Here, we present the results of the application of a real-time PCR assay for the diagnosis and follow-up of brucellosis in a large number of patients, and we discuss the potential pathophysiological significance of the findings.

MATERIALS AND METHODS

Patients. Peripheral blood specimens (both whole blood and serum) were collected from 39 patients who had received a diagnosis of acute brucellosis and from 50 healthy blood donors (control group). A minimum of 3 samples per patient were obtained (1 at diagnosis, 1 at the end of treatment, and at least 1 during the follow-up period). The control group, matched for age and sex, had no antibodies to *Brucella* species. All patients received a diagnosis of acute brucellosis during the period 2001–2004 in the University Hospital of Ioannina, reference center for the district of Epirus in northwestern Greece, where (like in nearby Greek and foreign regions) brucellosis is endemic [1, 10]. Epidemiological, clinical, and microbiological characteristics of the patient population are presented in table 1.

The diagnosis of acute brucellosis was established according to 1 of the following criteria: (1) isolation of *Brucella* species in blood culture or other clinical samples or (2) the presence of suggestive clinical characteristics together with the demonstration of specific antibodies at high titers, seroconversion, or an increase in antibodies in a serum sample obtained 15–20 days after the first sample was obtained.

The posttreatment phase varied from 2 to 36 months, according to the continuity of patient visits. Three patients experienced relapse during the follow-up period. Of the 39 study patients, 30 were treated with the standard regimen of doxycycline plus rifampin, 7 were treated with doxycycline plus ciprofloxacin, 1 was treated with moxalactamm, and 1 was treated with doxycycline plus streptomycin (the latter for 2 weeks). The duration of treatment was 6 weeks for all but 4 patients. Two patients were treated for 12 weeks (1 with doxycycline plus rifampin and 1 with doxycycline plus ciprofloxacin), and 2 patients were treated for 6 months (1 with doxycycline plus ciprofloxacin and 1 with doxycycline plus rifampin). Analysis of the evolution of the bacterial DNA load was performed at the time of initial diagnosis, at the end of treatment, and during the follow-up period (2, 6, 12–24, and 24–36 months after the end of treatment). The study underwent ethics review and approval.

Bacteriological and serological techniques. Serological tests—including rose Bengal plate (RBP) agglutination, Wright seroagglutination, and ELISA (Serion ELISA Classic Brucella IgG/IgM/IgA; Institut Virion\Serion; detecting IgM, IgG, and IgA antibodies)—were performed on all patient and control specimens; blood cultures were performed for 24 of the initial 39 patients.

The RBP agglutination and the Wright seroagglutination tests were performed in accordance with techniques described elsewhere [11]. The ELISA was performed in accordance with the manufacturer's instructions. Blood cultures were processed with BacT/Alert (bioMérieux) in accordance with standard techniques [12, 13] and were monitored for 10 consecutive

Table 1. Demographic characteristics and clinical and microbiological findings for patients with brucellosis.

Variable	Patients
No. of patients/no. of samples studied	39/130
Demographic characteristic	
Male	30 (77)
Female	9 (23)
Age, mean years (range)	41 (16–78)
Clinical characteristic	
Duration of symptoms, mean days (range)	32 (7–270)
Fever	36 (92.3)
Constitutive symptoms	23 (59)
Osteoarticular complications	16 (41)
Hepato/splenomegaly	7 (18)
Orchiepididymitis	3 (10)
CNS disorders	2 (5)
Diagnostic test result	
Titer ≥1:160, by Wright test	36 (92)
RBP test (from 2+ to 4+)	37 (95)
ELISA test	39 (100)
Proportion (%) of patients with positive blood culture results	13/24 (54)

NOTE. Data are no. (%) of patients, unless otherwise indicated. RBP, rose Bengal plate.

days. If the system failed to detect any growth, the vials were transferred to a conventional incubator for 10 additional days. Blind subcultures were performed on days 10 and 20 on *Brucella* agar (BBL; Becton Dickinson) and were incubated at 37°C in a 5%–10% carbon dioxide atmosphere for 3 days [12, 13]. If growth appeared, the suspected colonies were identified by colonial morphology; Gram staining; oxidase, catalase, and urease tests; and positive agglutination with specific antiserum. Identification and biotyping of *Brucella* species were performed in accordance with standard microbiological procedures [14].

Isolation of DNA from clinical blood specimens and bacteria strains. Peripheral blood samples were collected in EDTA tubes. DNA was extracted from whole blood (200 μL) with the QIAamp DNA Blood Mini Kit (Qiagen) in accordance with the manufacturer's instructions. The kit couples the selective binding properties of silica gels with a microcentrifugation step. The specimens were first lysed with protease in a buffer chosen to optimize the DNA-binding capacities on the QIAamp membrane. The use of the silica column allowed, after DNA coating, efficient washing of the samples to eliminate contaminants. After elution, DNA was stored at −80°C until PCR amplification analysis.

Hybridization probe–based quantitative real-time PCR assay. The real-time PCR assay was based on direct amplification of a 207–base pair DNA sequence of a gene that codes for the synthesis of an immunogenetic 31-kilodalton protein specific for the *Brucella* genus (BCSP31). The primer pair used was that published by Baily et al. [15]. The amplification product was detected by using fluorescence technique hybridization probes labeled with LightCycler Red 640 (detected in channel F2). A control amplification reaction in the third channel (F3) acted as an internal run control. A single-tube duplex LightCycler-PCR (LC-PCR) was performed using the FastStart DNA Hybridization Probes kit (Roche Diagnostics). To each LightCycler glass capillary, we added 20 μL reaction mixture containing 6.6 μL PCR-grade water, 2.5 mmol of magnesium chloride, 4.0 μL reagent mix (containing primers and probes), 2.0 μL FastStart mix, and 5.0 μL template (sample or standard). Primers and probes were designed and provided by TIB MOLBIOL. To detect any ampicon contamination or amplification failure, negative controls that contained 5 μL of PCR water instead of DNA and positive controls that contained DNA of *Brucella melitensis* biovar 1 were included in each real-time PCR run. Cycling conditions consisted of initial denaturation at 95°C for 10 min, followed by 55 cycles at 95°C for 10 s, at 55°C for 8 s, and at 72°C for 15 s in a LightCycler instrument (Roche Diagnostics). Fluorescence curves were analyzed with LightCycler software, version 3.5. After amplification, melting-curve analysis was performed to verify the specificity of PCR products (1 cycle consisted of 95°C for 20 s, 40°C for 20 s, and 85°C for 0 s). The BCSP31-derived product was identified by running the melting curve with a specific melting point of 67.5°C (concentration dependent). A standard curve, comprising 10-fold dilutions of *Brucella* BCSP31 DNA of 10^1–10^7 target equivalents, allowed quantification of unknown samples. In the provided standard row (TIB MOLBIOL), the lowest concentration (10 copies) was amplified in 35–36 cycles; 100, 10,000, and 1,000,000 copies were amplified after 32–33, 25–26, and 17–18 cycles, respectively (with the crossing point calculated by the "second derivative maximum"). With the method "fit points," the crossing point values were 32–33, 28–29, 22–23, and 15–16 cycles, respectively. The detection limit of the method spiked with serial dilution of *B. melitensis* DNA was 10 copies/5 μL DNA extract.

RESULTS

Brucella species was initially isolated in blood cultures from 13 (54%) of the 24 patients with available blood specimens. All strains isolated were identified as *B. melitensis* biotype 2. Of the 13 patients who had positive blood culture results, 12 also had positive results for all 3 serological tests used, whereas 1 had positive results only for 2 serological tests (RBP agglutination and ELISA). The remaining 26 (67%) of the 39 patients with acute brucellosis received their diagnoses on the basis of clinical and serological criteria. The RBP agglutination test result was positive for 37 patients (95%). The Wright seroagglutination test titers were within the diagnostic range (titers, >1:160) in 36 patients (92%). For the assessment of the ELISA results, samples with an optical density of 10% greater than the cutoff optical density were considered to be positive; samples from all 39 patients (100%) were positive. The types of antibodies detected by the ELISA were as follows for the 39 ELISA-positive samples: IgG, 27 (69%); IgA, 34 (87%); and IgM, 31 (80%).

All specimens obtained from 39 patients at initial diagnosis had positive real-time PCR assay results, conferring a sensitivity of 100%. All specimens obtained from the control group were negative for *B. melitensis*, conferring a specificity of 100%.

The evolution of the bacterial DNA load is shown in figure 1. The mean *B. melitensis* DNA load (±SD) for the 39 patients at the time of diagnosis was 803 ± 1236 copies/5 μL DNA extract (range, 26–4570 copies/5 μL DNA extract). At the end of treatment, samples from 34 patients (87%) remained positive for *B. melitensis*, with a mean bacterial DNA load (±SD) of 240 ± 314 copies/5 μL DNA extract (range, 0–1230 copies/5 μL DNA extract). Two months after the end of treatment, samples were collected from 34 patients, and the mean bacterial DNA load (±SD) was 192 ± 236 copies/5 μL DNA extract (range, 0–875 copies/5 μL DNA extract; results for 27 patients remained positive). Six months after the end of treatment, samples were collected from 26 patients, and the mean bacterial DNA load (±SD) was 96 ± 135 copies/5 μL DNA extract

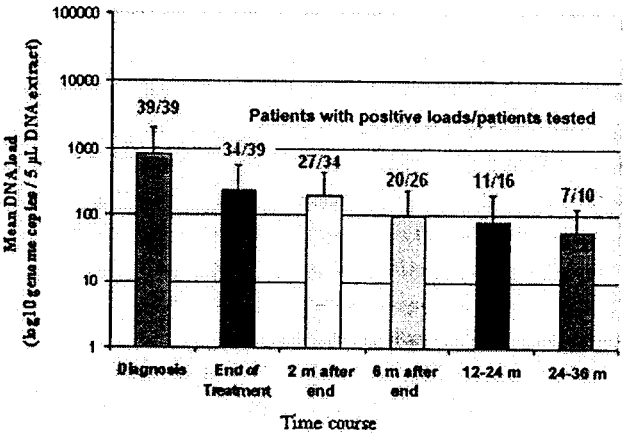

Figure 1. Evolution of *Brucella melitensis* DNA load at initial diagnosis and during the follow-up period. m, Months.

(range, 0–432 copies/5 μL DNA extract; results for 20 patients remained positive). Twelve to 24 months after the end of treatment, samples were collected from 16 patients, and the mean bacterial DNA load (± SD) was 80 ± 126 copies/5 μL DNA extract (range, 0–420 copies/5 μL DNA extract; results for 10 patients remained positive, and 1 patient had negative results for 2 consecutive specimens and then had a positive result but did not experience relapse). Twenty-four to 36 months after the end of treatment, samples were collected from 10 patients, and the mean bacterial DNA load (± SD) was 56 ± 74 copies/5 μL DNA extract (range, 0–220 copies/5 μL DNA extract; 7 patients continued to have positive results). Of 21 patients who were monitored for >1 year after therapy, 13 continued to have positive real-time PCR results but were asymptomatic. One patient had positive real-time PCR results 2 years after infection, although the patient had had negative real-time PCR results at the 1-year follow-up. Serological test results for these patients did not differ significantly between individuals with detectable and undetectable bacterial loads, with a mean Wright agglutination titer of 1:40, the presence of IgG antibodies determined by ELISA in all patients, and the presence of IgA antibodies in a minority of patients. During the follow-up phase of the study, only 3 patients experienced relapse. On relapse, 1 patient had positive blood culture results, whereas the other 2 relapses were diagnosed on the basis of clinical findings. In the 3 patients who experienced relapse, there was no increase in bacterial load during symptom reappearance, compared with their previous follow-up bacterial load measurement, although their titers did not decrease either. There were no statistically significant differences with regard to initial or posttreatment microbiological load between those who did and did not experience relapse. Results of blood cultures, when samples were obtained after treatment completion, were negative for all patients who did not experience relapse.

DISCUSSION

An emerging method for the detection and identification of a variety of infectious agents in the clinical laboratory is real-time PCR [16]. Real-time PCR was developed to improve the sensitivity, specificity, and speed of detecting PCR amplification products [17]. It does not require postamplification handling of PCR products, thereby reducing the risk of laboratory contamination and false-positive results. Moreover, real-time PCR has emerged as a powerful tool for quantification of the microbiological load; this is a concept that is valuable for numerous infectious disorders (e.g., hepatitis and HIV infection); real-time PCR has also blurred the traditional definitions of "eradication." Microbiological eradication (i.e., achievement of negative culture results) has long been used as an end point in clinical trials for several infections. However, these principles do not apply to a potentially chronic intracellular infection such as brucellosis. Therefore, microbiological load could serve as an indirect index of pathogen presence. One has to take into account, however, all of the potential problems of such an application; for example, isolation of DNA particles cannot discriminate between living and "eradicated" microorganisms. Furthermore, microbiological load could theoretically serve as an index of disease burden and thus allow for patient stratification by disease severity, relapse potential, and therapeutic regimens needed.

Our study revealed startling results. A significant number of patients continued to exhibit microbiological load even years after clinical cure and in the absence of any symptom indicative of disease persistence or relapse. We consider our results to be indicative of the long-term presence of viable bacteria in the human body in a cellular reservoir that needs further clarification. Could the results be explained as the outcome of particle shedding by dead bacteria? These dead bacteria could not be the result of antibiotic treatment administered <1 year earlier. Thus, in this case, bacteria indeed persisted for a long period after infection and clinical cure; these bacteria failed to elicit clinical manifestations, possibly because of a robust sustained immune response that eventually eliminates them by switching on the initially cancelled cellular apoptosis [18].

Even if we accept this alternative explanation, the only viable pathophysiological scenario is that, at least in the majority of patients, *Brucella* species persist inside the human body despite apparent clinical cure. The pathogen may replicate at low frequency. It may even cause transient, low-level bacteremia in a manner that can be handled by the body's immune system in such a way as to avoid the evolution of clinical disease. In that case, brucellosis should be considered to be only a chronic infection (much like tuberculosis), the clinical presentation of which depends on the equilibrium between the immune system and the microbial pathogenicity. Certain studies of patients with chronic brucellosis have indeed focused on such a dysregulation

[19]. The level at which this interaction occurs is vague; low-level bacteremia may not be detected easily, and follow-up through cultures of bone marrow specimens, theoretically sound and suggested to be diagnostically superior [20], is not convenient. Moreover, one could hypothesize that, with use of more-sensitive diagnostic techniques, it could be determined that patients with undetectable microbiological loads might actually have microbiological loads.

Contradictory results have emerged from the few relevant studies, with smaller sample numbers, that have recently been reported. Similar findings were elicited in a Spanish study [21] that showed that 4 of 7 individuals who experienced relapse and 3 of 11 who did not experience relapse also exhibited detectable bacterial loads after long-term follow-up. A similar result was also reported from a study in Peru [22] that used plain PCR for follow-up; the majority of the patients had PCR-positive samples even months after treatment completion. These results, however, were not reproduced in another Spanish study [23] that used a slightly different methodology. Two other real-time PCR studies did not evaluate evolution of the microbiological load during disease stages [24, 25].

One could support the hypothesis that patients with detectable microbiological loads were simply inadequately treated and were therefore candidates for relapse. However, the few who experienced relapse did not preferentially belong to the subgroup of patients with continually detected bacterial load. In addition, these patients did not exhibit higher loads or any statistically significant difference than did those of the group that did not experience relapse. Furthermore, relapses usually occur during the first few months after the end of treatment, and 90% of them usually occur during the first follow-up year, a cutoff point surpassed by our patients. No data can be extracted about whether a specific therapeutic regimen was related to long-term detectable microbiological loads because the majority of the patients were treated with the same regimen. In the Spanish study [21], numerous regimens were used, and the small statistical sample did not allow for any conclusions to be drawn.

One might argue that the significance of the study is marred by the fact that all patients with detectable DNA load during the follow-up period had negative results of blood cultures; thus, the PCR results may be considered to be dubious. Yet this fact (i.e., negative blood culture results) underlines the importance of our findings. If these patients had positive results of blood cultures, they would be considered de facto to have experienced relapse (reappearance of positive blood culture results is considered to indicate a relapse even in the absence of symptoms). These patients, however, met absolutely no criteria for relapse or disease in general, despite having detectable DNA load for *Brucella* species. Cases of reappearance of *Brucella* infection years after the initial course of symptoms are not rare, especially in the context of foreign body infection [26].

One might argue that there is selection bias in our results, because bacterial load was evaluated in only 10 patients in the 24–36-month period. However, the other study patients did not experience relapse, as assessed by telephone interview performed by 1 of the authors during this period; thus, the 10 patients for whom bacterial load data were available could be considered to be representative of the entire sample. Furthermore, a similar trend was observed among larger subsets of patients in the various follow-up time frames depicted in figure 1.

Recognizing that brucellosis is a chronic infection means that our understanding of the pathophysiology, diagnosis, and treatment of the disease may be drastically altered. First, one has to understand what suppresses *Brucella* species pathogenicity during the protracted posttreatment period when bacterial load is detectable. Moreover, one has to elucidate which are the critical components of this suppression and whether patients with chronic brucellosis exhibit a defect in these components. Second, one has to define whether the initial bacterial load is related to disease severity, tendency to relapse, or need for enhanced antibiotic treatment. Third, one has to seek other predictors of relapse, because real-time PCR did not exhibit any correlation in the present study. Finally, with regard to therapy, one has to redefine treatment goals. Eradication was never set as an issue in brucellosis, and the "acceptable" percentage of relapses was arbitrary [27]. The future question, however, will be whether to treat aggressively, with monitoring of the bacterial load, or to simply ignore the results and allow the pathogen to parasitize inside the human body.

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Potential conflicts of interest. All authors: no conflicts.

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研究報告の概要

○*Rickettsia felis*—ヒトへの新興世界的脅威

*Rickettsia felis*は、*TRG rickettsiae* (transitional group rickettsiae)に属する新興病原体である。*R. felis*は、イヌ、ネコ、齧歯類など世界中の様々な外部寄生生物から検出され、ネコノミは最も一般的な媒介生物と考えられている。ヒトにおける*R. felis*感染症の臨床症状は発疹熱やデング熱などに類似しており、ヒトの感染症は実際よりも少なく推定されている可能性が高い。ヒト症例は、1994年に第1例が米国で報告された後、ヨーロッパやアジアでも報告されている。*R. felis*は存続のためにノミだけを必要とする可能性があるが、ノミが媒介する紅斑熱リケッチアのライフサイクルにおける動物の役割についてはまだわかっていない。また、鑑別診断においては、ノミが媒介する紅斑熱を考慮すべきである。ヒトにおける*R. felis*感染症の実際の発現率、臨床症状の範囲、重症度を調べ、さらに公衆衛生への影響を評価するために、今後、試験を実施していく必要がある。

報告企業の意見	今後の対応
ネコノミが媒介する*Rickettsia felis*感染症のヒト症例は世界中で報告されているが、症状は発疹熱やデング熱などに類似しており、実際よりも少なく推定されている可能性が高く、今後調査が必要であるとの報告である。	日本赤十字社では、輸血感染症対策として問診時に海外渡航歴の有無を確認し、帰国(入国)後4週間は献血不適としている。また、発熱などの体調不良者を献血不適としている。今後も引き続き、新興・再興感染症の発生状況等に関する情報の収集に努める。

使用上の注意記載状況・その他参考事項等

合成血-LR「日赤」
照射合成血-LR「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

Rickettsia felis as Emergent Global Threat for Humans

Carlos E. Pérez-Osorio,* Jorge E. Zavala-Velázquez,* Juan José Arias León,* and Jorge E. Zavala-Castro*

Rickettsia felis is an emergent pathogen belonging to transitional group rickettsiae. First described in 1990, *R. felis* infections have been reported to occur worldwide in fleas, mammals, and humans. Because clinical signs of the illness are similar to those of murine typhus and other febrile illnesses such as dengue, the infection in humans is likely underestimated. *R. felis* has been found throughout the world in several types of ectoparasites; cat fleas appear to be the most common vectors. *R. felis* infection should be considered an emergent threat to human health.

Rickettsia felis is a member of the genus *Rickettsia*, which comprises intracellular pathogens that produce infections commonly called rickettsioses. Although the genus has no recognized subspecies, rickettsiae have traditionally been subdivided into 2 groups: the spotted fever group (SFG) and the typhus group. Infections produced by these 2 groups are clinically indistinguishable; however, groups can be differentiated by outer membrane protein OmpA (absent in the typhus group) and by vector. SFG members are transmitted by ticks; typhus group members, by fleas and lice (*1*,*2*). More recently, Gillespie et al. (*3*) added to this classification by designating the transitional group of rickettsiae and describing an ancestral group of rickettsiae.

In 1990, Adams et al. described a rickettsia-like organism, which resembled *R. typhi*, in the cytoplasm of midgut cells of a colony of cat fleas (*1*). The new rickettsia received the initial name of ELB agent after the company from which the fleas were obtained (El Labs, Soquel, CA, USA) (*4*). The first observations, such as reactivity with antibodies to *R. typhi* (*1*), the type of vector in which it was first discovered (*1*), and the apparent absence of OmpA (*5*), suggested that the new organism belonged to the typhus group of rickettsiae (*4*).

The molecular characterization of the organism described by Adams and reported by Bouyer et al. in 2001 provided sufficient evidence to support the designation of *R. felis* as a member of the SFG (*6*), and in 2002, La Scola et al. provided further characterization (*7*). One noticeable characteristic is the temperature-dependent growth of the bacterium, which requires incubation temperatures of 28°–32°C for optimal growth. However, the most striking characteristic of the novel rickettsia was the plasmid DNA in its genome (*8*).

World Distribution in Potential Host Vectors

Soon after the initial description of the typhus-like rickettsia, Williams et al. (*9*) reported that cat fleas collected from opossums in an urban setting in California were infected with the novel rickettsia, but no organism was detected in the tissues of the opossums. Since this report, this organism has been described in infected vectors from 20 countries on 5 continents (*9*). Not until 2002 did interest in *R. felis* increase, when the United States (*9*), Brazil (*10*), Mexico (*11*), and Spain (*12*) were among the first countries to describe cat fleas (*Ctenocephalides felis*) infected with *R. felis*. During the following 5 years, 28 additional reports appeared from all over the world (Table 1). These reports describe new potential vectors being infected with the emergent rickettsia, including the following: fleas, such as *C. canis* (*13–15*), *Anomiopsyllus nudata* (*16*), *Archaeopsylla erinacei* (*15*,*17*), *Ctenophthalmus* sp.

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Table 1. Potential vectors infected with *Rickettsia felis* reported worldwide, 1992–2007*

Year	Source of DNA sample	Animal†	Country	Reference
1992	*Ctenocephalides felis*	Opossum	USA	(*9*)
2002	*C. felis*	Cats and dogs	Brazil	(*10*)
2002	*C. felis*	Dogs	Mexico	(*11*)
2002	*C.felis*	Cats and dogs	Spain	(*12*)
2003	*Haemophysalis flava, H. kitaokai,* and *Ixodes ovatus*	Unknown (flagging)	Japan	(*19*)
2003	*C. felis*	Cats	France	(*22*)
2003	*C. felis*	Cats and dogs	UK	(*23*)
2004	*C. felis*	Dogs	Peru	(*24*)
2005	*Anomiopsyllus nudata*	Wild rodents	USA	(*16*)
2005	*C. felis*	Cats and dogs	New Zealand	(*25*)
2005	*C. felis*	Monkey	Gabon	(*26*)
2006	*C. felis* and *C. canis*	Dogs	Brazil	(*13*)
2006	*C. felis* and *C. canis*	Cats and dogs	Uruguay	(*14*)
2006	*Archaeopsylla erinacei* and *C. canis*	Hedgehog and rodents	Algeria	(*15*)
2006	*A. erinacei* and *Ctenophtalmus sp.*	Rodents and hedgehog	Portugal	(*17*)
2006	*Xenopsylla cheopis*	Rodents‡	Indonesia	(*18*)
2006	*C. felis, Rhipicephalus sanguineus,* and *Amblyommma cajennense*	Dogs and horse	Brazil	(*20*)
2006	Unknown flea	Gerbil	Afghanistan	(*27*)
2006	*C. felis*	Cats and dogs	Australia	(*28*)
2006	*C. felis*	Cats	Israel	(*29*)
2006	*C. felis*	Rodents	Cyprus	(*30*)
2007	Mites	Wild rodents	South Korea	(*21*)
2007	*C. felis*	Cats	USA	(*31*)
2007	*C. felis*	Cats	Chile	(*32*)

*PCR was used to detect *R. felis* infection with 1 noted exception.
†Animal host of potential vectors.
‡Quantitative PCR.

(*17*), and *Xenopsylla cheopis* (*18*); ticks, *Haemaphysalis flava* (*19*), *Rhipicephalus sanguineus* (*20*), and *Ixodes ovatus* (*19*); and mites from South Korea (*21*) (Table 1). Despite the large number of potential vectors reported, the only vector currently recognized is *C. felis* because it has been demonstrated that this flea is able to maintain a stable infected progeny through transovarial transmission (*4*). In addition, production of antibody to *R. felis* has been noted in animals after they have been exposed to infected cat fleas (*9*). Other evidence to be considered is the fact that 68.8% of the reports state that the cat flea is the most recurrent vector in which *R. felis* has been detected. These data further support the wide distribution of rickettsiae because they correlate with the worldwide distribution of *C. felis;* this distribution represents a threat to the human population because of lack of host specificity of the cat flea.

R. felis infection is diagnosed by PCR amplification of targeted genes. The genes most commonly amplified by researchers are *gltA* and *ompB*; followed by the 17-kDa gene. Also, 25% of published articles report that *R. felis* was detected by amplifying >2 genes, and all report that amplicons were confirmed as *R. felis* by sequencing. The animal hosts from which the infected ectoparasites were recovered represent a diversity of mammals (Table 1), which included 9 different naturally infested animal species. However, in 16 of 33 articles, ectoparasites were recovered from dogs. Other hosts for ectoparasites were cats (in 13 of 33 reports); rodents (5 of 33 reports); opossums and hedgehogs (2 reports each); and horses, sheep, goats, gerbils, and monkeys (1 report for each animal species).

In summary, the presence of *R. felis* in a diverse range of invertebrate and mammalian hosts represents a high potential risk for public health and the need for further studies to establish the role of ectoparasites other than *C. felis* as potential vectors. To date, whether any vertebrate may serve as the reservoir of this emergent pathogen has not been determined. However, preliminary data from our laboratory suggest that opossums are the most likely candidates.

World Distribution of Human Cases

In 1994, the first human case of infection with the new cat flea rickettsia was reported in the United States (*2*). This became the first evidence of *R. felis'* potential as a human pathogen. *R. felis* infection had a similar clinical manifestation as murine typhus (including high fever [39°–40°C], myalgia, and rash). Although the initial idea was that the murine typhus–like rickettsia had a transmission cycle involving cat fleas and opossums (*2,5,9*), no viable *R. felis* has yet been isolated from a vertebrate host.

Three more cases of *R. felis* infection were reported from southeastern Mexico in 2000. The patients had had contact with fleas or animals known to carry fleas. The clinical manifestations were those of a typical rickettsiosis: all patients had fever and myalgia; but the skin lesions, instead of a rash, were similar to those described for rickettsialpox. In addition, for 3 patients, central nervous system involvement developed, manifested as photophobia, hearing loss, and signs of meningitis (*33*).

As occurred with the fast-growing reports of the worldwide detection of *R. felis* in arthropod hosts, the reports of human cases of *R. felis* infection increased rapidly in the following years (Table 2). But, in contrast, only 11 articles reported human infection by *R. felis* compared with 32 that reported ectoparasite infection with the new rickettsia. Nevertheless, these findings indicate that an effective surveillance system is urgently needed to distinguish *R. felis* rickettsiosis from other rickettsial infections such as murine typhus and Rocky Mountain spotted fever, and from other febrile illnesses such as dengue. Although PCR is still a method of choice for many laboratories, its high cost prevents many from using the technique, particularly in developing countries. Important advances have been achieved in diagnostics, such as the recent establishment of a stable culture of *R. felis* in cell lines that allows its use as antigen in serologic assays differentiating the cat flea rickettsia from others. Use of this culture in the immunofluorecent assay has enabled detection of additional human cases (*38*).

The first autochthonous human case in Europe was reported in 2002, which demonstrated that *R. felis* has a potential widespread distribution and is not confined to the Americas. It also confirmed the risk for human disease anywhere in the world. After the first report in Europe of a human infection of *R. felis*, other human cases have appeared in other countries around the world, including Thailand (*36*), Tunisia (*38*), Laos (*39*), and Spain (*40*); additional cases have been reported in Mexico and Brazil (*34*). All the data support the conclusion that the incidence of *R. felis* rickettsiosis and the simultaneous worldwide distribution of the flea vector plausibly explain its endemicity.

At present, the involvement of domestic animals (e.g., dogs and cats) or wild animals coexisting in urban areas (e.g., opossums) maintains *R. felis* infection in nature. *C. felis* fleas serve as the main reservoir and likely have a central role in transmission of human illness.

Conclusions

R. felis is an emergent rickettsial pathogen with a worldwide distribution in mammals, humans, and ectoparasites. The clinical manifestations of *R. felis* infections resemble those of murine typhus and dengue, which makes them difficult to diagnose without an appropriate laboratory test. For this reason, infections due to this emergent pathogen are likely underestimated and misdiagnosed. Although *R. felis* may require only fleas for its maintenance in nature, we still do not know the role of animals in the life cycle of flea-borne spotted fever rickettsia. In addition, flea-borne spotted fever should be considered in the differential diagnosis of infectious diseases. Further research should be conducted to determine the actual incidence of *R. felis* infection in humans, the spectrum of clinical signs and symptoms, and the severity of this infection and also to assess the impact on public health.

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Table 2. Human cases of *Rickettsis felis* infection reported worldwide, 1994–2006*

Year	No. cases	Method	Country	Reference
1994	1	PCR	USA	(*2*)
2000, 2006	5	PCR	Mexico	(*33*)
2001, 2006	3	PCR	Brazil	(*34*)
2002	2	PCR/serology	Germany	(*35*)
2003	1	Serology (seroconversion)	Thailand	(*36*)
2005	3	Serology (Western blot)	South Korea	(*37*)
2006	8	Serology (IFAT/Western blot)	Tunisia	(*38*)
2006	1	Serology (seroconversion)	Laos	(*39*)
2006	33	Serology (IFAT)	Spain	(*40*)
Total	68			

*IFAT, indirect fluorescent antibody test.

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医薬品　研究報告　調査報告書

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一般的名称	別紙のとおり		研究報告の公表状況	ProMED-mail, 20080728.2306	公表国 オランダ	
販売名（企業名）	別紙のとおり					

研究報告の概要

問題点：オランダにおける調査において、2008年7月の時点でQ熱症例報告数が急激に増加している。

ブラバント州の公衆衛生局が行った調査では、2008年7月21日付けで491症例が報告されている。オランダ保健省によると、これはブランバント居住者5,000名が感染したことになる。Q熱は、ノールトブラバント州で急速に広がり、ナイメーヘン地域でもある程度広がった。感染症管理センター長であるRoel Coutinhoによると、実際の感染者数は報告された症例数の5倍というより10倍であると思われる。
労働党のブラバント州事務所は、この問題を評議会にかけた。評議会メンバーのNora Kasriouiによると、理事会がどう対処する予定かわからない。Kasriouiは、「Q熱は重篤な疾患であり、地域住民にとって、ますます大きな問題となっていて、政治的な働きかけが必要だと思います。」と述べた。Q熱には不明な点が多く、方針を打ち出し難いと認識しており、「団体は、違和感のある無しにかかわらず、いつでも経済的な援助やそれ以外の援助を受けることができます。」とも述べた。
Countinhoによると、Q熱を根絶することは不可能である。Q熱はヒツジの出産シーズン中からそれ以降に再発することが一般的であり、今のところ、ヤギが主な感染源であると考えられている。RIVM（国立衛生環境研究所）は、獣医学の専門家と共に、どのようにしてQ熱が動物から人に感染するのかを検討中であり、その後、詳細な予防対策が決定される。ちなみに、2007年までオランダにQ熱は存在しないも同然であった。

使用上の注意記載状況・その他参考事項等

記載なし

報告企業の意見	今後の対応
別紙のとおり	今後とも関連情報の収集に努め、本剤の安全性の確保を図っていきたい。

MedDRA/J ver.11.0

30

別紙

一般的名称	①人血清アルブミン、②人血清アルブミン、③人血清アルブミン*、④人免役グロブリン、⑤乾燥ペプシン処理人免疫グロブリン、⑥乾燥スルホ化人免疫グロブリン、⑦乾燥スルホ化人免疫グロブリン*、⑧乾燥濃縮人活性化プロテインC、⑨乾燥濃縮人血液凝固第Ⅷ因子、⑩乾燥濃縮人血液凝固第Ⅸ因子、⑪乾燥抗破傷風人免疫グロブリン、⑫抗 HBs 人免疫グロブリン、⑬トロンビン、⑭フィブリノゲン加第ⅩⅢ因子、⑮乾燥濃縮人アンチトロンビンⅢ、⑯ヒスタミン加人免疫グロブリン製剤、⑰人血清アルブミン*、⑱人血清アルブミン*、⑲乾燥ペプシン処理人免役グロブリン*、⑳乾燥人血液凝固第Ⅸ因子複合体*、㉑乾燥濃縮人アンチトロンビンⅢ
販売名(企業名)	①献血アルブミン20“化血研”、②献血アルブミン25“化血研”、③人血清アルブミン“化血研”*、④“化血研”ガンマーグロブリン、⑤献血静注グロブリン“化血研”、⑥献血ベニロンーⅠ、⑦ベニロン*、⑧注射用アナクトC2,500単位、⑨コンファクトF、⑩ノバクトM、⑪テタノセーラ、⑫ヘパトセーラ、⑬トロンビン“化血研”、⑭ボルヒール、⑮アンスロビンP、⑯ヒスタグロビン、⑰アルブミン20%化血研*、⑱アルブミン5%化血研*、⑲静注グロブリン*、⑳ノバクトF*、㉑アンスロビンP1500注射用
報告企業の意見	Q熱はリケッチアの一種コクシエラ・バーネッティ（*Coxiella burnetii*）による人畜共通感染症である。菌の大きさは0.2～0.4×1.0μmで、球菌の1/2～1/4である。感染源はおもに家畜や愛玩動物であるが、自然界では多くの動物やダニが保菌しており感染源となりうる。菌は感染動物の尿、糞、乳汁などに排泄され、環境を汚染する。ヒトは主にこの汚染された環境中の粉塵やエアロゾールを吸入し感染する。ヒトからヒトへの感染はほとんどおこらない。Q熱の患者は世界中で報告されている。日本では1999年4月から感染症法による届出が始まり、最近では2004年に7人、2005年に8人、2006年に2人の患者が報告されている。 Q熱の潜伏期は一般的には2～3週間で、感染量が多いと短くなる。発熱、頭痛、筋肉痛、全身倦怠感、呼吸器症状といったインフルエンザ様症状を示すが、主症状が肺炎、肝炎、あるいはその他の症状であったりと、その臨床像は多彩でQ熱に特徴的な症状や所見はない。また、患者の2～10%は心内膜炎を主徴とする慢性型に移行するといわれており、適切な治療をしないと致死率も高くなる。 本剤を含む当所で製造している全ての血漿分画製剤の製造工程には、約0.2μmの「無菌ろ過工程」および、本菌よりも小さいウイルスの除去を目的とした平均孔径19nm以下の「ウイルス除去膜ろ過工程」が導入されているので、仮に製造原料に本菌が混入していたとしても、これらの工程により除去されるものと考えられる。更に、これまでに本剤によるQ熱感染の報告例は無い。 以上の点から、本剤はQ熱感染に対して一定の安全性を確保していると考える。

*現在製造を行っていない

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Subject PRO/AH/EDR> Q fever - Netherlands (02): (NBR)

Q FEVER - NETHERLANDS (02): (NBR) (02)
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A ProMED-mail post
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[1]
Date: Fri 25 Jul 2008
Source: Agrarisch Dagblad [trans. from Dutch by Mod.AS, edited].
<http://www.agd.nl/1057422/Nieuws/Artikel/Forse-toename-meldingen-Q-koorts.htm>

A substantial increase in the number of reported Q-fever cases
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The number of reported cases of Q fever has risen sharply in recent weeks again [For the officially available data, indicating that the 2008 epidemic seems to have peaked by now, see the commentary. - Mod.AS]

The Public Health Service for Brabant had, in their last census on 21 Jul 2008, 491 known cases. That means that 5000 Brabanders have been actually infected, says the Ministry of Health. The disease spread rapidly in Noord-Brabant and, to a lesser extent, in the Nijmegen region. According to Roel Coutinho, head of the Centre for Infectious Disease Control, the actual number of victims is not 5-fold the number of reported cases but rather 10-fold.

The state branch of the Labour Party in Brabant has raised questions about the matter to the Executive Council. According to council member Nora Kasrioui, it is unclear what the directorate intends to do. "The disease is really a serious and growing problem for the population. We believe that the politics should go into action." Kasrioui acknowledges that it is difficult to make policies aimed at Q fever because much remains unclear about the disease. "Uncomfortable or not, organizations can always use help, financial or otherwise." [For the official government policy and background, see item 2].

According to Coutinho, the disease can never be fully eradicated. Normally it reappears during and following the lambing season. At present, goats are seen as the main source of infection. The RIVM (National Institute of Health and Environment), along with veterinary experts, is considering how the transfer from animal to man is established. Thereafter, a decision on further measures for disease prevention will be taken. Until last year [2007], Q fever was almost non-existent in the Netherlands.

[Byline: Jan Cees]

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[2]
Date: 10 Jun 2008
Source: Dutch government official document No VD. 2008/1191, "Measures for Q fever" [Trans. from Dutch by Mod.AS, edited]

<http://www.minlnv.nl/cdlpub/servlet/CDLServlet?p_file_id=28304>

A letter from the Ministers of Agriculture and of Health to the Parliament

Introduction

During the recent weeks, a significant increase in the number of Q-fever infections in humans has been observed again in the north-eastern region of the province Noord (north) Brabant. This has led to unrest among local people. With this letter we will bring you up to date with additional precautionary measures that we will undertake to prevent the spread of Q-fever as much as possible.

Q-fever

Q fever is a disease caused by the bacterium _Coxiella burnetii_. It is a zoonotic disease, which means that spread from animals to humans can take place. Q fever is traditionally present around the world and may affect many species -- not only farm animals but also species such as birds, dogs, cats, rats and wild animals. Ticks can be a vector in the transmission of Q fever between animals.

In particular, small ruminants are regarded as a major source of infection for humans. After excretion, the bacterium can survive a long time in the air and sometimes spread over long distances. People can be infected through various routes, including the inhalation of infectious, airborne particles. Human infection is often manifested by mild symptoms but a more serious course may occur.

The main clinical sign of Q fever in ruminants is abortion in pregnant animals, caused by the bacterium. During and after the abortion the animals excrete large quantities of the bacteria in their manure.

Small ruminants intended for milk production are held mainly in so-called pen barns. A pen barn is a shed where the manure is covered on a regular basis with a new layer of straw. When the mixture of manure and straw reaches a certain height, the shed is emptied. Especially during the manure removal process, bacteria are shed into the air with the consequent risk for both the public and animal health. Possibly, the spreading of manure on land is also a risk factor, but this procedure seems to be of less significance than the removal process of manure from the pen barns. This difference became apparent since manure from Noord Brabant farms has been used as fertilizer in other provinces without harmful results in humans.

Initiatives undertaken

Following the 2007 Q-fever outbreak in Herpen, Noord-Brabant, some steps were agreed between the Ministry of Health, Welfare and Sport (VWS) and the Ministry of Agriculture, Nature and Food Quality (LNV) to obtain better insight regarding the Q-fever problem and to prevent, as far as possible, its spread to man. In this framework, advisory information on the hygiene measures to be applied in small-ruminant farms has been prepared and published on the sites of the Health, Welfare and Sports Ministry, the Agriculture Ministry, and the Animal Health Service (GD).

Research by the Health Services has been undertaken in both large and small ruminant sectors to obtain better understanding of the extent of the problem. This research is funded by both sectors and by the government. There is also research under way into the risk factors for the spread of Q-fever.

The relevant research institutes, namely the National Institute of Health and Environment (RIVM), the Central Veterinary Research Institute (CVI) and the Health Service (GD) are also in the process of development and validation of testing methods suitable for the detection and identification of the bacterium.

Finally, a research initiative is ongoing regarding intervention strategies. Special attention is paid to a vaccine which is currently

being tested in Denmark and France, considering its possible experimental application in the Netherlands as well.

Designating Q fever as an infectious, reportable animal disease
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In order to be able to apply preventive and control measures on animal holdings, Q fever should be designated a reportable infectious animal disease. Indeed, this has been carried out by the Minister of Agriculture, adding Q fever to the list of animal diseases (including zoonoses) for which compulsory prevention, control and monitoring are regulated. Holders of small ruminants kept in pen barns are required to report signs which may indicate Q fever. This requirement obliges the veterinarians as well.

Measures regarding manure

Experts agree that manure probably plays an important role in the dissemination of the Q-fever agent in the province of Noord Brabant.

As a meaningful, provisional measure based on the precautionary principle, we plan to ban, for the duration of 3 months, the use of manure from small ruminant holdings in pen barns where serious infection has been established. A period of 3 months is regarded sufficient for a significant reduction of the infection load in the manure. Since the removal of manure from the pen barns is unavoidable as soon as the installation runs full, a practical solution is to be sought and finalized soon.

Other measures and consultations

In addition to the specific measures for the treatment of manure on infected holdings, further sector-related advice will be given in order to prevent future spread of Q fever. One of the ideas is to prescribe an advanced timetable for an earlier-in-season spreading of manure in the fields, preceding the lambing season. The aim is to prevent the utilization of the manure until at least 3 months after the lambing season, allowing significant reduction of its infection load.

Holdings with small ruminants are often frequented by recreation visitors and others interested. Contacts of people with infected premises are also undesirable. Temporarily preventing visits to such holdings seems to us advisable.

There are also a certain number of sheep and goat farms which produce their own cheese. This is often made with raw milk. The consumption of raw products from infected farms is discouraged by the RIVM (National Institute for Healthcare and the Environment). It seems therefore primarily useful to prescribe pasteurization in certain cases. The Minister for Health, Welfare and Sport will take these measures in consultation with RIVM.

With the above mentioned steps we try to limit, as far as possible, the spread of Q fever. The measures are aimed at the earliest possible action to diminish the risk of further spread. The development of the policy is being continued.

[Byline:
G. Verburg, Minister of Agriculture, Nature and Food Quality, and
Dr. A. Klink, Minister of Health, Leisure and Sport]

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[The above ministerial letter, addressed to the Dutch parliament, provides interesting and useful information on the epidemiology of the disease in the Netherlands and the preventive and control measures undertaken. It is also useful for those engaged in any handling of emergency situations related to zoonotic diseases. Hopefully, action plans and contemplated research will be accomplished according to plan.

In our previous posting (see ProMED archive below), data on the disease incidence from different media sources were inconsistent; we are grateful to Naomi Bryant, National Travel Health Network and

Centre (NaTHNaC), for drawing our attention to that. Official Q fever data for the first 28 weeks of 2008 (1 Jan - 23 Jul 2008) are available on the official website of the Public Health Service for Brabant (GGD Hart voor Brabant). The total number of reported human cases during the said period was 538. The 1st cases appeared during week 3, remaining under 10/week until the 15th week, when it began to rise, peaking during week 22 (72 cases). During the weeks 27-28, the number is again below 10; the outbreak seems to be dying out. The said data can be found (in Dutch) at <http://www.rivm.nl/cib/infectieziekten-A-Z/infectieziekten/Q_koorts/FAQ_Q-koor

According to the said website, prior to 2007 the mean annual number of human Q fever cases, on national level, was 15. Since the disease in animals was not reportable, there is no information on its incidence in animals during the said years. The source indicates that the main animal species responsible for the current outbreak are goats, followed by sheep. - Mod.AS]

[see also:
Q fever - Netherlands: (NBR) 20080725.2267]
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別紙様式第2

別紙(3)-4

医薬品　研究報告　調査報告書

識別番号・報告回数			報告日	第一報入手日 2008年9月9日	新医薬品等の区分 該当なし	厚生労働省処理欄
一般的名称	乾燥 pH4 処理人免疫グロブリン		研究報告の公表状況	Variant of Mad Cow Disease May Be Transmitted by Blood Transfusions, According to Animal Study http://www.hematology.org/media/08282008.cfm	公表国 英国	
販売名（企業名）	①サングロポール ②サングロポール点滴静注用 2.5g （CSL ベーリング株式会社）					

研究報告の概要

問題点（動物実験で輸血により vCJD が感染することが報告）
Blood Online の"Press Releases"に本研究の概要が報告された。しかし概要のため実験系の情報が少ないが、今回報告する。

英国グラスゴー大学獣医学部のヒューストン教授は、BSE とスクレイピーに感染したヒツジの輸血による感染について 9 年間研究している。
その結果、ヒツジ間において BSE とスクレイピーは、輸血により効率的に感染することが示された。特に、疾患の兆候が発現する前のドナーから採取された血液でも感染が伝播し、感染の後期ではより感染していた。
BSE ドナー群の感染した血液を投与されたヒツジ 22 頭のうち、5 頭が TSE の兆候を示し、3 頭は臨床症状の発現なしで、感染のエビデンスを示し、全体で 36%の感染率であった。
スクレイピー感染した血液を投与された 21 頭のうち、9 頭がスクレイピーの症状を発現し、全体で 43%の感染率であった。
これらの結果は、ヒトの輸血により vCJD に感染した 4 症例と一致している。
ドナーの感染期に加え、疾病感受性の遺伝的多様性や輸血成分などの要因が、ヒツジやヒトでの輸血による感染率に影響する。
BSE やスクレイピー感染したヒツジで、輸血による感染率は高い、特にドナーが感染後期の場合は高い。
以上の結果がヒトでの感染と一致していることから、輸血はこれらの疾患が効率的に感染する経路の代表であることが示された。
また、どの血液成分が重篤に感染するかを解明したり、切望されている診断試験を開発するために、BSE やスクレイピーに感染したヒツジの血液は効率的に利用されるであろうことが示された。

使用上の注意記載状況・その他参考事項等

報告企業の意見	今後の対応
これまで血漿分画製剤によって vCJD が伝播した報告はない。製造工程において異常プリオンを低減し得るとの報告があるものの、理論的な vCJD 等の伝播のリスクを完全には排除できないので、投与の際には患者への説明を十分行い、治療上の必要性を十分検討の上投与することを添付文書に記載し、注意喚起している。	今後とも新しい感染症に関する情報収集に努める所存である。

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Press Releases

Variant of Mad Cow Disease May Be Transmitted by Blood Transfusions, According to Animal Study

(WASHINGTON, August 28, 2008) – Blood transfusions are a valuable treatment mechanism in modern medicine, but can come with the risk of donor disease transmission. Researchers are continually studying the biology of blood products to understand how certain diseases are transmitted in an effort to reduce this risk during blood transfusions. According to a study in sheep prepublished online in *Blood*, the official journal of the American Society of Hematology, the risk of transmitting bovine spongiform encephalopathy (BSE, commonly known as "mad cow disease") by blood transfusion is surprisingly high.

BSE is one of a group of rare neurodegenerative disorders called transmissible spongiform encephalopathies (TSEs), and there is no reliable non-invasive test for detecting infection before the onset of clinical disease. In addition to BSE, these diseases include scrapie, a closely related disease in sheep, and Creutzfeldt-Jakob disease (CJD) in humans, which causes neurological symptoms such as unsteadiness and involuntary movements that develop as the illness progresses, rendering late-stage sufferers completely immobile at the time of death.

A new variant of CJD (termed vCJD) was recognized in the United Kingdom in the mid-1990s, apparently as a result of the transmission of BSE to humans. Because the symptoms of this disease can take many years to appear, it was not known how many people might have been infected, and without a reliable test for identifying these individuals, clinicians were very concerned that the infection could be transmitted between people by blood transfusion or contaminated surgical and dental instruments. As a result, costly control measures were introduced as a precautionary measure to reduce the risk of disease transmission, although at the time it was unclear whether there really was a significant risk or whether the control measures would be effective. This sheep study sought to better understand how readily TSEs could be transmitted by blood transfusion in order to help develop more targeted controls.

"It is vitally important that we better understand the mechanisms of disease transmission during blood transfusions so we can develop the most effective control measures and minimize human-to-human infections," said Dr. Fiona Houston, now a Faculty of Veterinary Medicine, University of Glasgow, UK, and lead author of the study.

The nine-year study conducted at the University of Edinburgh compared rates of disease transmission by examining blood transfusions from sheep infected with BSE or scrapie; the BSE donors were experimentally infected, while the scrapie donors had naturally acquired the disease. While scrapie is not thought to transmit to humans, it was included as an infection acquired under field conditions, which could possibly give different results than those obtained from experimentally infected animals. Because of the similarity in size of sheep and humans, the team was able to collect and transfuse volumes of blood equivalent to those taken from human blood donors.

The outcome of the experiment showed that both BSE and scrapie could be effectively transmitted between sheep by blood transfusion. Importantly, the team noted that transmission could occur when blood was collected from donors before they developed signs of disease, but was more likely when they were in the later stages of infection. Of the 22 sheep who received infected blood from the BSE donor group, five showed signs of TSEs and three others showed evidence of infection without clinical signs, yielding an overall transmission rate of 36 percent. Of the 21 infected scrapie recipients, nine developed clinical scrapie, yielding an overall transmission rate of 43 percent.

Investigators noted that the results were consistent with what is known about the four recorded cases of vCJD acquired by blood transfusion in humans. In addition to the stage of infection in the donor, factors such as genetic variation in disease susceptibility and the blood component transfused may influence the transmission rate by transfusion in both sheep and humans.

"The study shows that, for sheep infected with BSE or scrapie, transmission rates via blood transfusion can be high, particularly when donors are in the later stages of infection. This suggests that blood transfusion represents an efficient route of transmission for these diseases," said Dr. Houston. "Since the results are consistent with what we know about human transmission, the work helps justify the control measures put in place to safeguard human

blood supplies. It also shows that blood from BSE- and scrapie-infected sheep could be used effectively in non-human experiments to answer important questions, such as which blood components are most heavily infected, and to develop much-needed diagnostic tests."

Reporters who wish to receive a copy of the study or arrange an interview with lead author, Dr. Houston, may contact Becka Livesay at 202-776-0544 or rlivesay@hematology.org.

The American Society of Hematology (*www.hematology.org*) is the world's largest professional society concerned with the causes and treatment of blood disorders. Its mission is to further the understanding, diagnosis, treatment, and prevention of disorders affecting blood, bone marrow, and the immunologic, hemostatic, and vascular systems, by promoting research, clinical care, education, training, and advocacy in hematology.

Back to Press Release Listing

医薬品
医薬部外品　　研究報告　調査報告書
化粧品

識別番号・報告回数			報告日	第一報入手日 2008 年 7 月 14 日	新医薬品等の区分 該当なし	厚生労働省処理欄
一般的名称	乾燥濃縮人血液凝固第Ⅷ因子		研究報告の公表状況	Annals of Neurology 2008; 63(6): 697-708	公表国 アメリカ	
販売名（企業名）	コンコエイト-HT（ベネシス）					

研究報告の概要

＜目的＞
明確な組織病理および免疫染色所見によって特徴付けられ、また、通常のプリオン病とは異なりプロテアーゼ消化に感受性の高いプリオンたん白（PrP）の異常なアイソフォームに関連した新規プリオン病を報告する。
＜方法＞
National Prion Disease Pathology Surveillance Center において、11 名の被験者の臨床的、組織病理学的および免疫組織化学的特徴、遺伝型ならびに PrP の特徴を調査した。
＜結果＞
患者らは平均年齢 62 歳で行動的及び精神医学的症状を示し、その平均罹病期間は 20 ヶ月であった。海綿状変性のタイプ、PrP の免疫染色パターン、マイクロプラークの存在は、知られたプリオン病のものとは異なっていた。典型的なプロテアーゼ抵抗性 PrP は標準的診断では脳の新皮質に検出されなかった。異常 PrP は、濃縮すると通常のプリオン病の 16 倍低い濃度で検出された。それはプロテアーゼ抵抗性 PrP が約 4 倍低く、特徴的な電気泳動像を示した。検査した被験者は、National Prion Disease Pathology Surveillance Center によって評価された孤発性症例の約 3%である。数人の被験者は痴呆の家族歴を有したが、PrP 遺伝子のオープン・リーディング・フレームの変異は見つからなかった。
＜解釈＞
その明確な組織病理学的、PrP 免疫組織化学的、物理化学的特徴は、同じ遺伝型と併せて、以前に確認されていない、PrP が関わる疾患であることを示しており、われわれはこれをプロテアーゼ感受性プリオン病（PSPr）と名付けた。PSPr は、プリオン病の中では稀ではなく、われわれのデータが示すよりもさらに多い可能性がある。その理由は、PSPr の症例がアルツハイマー以外の痴呆症のグループに分類されている可能性があるからである。

使用上の注意記載状況・その他参考事項等

2. 重要な基本的注意
(1) 略
1) 略
2) 略
3) 現在までに本剤の投与により変異型クロイツフェルト・ヤコブ病（vCJD）等が伝播したとの報告はない。しかしながら、製造工程において異常プリオンを低減し得るとの報告があるものの、理論的な vCJD 等の伝播のリスクを完全には排除できないので、投与の際には患者への説明を十分行い、治療上の必要性を十分検討の上投与すること。

報告企業の意見	今後の対応
プロテアーゼ感受性のプリオンたん白と関連した新規プリオン病に関する報告である。 これまで血漿分画製剤によってvCJD、スクレイピー及びCWDを含むプリオン病が伝播したとの報告はない。しかしながら、万一vCJD感染者の血漿が本剤の原料に混入した場合には、製造工程においてプリオンを低減し得るとの報告があるものの、製剤から伝播する可能性を完全には否定し得ない。そのため、弊社の血漿分画製剤の製造工程におけるTSE感染性低減に関する検証実験を加速し、自社データを早期に取得し、工程評価を行い、必要に応じて工程改善を実施する予定である。	本報告は本剤の安全性に影響を与えないと考えるので、特段の措置はとらない。

ORIGINAL ARTICLE

A Novel Human Disease with Abnormal Prion Protein Sensitive to Protease

Pierluigi Gambetti, MD,[1] Zhiqian Dong, PhD,[1] Jue Yuan, BA,[1] Xiangzhu Xiao, PhD,[1] Mengjie Zheng, PhD,[1] Amer Alshekhlee, MD,[1] Rudy Castellani, MD,[2] Mark Cohen, MD,[1] Marcelo A. Barria, PhD,[3] D. Gonzalez-Romero, PhD,[3] Ermias D. Belay, MD,[4] Lawrence B. Schonberger, MD, MPH,[4] Karen Marder, MD,[5] Carrie Harris, BA,[1] James R. Burke, MD, PhD,[6] Thomas Montine, MD,[7] Thomas Wisniewski, MD,[8] Dennis W. Dickson, MD,[9] Claudio Soto, PhD,[3] Christine M. Hulette, MD,[10] James A. Mastrianni, MD, PhD,[11] Qingzhong Kong, PhD,[1] and Wen-Quan Zou, MD, PhD[1]

Objective: To report a novel prion disease characterized by distinct histopathological and immunostaining features, and associated with an abnormal isoform of the prion protein (PrP) that, contrary to the common prion diseases, is predominantly sensitive to protease digestion.

Methods: Eleven subjects were investigated at the National Prion Disease Pathology Surveillance Center for clinical, histopathological, immunohistochemical, genotypical, and PrP characteristics.

Results: Patients presented with behavioral and psychiatric manifestations on average at 62 years, whereas mean disease duration was 20 months. The type of spongiform degeneration, the PrP immunostaining pattern, and the presence of microplaques distinguished these cases from those with known prion diseases. Typical protease-resistant PrP was undetectable in the cerebral neocortex with standard diagnostic procedures. After enrichment, abnormal PrP was detected at concentrations 16 times lower than common prion diseases; it included nearly 4 times less protease-resistant PrP, which formed a distinct electrophoretic profile. The subjects examined comprised about 3% of sporadic cases evaluated by the National Prion Disease Pathology Surveillance Center. Although several subjects had family histories of dementia, no mutations were found in the PrP gene open reading frame.

Interpretation: The distinct histopathological, PrP immunohistochemical, and physicochemical features, together with the homogeneous genotype, indicate that this is a previously unidentified type of disease involving the PrP, which we designated "protease-sensitive prionopathy" (or PSPr). Protease-sensitive prionopathy is not rare among prion diseases, and it may be even more prevalent than our data indicate because protease-sensitive prionopathy cases are likely also to be classified within the group of non-Alzheimer's dementias.

Ann Neurol 2008;63:697–708

Human prion diseases or transmissible spongiform encephalopathies may be sporadic, inherited, or acquired by infection.[1] Creutzfeldt–Jakob disease (CJD) is the most common phenotype and occurs in all three forms. In the sporadic form, CJD is classified into five subtypes, which can be readily distinguished based on clinical features, type and distribution of brain lesions, and pattern of prion protein (PrP) immunostaining.[2,3] Fatal insomnia, a much rarer phenotype, includes sporadic and inherited forms, and is characterized by loss of ability to sleep and preferential thalamic degeneration.[4] Gerstmann–Sträussler–Scheinker disease (GSS), the third phenotype, occurs exclusively as a heritable disease invariably associated with a mutation in the PrP gene open reading frame (ORF) and is characterized by the presence of prion amyloid plaques.[4]

Despite their heterogeneity, all sporadic human prion diseases described to date have been associated with abnormal PrP (commonly called PrP^{Sc} but henceforth referred to as PrPr), which is resistant to treatment with proteases and is considered the diagnostic hallmark of these diseases.[1] PrPr is derived from normal or cellular PrP (PrP^{C}) via a posttranslational tran-

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sition from α-helical to β-sheet–rich conformations. PrP^C and PrPr are quite different. Whereas PrP^C is soluble in nondenaturing detergents and is completely digested when exposed to appropriate concentrations of proteinase K (PK), PrPr is detergent insoluble and its C-terminal region resists PK treatment.[5] Based on the size of their PK-resistant fragments, at least three major PrPr types are recognized, which codistribute with specific disease phenotypes: (1) PrPr type 1, which on PK treatment generates an approximately 21kDa fragment; (2) PrPr type 2, generating an approximately 19kDa fragment; and (3) PrP7-8, a PrP internal fragment of 7 to 8kDa.[4–6] Both PrPr types 1 and 2 have been observed associated with distinct subtypes of CJD. To date, PrP7-8 has been consistently observed only in GSS. Therefore, the conformational changes, which render PrPr pathogenic and in many but not all cases infectious, may engender different species or strains of PrPr that can be recognized based on their distinct protease-resistant fragments and by their associated clinicopathological phenotype.[5,7–12]

Studies mostly based on experimental models recently have shown that PK-resistant PrP (PrPr) is associated with varying quantities of a PrP isoform that, as PrPr, is detergent insoluble but sensitive to protease digestion (PrPs).[11–15] The relation of PrPs with PrPr and the role that PrPs plays in the pathogenesis of prion diseases remains uncertain.[16–18]

Here we report 11 patients with a human disease characterized by the presence of detergent-insoluble PrP that is predominantly sensitive to protease digestion and forms unusual immunohistochemical patterns. Furthermore, the small amount of PrPr present generates a distinct profile on immunoblot. Several affected patients have family histories of dementia but lack mutations in the PrP gene ORF. We refer to this condition as protease-sensitive prionopathy (PSPr). PSPr broadens the spectrum of human prion diseases and raises several important issues related to the nature of these diseases in light of their association with different PrP isoforms. Among prion diseases, PSPr is not rare. Because the presenting clinical signs often suggest the diagnosis of non-Alzheimer's dementia, PSPr may be even more prevalent than our data indicate because many PSPr cases might currently be classified within this group of dementias. Parts of this study have been presented previously.[19]

Subjects and Methods

Subjects

The 11 (10 autopsy and 1 biopsy) patients and the control subjects were referred to the National Prion Disease Pathology Surveillance Center between May 2002 and January 2006. Consent was obtained to use tissues for research, including genetic analyses.

General Tissue Processing

Fixed and frozen brain tissues were obtained from all subjects and processed as described previously.[20]

Histopathology and Immunohistochemistry

Samples obtained from up to 18 brain regions were processed as described previously.[2,3] Lesion profiles were constructed using semiquantitative evaluation of spongiform degeneration (SD) and astrogliosis in 12 brain regions from 6 subjects, and 4 or 5 regions from 2 subjects. SD and astrogliosis were scored (Fig 1), and the scores from each of the brain regions were summed for each subject separately; values were averaged, and standard deviations were determined and plotted according to the brain region.[2] Vacuoles with larger than 4μm diameter were measured individually on random photomicrographs of frontal neocortex (10/subject, ×180) using Spot-software version 4.6 after calibration (Diagnostic Instruments, Sterling Heights, MI). Sections from the frontal and occipital neocortices, hippocampus, basal ganglia, thalamus, cerebellar hemisphere, and midbrain were processed for PrP immunohistochemistry with the monoclonal antibody (Mab) 3F4 or 1E4 (Cell Sciences, Canton, MA).[2,20–23] Selected brain regions were also immunostained with the Mabs 4G8 to amyloid β.[24]

Electron Microscopy

Formalin-fixed postmortem brain tissue was processed for conventional electron microscopy and for PrP immunohistochemistry according to standard techniques using peroxidase-antiperoxidase Mab 3F4 to PrP.[25]

Molecular Genetics

The entire PrP ORF was amplified by polymerase chain reaction using genomic DNA extracted from unfixed brain tissue or blood and the primers PrPO-F [GTCATYATGGCGAACCTTGG (Y = C + T)] and PrPO-R [CTCATCCCACKATCAGGAAG (K = T + G)]; sequencing was done directly or after cloning into plasmid pSTBlue 1 (Novagen, Madison, WI) by automated sequencing.[22]

Prion Protein Characterization

CONVENTIONAL IMMUNOBLOT.

Five to 20μl 10% wt/vol brain homogenates with or without PK digestion (Sigma Chemical, St. Louis, MO) were loaded onto 15% Tris-HCl Criterion precast gels (Bio-Rad Laboratories, Hercules, CA) for sodium dodecyl sulfate polyacrylamide gel electrophoresis, and immunoblotted with 3F4 and 1E4 to human PrP residues 109 to 112 and 97 to 108, respectively.[23] PrP was deglycosylated with PNGase F (New England Biolabs, Beverly, MA) following manufacturer's instructions.

ENRICHMENT OF THE ABNORMAL PRION PROTEIN.

Two procedures were utilized: (1) capture of the abnormal PrP with the gene 5 protein (g5p), as described previously[13,23]; and (2) abnormal PrP precipitation with sodium phosphotungstate.[26]

SEDIMENTATION OF PRION PROTEIN IN SUCROSE GRADIENTS.

Brain homogenates were incubated with 2% Sarkosyl for 30 minutes on ice, loaded atop a 10 to 60% step sucrose gra-

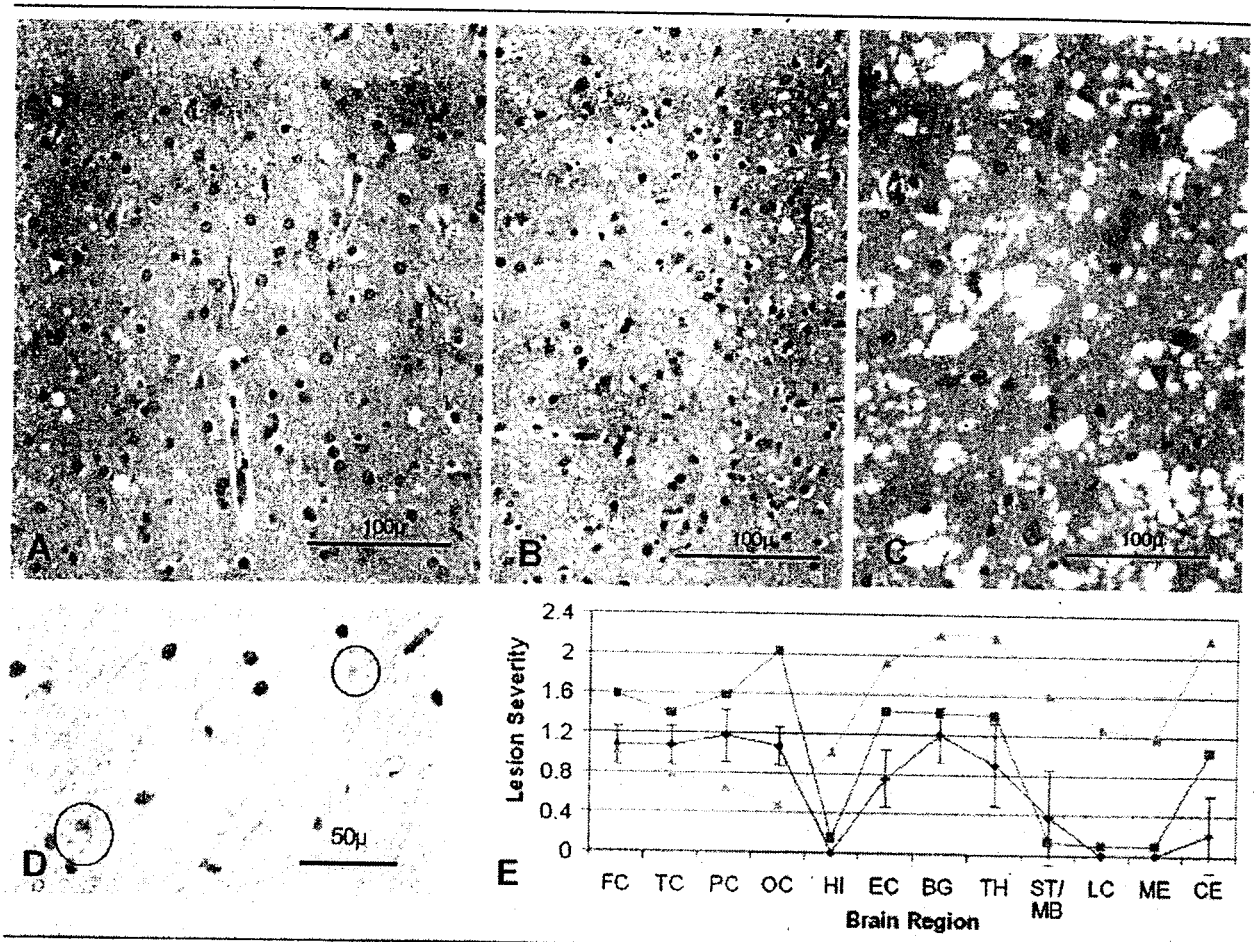

Fig 1. Histopathology and lesion profile. The spongiform degeneration of protease-sensitive prionopathy (PSPr) (A) is characterized by a mixture of small and intermediate size vacuoles, whereas the vacuoles of two subtypes of sporadic Creutzfeldt–Jakob disease (CJD), sCJDMM1 (B) and sCJDMM2 (C), are mostly small (sCJDMM1) or much larger and confluent (sCJDMM2). (D) Eosinophilic microstructures surrounded by a pale halo (circle) *in the cerebellar molecular layer; (A–D) Hematoxylin and eosin staining. (E) Lesion profiles of PSPr* (diamonds), *sCJDMM1* (squares), *and sCJDVV2* (triangles). Vertical bars *refer to standard deviations. In sCJDMM1 and sCJDVV2, for which data were adapted from Parchi and colleagues,[2] standard deviations were omitted for clarity. Spongiform degeneration was scored on a 0 to 4 scale (0 = not detectable; 1 = mild; 2 = moderate; 3 = severe; 4 = confluent); astrogliosis was scored on a 0 to 3 scale (0 = not detectable; 1 = mild; 2 = moderate; 3 = severe). FC = front cortex; TC = temporal cortex; PC = parietal cortex; OC = occipital cortex; HI = CA1 of hippocampus; EC = entorhinal cortex; BG = basal ganglia; TH = thalamus mediodorsal nucleus; MB/ST = midbrain in PSPr, substantia nigra in sCJDMM1 and sCJDVV2; LC = pons; ME = medulla; CE = cerebellar cortex.*

dient and centrifuged 1 hour at 200,000*g* in a SW55 rotor (Beckman Coulter, Fullerton, CA).[16,23,27]

Statistics

Analyses were performed with the two-tail Student's *t* test.

Results

Clinical Features

Mean age of onset and disease duration were 62 years (range, 48–71 years) and 20 months (range, 10–60 months), respectively (Table 1). Presentation and course were dominated by neurobehavioral and psychiatric signs, with progressive motor and cognitive decline. Seven patients were ataxic. Other consistent features included absence of periodic complexes on the electroencephalogram and nondiagnostic 14-3-3 protein test in the cerebrospinal fluid. Magnetic resonance imaging showed diffuse atrophy without restricted diffusion signals in all 10 patients examined. No subject had known history of prion exposure; probable familial occurrence of dementia was reported in 6 of 10 investigated patients (see Table 1).

Neurohistopathology

SD and astrogliosis of moderate severity were present in the cerebral cortex, basal ganglia, and thalamus of

Table 1. Clinical Findings

Case No.	Sex	Age (yr)	Disease Duration (mo)[a]	Symptoms at Onset[b]	Symptoms during Illness Evolution	EEG	MRI Atrophy/ Diffusion[c]	Family History of Dementia	Other Information
1	M	62	60	Behavioral and mood swings, psychosis (patient diagnosed with bipolar illness)	Dementia, aphasia, ataxia, and seizure	Slowing right > left	+/−	Mother died of dementia at age 70	(1) Right hemispheric hypoperfusion on SPECT study; (2) CSF 14-3-3 (not performed)
2	F	71	33	Depression and dementia	Dementia, ataxia, and Parkinsonism	Normal	+/−	Mother with dementia	CSF 14-3-3 (not performed)
3	M	70	12	Dementia and apathy	Aphasia, Parkinsonism, hyperreflexia, and prominent frontal release signs	Normal	+/−	Father with dementia at age 60	(1) Negative CSF 14-3-3; (2) increased CSF proteins 175mg/dl without cells
4	M	50	7 (died in a fall)	Dementia and mood swings	Psychosis, aphasia, patient fell and died of subdural hematoma	Diffuse slowing	NA	NA	Ambiguous CSF 14-3-3
5	F	67	11	Dementia and aphasia	Ataxia and depression	Not performed	+/−	Dementia in a paternal aunt and sister died of dementia at age 69	CSF 14-3-3 (not performed)
6	M	60	13	Dementia	Ataxia, psychosis, and incontinence	NA	+/−	No family history of dementia	CSF 14-3-3 (not performed)
7	F	48	17	Dementia, emotional lability, and outbursts	Motor decline	Diffuse slowing	+/−	Mother with early dementia at age 60	(1) Negative CSF 14-3-3; (2) patient had VP shunt without response
8	F	64	10	Dementia, depression, and psychosis	Ataxia, Parkinsonism, and tremor	Diffuse slowing	+/−	Mother with dementia	Negative CSF 14-3-3
9	M	63	23 (patient alive)	Dementia, personality and behavioral changes	Motor decline, Parkinsonism, and psychosis	Diffuse slowing	+/−	Mother died at age 83 with mild dementia	(1) Global hypoperfusion on SPECT study; (2) negative CSF 14-3-3; (3) increased CSF protein 126mg/dl without cells
10	F	68	17	Insomnia, tremor, and slurred speech	Dementia, ataxia, worsening depression with psychosis and agitation, hyperreflexia	Diffuse slowing	+/−	No family history of dementia	History of bipolar illness with suicidal attempts
11	M	52	13	Decreased verbal output, and progressive motor decline	Dementia, ataxia, and Parkinsonism	Normal	+/−	No family history of dementia	NA

[a]Average disease duration (20.4 ± 15.4) excludes patients #4, who died of subdural hematoma caused by a fall, and #9 still alive at last report. [b]The neurobehavioral and psychiatric manifestations included insomnia, apathy, personality changes, mood swings, emotional outburst, depression, and psychosis. [c]Plus and minus signs, respectively, indicate the presence and absence of atrophy or restricted diffusion signals on brain magnetic resonance imaging (MRI). EEG = electroencephalography; SPECT = single-photon emission computerized tomography; CSF = cerebrospinal fluid; NA = not available; VP = ventriculoperitoneal.

the PSPr cases without severe neuronal loss. SD comprised a mixture of fine vacuoles, comparable with those seen in sCJDMM1 (the most common sCJD subtype), and slightly larger vacuoles that resulted in a mean vacuolar diameter greater than that of sCJDMM1 (7.8 ± 2.7 vs 5.8 ± 1.2μm). But the "larger" vacuoles clearly were smaller than the "coarse" vacuoles characteristic of sCJDMM2 (see Figs 1A–C).[2,3] The hippocampal pyramidal cell layer appeared unaffected; the molecular layer of the dentate gyrus and the stratum lacunosum moleculare showed mild SD, which extended into the subiculum and the entorhinal and inferior temporal neocortices. No kuru plaques or multicore plaques were detected. In some subjects, structures suggestive of microplaques were observed in the molecular layer of the cerebellum (see Fig 1D). Lesion profiling identified the cerebral neocortex, basal ganglia, and thalamus as the regions most severely affected, whereas the brainstem and cerebellum were apparently spared (see Fig

1E). Congo red staining of selected cerebral and cerebellar cortices was negative.

Immunohistochemistry

PrP immunostaining with Mabs 3F4 and 1E4 of the cerebral cortex, basal ganglia, and thalamus from the PSPr cases was strong, and in the hippocampal formation was selective with strong immunoreactivity in the molecular layer of the dentate and stratum lacunosum moleculare, without pyramidal cell layer staining (Figs 2A, B). The staining pattern in the cerebrum was characterized by round, loose clusters of coarse granules quite evenly distributed over a background of smaller granules (see Fig 2C). The size of the cluster-forming granules often increased progressively toward the cluster's center, which generally contained a larger granule or a tight aggregate of small granules (see Fig 2D). Strongly immunostained globular structures were occasionally seen, rarely also in the white matter (see Fig 2D, inset). Immunoreactivity in cerebellum and brainstem was limited to minute, rounded structures or aggregates of a few granules in the cerebellar molecular layer and midbrain colliculi, except for one subject who displayed a large number of these structures (see Fig 2H). The immunostained clusters and globules could not be correlated with histologically detectable lesions except for the intense immunostaining of possible microplaques in the cerebellum of some cases (see Figs 1D and Fig 2H). The pattern of PrP immunostaining of cerebrum and cerebellum in the PSPr cases was readily distinguishable from those of sCJD subtypes and nonprion disease controls (see Figs 2E–J). Furthermore, on paraffin-embedded tissues, PrP immunoreactivity was virtually removed with PK treatment (50μg/ml, 37°C, 1 hour) in these cases, whereas it was only reduced in sCJD (data not shown). Amyloid-β immunostaining showed mostly diffuse plaques apparently compatible with the subject's age.

Electron Microscopy

The ultrastructural examination of the cerebellar molecular layer from the case shown in Figure 1D showed poorly defined, rounded structures with barely detectable filament-like profiles that were embedded in an amorphous-granular matrix. These formations strongly reacted with antibodies to PrP and overall had the features of poorly formed or immature PrP microplaques (Figs 3A, B).

Genetic Findings

All PSPr patients were homozygous for valine at codon 129 of the PrP gene, and none carried mutations in the PrP gene ORF; three subjects had silent polymorphisms (two at codon 117 and one at codon 122).

Prion Protein Characterization: Detergent-Insoluble, Protease-Resistant, and Protease-Sensitive Prion Protein

The total PrP immunoblot profile from all PSPr patients was indistinguishable from that of nonprion disease control subjects (Fig 4A). The glycoform ratios of the three PrP bands from the two groups were similar. Measured by densitometry in arbitrary units, the diglycosylated or upper band was 10.44 ± 1.78 (n = 3) in PSPr versus 7.83 ± 3.64 (n = 5) in nonprion disease control subjects ($p = 0.30$); the monoglycosylated or intermediate band was 4.40 ±1.88 (n = 3) in PSPr versus 3.40 ± 2.74 (n = 5) in control subjects ($p = 0.79$). Under our conditions, the unglycosylated or lower band was not measurable in both PSPr patients and control subjects (see Fig 4A). Furthermore, the mean amount of total PrP present in six subjects apparently did not significantly differ from that of the nonprion disease control subjects (n = 7) (1.69 ± 0.28 vs 1.57 ± 0.39; $p = 0.53$) and from that of cases with prion disease (n = 3) (1.69 ± 0.28 vs 2.03 ± 0.46; $p = 0.20$).

In conventional diagnostic immunoblot procedures using Mab 3F4, classic PrPr (PrP27-30) was undetectable in the brain homogenates from the frontal cortex of all 11 subjects, and from the occipital and cerebellar cortices of the 7 subjects in which these brain regions were tested (see Fig 4A). Treatment with various doses of PK showed no consistent difference between these subjects and nonprion disease control subjects in these brain regions (see Fig 4B). Barely detectable amounts of approximately 6kDa PK-resistant PrP (PrP~6) were present in the temporal cortex of three of the eight tested subjects. Of the eight subjects for whom subcortical regions (substantia nigra, putamen, and thalamus) were available, significant quantity of PK-resistant PrP27-30 was found in one case, and minimal amounts in two others (one showed small amounts of PrP~6 only), whereas no PrPr could be definitely detected in the other five subjects (see Fig 4C). In contrast, probing with Mab 1E4 demonstrated a ladder of PK-resistant PrP fragments ranging from approximately 29 to 6kDa in all PSPr cases examined (see Fig 4D). The ladder-like electrophoretic mobility of the PrPr fragments did not match those associated with common subtypes of CJD, except for an approximately 20kDa fragment, which, after deglycosylation, was tentatively identified as the unglycosylated form of PrPr (see Fig 4C; also data not shown).[2] The approximately 6kDa fragment was also unglycosylated and was reminiscent of the PrP~7 fragment of GSS.[1] These fragments were most obvious at PK concentrations of 5 to 10μg/ml and decreased at greater PK concentrations. The ladder-like electrophoretic profile of PrP treated with PK was highly reproducible and was observed in all 11 PSPr cases examined. In contrast, the PrPr frag-

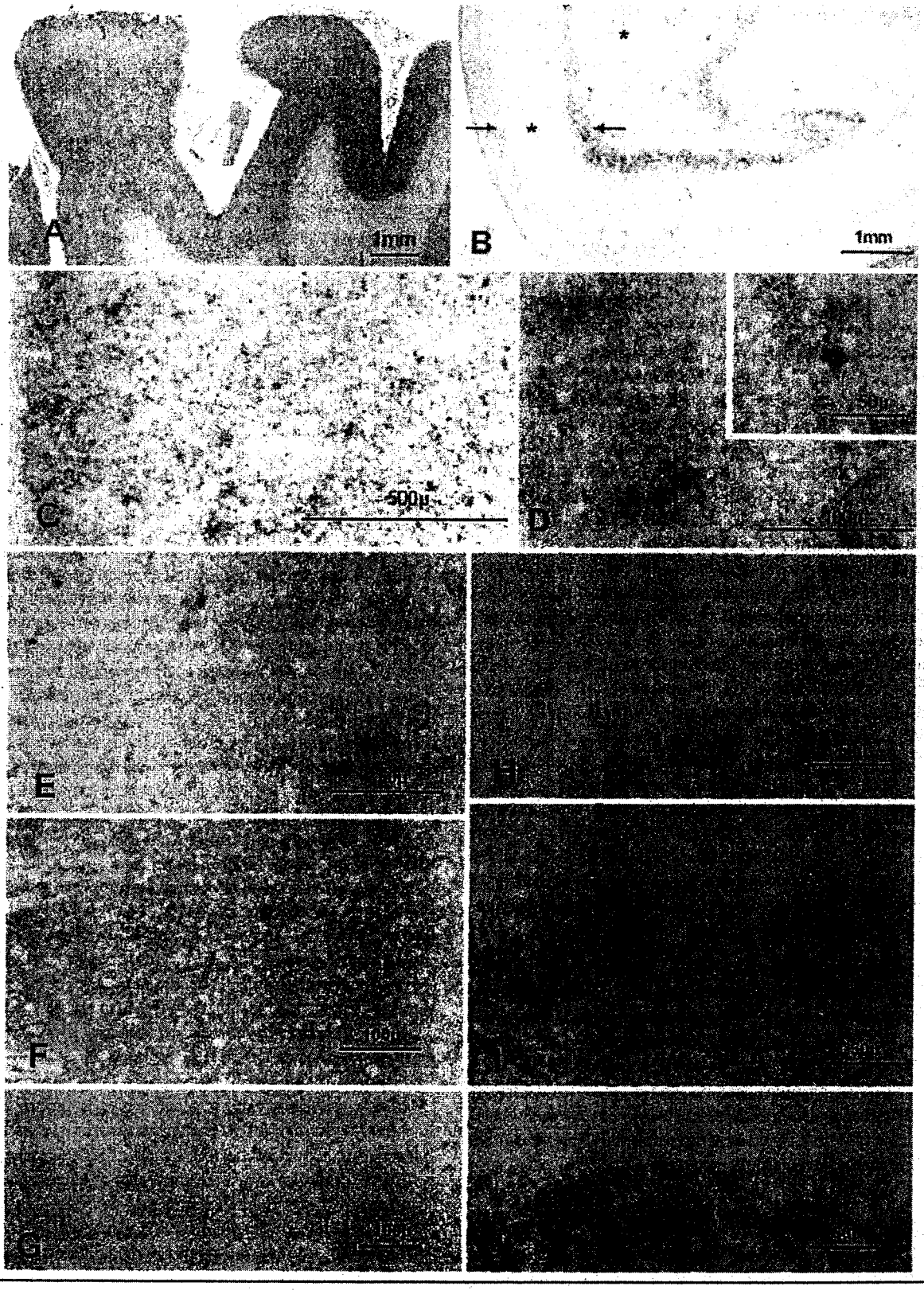

Figure 2.

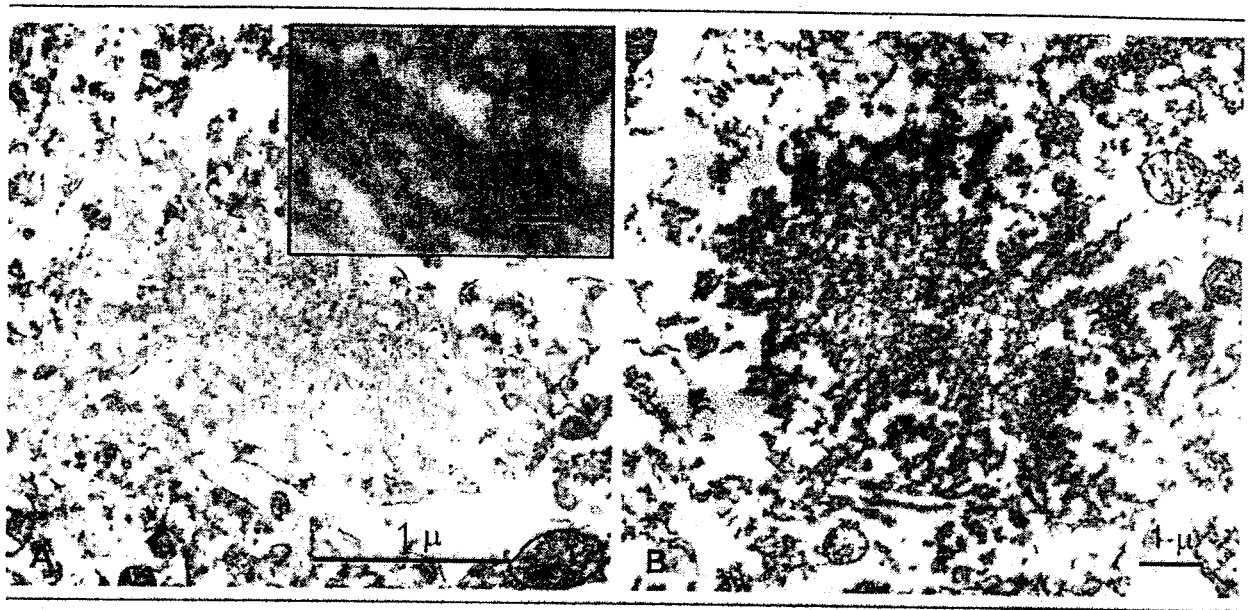

Fig 3. Electron microscopy (EM) of brain microstructures of protease-sensitive prionopathy (PSPr). (A) EM of the eosinophilic microstructures observed at light microscopy (Fig 1D) shows plaquelike formations with fuzzy filamentous appearance (inset). These structures are strongly reactive with antibodies to prion protein (PrP) (B) consistent with PrP microplaques (peroxidase anti-peroxidase with 3F4).

ments from sCJD were clearly detectable with both 3F4 and 1E4 Mab only after treatment with more than 10μg/ml PK, and increased with greater PK concentrations (see Fig 4C; also data not shown). Therefore, a small amount of PrPr detectable with Mab 3F4 is present mostly in the subcortical regions of these subjects. Moreover, most of the PrPr appears to have a different conformation from that of typical PrPr because on PK digestion it generates a unique set of fragments that are detected by 1E4 but not by 3F4.

Fig 2. Prion protein (PrP) immunohistochemistry. (A) Intense and widespread PrP immunostain of the cerebral cortex and (B) distinctive PrP immunostaining pattern in the hippocampal gyrus with staining of the molecular layers (arrows) *but not of the pyramidal cell layer or of the end plate* (asterisk). *(C, D) The cortical staining consists of coarse granules forming loose clusters with larger granules or a tighter aggregate of granules at the center; (D, inset) heavily stained globular structures are also present. (A–D) Protease-sensitive prionopathy (PSPr). (E, F) Immunostaining patterns of the cerebral cortex in sCJDVV2 (E) and sCJDVV1 (F) showing laminar staining and occasional perineuronal staining in sCJDVV2 and weak and fine widespread staining in sCJDVV1. (G) No immunostaining is detectable in the cerebral cortex of a nonprion disease control. (H–J) Cerebellar immunostaining patterns in PSPr (H), sCJDVV2 (I), and sCJDVV1 (J). There is intense and exclusive staining of large granules in the molecular layers in PSPr (H), presumably corresponding to the eosinophilic microstructures surrounded by a pale halo shown in Figure 1D; staining of irregular deposit limited to the granule cell layer in sCJDVV2 (I); no detectable staining in sCJDVV1 (the staining of the granule cell nuclei is nonspecific) (J). (A–I) Monoclonal antibody 3F4.*

Total abnormal PrP and the PrPr conformers were further characterized in abnormal PrP-enriched preparations after the capture of the abnormal PrP with g5p, a single-stranded DNA binding protein with a high affinity for abnormal PrP regardless of its PK resistance.[13,23] The amount of PrP captured by g5p in the PSPr subjects was three times greater than the amount of PrP captured in nonprion disease control subjects (data not shown), but it was nearly 16 times less than the g5p-captured PrP in typical sCJD. As measured by densitometry in arbitrary units, the mean PrP captured by g5p in eight of PSPr subjects was 3.44 ± 2.8% of the total PrP detected by direct gel loading compared with 53.55 ± 24.6% in sCJD ($n = 3$; $p = 0.00015$; Fig 5A). Furthermore, although nearly 90% of the g5p-captured PrP was resistant to PK digestion in sCJD, the PrPr accounted for only 24% of the total abnormal PrP captured in the PSPr subjects (87.59 ± 26.8% in four sCJD cases vs 24.23 ± 14.9% in nine PSPr cases; $p = 0.0001$) (see Fig 5A). The PK-resistant PrP obtained after PrP enrichment from the subjects was distributed in three major bands of approximately 26, 20, and 6kDa, which were detected by both 3F4 and 1E4, and matched the major bands of the immunoblot ladder detected with 1E4 on direct loading (see Fig 5A; also data not shown). A similar PrP banding pattern was obtained after sodium phosphotungstate precipitation, another method of abnormal PrP enrichment.[11,26] It was detected by both 3F4 and 1E4, al-

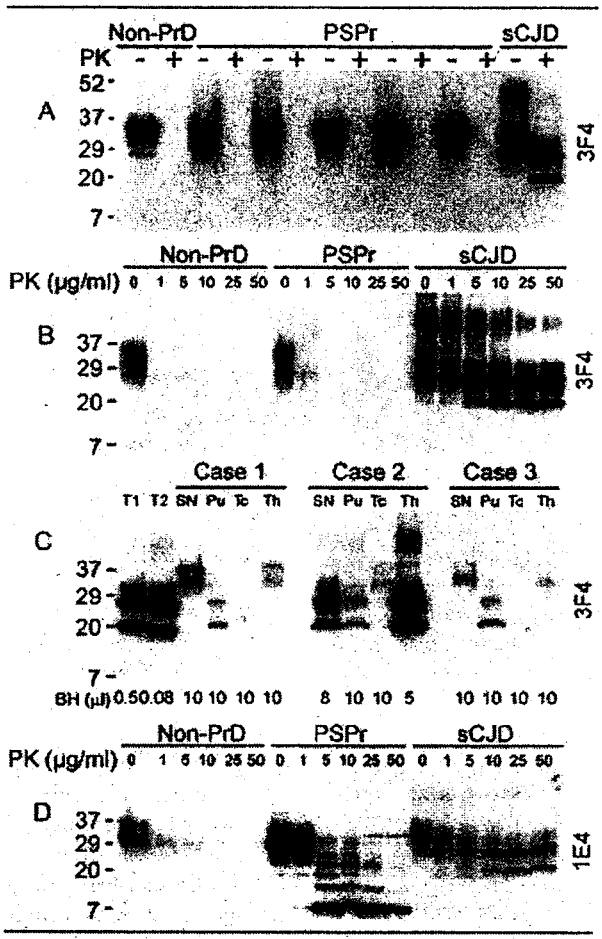

Fig 4. Characterization of prion protein (PrP) in protease-sensitive prionopathy (PSPr). (A) On conventional immunoblots, proteinase K (PK)–resistant PrP is undetectable in nonprion disease control subjects (non-PrD) and PSPr patients, although it is prominent in sporadic Creutzfeldt–Jakob disease (sCJD). (B) PK-resistant PrP from non-PrD and PSPr is not detectable even after treatment with low PK concentrations, but only in sCJD control when probed with the monoclonal antibody 3F4. (C) Subcortical regions of three PSPr cases treated with PK at 50μg/ml before Western blot analysis with 3F4 showed various amounts of PK-resistant PrP in three PSPr cases. Samples from temporal cortex (Tc) were used as controls. (D) When the same samples used in (B) are probed with 1E4, moderately PK-resistant PrP fragments forming a ladder are observed. (A, B, D) Tissues are from the frontal cortex. BH = brain homogenate; Pu = putamen; SN = substantia nigra; T1 = PrPr type 1 control; T2 = PrPr type 2 control; Th = thalamus.

though the bands were much more prominent when probed with 1E4 (see Fig 5B). The abnormal PrP enrichment experiments confirm that, in PSPr patients, there is much less abnormal PrP than in sCJD, and that the proportion of abnormal PrP that is PK resistant is much smaller.

Prion Protein Sedimentation in Sucrose Gradients

After sucrose gradient sedimentation, 30% of the total PrP from the PSPr patients was recovered in fractions 7 to 11 containing large aggregates, whereas these fractions accounted for only 5% of the total PrP in nonprion disease subjects (Figs 6A, B, E). The same fractions contained about 24 and 58% of the total PrP in GSS patients with the A117V mutation and sCJDVV1, respectively (see Figs 6C–E). Also, the percentages of PrP recovered in fractions 2 and 3 differed significantly between PSPr and nonprion disease. PSPr differed from GSS in fractions 7 and 8, and from

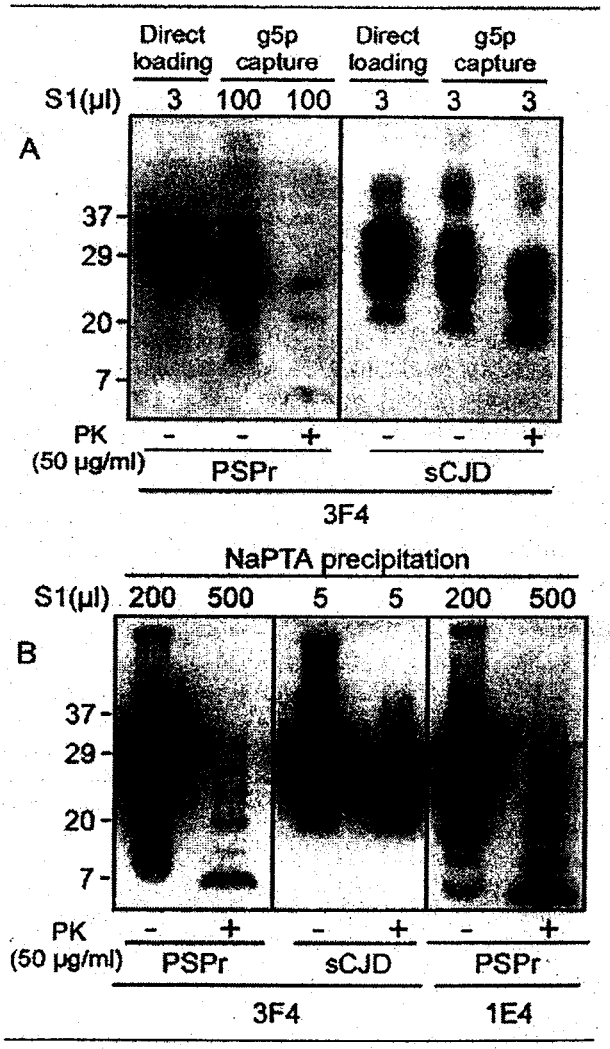

Fig 5. Capture by g5p (A) and sodium phosphotungstate (NaPTA) (B) of prion protein (PrP) from protease-sensitive prionopathy (PSPr) and sCJDMM1 (sporadic Creutzfeldt–Jakob disease). Probing with 3F4 or 1E4 after stripping. The same ladder of proteinase K (PK)–resistant PrP as in Figure 4D is detectable in PSPr preparations after heavy loading of the gel. S1 = supernatant of brain homogenate obtained after low-speed centrifugation (1,000g for 10 minutes).

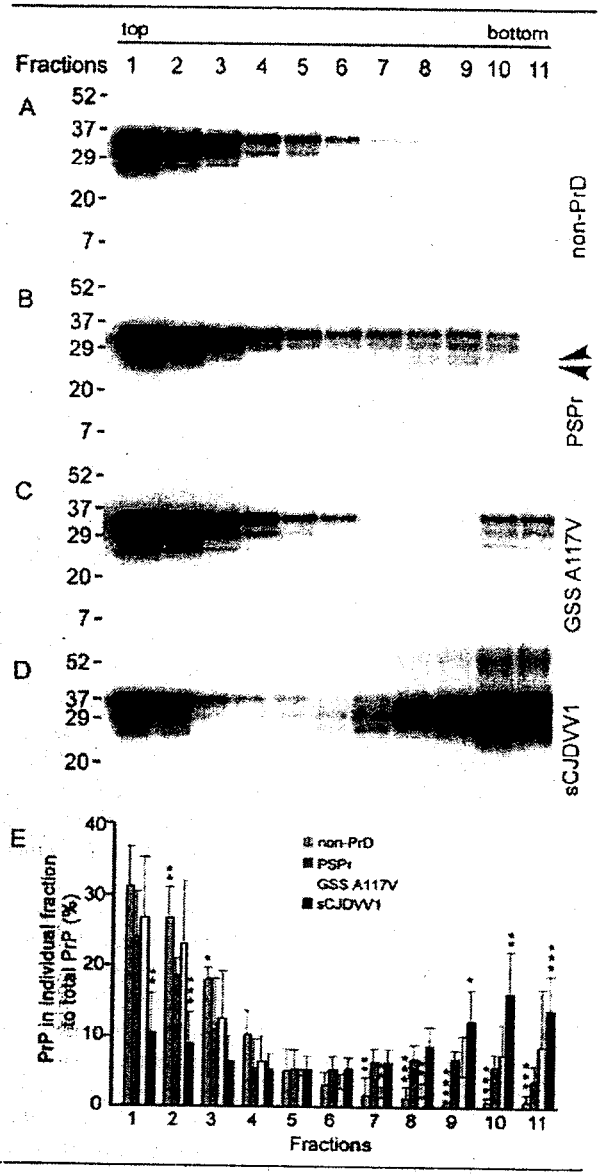

Fig 6. Prion protein (PrP) profiles in sucrose gradient sedimentation. (A) Nonprion disease (non-PrD); (B) protease-sensitive prionopathy (PSPr); (C) Gerstmann–Sträussler–Scheinker disease (GSS) with the A117V mutation (GSSA117V); (D) sCJDVV1; (E) PrP distribution in the fractions plotted as percentages of the total PrP. Although the amounts of PrP from PSPr are similar to those of non-PrD subjects in fractions 1 and 4 to 6, they differ significantly in fractions 2, 3, and 7 to 11, and also clearly differ from GSSA117V in fractions 7 and 8 and sCJDVV1 in fractions 1, 2, and 9 to 11. PSPr fractions 8 to 11 also have a distinctive low double band (B, arrowheads*) not present in the fractions from non-PrD, GSS, and sCJDVV1. n = 6 for non-PrD* (green bars); *n = 6 for PSPr* (red bars); *n = 3 for GSS* (yellow bars); *and n = 7 for sCJDVV1* (blue bars). Vertical bars *refer to standard deviations.* Asterisks *denote PrP fractions from non-PrD, GSS, and sCJDVV1 that by statistical analysis are significantly different from corresponding PSPr fractions.* $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

sCJD in fractions 1, 2, and 9 to 11 (see Fig 6E). In addition to the quantitative differences, the electrophoretic profiles of the high-molecular-weight aggregates from PSPr also differed from those of nonprion disease, GSS, and sCJDVV1 subjects: the lower band was double in PSPr but single in other conditions (see Figs 6A–D). Comparable data were obtained after gel filtration fractionation, which demonstrated that PrP aggregates exceeding 2,000kDa were more abundant in PSPr than in nonprion disease control subjects but much fewer than in sCJD (data not shown).

Discussion

We report 11 patients affected by a disease that involves abnormal PrP and has homogeneous and distinctive features (Table 2). Based on several lines of evidence, we argue that these features allow for the separation of this condition from all known forms of human prion disease. First, the abnormal PrP associated with this disease is predominantly, and in several brain regions almost exclusively, sensitive to protease or PrPs, and the PK-resistant PrP isoform or PrPr has a distinctive electrophoretic profile. The high sensitivity to PK and the distinctive electrophoretic profile of the abnormal PrP clearly distinguish these cases from each of the five subtypes of sCJD and from sporadic fatal insomnia (sFI), the known human sporadic prion diseases.[1] For example, compared with sCJDMM1, the most common and typical sCJD,[2] these cases have 16 times less total abnormal PrP, and the fraction of the total abnormal PrP that is PK resistant is nearly 4 times less. Furthermore, the ladder-like electrophoretic profile of the PrPr associated with this condition has not been observed in either sCJD or sFI, all of which instead are characterized by the presence of the well-known PrPr type 1 or 2.[1] When present, the traditional PrPr, commonly called *PrP27-30,* was located in subcortical regions and was of type 1, another combination not observed in sporadic human prion diseases.[1] Second, these cases are also homogeneous as for the PrP coding genotype because they are all homozygous for valine at codon 129 of the PrP gene, the site of a common methionine/valine polymorphism.[28] Valine homozygosity in white individuals is the rarest 129 genotype, being found only in 12% of people.[28] The sCJD subtypes associated with valine homozygosity, sCJDVV1 and sCJDVV2, have been well characterized and differ from these cases phenotypically and for the characteristics of the abnormal PrP.[1] Third, the pattern of PrP immunostaining and the presence of structures with the features of poorly formed plaques that we observed in the cerebellum are to our knowledge unprecedented. Lastly, the clinical presentation and initial course that prominently features relatively slow cognitive deterioration, occasional gait impairment, and incontinence has evoked the diagnoses of normal pressure hydrocepha-

Table 2. **Summary of Protease-Sensitive Prionopathy Common Features**

Mean Age at Onset (range), yr	Mean Duration (range), mo	Clinical Presentation	Histopathology	PrP IHC	Abnormal PrP	Family History	PrP Genetics
62 (48-71)	20 (10-60)[a]	Cognitive decline (8/11)[b] and mood/behavioral changes (7/11)[b]	Minimal spongiform degeneration with vacuoles larger than typical CJD, and minimal astrogliosis	Intense staining with distinct target pattern in cerebral gray matter; and dot pattern in cerebellar molecular layer	Minimal amount of PK-resistant PrP forming a ladder-like pattern on Western blot	Dementia (8/10)[b] Dementia with age at onset < 61 yr (2/4)[b]	Valine homozygosity at codon 129 No mutation in the PrP gene coding region

[a]One patient alive after 23-month duration and one dead 7 months from onset for other causes excluded. [b]Positive cases/total number of cases. PrP = prion protein; IHC = immunohistochemistry; CJD = Creutzfeldt-Jakob disease; PK = proteinase K.

lus, diffuse Lewy body disease, or frontotemporal dementia, whereas prion disease was suspected only at a later stage based on the relatively short duration.

Although these cases can be easily distinguished from sporadic prion diseases, some of their features such as overrepresentation of PrPs and the multiple PK-resistant PrP fragments, have been reported in GSS.[4] However, all cases of GSS reported to date are associated with a mutation in the coding region of the PrP gene or immediately adjacent to it.[4] None of these cases carried such mutation. Moreover, the ladder-like, PK-resistant, PrP fragments observed in our cases are preferentially detected with 1E4 but not with 3F4, which obviously separates these cases from GSS carrying the multiple PK-resistant PrP fragments. In a recent study, we observed that although 1E4 and 3F4 have adjacent epitopes along human PrP residues 97-112, their accessibility to these epitopes is different because of different neighboring N-terminal residues.[29] It is possible that the 1E4 selectively detected PK-resistant PrP fragments have N-terminal starting sites that are different from those of the well-characterized PrPr types 1 and 2. The earlier evidence clearly indicates that this condition differs from GSS, although the possibility that it represents the long-sought sporadic form of GSS remains to be excluded. Six of the 10 patients with obtainable pedigree had a family history of dementia that cannot be ignored, yet none carried a mutation in the PrP gene ORF. Therefore, at least in some cases, a causative mutation may be located outside the ORF of the PrP gene, a condition never observed in human prion diseases.[1]

All these considerations argue that the 11 patients were affected by a novel condition involving the PrP that cannot be classified within the spectrum of currently known human prion diseases. We suggest the designation of PSPr to emphasize a major distinctive feature (see Table 2).

Compared with other human prion diseases, PSPr is not exceedingly rare, because it accounts for about 3% of all sCJD and 16% of all valine homozygous CJD accessioned by the National Prion Disease Pathology Surveillance Center during the same time period as these 11 patients, making PSPr about as common as some of the well-known sporadic prion diseases (such as sCJDMM2, sFI, and sCJDVV1).[2] Furthermore, because the clinical presentation and the duration of PSPr often do not point to the diagnosis of prion disease, some cases of PSPr may currently be classified within the group of non-Alzheimer's dementias and not be investigated further. Should this be the case, PSPr may be more common than this study suggests.

The small amount of PrPr associated with PSPr and the finding that about 76% of the detectable abnormal PrP is PK sensitive not only hinders the diagnosis but also has implications concerning origin, pathogenicity, infectivity, and classification of PSPr.

The discovery of PrPs has opened a new chapter in prion diseases.[11–15] The demonstration that PrPs forms smaller aggregates than the PrPr counterpart,[16] and that apparently it is competent to convert PrP^C to PrPr in vitro, as well as to seed the polymerization of recombinant PrP into amyloid,[17,18] suggests that PrPs shares defining features with PrPr. However, the pathogenetic mechanisms of PrPs in the absence of PrPr and, therefore, the nature of the prion diseases associated with PrPs currently remain conjectural.

Prion diseases associated with PrPs, in the presence of minimal or no PrPr, have been modeled and studied in detail in a variety of transgenic (Tg) mouse lines carrying mouse homologues of human PrP gene mutants or overexpressing PrP^C.[12,30–33] Two Tg mouse models appear relevant to these cases.

In the first model, Tg mice expressing high levels of mouse PrP carrying the P101L mutation, the mouse equivalent of the human P102L mutation associated with a GSS phenotype,[4,34,35] spontaneously developed a neurodegenerative process characterized by SD and prion plaque formation. After inoculation, they transmitted a disease phenotypically similar to P101L-mutated Tg mice but not to wild-type mice. As in our cases, the affected mice had PrPs but no, or minimal amounts of, PrPr, indicating that PrPs can be associated with a prion disease that is under certain condi-

tions transmissible and has a histopathological phenotype displaying general features of prion diseases.[12]

In the second model, Tg mice carrying the P101L mutation were inoculated with brain homogenate from patients affected by a subtype of GSS P102L characterized by the exclusive presence of an approximately 8kDa PK-resistant fragment reminiscent of the approximately 6kDa fragment observed in small amounts in our cases. The inoculated Tg mice remained largely asymptomatic, but at histological examination, they displayed PrP plaques and had minimal amounts of PrPr.[33] They failed to transmit the disease to wild-type mice, but inoculation to P101L-mutated mice resulted in the formation of PrP plaques in the absence of clinical disease.

These mouse models and now our cases raise issues with the definition of prion diseases. Currently, it is unclear whether PSPr is transmissible because time-consuming transmissibility experiments to different lines of Tg mice and in vitro PrP replication are still ongoing. Should PSPr not be transmissible, the question is whether it is a prion disease. A similar question can be raised for GSS, of which to date only one subtype has been shown to be consistently transmissible.[4] The issue is further compounded by the recent evidence that amyloid β, the pathogenic peptide of Alzheimer's disease, has the propensity to replicate after inoculation into susceptible Tg mice in a conformation-dependent fashion reminiscent of prions.[36] These findings appear to blur the once tight association of prion diseases and transmissibility. It may be more practical to apply the label of prion diseases to all conditions in which the PrP is abnormal and appears to play a central role in the pathology, as in all prion diseases known to date and in PSPr.[37] In contrast, one might reserve the qualification of transmissible to those prion diseases that can be transmitted to recipients expressing relatively normal amounts of wild-type PrP.[36]

The finding that several PSPr patients had first-degree relatives diagnosed with dementia necessitates a search for an underlying genetic cause. In AD, the discovery of mutations outside the gene of the amyloid precursor protein (the central protein in AD, as PrP is in prion diseases) has provided a wealth of information regarding pathogenetic mechanisms of AD.[38] Similarly, the discovery of a mutation outside the PrP gene ORF capable of generating a prion disease may greatly expand our understanding of pathogenetic mechanisms and the role of PrP in prion diseases.

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研究報告の概要

（in vitroでのPMCA増幅によりPrP^{Sc}の異常折り畳み構造が種の壁を超えて伝播し感染性プリオンが生成）
プリオンは異常な折り畳み構造のたん白（PrP^{Sc}）のみから構成される今までにない感染性病原体であり、細胞プリオン蛋白（PrP^{C}）にその異常構造を蔓延させることにより疾患が伝播する。プリオンが有する重要な特質はその種の壁であり、種の壁があることによって1つの種のプリオンは限られた数の別の種にしか感染を起こすことができない。ここで我々は、in vitroにおけるPMCA(protein misfolding cyclic amplification) 増幅によって、PrP^{Sc}異常折り畳み構造が種の間で伝播し感染性プリオンが生成されることを報告する。マウスPrP^{Sc}と混合させることによって異常折り畳みが起こったハムスターPrP^{C}は、野生型ハムスターに対して感染性を有する新規なプリオンを生成した。同様の結果は、反対の方向でも得られた。PMCA増幅を繰り返すとin vitro産生プリオンの順応が起こるが、そのプロセスは、in vivoでの連続継代の際に観察される株の安定化を暗示させるものであった。我々の結果から、PMCAが種の間の伝播を調査するための価値のあるツールであることが示された。また、種の壁と株の生成がPrPの異常折り畳み構造の蔓延によって決定されることが示唆された。

使用上の注意記載状況・その他参考事項等

代表としてノイアート（献血）の記載を示す。
2. 重要な基本的注意
(1) 略
1) 略
2) 現在までに本剤の投与により変異型クロイツフェルト・ヤコブ病（vCJD）等が伝播したとの報告はない。しかしながら、製造工程において異常プリオンを低減し得るとの報告があるものの、理論的なvCJD等の伝播のリスクを完全には排除できないので、投与の際には患者への説明を十分行い、治療上の必要性を十分検討の上投与すること。

報告企業の意見	今後の対応
in vitroでのPMCA増幅によりPrP^{Sc}の異常折り畳み構造が種の壁を超えて伝播し感染性プリオンが生成されるとの報告である。 これまで血漿分画製剤によってvCJD、スクレイピー及びCWDを含むプリオン病が伝播したとの報告はない。しかしながら、万一vCJD感染者の血漿が本剤の原料に混入した場合には、製造工程においてプリオンを低減し得るとの報告があるものの、製剤から伝播する可能性を完全には否定し得ない。そのため、弊社の血漿分画製剤の製造工程におけるTSE感染性低減に関する検証実験を加速し、自社データを早期に取得し、工程評価を行い、必要に応じて工程改善を実施する予定である。	本報告は本剤の安全性に影響を与えないと考えるので、特段の措置はとらない。

Cell

Crossing the Species Barrier by PrP^{Sc} Replication In Vitro Generates Unique Infectious Prions

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SUMMARY

Prions are unconventional infectious agents composed exclusively of misfolded prion protein (PrP^{Sc}), which transmits the disease by propagating its abnormal conformation to the cellular prion protein (PrP^{C}). A key characteristic of prions is their species barrier, by which prions from one species can only infect a limited number of other species. Here, we report the generation of infectious prions by interspecies transmission of PrP^{Sc} misfolding by in vitro PMCA amplification. Hamster PrP^{C} misfolded by mixing with mouse PrP^{Sc} generated unique prions that were infectious to wild-type hamsters, and similar results were obtained in the opposite direction. Successive rounds of PMCA amplification result in adaptation of the in vitro-produced prions, in a process reminiscent of strain stabilization observed upon serial passage in vivo. Our results indicate that PMCA is a valuable tool for the investigation of cross-species transmission and suggest that species barrier and strain generation are determined by the propagation of PrP misfolding.

INTRODUCTION

Prion diseases also known as transmissible spongiform encephalopathies (TSEs) are infectious neurodegenerative diseases affecting the brain of humans and several species of mammals (Collinge, 2001). Creutzfeldt-Jakob disease (CJD) is the most common TSE in humans, and scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, and chronic wasting disease (CWD) in cervids are the most prevalent prion diseases in animals. Unlike conventional infectious microorganisms, the TSE agent appears to be devoid of genetic material and instead composed exclusively by a misfolded form of the prion protein (PrP^{Sc}) (Prusiner, 1998). PrP^{Sc} has the unprecedented ability to replicate in the body by inducing the misfolding of the cellular form of the prion protein (PrP^{C}).

One of the characteristics of the agent responsible for prion diseases is its ability to infect some species and not others (Hill and Collinge, 2004; Moore et al., 2005). This phenomenon is known as species barrier. Even between close species, the species barrier is manifested as an incomplete attack rate and a prolongation of the time it takes for animals to develop the clinical disease when injected with another species' infectious material (Hill and Collinge, 2004). Primary interspecies transmission is usually not very efficient, and it takes a long time for the prion replication process to reach the point at which full-blown clinical disease appears. After sequential passages, the PrP^{Sc} in the new host adapts, resulting in a shortage of the incubation period and stabilization of the new strain (Hill and Collinge, 2004).

Compelling evidence indicates that the species barrier is largely controlled by the sequence of PrP (Moore et al., 2005). Unfortunately, we cannot predict the degree of a species barrier simply by comparing the prion proteins from two species. The barrier has to be measured by experimental studies in animals. These studies are long and costly, and in the case of the human species barrier, the studies have to be done with experimental models, the validity of which is not absolutely guarantied. Evaluation of the species barrier is of tremendous medical importance for risk assessment and to implement regulatory measures to avoid spreading of diseases (Moore et al., 2005). At this time, the epidemiological evidence suggests that among animal TSEs only cattle BSE has been transmitted to humans, generating a variant form of CJD (vCJD) (Will et al., 1996). It is unlikely that sheep scrapie is a concern for humans, because the disease has been described for centuries and no increased prevalence of human prion diseases has been found in scrapie-endemic areas (Caramelli et al., 2006; Hunter, 1998). However, the appearance of "atypical" strains of scrapie, as well as the known transmission of BSE to sheep, has generated new concerns of human infections with sheep-derived material (Buschmann and Groschup, 2005; Hunter, 2003). Similarly, the possibility that some of the newly identified animal prion diseases, such as CWD, could be transmitted to humans cannot be ruled out at the present time (Williams, 2005; Xie et al., 2005).

Recently, we reported the generation of infectious prions in vitro by amplification of PrP^{Sc} misfolding in the test tube (Castilla et al., 2005). For these experiments, we used a technology termed PMCA (protein misfolding cyclic amplification) that mimics in vitro some of the fundamental steps involved in PrP^{Sc} replication in vivo at an accelerated rate (Saborio et al., 2001). During PMCA, small quantities of PrP^{Sc} are mixed with excess of PrP^{C}, and through a cyclical process involving incubation and sonication, prion propagation occurs in an autocatalytic way. With this procedure, prions can replicate indefinitely in the test tube and, after successive rounds of dilutions followed by PMCA amplification, PrP^{Sc} used to begin the reaction can be eliminated, and only in vitro-generated misfolded protein remains in the sample (Castilla et al., 2005). Inoculation of PMCA-generated prions into wild-type animals resulted in a disease with the same clinical, neuropathological, and biochemical features as the disease produced by brain-derived infectious material (Castilla et al., 2005). The conclusion drawn from these findings is that all of the information required to propagate the infectious properties is enciphered in the structure of PrP^{Sc}. This is further supported by recent studies from Supattapone and coworkers in which infectious prions were generated in vitro by PMCA with purified PrP^{C} and PrP^{Sc} with the sole addition of synthetic polyanions (Deleault et al., 2007).

The goal of this study was to attempt crossing the species barrier in vitro to generate unique infectious prions in a cell-free system. For these studies, we used mice and hamsters, two experimental rodent systems widely employed in TSE studies and for which several prion strains are available (Bruce, 2003; Kimberlin and Walker, 1988). The PrP sequence shows nine differences between these two animal species (Figure 1A). Infectivity studies have shown that there is a large barrier for prion transmission between these species (Kimberlin et al., 1989; Kimberlin and Walker, 1988; Race et al., 2002). Our findings show that incubation of PrP^{C} from one of the species with PrP^{Sc} from the other resulted in new PrP^{Sc} that was infectious to wild-type animals. Interestingly, a detailed examination of the infectious, neuropathological, and biochemical features of the disease that was produced revealed characteristics that were different from other known prion strains. These results indicate that the prions generated in vitro by crossing of the mouse-hamster barrier represent new strains. Strikingly, studies of the infectious characteristics of these newly generated prions after different rounds of PMCA showed that the procedure not only enabled crossing of the species barrier but also resulted in stabilization of the new strain in vitro by successive rounds of amplification. Our findings show that prions can be propagated in vitro across the species barrier, leading to the generation and adaptation of unique prion strains.

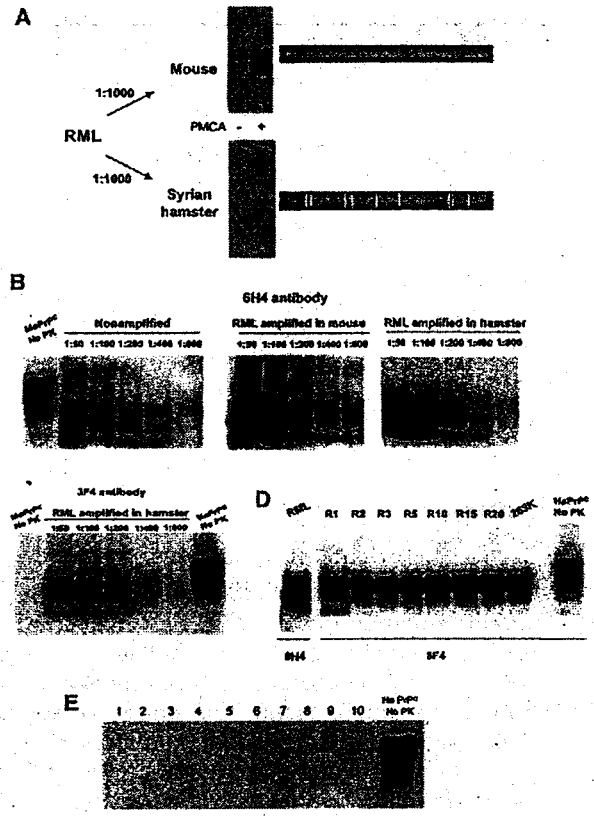


Figure 1. In Vitro Conversion of Hamster PrP^{C} Induced by Mouse RML PrP^{Sc}

(A) RML brain homogenate was diluted 1000-fold into either mouse or hamster normal brain homogenate and subjected to 96 PMCA cycles. The blot shows the results with and without PMCA in eabh species. At the right side, we show a scheme of PrP indicating the position in which there are amino acid differences between mice and hamsters.

(B) To attempt forcing conversion, we incubated larger quantities (dilutions 1:50 through 1:800) of RML PrP^{Sc} with mouse (central panel) or hamster (right panel) PrP^{C}. All samples (except for the control samples in the left panel labeled "nonamplified") were subjected to 96 PMCA cycles, and PrP^{Sc} signal was detected after PK digestion by western blot with the 6H4 antibody.

(C) The same samples as those in the right panel of (B) were developed with the 3F4 antibody.

(D) The newly generated RML-Ha PrP^{Sc} was serially passed in hamster brain homogenate by a series of 1:10 dilution followed by 48 PMCA cycles. "R" indicates the number of rounds of PMCA; i.e., R5 represent the samples after five serial rounds of PMCA.

(E) For the assessment of spontaneous generation of PrP^{Sc} by PMCA, samples from brain of ten different hamsters were subjected to the same process of serial PMCA as in (D). PrP^{Sc} formation was analyzed by western blot after PK treatment in each PMCA round. The figure shows the results obtained after 20 rounds of PMCA. In the experiments shown in this figure, all samples were treated with PK, except when indicated.

RESULTS

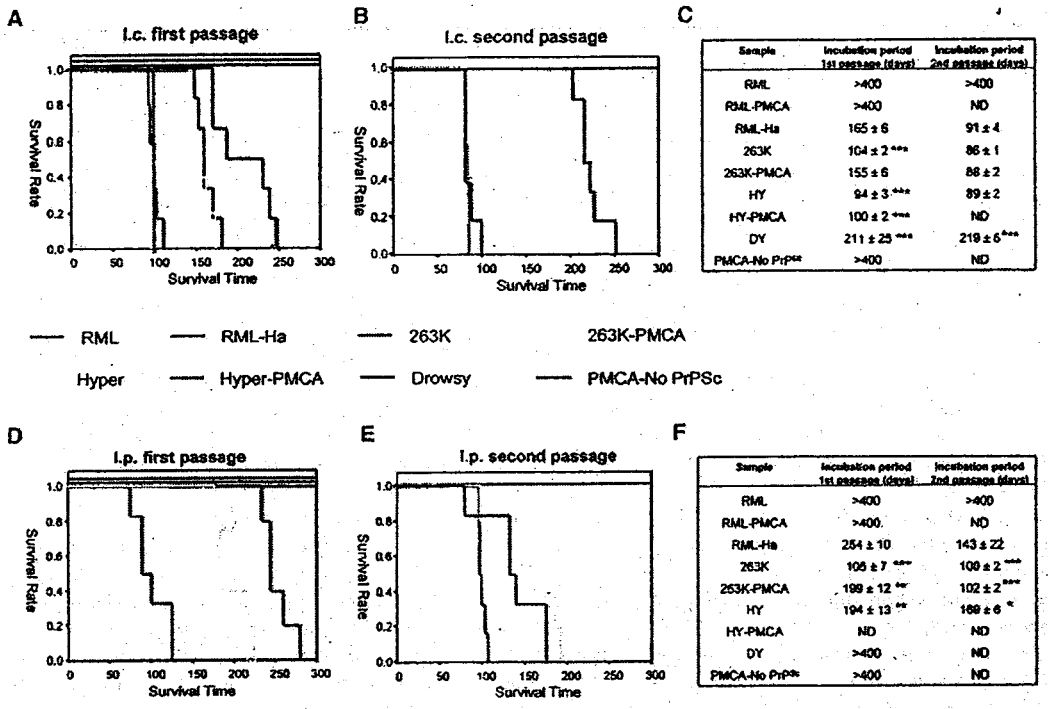
Crossing the Mouse-Hamster Species Barrier to Generate New Hamster Prions

To assess whether prions can be generated in vitro across the species barrier, we used hamsters and mice, two widely studied rodent experimental models of TSEs (Bruce, 2003; Kimberlin and Walker, 1988; Morales et al., 2007). A PMCA experiment done with our standard conditions for amplification of mouse RML prions showed no detectable formation of PrP^{Sc} when hamster PrP^{C} was used as a substrate (Figure 1A). Conversely, a robust PrP^{Sc} generation was observed with mouse PrP^{C} substrate. For this experiment, we mixed a 1000-fold dilution of RML PrP^{Sc}

into 10% brain homogenates of healthy hamsters and mice, respectively. We reasoned that if in vivo it takes longer for prions to replicate across species barriers, then in PMCA we should also encounter more difficulties to convert PrP^{C} when using PrP^{Sc} from a different species. To attempt forcing the in vitro conversion, we added a higher proportion of PrP^{Sc}-containing mouse brain homogenate into the hamster substrate. A range of dilutions from 50- to 800-fold were tested, but the problem with these experiments is that the large concentration of RML PrP^{Sc} used as inoculum makes it difficult to estimate convincingly whether new PrP^{Sc} generation was obtained (Figure 1B). Fortunately, the 3F4 monoclonal antibody can recognize hamster but not mouse PrP (Lund et al., 2007). Using this antibody for western blot, we could clearly observe that protease-resistant hamster PrP^{Sc} was being produced when the reaction was done with low dilutions (from 1:50 to 1:200) of mouse RML PrP^{Sc} (Figure 1C). When the amplification was attempted with 800-fold diluted PrP^{Sc}-containing mouse brain homogenate, only a very faint signal was observed, confirming the results obtained in Figure 1A and the idea that the combination of PrP^{C} and PrP^{Sc} from different species impairs PMCA efficiency.

Newly generated hamster PrP^{Sc} starting from RML prions was propagated many times in vitro by serial PMCA in order to remove by dilution the initial amount of mouse scrapie brain material added to begin prion replication (Figure 1D). As described before, using this procedure, we can completely remove all molecules of brain-derived PrP^{Sc} from the sample (Castilla et al., 2005). Hamster PrP^{Sc} of RML origin efficiently propagates in vitro at the expense of hamster PrP^{C}. Interestingly, in the first PMCA round, the glycoform distribution pattern of the in vitro-generated hamster PrP^{Sc} was comparable to the RML profile showing the three glycoform bands (Figure 1D). After further PMCA rounds, this pattern changed to become undistinguishable from PrP^{Sc} associated to the typical hamster strains, such as 263K (Figure 1D) or Hyper (HY), in which the diglycosylated band is highly predominant. This result suggests that the characteristics of the newly generated PrP^{Sc} are being adapted to the new species during successive PMCA cycling, reminiscent of the adaptation process occurring in vivo upon serial passage of the infectious material. After 20 serial rounds of PMCA, representing a dilution equivalent to 10^{-22} with respect to the brain (since the first round contains a 100-fold dilution of the material), our estimation is that no molecules of mouse brain PrP^{Sc} should be present in the sample. This in vitro-generated material was termed RML-Ha PrP^{Sc} to emphasize the RML origin of this new hamster misfolded prion protein. To make sure that newly formed PrP^{Sc} was indeed coming from conversion of hamster PrP^{C} induced by mouse PrP^{Sc} and not just spontaneous "de novo" formation of PrP^{Sc} in hamsters (Deleault et al., 2007), we did a large experiment to analyze in detail the possibility of spontaneous generation of PrP^{Sc} and infectivity under our experimental conditions. Samples of healthy brain homogenate from ten different hamsters were subjected to serial rounds of PMCA amplification in the absence of PrP^{Sc} seed. After up to 20 serial rounds of PMCA, we did not observe de novo formation of PrP^{Sc} in any of the samples (Figure 1E).

Inoculation of wild-type hamsters with RML-Ha PrP^{Sc} (produced after a 10^{-22} dilution of RML scrapie brain homogenate) produced disease in 100% of the animals by both intracerebral (i.c.) and intraperitoneal (i.p.) routes (Figure 2). The disease exhibits the clinical characteristics typical of hamster scrapie, including hyperactivity, motor impairment, head wobbling, muscle weakness, and weight loss. The incubation time in the first passage was 165 ± 6 days by i.c. inoculation (Figures 2A and 2C). This is longer than the incubation time obtained with hamster scrapie strains, such as 263K and HY, in which a similar quantity of PrP^{Sc} produces disease at around 100 days by this route (Figures 2A and 2C). However, in agreement with our previously reported data (Castilla et al., 2005), when hamster 263K prions were replicated in vitro by PMCA, the newly generated PrP^{Sc} produced disease with a delay similar to that observed with the RML-Ha material (Figures 2A and 2C). The delay in our previous study was eliminated upon a second passage in vivo, in which the new infectious material was stabilized to acquire properties undistinguishable from in vivo-derived 263K (Figures 2B and 2C). Interestingly, in the HY hamster prion strain, PMCA-generated material did not show any statistically significant difference compared to in vivo-produced prions (Figures 2A and 2C). These results suggest that in vitro replication of prions by PMCA maintains the strain characteristics, at least in respect to the incubation periods. To assess the stability of RML-Ha and estimate the stabilized incubation period, we performed a second passage. As shown in Figure 2B, the incubation time of RML-Ha prions was decreased to around 90 days, which is very similar to that obtained with 263K and HY but different from the Drowsy (DY) strain. These results suggest that RML-Ha prions behave similarly to the 263K strain; both in vitro-generated prions show a delay in the first passage that gets corrected upon a second in vivo passage. This feature is not displayed by other hamster prion strains, such as HY, or other species of prions (see below for the results in mice), where PMCA-generated prions exhibited the same incubation period in the first passage as in vivo-produced infectious material. As expected, hamsters inoculated with RML prions did not develop disease during the time of the experiment (>400 days). Animals inoculated with hamster brain homogenate subjected to 20 rounds of PMCA in the absence of PrP^{Sc} (control for the de novo generation of PrP^{Sc}) did not develop disease more than 400 days after inoculation (Figures 2A and 2C). Intraperitoneal inoculations of the infectious material showed a clear difference between the three prion strains used as reference, with 263K being the fastest and DY not producing disease by this route (Figure 2D). The incubation period produced by i.p. inoculation of RML-Ha prions was longer than that of the 263K and HY strains, with an average of 254 days in the first passage. This is also longer than 263K prions amplified in vitro by PMCA, which produced disease after 199 days postinoculation in the first passage (Figures 2D and 2F). A second in vivo passage again stabilized PMCA-generated 263K prions to produce disease at a time indistinguishable from that of brain-derived 263K infectious material. The second passage of RML-Ha prions showed that the stabilized incubation period for the i.p. route was on average around 140 days, which is significantly higher than 263K or 263K-PMCA material but shorter than HY prions (Figures 2E and 2F). The differences remained stable in a third passage (data not shown). These results indicate that in some aspects, RML-Ha prions are similar to the agent in the 263K strain but in other features are intermediate between 263K



Sample	Incubation period 1st passage (days)	Incubation period 2nd passage (days)
RML	>400	>400
RML-PMCA	>400	ND
RML-Ha	165 ± 6	91 ± 4
263K	104 ± 2 ***	86 ± 1
263K-PMCA	155 ± 6	88 ± 2
HY	94 ± 3 ***	89 ± 2
HY-PMCA	100 ± 2 ***	ND
DY	211 ± 25 ***	219 ± 6 ***
PMCA-No PrP^{Sc}	>400	ND

Sample	Incubation period 1st passage (days)	Incubation period 2nd passage (days)
RML	>400	>400
RML-PMCA	>400	ND
RML-Ha	254 ± 10	143 ± 22
263K	105 ± 7 ***	100 ± 2 ***
263K-PMCA	199 ± 12 **	102 ± 2 ***
HY	194 ± 13 **	169 ± 6 *
HY-PMCA	ND	ND
DY	>400	ND
PMCA-No PrP^{Sc}	>400	ND

Figure 2. Infectivity of Newly Generated RML-Ha PrP^{Sc} after Crossing the Species Barrier

RML-Ha PrP^{Sc} samples amplified by 20 serial PMCA rounds were inoculated i.c. or i.p. into six wild-type hamsters. For controls, we inoculated similar quantities of PrP^{Sc} from RML or three distinct hamster strains (263K, Hyper, and Drowsy). We also show the data obtained by inoculation of in vitro-generated prions through 20 serial rounds of PMCA by incubation of 263K (263K-PMCA) or Hyper (HY-PMCA) PrP^{Sc} with healthy hamster brain homogenate and RML replicated at expenses of mouse PrP^{C} (RML-PMCA). The figure also show the results obtained by inoculation of the material produced after 20 rounds of PMCA with unseeded normal hamster brain homogenate (PMCA-No PrP^{Sc}). (A) and (D) show the survival curves obtained after i.c. and i.p. inoculation, respectively, of the in vitro-generated RML-Ha after 20 rounds of PMCA. (B) and (E) show the survival curves of the second passage (i.e., animals were inoculated with material obtained from the brain of sick animals in the experiments depicted in [A] and [D]) after i.c and i.p inoculation, respectively. (C) and (F) show the average incubation periods of the experiments done by i.c. and i.p. inoculation of various samples. The values correspond to the average ± standard error. The data was analyzed by ANOVA and the Dunnett multiple comparison post-test. Each set of data was compared to the results obtained with the RML-Ha strain, and significant differences are highlighted with asterisks (* = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$). ND, not done.

and HY prions, providing a first indication that the material obtained by crossing of the mouse-hamster species barrier represents a unique hamster prion strain.

To further assess the characteristics of the disease produced by in vitro-generated RML-Ha prions, we studied in detail the neuropathological and biochemical features of the brain damage. Histopathological studies showed that animals inoculated with RML-Ha prions exhibit the typical brain lesions of scrapie, including spongiform degeneration, astrogliyosis, and PrP^{Sc} deposition (Figures 3A–3C). Quantitative studies of the vacuolation profile in different brain areas showed that RML-Ha-infected hamsters showed the largest extent of spongiosis in medulla and cerebellum and less damage in hippocampus, cortex, and colliculum (Figure 3D). This pattern of brain damage was similar to that observed in 263K-inoculated animals and statistically different from that obtained in hamsters injected with HY and DY (Figure 3D). However, the extent of both astrogliyosis (Figure 3B) and PrP^{Sc} accumulation (Figure 3C) in the medulla of RML-Ha-infected animals was lower than that in 263K-sick animals and similar to that observed in HY-injected hamsters (Figures 3B and 3C). These data suggest again that the RML-Ha prions are a unique strain with properties intermediate between the previously known 263K and HY hamster strains.

Comparative studies of the biochemical characteristics of PrP^{Sc} obtained from the brain of sick animals after inoculation with RML-Ha, 263K, HY, and DY were done by analysis of the electrophoretical pattern of the protein, its susceptibility to proteolytic degradation, and its resistance to denaturation. For comparison of the protease resistance profile, similar quantities of PrP^{Sc} from the new RML-Ha prions and PrP^{Sc} obtained from the brain of sick hamsters inoculated with the prion strains 263K, HY, and DY were treated for 60 min with various concentrations of proteinase K (PK) (Figure 4A). RML-Ha PrP^{Sc} was highly resistant to large PK concentrations. The misfolded protein associated to the newly generated strain was more resistant than HY or DY and similarly (but still significantly more) susceptible to PK digestion than 263K PrP^{Sc} (Figure 4A). The PK concentration in which 50% of the protein was degraded (PK50) was highest for PrP^{Sc} associated to RML-Ha, followed by 263K, HY, DY, and RML (Table S1 available online).

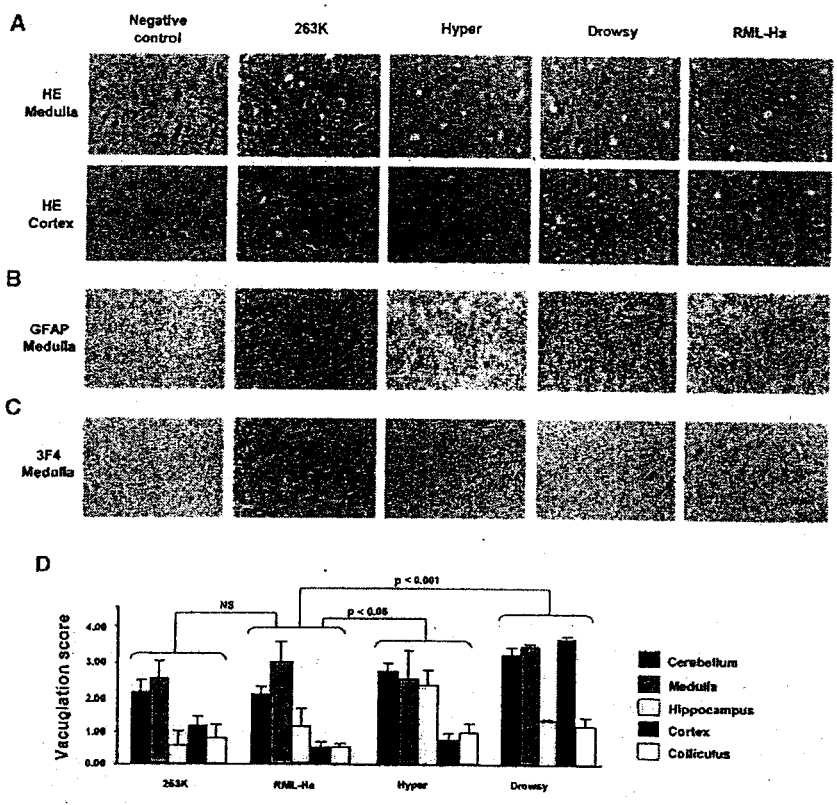


Figure 3. Histopathological Features of the Disease Induced by Inoculation of Hamsters with PMCA-Generated RML-Ha PrP^{Sc}

Brain from sick animals in which disease was produced by inoculation with the in vitro-generated RML-Ha PrP^{Sc} (first passage) or the known hamster strains 263K, Hyper, and Drowsy were analyzed by histological studies. As a control, we used the brain of a hamster inoculated with PBS and sacrificed without disease at 350 days after inoculation.

(A) Spongiform degeneration was evaluated after hematoxilin-eosin staining of medulla and occipital cortex sections and visualized by microscopy at a 40× magnification.

(B) Reactive astrogliosis was evaluated by histological staining with glial fibrillary acidic protein antibody.

(C) PrP accumulation in these animals was evaluated by staining of the tissue with the 3F4 anti-PrP monoclonal antibody.

(D) The vacuolation profile in each brain area was estimated with a semiquantitative scale, as described in the Experimental Procedures. The brain areas used were the following: occipital cortex, cerebellum (mostly white matter), medulla (spinal 5 nucleus, interpolar part), inferior colliculum, and hippocampus (CA1 and CA2 regions). We also included in the analysis brain sections from animals inoculated with the other hamster prion strains. The values represent the average ± standard error of the extent of vacuolation from the five animals analyzed in each set. Statistical analysis by two-way ANOVA with brain regions and prion origin as the variables indicated that differences were highly significant ($p < 0.001$). To assess the significance of the differences between each known prion strain and RML-Ha, we used the Dunnett multiple comparison post-test, and the p values for each combination are shown.

Another characteristic we studied was the electrophoretic mobility and glycosylation pattern of PrP^{Sc} associated to distinct strains. The predominant glycoform for the hamster strains (including the newly generated RML-Ha) is the diglycosylated band, whereas mouse RML PrP^{Sc} shows a more even distribution of the three bands with the main one being the monoglycosylated form. To assess the size of the protein after PK cleavage, we performed endoglycosidase treatment to remove the glycosylated chains (Figure 4B). Whereas PrP^{Sc} associated to the DY strain has a higher electrophoretical mobility, no significant differences were observed among the other proteins. Another biochemical property of misfolded PrP often used to differentiate prion strains is its resistance to chemical denaturation (Safar et al., 1998). Clear differences were observed in the guanidine concentrations required to denature PrP^{Sc} associated to different strains (Figure 4C). The concentration of the chaotropic agent needed to denature 50% of PrP^{Sc} RML-Ha was 1.11 M, substantially different from the 1.69, 1.56, and 1.72 M required for the proteins associated to HY, DY, and RML, respectively (Table S1).

Crossing the Hamster-Mouse Species Barrier to Generate and Stabilize New Mouse Prions

To study the barrier between these rodent species in the opposite direction, we mixed 263K hamster prions with mouse healthy brain homogenate. As before, when a standard PMCA assay was done by dilution of 263K brain homogenate 1000-fold into mouse healthy brain material, we did not see detectable generation of mouse PrP^{Sc} (data not shown). However, when a higher quantity of hamster PrP^{Sc} was added, we were able to generate new mouse PrP^{Sc} (termed 263K-Mo) that could be propagated by serial rounds of PMCA to reach a dilution of the hamster brain homogenate equivalent to 10^{-17} (Figure 5A). Since there are not available antibodies capable of recognizing mouse PrP but not hamster PrP, we could not compare the electrophoretical pattern of PrP^{Sc} generated in the first rounds of PMCA with the profile of PrP^{Sc} typically observed in mouse and hamster strains. However, the western blot pattern of 263K-Mo after 15 rounds of PMCA (when no more molecules of 263K PrP^{Sc} are present) is similar to the one observed for RML and other ovine-derived mouse strains, despite a slightly faster migration (Figure S1A) that will be investigated in more detail later. To assess whether newly generated PrP^{Sc} was indeed coming from conversion of mouse PrP^{C} induced by 263K hamster PrP^{Sc} and not just spontaneous "de novo" formation of PrP^{Sc} in mice, we did an experiment to analyze the possibility of spontaneous generation of PrP^{Sc} and infectivity under our experimental conditions. Samples of healthy brain homogenate from ten different mice were subjected to serial rounds of PMCA amplification in the absence of PrP^{Sc} seed. After up to 20 serial rounds of PMCA, we did not observe de novo formation of PrP^{Sc} in any of the samples (Figure S1B).

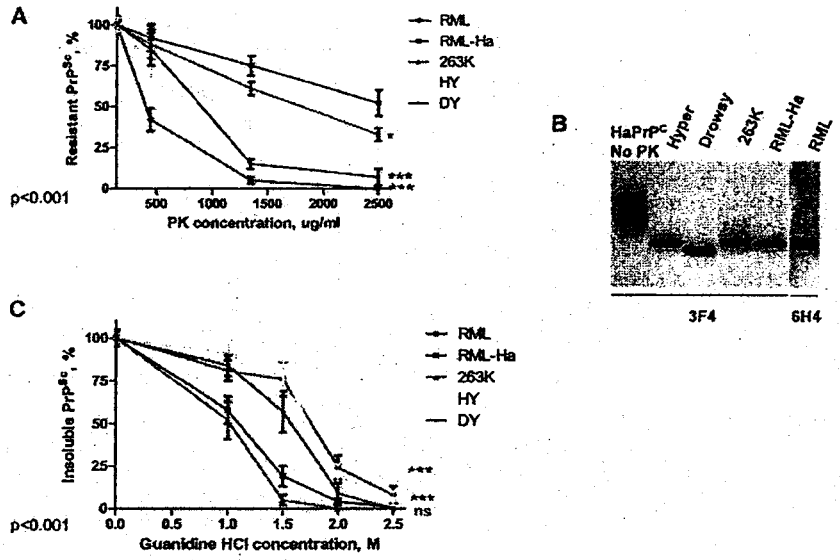


Figure 4. Biochemical Characterization of RML-Ha PrP^{Sc}

Samples from brains of animals inoculated with RML-Ha PrP^{Sc} (first passage in vivo) were used to study the PK resistance profile (A), the relative mobility after deglycosylation and PK treatment (B), and the susceptibility to guanidine denaturation (C). For controls, we used samples from RML or three distinct hamster strains (263K, Hyper, and Drowsy). The results in (A) and (C) correspond to the quantitative evaluation of western blots by densitometric analysis from three independent animals. The data represent the average ± standard error. The data were analyzed by ANOVA and the Dunnett multiple comparison post-test. Each set of data was compared to the results obtained with the RML-Ha strain, and significant differences are highlighted with asterisks (* = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$).

To assess whether mouse PrP^{Sc} generated in vitro from hamster 263K is infectious to wild-type mice and to determine whether the infectious properties are being adapted upon serial PMCA passages, we inoculated several rounds of in vitro-generated material into mice (Figure 5A). Despite the fact that the same amount of PrP^{Sc} was inoculated (as determined by western blot), striking differences in the infectious properties were seen among in vitro-generated prions in distinct rounds of PMCA (Figure 5B). Only two of the six mice inoculated with material produced in the first round of PMCA showed disease symptoms, which appear at a very long time after inoculation (around 500 days) (Figures 5B and 5C). A complete attack rate was observed when animals were inoculated with material produced after three serial rounds of PMCA. However, the incubation period was long (around 310 days on average), and there was a large dispersion among animals (Figures 5B and 5C). The incubation period became stable, short (around 165 days), and there was little dispersion after the six serial rounds of PMCA. These findings indicate that upon successive rounds of PMCA, the newly generated prion, after crossing the species barrier, is becoming adapted and stabilized to the new host, a process very similar to what is seen after several passages in vivo. The large dispersion of incubation times observed in the third round of PMCA suggests that more than one strain has been generated upon crossing of the species barrier and that successive in vitro amplification leads to the selection and cloning of the most efficient of these strains. The incubation time for 263K-Mo after 15 rounds of PMCA (equivalent to a 10^{-17} dilution of the 263K inoculum) was around 165 days, similar to the one produced by scrapie-adapted mouse strains, such as RML, but different from that of the bovine strain 301C (Figure 5D). In vitro replication of the mouse strains RML and 301C at expense of mouse PrP^{C} produced PrP^{Sc} with identical properties as the brain-derived material, reflected as an indistinguishable incubation period (Figure 5D). As expected, mice inoculated with hamster 263K prions did not develop disease during the time of the experiment (>500 days). No disease was also observed in animals inoculated with mouse brain homogenate subjected to 20 rounds of PMCA in the absence of PrP^{Sc}, which corresponds to the control experiment for the de novo generation of PrP^{Sc} (Figure 5D).

To analyze whether the newly generated 263K-Mo infectious material corresponded to a new strain of mouse prions, we studied the histopathological and biochemical features of the brain damage. Animals affected with the disease produced by inoculation of 263K-Mo showed extensive vacuolation in the medulla and hypocampus and moderate but clearly detectable damage in the cerebellum (Figures 6A and 6D). The pattern of spongiform degeneration does not correspond with any of the previously known mouse strains studied and indeed is statistically significantly different to the vacuolation profile produced by RML and 301C prions (Figure 6D). Differences were also detected in the extent of brain inflammation produced by 263K-Mo, since the degree of astroglyosis was less prominent than the one observed in animals inoculated with RML or 301C prions (Figure 6B). The profile of PrP^{Sc} accumulation consisted mostly of diffuse deposition and was not clearly different from the one observed in the other strains (Figure 6C). Then we studied the biochemical characteristics of PrP^{Sc} obtained from the brain of animals infected with 263K-Mo. Electrophoretical migration was assessed after PK digestion and endoglycosidase treatment to remove glycosylation chains. The PK-resistant core of PrP^{Sc} migrated slightly faster than RML but slightly slower than 301C, with an estimated molecular weight of 20 KDa (Figures 7A and 7B). These results indicate that the cleavage site after PK digestion is different from all of the currently known mouse strains. This is important because it is thought that differences in the PK cleavage site reflect disparities in the folding or aggregation of the protein (Chen et al., 2000; Collinge et al., 1996). To further search for biochemical differences, we subjected the protein to proteolytic degradation by using various concentrations of PK. 263K-Mo PrP^{Sc} was much more resistant to PK than to RML (Figure 7C), with a PK_{50} (the PK concentration needed to

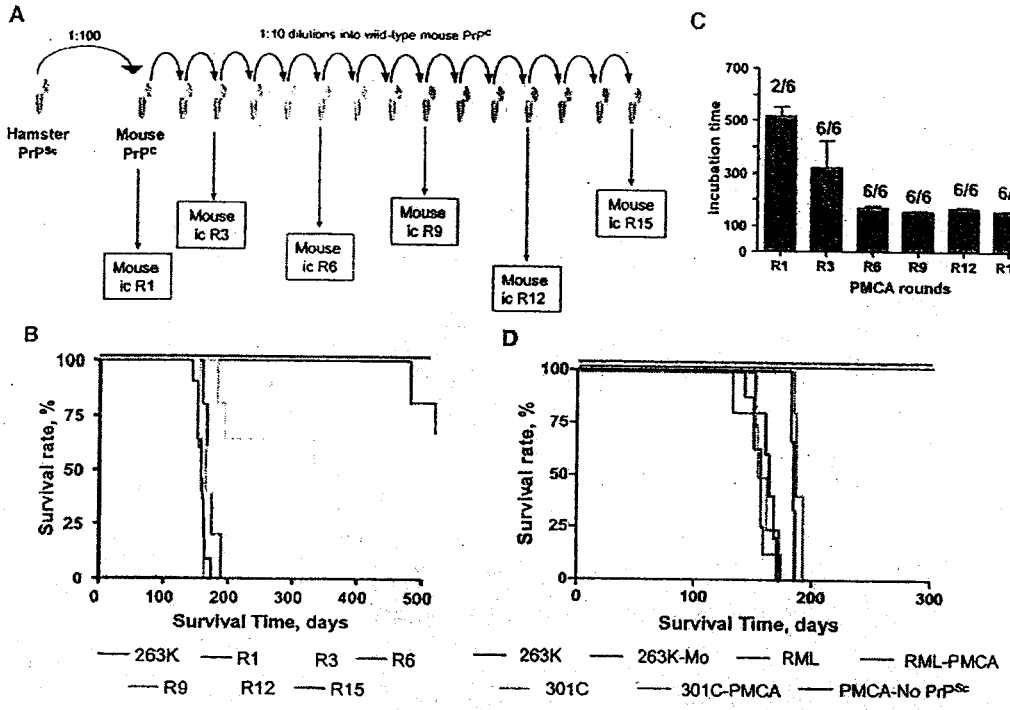


Figure 5. In Vitro Conversion of Mouse PrP^C Induced by Hamster 263K PrP^{Sc} Generates Infectious Prions

(A) Schematic representation of the dilutions done and the PMCA rounds used for our in vivo infectivity experiments.

(B) Survival curve observed after inoculation of six wild-type mice with the material generated after several rounds of PMCA. "R" indicates the number of rounds of PMCA. As a control, the animals were inoculated with 263K hamster prions.

(C) Average and standard error of the incubation times and attack rates observed after inoculation of wild-type mice with the material produced after different rounds of PMCA.

(D) Comparison of survival curves for the stabilized 263K-Mo infectious material (after 15 rounds of PMCA) with those obtained with RML and 301C, two mouse strains of different origin. We also show the data obtained by inoculation of in vitro-generated prions through 20 serial rounds of PMCA by incubation of RML (RML-PMCA) or 301C (301C-PMCA) PrP^{Sc} with healthy mouse brain homogenate. The figure also show the results obtained by inoculation of the material produced after 20 rounds of PMCA with unseeded normal mouse brain homogenate (PMCA-No PrP^{Sc}), which correspond to the control for de novo generation of prions. For all of these experiments, the material was inoculated i.c. as described in the Experimental Procedures.

degrade half of the protein) of 1450 μg/ml (Figure 7D), much larger than the values obtained for RML (240 ug/ml) and 301C (430 ug/ml) (Table S2). Interestingly, the high resistance of PrP^{Sc} is typical of the hamster prions (Table S1), and indeed, 263K, the parental strain of the newly generated mouse prions, has a PK_{50} of around 1700 μg/ml.

DISCUSSION

The phenomenon of the species barrier, by which the agent coming from one species can infect only a limited number of other species, is a typical feature of prion diseases. The molecular basis of this process is not well-understood, but it is thought to be controlled by the structure and folding of the prion protein (Moore et al., 2005; Vanik et al., 2004). As with the related phenomenon of prion strains, it is difficult to imagine how an infectious agent lacking genetic material and composed by a single protein can encode the structural diversity and specificity required to control strains variability and species selectivity (Soto and Castilla, 2004).

In addition to the intriguing molecular mechanism behind the species barrier, understanding this phenomenon has profound implications for public health. Indeed, one of the scariest medical problems of the last decades has been the emergence of a new and fatal human prion disease (variant CJD) originated by cross-species transmission of BSE from cattle (Will et al., 1996). BSE has not only been transmitted to humans. The extensive use of cow-derived material for feeding other animals led to the generation of new diseases in exotic felines, nonhuman primates, and domestic cats (Doherr, 2003). Worrisomely, the transmission of BSE into these different species could create new prion strains with unique biological and biochemical characteristics and thus a potentially new hazard for human health. More frightening is perhaps the possibility that BSE has been passed into sheep and goats. Studies have already shown that this transmission is possible and actually relatively easy (Foster et al., 1993). The disease produced is clinically similar to scrapie, but since it comes from BSE it has the potential to be infectious to humans. Another concern is CWD, a disorder affecting farm and wild species of cervids (Sigurdson and Aguzzi, 2006; Williams, 2005). The

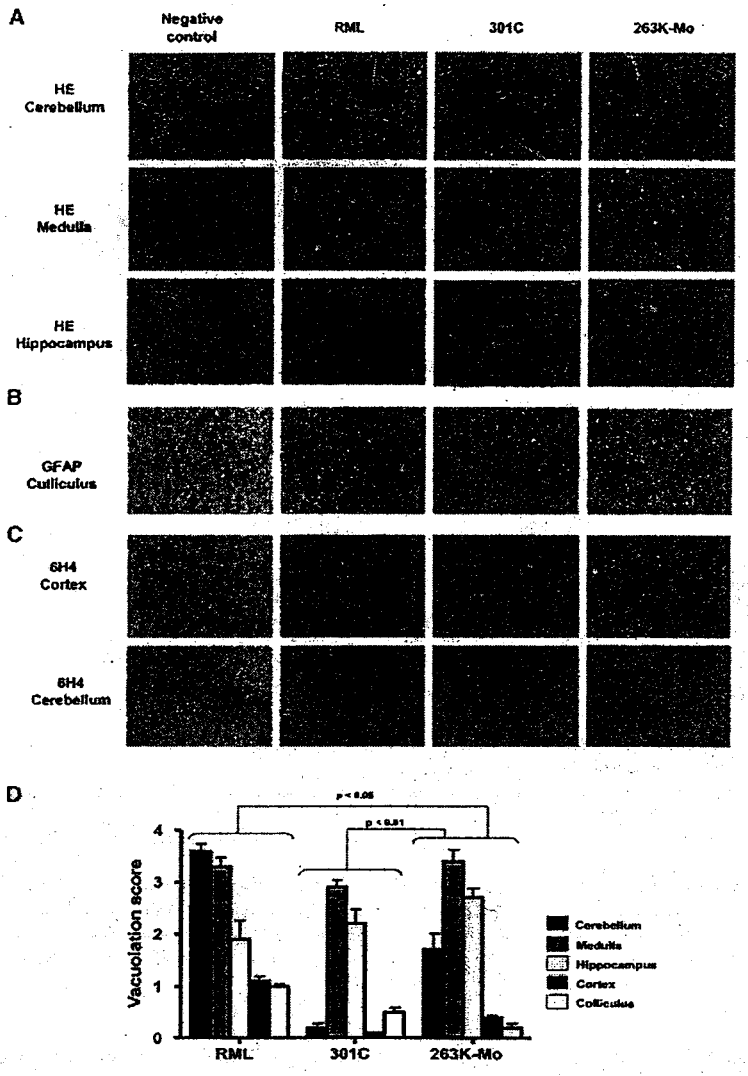


Figure 6. Histopathological Features of the Disease Induced by Inoculation of Mice with PMCA-Generated 263K-Mo PrP^{Sc}

Brains from sick mice in which disease was produced by inoculation with the newly generated 263K-Mo prions after 15 rounds of PMCA (first passage) or the known mouse strains RML and 301C were analyzed by histological studies. As a control, we used brain of a mouse inoculated with PBS and sacrificed without disease at 350 days after inoculation.

(A) Spongiform degeneration was evaluated after hematoxilin-eosin (HE) staining of three different brain areas (cerebellum, medulla, and hippocampus) and was visualized at a 40× magnification.

(B) Reactive astroglyosis was evaluated in the inferior culliculus by staining with glial fibrillary acidic protein antibody.

(C) PrP accumulation in these animals was evaluated in the occipital cortex and cerebellum by staining of the tissue with the 6H4 antibody.

(D) The vacuolation profile in each brain area was estimated with a semiquantitative scale, as described in the Experimental Procedures. The brain areas used were the following: occipital cortex, cerebellum (mostly white matter), medulla (spinal 5 nucleus, interpolar part), inferior colliculum, and hippocampus (CA1 and CA2 regions). We also included in the analysis brain sections from animals inoculated with RML and 301C. The values represent the average ± standard error of the extent of vacuolation from the five animals analyzed in each set. Statistical analysis by two-way ANOVA with brain regions and prion origin as the variables indicated that differences were highly significant ($p < 0.001$). To assess the significance of the differences between each known prion strain and 263K-Mo, we used the Dunnett multiple comparison post-test, and the p values for each combination are shown.

origin of CWD and its potential to transmit to humans are currently unknown. This is worrisome, considering that CWD has became endemic in some parts of the USA and that the number of cases continues to increase (Williams, 2005). CWD transmissibility studies have been performed in many species in order to predict how this disease could be spread by the consumption of CWD meat (Sigurdson and Aguzzi, 2006). Transmission of CWD to humans cannot be ruled out at present, and a similar infective episode to BSE involving CWD could result in catastrophic consequences.

The exciting scientific problem coupled with the relevant public-health issue prompted us to develop strategies to reproduce the species-barrier phenomenon in the test tube. We reported previously the generation of infectious prions in vitro by cyclic replication of the protein misfolding process featuring the pathogenesis of prion diseases (Castilla et al., 2005). These results were reproduced and extended by other groups to better dissect the elements required for prion replication (Deleault et al., 2007; Weber et al., 2007). The PMCA technology has been adapted to replicate prions from various species (Deleault et al., 2005; Jones et al., 2007; Kurt et al., 2007; Murayama et al., 2007; Sarafoff et al., 2005; Soto et al., 2005) and even to use bacterially produced recombinant PrP as substrate (Atarashi et al., 2007). The conclusion drawn from these studies together with the findings reported in this manuscript is that propagation of the PrP^{Sc} misfolding results in formation of infectious material, which maintains the strains and species-barrier properties of the original prions. Qualitatively similar conclusions have been obtained for yeast prions, which are a group of "infectious proteins" that behave as a non-Mendelian genetic element and transmit biological information in the absence of nucleic acid (Wickner et al., 1995). Recent studies showed that bacterially produced N-terminal fragments of the yeast prions Sup35p and Ure2p when transformed into amyloid fibrils were able to propagate the prion phenotype to yeast cells (Brachmann et al., 2005; King and Diaz-Avalos, 2004; Tanaka et al., 2004). Infection of yeast with different conformers led to generation of distinct prion strains in vivo (Brachmann et al., 2005; Tanaka et al., 2004). Remarkably, yeast prions also show the species-barrier phenomenon, and recent data indicate that strain conformation is the critical determinant of cross-species prion transmission (Tanaka et al., 2005).

In the current study, we demonstrate the generation of new infectious prions across the species barrier. For this purpose, we

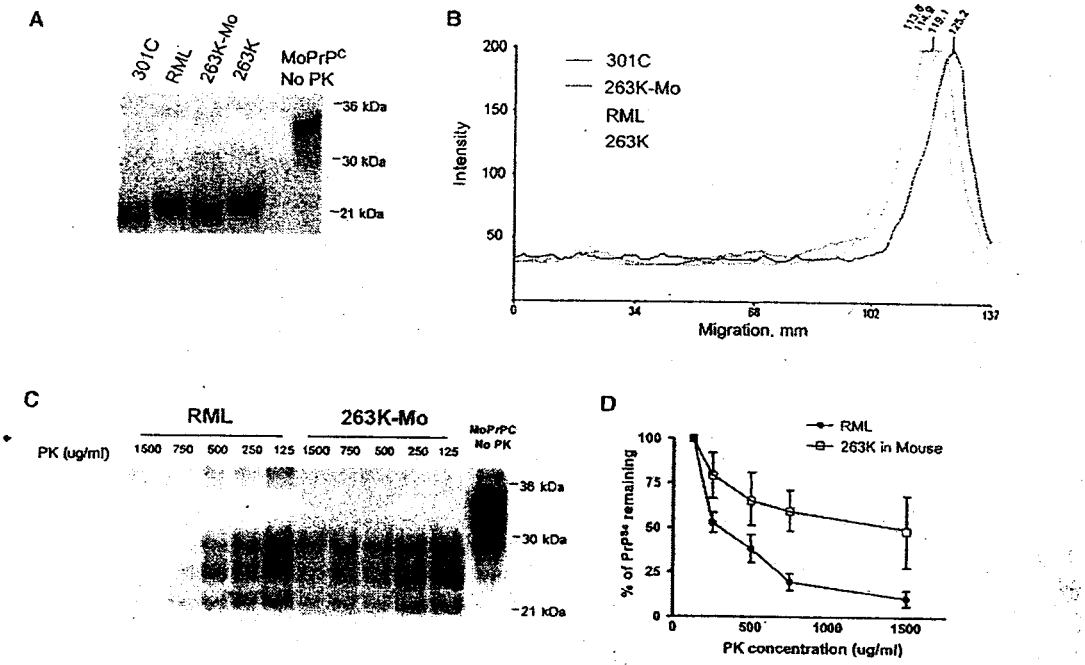


Figure 7. Biochemical Characteristics of 263K-Mo PrP^{Sc}

(A) Samples from brains of mice inoculated with 263K-Mo, RML, or 301C were used to study the electrophoretical migration after deglycosylation and PK treatment.

(B) For assessment of the electrophoretical differences among distinct strains, the blot in (A) was scanned and analyzed by software included in the UVP image analysis system to locate the exact position of the bands.

(C) The PK resistance profile of 263K-Mo PrP^{Sc} was studied and compared with RML.

(D) The results of the experiment shown in (B) were quantitated by densitometric analysis. The data in the figure represent the average ± standard error from three independent animals. The differences were statistically significant as evaluated by one-way ANOVA ($p < 0.01$).

mixed PrP^{Sc} from one species with PrP^{C} from a different animal species and subjected the mixture to serial rounds of PMCA to generate, propagate, and stabilize new prion strains. Hamster PrP^{Sc} generated from mouse RML prions was infectious to wild-type hamsters. Detailed analysis of the disease characteristics and comparison with the illness produced by several known hamster prion strains indicate that the in vitro-generated infectious material across the species barrier corresponds to a new prion strain in hamsters (termed RML-Ha). The main differences of the RML-Ha were on the incubation times after i.p. inoculation, the extremely high resistance to PK degradation, and the pattern of brain damage (Table S1). Similarly, PrP^{Sc} generated by conversion of mouse PrP^{C} with hamster PrP^{Sc} from the 263K strain was shown to be infectious to wild-type mice, with an incubation period comparable to that obtained after inoculation with some of the mouse-adapted scrapie strains, such as RML. Again, the disease produced by the new prions (termed 263K-Mo) was clearly distinguishable from the one produced by some of the currently known mouse prion strains. The major differences were seen in the electrophoretical migration, extremely high resistance to proteolytic degradation, and pattern of brain spongiform degeneration (Table S2). To rule out that newly generated PrP^{Sc} in these experiments was coming from "de novo" spontaneous conversion of PrP^{C} into PrP^{Sc} during PMCA, we used samples of healthy brain homogenate from ten different mice and hamsters that were subjected to serial rounds of PMCA amplification in the absence of PrP^{Sc} seed. After up to 20 serial rounds of PMCA, we did not observe de novo formation of PrP^{Sc} in any of the samples. This material was inoculated into wild-type animals, and no disease was observed more than 400 days after inoculation. These results strongly indicate that the generation of PrP^{Sc} reported in the present study was due to interspecies prion conversion. Nevertheless, we would like to highlight that recently we have been able to generate in vitro PrP^{Sc} de novo without addition of PrP^{Sc} seed (data not shown). However, to reach this aim, the PMCA conditions need to be modified. The modifications include changes on the PMCA parameters (length of incubation and potency of sonication), preincubation, or pretreatment of the normal brain homogenate to induce/stabilize PrP misfolding prior to PMCA. These findings suggest that de novo formation of PrP^{Sc} can be experimentally distinguished from replication of preformed PrP^{Sc}, indicating that the biochemical, conformational, or stability properties of the PrP structures involved in both processes are probably different. Standard PMCA conditions, as those used in the current study, do not result in spontaneous PrP^{Sc} formation.

Interestingly, in our serial PMCA amplifications of RML PrP^{Sc} into hamster PrP^{C}, we observed a progressive change on the western blot profile of the newly generated RML-Ha PrP^{Sc}. Indeed, in the first round of PMCA, the glycoform distribution

pattern was reminiscent of RML and later switched to a profile typical of the hamster strains, characterized by the predomination of the diglycosylated form (Figure 1D). Our interpretation of this result was that consecutive rounds of PMCA may enable the new prion strain to adapt and stabilize. To further study this possibility in our experiments in which mouse prions were generated from 263K hamster prions, we inoculated the material generated after various rounds of PMCA. Strikingly, similar amounts of PrP^{Sc} generated after one and three rounds of PMCA produced disease with incomplete attack rates and/or very long incubation periods (Figures 5B and 5C). Incubation time stabilized after six rounds of serial PMCA, suggesting that at this point the new strain is fully adapted. These findings suggest that PMCA is not only able to reproduce the interspecies transmission of prions but is also able to mimic the strain adaptation process observed in vivo. In vivo adaptation and stabilization of prions generated after crossing the species barrier takes at least four consecutive passages, which requires several years of work (Race et al., 2001, 2002). Conversely, strain adaptation by PMCA takes only 2 or 3 weeks. Importantly, the kinetics of adaptation in vitro and in vivo, as well as the characteristics of the stabilized material, are very similar. Indeed, it has been reported that three serial passages of 263K in mice produce disease in all animals, with an incubation time of around 300 days (Race et al., 2002). This result is very similar to the data obtained with the material generated in vitro after three successive rounds on PMCA replication (Figures 5B and 5C). Moreover, less than three in vivo passages produced an incomplete attack rate, and more than three passages are needed to obtain a stable and low incubation period (Race et al., 2002), which is in the same range of our 263K-Mo infectious material. Finally, similar to our in vitro data, the in vivo cross-species transmission between hamsters and mice also led to the generation of unique prion strains (Race et al., 2001, 2002). Although we are tempted to speculate that each PMCA round has the same effect on strain adaptation as did each in vivo passage, more experiments with other species combinations are needed to reach this conclusion.

In summary, our results show that all elements controlling interspecies transmission of prions are contained in a cell-free system and that new prion strains can be generated, adapted, and stabilized upon crossing the species barrier in vitro by PMCA. These findings provide additional support for the prion hypothesis, suggesting that species-barrier transmission and strain generation are determined by the propagation of PrP misfolding. Furthermore, the data demonstrate that PMCA is a valuable tool for the investigation of the strength of the barrier between diverse species, its molecular determinants, and the expected features of the new infectious material produced. Finally, our findings suggest that the universe of possible prions is not restricted to those currently known but that likely many unique infectious foldings of the prion protein may be produced and that one of the sources for this is cross-species transmission.

EXPERIMENTAL PROCEDURES

Preparation of Tissue Homogenates

Healthy and sick animals were perfused with phosphate-buffered saline (PBS) plus 5 mM ethylenediaminetetraacetic acid (EDTA) before the tissue was harvested. Ten percent brain homogenates (w/v) were prepared in conversion buffer (PBS containing 150 mM NaCl, 1.0% Triton X-100, and the complete cocktail of protease inhibitors from Boehringer Mannheim, Mannheim, Germany). The samples were clarified by a brief, low-speed centrifugation (1500 rpm for 30 s) with an Eppendorf centrifuge (Hamburg, Germany), model 5414.

Serial Replication of Prions In Vitro by PMCA

Aliquots of 10% brain homogenate from clinically sick mice infected with RML or 301C and hamsters infected with 263K, HY, or DY prions were diluted into 10% hamster or mouse healthy brain homogenate. Samples were loaded onto 0.2 ml PCR tubes and positioned on an adaptor placed on the plate holder of a microsonicator (Misonix Model 3000, Farmingdale, NY). Each PMCA cycle consisted of 30 min incubation at 37°C followed by a 20 s pulse of sonication set at potency of 7. Samples were incubated without being shaken immersed in the water of the sonicator bath. After a round of PMCA cycles, a 10 µl aliquot of the amplified material was diluted into 90 µl of more normal brain homogenate, and a new round of PMCA cycles was performed. This procedure was repeated several times to reach the final dilutions indicated in the text. The detailed protocol for PMCA, including reagents, solutions, and troubleshooting, has been published elsewhere (Castilla et al., 2006; Saa et al., 2005).

Proteinase K Degradation Assay

The standard procedure for digestion of PrP^{Sc} consists of subjecting the samples to incubation in the presence of PK (50 µg/ml) for 60 min at 37°C. The digestion was stopped by addition of electrophoresis sample buffer, and the protease-resistant PrP was revealed by western blotting. So that the profile of PK sensitivity for in vitro- and in vivo-generated PrP^{Sc} could be studied, the samples were incubated for 60 min at 37°C with different concentrations of PK ranging from 0 to 2500 µg/ml. The PK_{50} values represent the concentration of PK needed to digest half of the protein, and these values are estimated on the basis of the densitometric analysis of three replicated western blots.

Guanidine Denaturation Assay

Samples were incubated with different concentrations of guanidine hydrochloride for 2 hr at room temperature with shaking. Thereafter, samples were incubated in the presence of 10% sarkosyl for 30 min at 4°C and centrifuged at 100,000 × g for 1 hr in a Biosafe Optima MAX ultracentrifuge (Beckman Coulter, Fullerton, CA). The pellet of the centrifugation was resuspended in conversion buffer and treated with PK as described above. Equivalent aliquots of pellet were analyzed by western blot. The Gdn_{50} value corresponds to the concentration of guanidine hydrochloride required to denaturate 50% of the protein, and these values were estimated on the basis of the densitometric analysis of three replicated western blots.

Protein Deglycosylation Assay

PrP^{Sc} samples were first digested with PK as describe above. After addition of 10% sarkosyl, samples were centrifuged at 100,000 × g for 1 hr at 4°C, supernatant was discarded, and the pellet resuspended in 100 µl of glycoprotein denaturing buffer (New England Biolabs, Beverly, MA) and incubated for 10 min at 100°C. Thereafter, 26 µl of 50 mM sodium phosphate (pH 7.5) containing 1% nonidet P-40 and 3 µl of peptide N-glycosidase F (New England Biolabs, Beverly, MA) were added. Samples were incubated for 2 hr at 37°C, and the reaction was stopped by the addition of electrophoresis buffer and samples were analyzed by western blot.

Western Blot

Proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, electroblotted into nitrocellulose membrane, and probed with 6H4 (for mouse samples) and 3F4 (for hamster samples) antibodies at a 1:5000 dilution. The immunoreactive bands were visualized by enhanced chemoluminesence assay (Amersham, Piscataway, NJ) with an UVp image analysis system. So that the quantity of PrP^{Sc} in the western blot would be assessed, densitometric analyses were done by triplicate.

PrP^{Sc} Quantification

To inject the same quantity of PrP^{Sc} from each preparation, we compared the samples by western blotting after PK digestion. To obtain a reliable and robust quantification, we ran several different dilutions of the sample in the same gel, to avoid artifacts due to saturation of the signal or to too weak of a signal.

Infectivity Studies

In vivo infectivity studies were done in C57Bl6 female mice or Golden Syrian female hamsters, purchased from Charles river. Animals were 4 to 6 weeks old at the time of inoculation. Anesthesized animals were injected stereotaxically into the right hippocampus with 2 or 4 µl of the mouse or hamster infectious material, respectively. For the i.p. infectivity studies, 100 µl of the sample were injected into the peritoneal cavity. The quantity of infectious material injected corresponds to the plateau portion of the incubation period; therefore, small differences in the amount of infectivity should not change incubation period unless there are strain differences. The onset of clinical disease was measured by scoring of the animals twice a week. For mice, the following scale was used: 1, normal animal; 2, roughcoat on limbs; 3, extensive roughcoat, hunckback, and visible motor abnormalities; 4, urogenital lesions; and 5, terminal stage of the disease in which the animal presented with cachexia and lies in the cage with little movement. For hamsters, the following scoring scale was used: 1, normal animal; 2, mild behavioral abnormalities including hyperactivity and hypersensitivity to noise; 3, moderate behavioral problems including tremor of the head, ataxia, wobbling gait, head bobbing, irritability, and aggressiveness (or lethargy in case of the DY strain); 4, severe behavioral abnormalities including all of the above plus jerks of the head and body and spontaneous backrolls; and 5, terminal stage of the disease in which the animal lies in the cage and is no longer able to stand up. Animals scoring level 4 during two consecutive weeks were considered sick and were sacrificed to avoid excessive pain via exposition to carbonic dioxide. Brains were extracted, the right cerebral hemisphere was frozen and stored at −70°C for biochemical examination of PrP^{Sc} with western blots, and the left hemisphere was used for histology analysis.

Histopathological Studies

Brain tissue was fixed in 10% formaldehyde solution, cut in sections, and embedded in paraffin. Serial sections (6 µm thick) from each block were stained with hematoxylin-eosin, or incubated with monoclonal antibodies recognizing PrP or the glial fibrillary acidic protein, via our previously described protocols (Castilla et al., 2005). Samples were visualized with a Zeiss microscope. The vacuolation profile was estimated by consideration of both number and size of spongiform degeneration in five different brain areas: occipital cortex, cerebellum (mostly white matter), medulla (spinal 5 nucleus, interpolar part), inferior colliculum, and hippocampus (CA1 and CA2 regions). Each analyzed brain area was scored from 0 to 4 according to the extent of vacuolation in slides stained with hematoxilin-eosin and visualized at a 40× magnification. Samples were analyzed blindly by two different persons, and the scores represent the average of the two determinations.

Statistical Analysis

The differences in incubation periods, histopathological profile of brain damage, and biochemical characteristics of PrP^{Sc} were analyzed by ANOVA, followed by the Dunnett Multiple Comparison post-test to estimate the significance of the differences between the newly generated strains and each of the other hamster and mouse prion strains studied. For these studies, the data were analyzed with the GraphPad Instat, version 3.05 software.

SUPPLEMENTAL DATA

Supplemental Data include one figure and two tables and can be found with this article online at http://www.cell.com/cgi/content/full/134/5/757/DC1/.

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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

○伝達性海綿状脳症感染性の尿への排出
伝達性海綿状脳症の自然感染経路は未だに仮説段階である。TSE暴露の潜在的要因としての尿を調査するため、高感度検出法とTSE感染性の定量を行った。263Kスクレイピーの臨床症状を呈しているハムスター22匹からプールした尿は、3.8 ± 0.9 感染量/mLの感染性を含んでいた。同じ動物由来の腎臓と膀胱のホモジネートの滴定は2万倍の濃度を示した。これら同じ組織の組織学的、免疫組織化学的分析では、腎臓における疾患関連プリオンタンパク質の散発的な沈着を除いて、炎症あるいは他の病変は見られなかった。尿におけるTSE感染源は未だに解明されていないが、これらの結果は、TSE感染性が尿中に排出されており、その結果自然のTSEの水平感染において何らかの役割を果たしていることを立証している。また、ヒトの尿由来ホルモンや他の医薬品からのTSE伝播の潜在的リスクを示している。

使用上の注意記載状況・その他参考事項等

新鮮凍結血漿「日赤」
新鮮凍結血漿-LR「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
263Kスクレイピーの臨床症状を呈しており、腎臓・膀胱に炎症のないハムスター22匹からプールした尿にTSEの感染性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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RESEARCH

Excretion of Transmissible Spongiform Encephalopathy Infectivity in Urine

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

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

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

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

Materials and Methods

Urine Collection and Processing

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

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