PrP^{Sc}-soil suspension was placed over a 750 mM sucrose cushion prepared in a solution of the same composition as the background solution in the sorption experiment, and centrifuged at 800 *g* for 7 min to sediment mineral or soil particles and adsorbed PrP^{Sc}. A sucrose cushion was found necessary to prevent a fraction of unbound PrP^{Sc} from sedimenting during centrifugation. Clarified PrP^{Sc} did not sediment through the sucrose cushion (Figure S1).

Unbound PrP^{Sc} remaining in the supernatant was precipitated with four volumes of cold methanol and resuspended in SDS-PAGE sample buffer (100 mM Tris [pH 8.0], 10% SDS, 7.5 mM EDTA, 100 mM dithiothreitol, and 30% glycerol). PrP^{Sc} was extracted from pelleted mineral particles with SDS-PAGE sample buffer at 100 °C for 10 min. The same procedure was followed for PrP^{Sc}-soil suspensions. To determine mineral adsorption capacities for prion protein, varying volumes of clarified PrP^{Sc} preparation were added to a 1:100 dilution of each mineral suspension. All adsorption experiments were repeated at least three times.

For BH sorption experiments, 10% BH was clarified by collecting supernatants from two sequential 5-min centrifugations at 800 *g*. Aliquots (10 or 30 μl) of clarified BH were rotated with Mte in 10 mM NaCl at ambient temperature for 2 h; complexes of Mte and BH constituents were then sedimented through a sucrose cushion and processed as described in the preceding paragraphs.

All samples prepared for SDS-PAGE were separated on 4%–20% precast gels (BioRad, Hercules, California, United States) under reducing conditions. Proteins were transferred to polyvinyl difluoride membranes and immunoblotted with mAb 3F4 (1:40,000 dilution), R20 N-terminal pAb (1:10,000 dilution), Rab 9 pool 2 full-length PrP pAb (1:10,000 dilution), or anti-20*S* proteosome subunit C2 pAb (1 μg ml^{-1}; A.G. Scientific, San Diego, California, United States). Detection was achieved with an HRP-conjugated goat anti-mouse immunoglobulin G (IgG) (BioRad) for mAb 3F4 and an HRP-conjugated goat anti-rabbit IgG (BioRad) for all pAbs.

X-ray diffraction analysis. PrP^{Sc} preparation (10 μg) was added to 50 μg of Mte in 10 mM NaCl (final volume of 0.5 ml). Samples were rotated at ambient temperature for 2 h and centrifuged at 16,100 *g* for 7 min. After centrifugation, the bulk of the supernatant was removed, leaving a small amount of solution above the clay pellet. The clay was resuspended in the remaining supernatant, and the slurry was placed on silica wafer slides and stored in a desiccator for over 12 h. The basal d_{001} spacings of near homoionic Na^+-SWy-2 before and after adsorption of PrP^{Sc} were determined by X-ray diffraction on a Scintag PAD V diffractometer (Cupertino, California, United States) using CuKα radiation and continuous scanning from 3° to 15° 2θ with a step size of 0.02° and a dwell time of 2 s.

Extraction experiments. PrP^{Sc} adsorbed to Mte was incubated for 30 min at room temperature in 8 M urea or 8 M guanidine HCl (50 μl per pellet), 0.1 or 1 M NaCl (25 μl per pellet), or 100 mM sodium phosphate (pH 2.5 or 11.5; 25 μl per pellet). Primary extractions with these solutions were followed by secondary extractions with SDS-PAGE sample buffer at 100 °C to assess the efficacy of the primary extraction. Urea and guanidine primary extracts were dialyzed against double distilled water for 2 h (nominal molecular weight cutoff, 12–14 kDa; Fisher Scientific, Pittsburgh, Pennsylvania, United States) prior to SDS-PAGE analysis.

Infectivity bioassay. PrP^{Sc}-Mte pellets prepared as above were resuspended in pH 7.4 PBS (50 μl per pellet) and intracerebrally inoculated into male, weanling Syrian hamsters (Harlan, Indianapolis, Indiana, United States). Equivalent amounts of PrP^{Sc} starting material or Mte without PrP^{Sc} were inoculated into control animals. Hamsters were monitored every 3 d for the onset of clinical symptoms [32,44]. Brains from clinically positive hamsters and uninfected controls were analyzed for protease-resistant PrP by immunoblotting.

Supporting Information

Figure S1. Sucrose Cushion Prevented Sedimentation of Unbound PrP^{Sc} under Conditions Necessary to Pellet Soil Minerals

A substantial amount of unbound PrP^{Sc} pelleted when centrifuged under conditions required to remove Na^+-Mte from suspension, but was prevented from sedimenting by a sucrose cushion. Sucrose cushions were therefore employed in batch sorption experiments to prevent sedimentation of unbound PrP^{Sc}. Results from representative mock adsorption experiments are shown. PrP^{Sc} was rotated in a solution of 10 mM NaCl in the absence of soil minerals for 2 h and was either placed above a 750 mM sucrose cushion and centrifuged (two right lanes), or centrifuged without a sucrose cushion (two left lanes). Supernatants (Sup) and pellets (Pel) were analyzed by immunoblotting with mAb 3F4.

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Table S1. Characteristics of Minerals Used in PrP^{Sc} Sorption Experiments

Found at DOI: 10.1371/journal.ppat.0020032.st001 (25 KB DOC).

Table S2. Characteristics of Soils Used in PrP^{Sc} Sorption Experiments
Found at DOI: 10.1371/journal.ppat.0020032.st002 (26 KB DOC).

Accession Numbers

The GenBank (http://www.ncbi.nlm.nih.gov/) accession number for PrP^{Sc} is M14054.

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Author contributions. CJJ, DM, JMA, and JAP conceived and designed the experiments. CJJ, KEP, and PTS performed the experiments. CJJ, KEP, PTS, DM, JMA, and JAP analyzed the data. JMA and JAP contributed reagents/materials/analysis tools. CJJ, DM, JMA, and JAP wrote the paper.

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Competing interests. The authors have declared that no competing interests exist.

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医薬品 研究報告 調査報告書

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研究報告の概要	使用上の注意記載状況・その他参考事項等
○慢性消耗病（CWD）に感染したシカの骨格筋におけるプリオン シカやエルクにおけるCWDの流行は広い地域に広がっており、ウシ海綿状脳症が変異型クロイツフェルト・ヤコブ病としてヒトへ種間伝播したことと同様に、CWDが人畜共通感染を起こすのではないかという懸念が起こっている。食肉の摂取が最も可能性の高い暴露の経路であるため、感染したシカ科の動物の骨格筋に感染性プリオンが含まれているかを明らかにすることが重要である。シカプリオン蛋白を発現したトランスジェニックマウスにおける動物実験で、CWDに感染したシカの骨格筋に感染性プリオンが存在することが明らかになり、CWDに感染したシカ肉を摂取あるいは取り扱う人はプリオンへの暴露のリスクがあることが示された。	解凍赤血球濃厚液「日赤」 照射解凍赤血球濃厚液「日赤」 血液を介するウイルス、細菌、原虫等の感染 vCJD等の伝播のリスク

報告企業の意見	今後の対応
CWDに感染したシカの骨格筋に感染性プリオンが存在することが明らかになり、CWDに感染したシカ肉を摂取あるいは取り扱う人はプリオンへの暴露のリスクがあることが示されたとの報告である。	今後も引き続き、プリオン病に関する新たな知見及び情報の収集に努める。

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Prions in Skeletal Muscles of Deer with Chronic Wasting Disease

Rachel C. Angers,[1*] Shawn R. Browning,[1*†] Tanya S. Seward,[2] Christina J. Sigurdson,[4‡] Michael W. Miller,[5] Edward A. Hoover,[4] Glenn C. Telling[1,2,3§]

Prions are transmissible proteinaceous agents of mammals that cause fatal neurodegenerative diseases of the central nervous system (CNS). The presence of infectivity in skeletal muscle of experimentally infected mice raised the possibility that dietary exposure to prions might occur through meat consumption (*1*). Chronic wasting disease (CWD), an enigmatic and contagious prion disease of North American cervids, is of particular concern. The emergence of CWD in an increasingly wide geographic area and the interspecies transmission of bovine spongiform encephalopathy (BSE) to humans as variant Creutzfeldt Jakob disease (vCJD) have raised concerns about zoonotic transmission of CWD.

To test whether skeletal muscle of diseased cervids contained prion infectivity, Tg(CerPrP) mice (*2*) expressing cervid prion protein (CerPrP) were inoculated intracerebrally with extracts prepared from the semitendinosus/semimembranosus muscle group of CWD-affected mule deer or from CWD-negative deer. The availability of CNS materials also allowed for direct comparisons of prion infectivity in skeletal muscle and brain. All skeletal muscle extracts from CWD-affected deer induced progressive neurological dysfunction in Tg(CerPrP) mice, with mean incubation times ranging between 360 and ~490 days, whereas the incubation times of prions from the CNS ranged from ~230 to 280 days (Table 1). For each inoculation group, the diagnosis of prion disease was confirmed by the presence of disease-associated, protease-resistant PrP (PrP^{Sc}) in the brains of multiple infected Tg(CerPrP) mice [see (*3*) for examples]. In contrast, skeletal muscle and brain material from CWD-negative deer failed to induce disease in Tg(CerPrP) mice (Table 1), and PrP^{Sc} was not detected in the brains of asymptomatic mice as late as 523 days after inoculation (*3*).

Our results show that skeletal muscle as well as CNS tissue of deer with CWD contains infectious prions. Similar analyses of skeletal muscle from BSE-affected cattle did not reveal high levels of prion infectivity (*4*). It will be important to assess the cellular location of PrP^{Sc} in muscle. Although PrP^{Sc} has been detected in muscles of scrapie-affected sheep (*5*), previous studies failed to detect PrP^{Sc} by immunohistochemical analysis of skeletal muscle from deer with natural or experimental CWD (*6*, *7*). Because the time of disease onset is inversely proportional to prion dose (*8*), the longer incubation times of prions from skeletal muscle extracts compared with those from matched brain samples indicated that prion titers were lower in muscle than in the CNS, where infectivity titers are known to reach high levels. Although possible effects of CWD strains or strain mixtures on these incubation times cannot be excluded, the variable 360- to ~490-day incubation times suggested a range of prion titers in skeletal muscles of CWD-affected deer. Muscle prion titers at the high end of the range produced the fastest incubation times, which were ~30% longer than the incubation times of prions from the CNS of the same animal. Because all mice in each inoculation group developed disease, prion titers in muscle samples producing the longest incubation times were higher than the end point of the bioassay, defined as the infectious dose at which half the inoculated mice develop disease. Although the risk of exposure to CWD infectivity after consumption of prions in muscle is mitigated by relatively inefficient prion transmission via the oral route (*9*), our results show that semitendinosus/semimembranosus muscle, which is likely to be consumed by humans, is a major source of prion infectivity. Humans consuming or handling meat from CWD-infected deer are therefore at risk to prion exposure.

Table 1. Incubation times after inoculation of Tg(CerPrP) mice with prions from skeletal muscle and brain samples of CWD-affected deer. PBS, phosphate buffered saline.

Inocula	Incubation time, mean days ± SEM (n/n_0)*	
	Skeletal muscle	Brain
	CWD-affected deer	
H92	360 ± 2 (6/6)	283 ± 7 (6/6)
33968	367 ± 9 (8/8)	278 ± 11 (6/6)
5941	427 ± 18 (7/7)	
D10	483 ± 8 (8/8)	231 ± 17 (7/7)
D08	492 ± 4 (7/7)	
Averages	426	264
	Nondiseased deer	
FPS 6.98	>523 (0/6)	
FPS 9.98	>454 (0/7)	>454 (0/6)
None	>490 (0/6)	
PBS	>589 (0/5)	

*The number of mice developing prion disease (n) divided by the original number of inoculated mice (n_0) is shown in parentheses. Mice dying of intercurrent illnesses were excluded.

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Supporting Online Material

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医薬品 研究報告 調査報告書

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研究報告の概要	使用上の注意記載状況・その他参考事項等
○CJD専門家が「ヒツジのBSE」を警告 非定型スクレイピーと呼ばれるヒツジやヤギの脳疾患は、BSEに似ており、2003年に流行が始まった。今では英国中で82,000頭ものヒツジが罹患していると見積もられており、他の欧州諸国でも症例が報告されている。 現在、生後18ヶ月を越えたヒツジ20,000頭に対しては毎年TSEの検査を行っており、今までに非定型スクレイピー108例が発見された。しかしvCJD専門家のDr. Stephen Deallerは、この疾患がどの程度まで広がっているかを把握するために、もっと若い動物に対して緊急に検査を行うよう求めている。彼は、農業への影響を懸念して大規模な検査が行えないのではないかと示唆している。Deallerは、政府が人への感染の危険があると認める6年前に、共同研究者とともにBSEに関して警告を発している。彼の調査要求は他の消費者団体からも支持されている。 現在の消費者保護規定では、BSEの感染性が高いと考えられる動物の部位(脳など)は流通工程から取り除かれる。しかし、非定型スクレイピーが他の部位から感染するかどうかは不明である。 政府に対して助言する独立科学委員会は人や動物の健康への影響について確実なリスク分析をするにはデータが不十分であると話した。海綿状脳症諮問委員会は、より多くの情報を提供するために綿密な調査が重要でありすぐに行うべきだと述べた。 食品基準庁(FSA)は今後この問題を検討する予定であり、「理論上は危険」があるとしながらも、消費者にヒツジやヤギの肉を食べないよう推奨することはしていない。 微生物学会の会長で食品基準の専門家であるHugh Pennington教授は非定型スクレイピーが人に害をもたらすとは言えないと話している。「人間は200年スクレイピーのヒツジを食べてきたが、誰も感染していない」	解凍赤血球濃厚液「日赤」 照射解凍赤血球濃厚液「日赤」 血液を介するウイルス、細菌、原虫等の感染 vCJD等の伝播のリスク

報告企業の意見	今後の対応
非定型スクレイピーと呼ばれるヒツジやヤギの脳疾患に関して、専門家が緊急に検査を行うよう求めているとの報告である。	今後も引き続き、プリオン病に関する新たな知見及び情報の収集に努める。

MedDRA/J Ver.9.0J

Sunday Herald – 05 March 2006

CJD expert warns of 'BSE in sheep'

Scientist who told of threat to humans from cattle calls for urgent study to find out how many animals have new disease

By Judith Duffy, Health Correpondent

A leading vCJD expert who sounded the alarm on BSE has called for the government to "take action right now" over fears that a recently discovered brain disease in sheep and goats could pose a risk to human health.

The disease, known as atypical scrapie, is similar to BSE in cattle and first emerged in 2003. It is now estimated that as many as 82,000 sheep could be infected in the UK and cases have been reported in other European countries.

The Food Standards Agency (FSA), has admitted there is a "theoretical risk" but it is not recommending that consumers stop eating sheep or goat meat.

However, vCJD expert Dr Stephen Dealler has demanded an immediate investigation to determine the extent of the disease. Lancaster-based microbiologist Dealler and his colleague Professor Richard Lacey warned the government about the dangers of BSE in cattle six years before ministers conceded there was a risk to humans.

"The worry is, of course, that atypical scrapie will be infectious to humans, but we don't know," Dealler said.

"All I can say at the moment is that with atypical scrapie, let's wait and see - but should we, in this wait-and-see period, be taking more aggressive action?

"Lots of people are saying we shouldn't just stand here and wait, lots of people are saying take action right now."

Under current regulations, 20,000 sheep in the UK over 18 months old are tested annually for brain diseases known as transmissible spongiform encephalopathies (TSE). These include atypical scrapie as well as the more common form of scrapie and BSE.

To date, a total of 108 cases of atypical scrapie have been detected via this testing programme. But Dealler called for further testing to be urgently carried out, particularly in younger animals, to determine exactly how widespread it is.

"At the moment, without the data on how much disease is out there, it is difficult to know what to do and how fast to act," he said. "That is why I say we need a survey right now.

"What they could certainly do is to do surveys and take so many sheep, test them when they are being slaughtered, and then see what proportion of those is atypical form.

"You can find BSE in the brains of cows long, long before they showed any symptoms at all and this will almost certainly be true with scrapie as well."

He suggested that concerns about the impact on farming were likely to be hindering an expansion in testing.

Current controls to protect consumers mean that parts of animals most likely to carry BSE infectivity - such as brains - are removed from sheep and cattle before entering the food chain. But it is uncertain if atypical scrapie could be carried in other tissue.

Dealler's calls for an investigation have been backed by consumer groups.

Sue Davies, Which? chief policy adviser, said: "We need urgent answers as to the many uncertainties surrounding this finding as quickly as possible so that there is a better understanding of whether there are any human health implications and, if so, whether existing control measures are adequate."

An independent scientific committee that advises the government said last week there is "insufficient data, as yet, to make reliable risk assessments for human health or animal health and welfare". In a statement, the Spongiform Encephalopathy Advisory Committee (Seac) also concluded that rigorous studies are "critical and urgent" to provide more information.

The FSA is due to initially examine the issue at a board meeting on Thursday . Possible options for precautionary risk reduction measures will be then discussed next month. An FSA spokeswoman said she could not pre-empt discussions by suggesting what - if any - measures might be taken.

"We can't rule out any theoretical risk, but we won't be changing our advice at this stage ," she said. "Based on the information we have, we are not recommending people change their eating habits on sheep or goats."

Professor Hugh Pennington, president of the Society for General Microbiology and an expert on food standards, said current evidence did not suggest atypical scrapie was a threat to humans.

He added: "The big question is: what implications does it have for human health? As far as we know, there are none basically, but of course we have to keep on doing research on this.

"One certain thing is that we have been eating scrapied sheep for 200 years and nobody has come to any harm."

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