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Scrapie and Creutzfeldt–Jakob disease prion proteins share physical properties and antigenic determinants

(slow infections/prion diseases/immunoblots/subviral pathogens/protease-resistant proteins)

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ABSTRACT **Scrapie of sheep and goats as well as Creutzfeldt–Jakob disease (CJD) of humans are neurologic disorders caused by slow infectious pathogens. The novel molecular properties of the pathogen causing scrapie have prompted introduction of the term "prion" to denote a small proteinaceous infectious particle that resists inactivation by nucleic acid-modifying procedures. Antiserum to the major hamster scrapie prion protein (PrP 27–30) was found to cross-react with murine CJD proteins. The CJD proteins had molecular weights similar to those observed for scrapie prion proteins as determined by NaDodSO$_4$ gel electrophoresis. In addition, the CJD proteins were resistant to digestion by proteinase K and appear to polymerize into rod-shaped particles. The purification procedure developed for scrapie prions was found to be useful in purifying the CJD agent. Purification of the two infectious pathogens by virtually identical procedures provided further evidence for similarities in their molecular structures. We conclude that the molecular and biologic properties of the CJD agent are sufficiently similar to those of the scrapie prion protein that CJD should be classified as a prion disease.**

Scrapie of sheep and goats as well as Creutzfeldt–Jakob disease (CJD) of humans are slow neurologic diseases caused by infectious pathogens (1). The novel biologic and molecular properties of these pathogens led to introduction of the term "prion" to denote a small proteinaceous infectious particle that resists inactivation by nucleic acid-modifying procedures (2). Similar pathogens may also cause kuru and Gerstmann–Sträussler syndrome in humans, transmissible mink encephalopathy, as well as chronic wasting disease of mule deer and elk (1, 3–5). The molecular properties of prions distinguish them from both viruses and viroids (2, 6).

Scrapie and CJD share long incubation periods, transmissibility to experimental hosts, absence of a detectable inflammatory or immunologic response, and characteristic pathologic changes (1, 2, 7). Incubation periods are measured in months, years, and occasionally decades (1, 8). Although the natural host range appears to be limited for these diseases, they have been transmitted to a variety of laboratory animals (1, 9). A considerable overlap of these experimental host ranges exists and, in fact, experimental CJD in goats is indistinguishable both clinically and pathologically from natural scrapie (10).

In both scrapie and CJD, the immune system remains intact; no humoral or cell-mediated responses to either of these pathogens have been observed (1, 11–15). Pathologically, only the central nervous system is affected, although many tissues harbor infectivity (16). The hallmarks of the histological changes are a dense astrocytosis, neuronal loss, spongiform changes, and amyloid plaque formation, although the degree of each of these changes varies from species to species and only astrocytic proliferation is a constant feature (15, 17). Recent studies suggest that genetic control of the incubation period in mice for scrapie and CJD may occur through the same genetic locus (18).

Studies on the molecular properties of the CJD agent have shown striking similarities to those reported for scrapie prions. Both infectious pathogens exhibit extreme resistance to inactivation by ionizing irradiation (19–21). The apparent size of the CJD agent seems to be similar to that of the scrapie prion based on sedimentation data as well as target size estimates (22, 23). In addition, both pathogens show similar patterns of inactivation by chaotropic ions, alkali, and heat (2, 24–27).

We report here that the CJD agent can be purified by using a protocol that is virtually identical to that developed for scrapie prions (28) and that antiserum raised against the hamster scrapie prion protein (PrP 27–30) (29) cross-reacts with similar protease-resistant proteins in murine CJD fractions. The antiserum appears to have a lower affinity for CJD proteins than for the comparable scrapie proteins; however, its cross-reactivity allowed the first direct molecular comparison between the protein components of the two prions causing these slow transmissible encephalopathies. Additionally, the rod-shaped structures found in purified CJD fractions indicate that CJD prions, like scrapie prions, aggregate into linear forms.

MATERIALS AND METHODS

Materials. All chemicals were of the highest grades commercially available. The sources of most chemicals have been described (28, 30).

Source and Propagation of the Scrapie and CJD Prions. Throughout the course of this study, the propagation and purification of the two infectious pathogens was done in two facilities separated by several miles in order to eliminate the possibility of any cross-contamination. Likewise, the experimental procedures were done by two different groups of investigators.

The hamster-adapted isolate of the scrapie agent (31) was passaged in LVG/LAK Golden hamsters obtained from the Charles River Breeding Colony (Lakeview, NJ) as described (32). The K.Fu. isolate of the CJD agent (33), previously adapted to mice (23), was passaged in the NAMRU strain of random-bred Swiss–Webster mice by intracerebral inoculation. The mice were sacrificed, and their brains were removed at the time of clearly defined clinical neurologic disease, ≈130–140 days postinoculation.

Purification of the Prions. The isolation procedure used for both the scrapie and CJD prions was based on the procedure

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Abbreviation: CJD, Creutzfeldt–Jakob disease.
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described by Prusiner et al. (28, 30). Homogenates of infected brain (10% wt/vol) were prepared in 320 mM sucrose and a postmitochondrial supernatant prepared by two low-speed centrifugations. After the addition of Triton X-100 and deoxycholate, the infectious particles were precipitated by the addition of polyethylene glycol, and the precipitate was collected by centrifugation. After resuspension, the pelleted material was sequentially digested with micrococcal nuclease (5 or 12.5 units/ml for 16 hr) and proteinase K (100 μg/ml for 8 hr) at 4°C. Sodium cholate was added, and the prions were precipitated by the addition of solid ammonium sulfate (30% saturation for scrapie and 24% for CJD prions). The precipitate was collected by centrifugation and resuspended in Triton X-100 and $NaDodSO_4$. The prions were then sedimented through a 25% (wt/vol) sucrose solution onto a cushion of 60% sucrose. The sucrose gradient fractions collected at the 25%/60% interface contained the highest concentration of purified prions.

Uninfected control brain fractions were purified by an identical protocol. The control animals were the same sex and age as their infected counterparts.

Radioiodination of Proteins. Sucrose gradient fractions obtained at the 25%/60% sucrose interface were concentrated by precipitation with $NaDodSO_4$ and quinine hemisulfate, and the pellet was washed with 80% acetone. The proteins were Bolton–Hunter reagent-labeled by resuspension in 0.1 M sodium borate, pH 8.5/0.1% $NaDodSO_4$, followed by the addition of N-succinimidyl 3-(4-hydroxy-5-[^{125}I]iodophenyl)-propionate (34). The radioiodination reaction was terminated through the addition of 0.2 M glycine/0.1 M sodium borate, pH 7.5. The iodinated proteins were removed from the reaction mixture by reprecipitation with $NaDodSO_4$ and quinine hemisulfate.

Polyacrylamide Gel Electrophoresis and Electroblot Transfer. Radiolabeled proteins were resuspended in electrophoresis sample buffer (62.5 mM Tris·HCl, pH 6.8/2% $NaDodSO_4$/5% 2-mercaptoethanol/10% glycerol/0.002% bromphenol blue) and boiled for 2 min. Protein separation was accomplished by electrophoresis through 12% or 15% polyacrylamide gels as described by Laemmli (35). After electrophoresis the proteins were transferred to nitrocellulose membranes by overnight electrotransfer in a Trans-Blot apparatus (Bio-Rad) containing 25 mM Tris·HCl (pH 8.3), 192 mM glycine, and 20% (vol/vol) methanol.

Immunoblot Analysis. Dilutions of purified scrapie and CJD prions in 100 μl of 20 mM Tris OAc, pH 7.4/1 mM EDTA were spotted onto preequilibrated nitrocellulose membranes under mild vacuum in a Minifold apparatus (Schleicher & Schuell). Each spot was washed with several hundred microliters of buffer prior to drying the filter for immunoassay (36–38). Filter binding sites were blocked from further reactivity by incubation in a buffer containing phosphate-buffered saline, 3% bovine serum albumin, and 10% fetal bovine serum (blocking buffer) for 90 min at 37°C. All other incubations were carried out at room temperature with shaking. The filter was then incubated for 2 hr in a 1:1000 dilution of rabbit anti-scrapie PrP 27–30 antiserum in phosphate-buffered saline containing 0.3% bovine serum albumin. The filters were subsequently washed for 1 hr in four changes of phosphate-buffered saline containing 0.3% bovine serum albumin, followed by incubation for an additional 2 hr with 1:1000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG in blocking buffer. The filters were washed as above, and the peroxidase reaction was initiated by the addition of the substrate (0.02% 4-chloro-1-naphthol/0.005% H_2O_2/75 mM Tris·HCl, pH 7.4). Color development of the enzyme assay was terminated by washing in deionized water and drying of the filter.

Electron Microscopy. Samples for electron microscopy were prepared as described (28). The specimens were negatively stained with uranyl formate and examined in a JEOL 100B electron microscope at 80 keV.

Production of Scrapie PrP 27–30 Antiserum. Antiserum was produced in rabbits by injection of ≈80 μg of electrophoretically purified scrapie PrP 27–30 emulsified in complete Freund's adjuvant into the popliteal lymph nodes and subcutaneous sites (29). Subsequent booster injections in incomplete Freund's adjuvant were performed subcutaneously. The antiserum used in these studies was obtained after the third booster.

RESULTS

Scrapie and CJD prions were purified by a series of detergent extractions, differential centrifugations, and enzyme digestions, followed by ammonium sulfate precipitation and sucrose gradient sedimentation. Gradient fractions prepared from scrapie-infected hamster brains contained $\approx 10^{9.5}$ ID_{50} units/ml, whereas those from CJD-infected murine brains contained $\approx 10^{6.2}$ units/ml. These fractions enriched for prions were labeled with ^{125}I-labeled Bolton–Hunter reagent and then digested with proteinase K for 30 min at 37°C. The digestion was terminated by boiling the samples in $NaDodSO_4$ prior to electrophoresis into polyacrylamide gels. The CJD sample was found to contain three protease-resistant proteins of M_rs 26,000–29,000, 22,000–24,000, and 18,000–20,000 (Fig. 1A). The scrapie sample contained primarily PrP 27–30 and two other protease-resistant proteins (Fig. 1B). Thus, application of the same purification protocol to CJD- and scrapie-infected rodent brains yielded samples that were enriched for prions and contained protease-resistant proteins of similar molecular weight.

Scrapie and CJD Prion Proteins Form Rods. Recent studies (30) have shown that scrapie prion protein molecules polymerize to form rod-shaped particles measuring 10–20 nm in diameter and 100–200 nm in length (Fig. 2A). Fragmentation of the rods by prolonged sonication into particles measuring <100 nm in length did not alter the titer (unpublished data). Rod-shaped particles were identified in sucrose gradient fractions containing CJD prions (Fig. 2B). These rods gener-

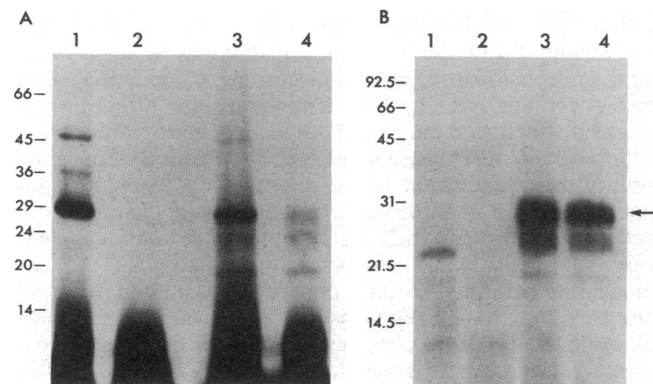

FIG. 1. Radioiodinated protease-resistant proteins in purified preparations of CJD and scrapie prions. (A) Murine CJD prion proteins. (B) Hamster scrapie prion proteins. Sucrose gradient fractions were labeled with ^{125}I-labeled Bolton–Hunter reagent and boiled for 2 min in electrophoresis sample buffer containing 2% (wt/vol) $NaDodSO_4$. The denatured samples were electrophoresed into 12% (A) and 15% (B) polyacrylamide gels, and autoradiograms were produced by exposure at room temperature for 30 and 25 hr, respectively. Lanes: 1 and 2, fractions purified from uninfected control animals; 3 and 4, fractions purified from prion-infected animals; 2 and 4, fractions digested with 25 μg of proteinase K per ml for 30 min at 37°C. The arrow denotes the position of scrapie PrP 27–30. The molecular masses of reference proteins are given in kDa.

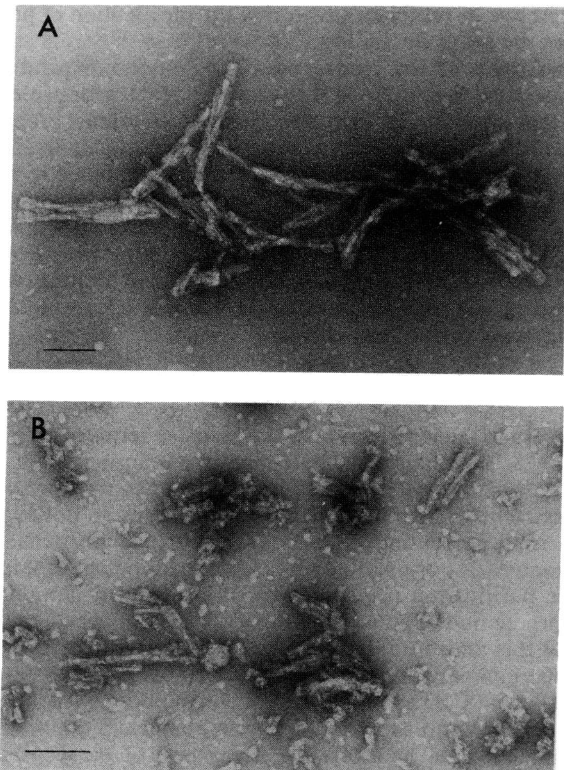

FIG. 2. Electron micrographs of scrapie and CJD prion rods. Fractions were obtained by discontinuous sucrose gradient sedimentation. Aliquots were negatively stained with 1% uranyl formate. (*A*) Scrapie rods. (*B*) CJD rods. (Bars = 100 nm.)

ally measured 8–16 nm in diameter and 50–150 nm in length (Table 1).

Antiserum to Scrapie PrP 27–30 Cross-Reacts with CJD Proteins. Antiserum produced in a rabbit against scrapie PrP 27–30 was found to cross-react with proteins in purified fractions containing CJD prions. The antiserum failed to react with proteins in analogous fractions purified from normal hamster and mouse brains (Fig. 3, column 1, rows a and b, respectively). As little as 25 ng of scrapie prion proteins was detected (Fig. 3, column 2, row a), whereas 5 μg of CJD prion protein was required for detection by this dot-blot immunoassay (Fig. 3, column 4, row b). Thus, the antiserum was at least 200 times more reactive with scrapie prion preparations compared to those of CJD. The preparations shown here were in a native, infectious state when applied to the nitrocellulose filters. Similar results were obtained with denatured, uninfectious preparations. Preimmune serum at a dilution of 1:100 gave background reactivity, but at 1:1000 no reactivity was seen. In all other studies, the antiserum diluted 1:20,000 gave detectable reactivity against scrapie PrP 27–30 (29).

Table 1. Dimensions of murine CJD prion rods negatively stained

Dimension, nm	Observations, no.	% of total
Length		
25–50	3	8.8
50–75	10	29.4
75–100	7	20.6
100–125	6	17.6
125–150	5	14.7
150–175	2	5.7
200–225	1	2.9
Width		
2–4	1	2.9
4–6	1	2.9
6–8	1	2.9
8–10	7	20.0
10–12	8	22.9
12–14	4	11.4
14–16	11	31.4
16–18	2	5.7

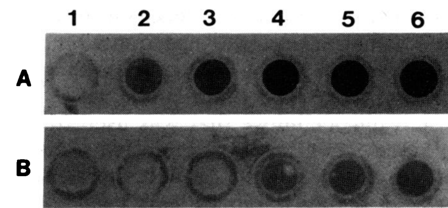

FIG. 3. Immunoblot of native scrapie and CJD fractions. Purified preparations of scrapie and CJD prions were blotted onto nitrocellulose. Row A: column 1, normal hamster brain fraction (5 μg of protein); columns 2–6, scrapie-infected hamster brain fraction (0.025, 0.05, 0.25, 0.5, and 1 μg). Row B: column 1, normal mouse brain fraction (10 μg of protein); columns 2–6, CJD-infected mouse brain fraction (0.5, 1, 5, 10, and 20 μg). Rabbit anti-scrapie PrP 27–30 antiserum was used at a 1:1000 dilution, followed by horseradish peroxidase-conjugated goat anti-rabbit IgG.

Immunoblots of CJD Prion Proteins. As reported previously (29), the antiserum to scrapie prions recognized PrP 27–30 and several lower molecular weight proteins (23,000–26,000, 19,000–20,500, 17,000, and 14,500) in fractions purified from scrapie-infected hamster brains. It did not react with proteins in analogous purified fractions prepared from uninfected hamster brains. Immunoblots of CJD prion proteins purified from infected murine brains displayed a similar pattern of immunoreactive polypeptides (Fig. 4). An autoradiogram

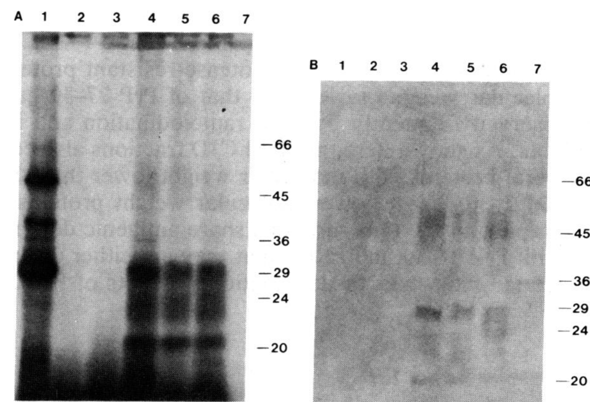

FIG. 4. Analysis of proteins from purified CJD preparations by $NaDodSO_4$/polyacrylamide gel electrophoresis using radioiodination and immunoblotting. Purified preparations of infected and normal mouse brain were labeled with ^{125}I-labeled Bolton–Hunter reagent. Radiolabeled samples of normal (lanes 1–3) and CJD-infected fractions (lanes 4–6) were digested with proteinase K at 25 μg/ml (lanes 2 and 5) or trypsin at 500 μg/ml (lanes 3 and 6) for 30 min at 37°C. Unlabeled proteinase K and trypsin were run as the control (lane 7). Digestion was terminated by boiling in an equal volume of 2× $NaDodSO_4$ sample buffer for 2 min. The samples were electrophoresed in 12% polyacrylamide gels. Proteins were transferred to nitrocellulose filter by running overnight at 100 mA in a Trans-Blot apparatus as described. (*A*) Radiolabeled normal and infected fractions. Autoradiogram exposure of the filter was performed at room temperature for 30 hr. (*B*) Immunoblot of the same filter. Rabbit anti-scrapie PrP 27–30 antiserum was used at a 1:1000 dilution followed by horseradish peroxidase-conjugated goat anti-rabbit IgG.

of ^{125}I-labeled proteins in purified gradient fractions of CJD prions before and after protease digestion with proteinase K or trypsin is shown in Fig. 4*A*. The radioiodinated proteins were transferred from a polyacrylamide gel by electroblotting them onto nitrocellulose that was used to produce the autoradiogram. After allowing the blotted proteins to react with antiserum to scrapie PrP 27–30, horseradish peroxidase-conjugated second antibody was used to identify the immunoreactive species (Fig. 4*B*). The antiserum reacted with proteins only from CJD-infected brains, not from uninfected controls. The major proteins detected had M_rs of 18,000–20,000, 22,000–24,000, 25,000–27,000, and 27,500–30,000. Whether or not the lower molecular weight proteins are degradation products of CJD PrP 27.5–30 remains to be established. A higher molecular weight protein (44,000–46,000) also was detected and may represent a precursor or undissociated oligomer of one or more of the lower molecular weight proteins. Like their scrapie prion counterparts, these murine CJD proteins exhibited microheterogeneity with respect to molecular weight and relative resistance to protease digestion.

A comparison of the CJD and scrapie prion proteins by immunoblots with scrapie PrP 27–30 antiserum (Fig. 5) shows that the immunoreactive protease-resistant proteins purified from CJD murine brains were virtually identical to those from scrapie hamster brains with respect to their migration in $NaDodSO_4$/polyacrylamide gels.

A protein of M_r 43,000–46,000 was clearly seen in the scrapie prion preparation. It is unknown whether this higher molecular weight protein is a precursor of PrP 27–30, an oligomer of lower molecular weight proteins that is not dissociated, or an unrelated protease-resistant protein. The latter possibility seems unlikely because the immunoblot of scrapie prions showed that this protein shares antigenic determinants with PrP 27–30. A protein of similar molecular weight was observed in some purified CJD fractions (Fig. 4). In purified fractions of both scrapie and CJD preparations, these higher molecular weight proteins did not seem to be a constant finding. When they were detectable, their concentration was <5% of that of the major protein.

In scrapie prion preparations, protease-resistant proteins with molecular weights lower than that of PrP 27–30 have been observed frequently by both radioiodination and immunoblots. As shown here, purified CJD fractions also contain several proteins with molecular weight lower than that of PrP 27.5–30. These lower molecular weight proteins in both scrapie and CJD preparations share antigenic determinants with PrP 27–30, indicating that they are either distinct prion-related molecules or degradation products of PrP 27–30. The use of proteinase K during the purification of prions favors the latter possibility.

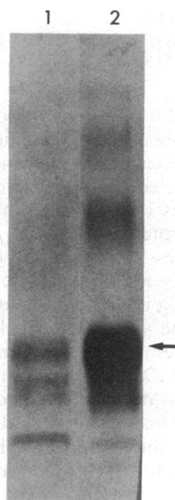

FIG. 5. Comparison of CJD and scrapie prion proteins by immunoblotting. Lanes: 1, CJD prion proteins; 2, scrapie prion proteins. The arrow denotes the position of scrapie PrP 27–30. Samples were electrophoresed into a $NaDodSO_4$/12% polyacrylamide gel. The CJD prion sample contained 2.5 times as much protein as did the scrapie prion sample. After transfer of the proteins to nitrocellulose, serum was used at a 1:1000 dilution, followed by horseradish peroxidase-conjugated goat anti-rabbit IgG.

Specificity of the Antiserum. The antiserum produced against scrapie PrP 27–30 was found to react with protease-resistant proteins in partially purified fractions prepared from murine and guinea pig brains with experimental CJD as well as human CJD brain (54). The antiserum failed to react with proteins in analogous fractions prepared from uninfected control hamster, murine, and guinea pig brains. In addition, the antiserum failed to react with proteins in analogous fractions prepared from a human brain exhibiting widespread gliosis due to repeated anoxic episodes associated with poorly controlled seizures.

DISCUSSION

Progress in the purification of the hamster scrapie prion proteins (28, 39) enabled the detection of a unique protein, PrP 27–30, which was determined to be a major component of the infectious particle (40). PrP 27–30 was found to polymerize into rods possessing the ultrastructural and histochemical characteristics of amyloid (30). The development of a large-scale purification protocol provided sufficient immunogen for production of antiserum to PrP 27–30 (29). The availability of this antiserum has provided a means for the first molecular comparison between the infectious particles causing scrapie and CJD as reported here. In addition, we have used the antiserum to show that amyloid plaques in the brains of scrapie-infected hamsters are composed of paracrystalline arrays of prions (29). Other studies using immunoelectron microscopy have shown directly that scrapie and CJD prion rods contain prion protein molecules (unpublished data).

Our findings that purified scrapie and CJD prion preparations contain protease-resistant proteins of similar molecular weights and that these proteins share antigenic determinants (Fig. 5) indicate the primary structures of these proteins must be similar. The apparent lower affinity of the CJD prion proteins for the antiserum compared to scrapie prions shows that the two groups of prions are similar, but probably not identical. How much these differences are due to the molecular structures of the prions, the hosts in which each prion was replicated, or simply the specific infectivities of the preparations remains to be determined. The scrapie prion preparations described here were purified from hamster brain, while the CJD prions were isolated from murine brain. Protease-resistant proteins of scrapie prions purified from murine brain have recently been identified by radioiodination (41).

The polymerization of scrapie and CJD prion proteins into rod-shaped particles indicates that these proteins share tertiary and quaternary structural features. In both scrapie and CJD preparations, the rods form aggregated arrays. It is unclear how much of the protease resistance of prion proteins arises from its conformation or tertiary structure and how much is due to its polymerization into rods.

Polymerized scrapie prion protein molecules appear to contain an extensive β pleated sheet structure (30); presumably murine CJD prion proteins possess a similar secondary structure. Human CJD prion rods were found to bind to Congo red dye and exhibit green birefringence by polarization microscopy (unpublished data). These structural similarities suggest that amyloid plaques in CJD brain (42) are composed of paracrystalline arrays of prions.

Cylindrical particles described as tubular or rod-shaped have been reported in brain sections from animals with scrapie (43–48) and humans with CJD (49, 50). One report (51) has suggested that scrapie prion rods found in purified preparations are related to longer helically twisted fibrils observed in crude extracts (52). These long fibrils measure up to 1000 nm in length and also have been observed in extracts

of brain derived from mice with experimental CJD and from humans with CJD (53). Based on ultrastructural characteristics, some investigators have been able to distinguish the fibrils from amyloid (52). Whether or not these fibrils represent an elongated form of the prion rods remains to be established. Clearly, the long fibrils are not found in purified preparations of either scrapie or CJD prions; thus, the fibrils are not required for infectivity.

The scrapie PrP 27–30 antiserum not only cross-reacts with protease-resistant proteins purified from the brains of mice with experimental CJD but also with proteins purified from the brains of two patients dying of CJD (54). The purification protocol for the human CJD prion proteins was similar to that used for hamster scrapie and murine CJD prions. Purification of scrapie and CJD prions by the same protocol provides further evidence for the similarities in their physical and chemical structures. Electron microscopic examination of the human CJD fractions has shown rod-shaped particles of dimensions similar to those found in hamster scrapie and murine CJD prion preparations.

From studies reported here, we conclude that the prions causing scrapie and CJD share antigenic determinants and structural features. These similarities are consistent with minor yet significant differences in the biologic properties of these slow infectious pathogens. We believe that the molecular and biologic properties of the CJD agent are sufficiently similar to those of the scrapie prion that CJD should be classified as a prion disease. Since no polynucleotide has been found to date within prions, determination of the extent to which host cells modify prion proteins may be important.

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