JOURNAL OF VIROLOGY, Aug. 1988, p. 2845–2849
0022-538X/88/082845-05$02.00/0

Vol. 62, No. 8

Analyses of Frequency of Infection, Specific Infectivity, and Prion Protein Biosynthesis in Scrapie-Infected Neuroblastoma Cell Clones

RICHARD E. RACE,* BYRON CAUGHEY, KENNETH GRAHAM, DARWIN ERNST, AND BRUCE CHESEBRO

Laboratory of Persistent Viral Diseases, National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratories, Hamilton, Montana 59840

Received 2 February 1988/Accepted 3 May 1988

Scrapie, a spongiform encephalopathy of sheep and goats, is caused by a poorly understood transmissible agent in which no nucleic acid has been conclusively identified. Biochemical characterization of agent derived from animal tissues has not been precise because of the tenacious association of the agent with tissue components. As an approach toward obtaining homogeneous preparations of agent generated in vitro, we cloned scrapie-infected neuroblastoma cells. By frequency analysis, nearly every cell in expanded cultures contained scrapie agent. We also analyzed cell-dose infectivity relationships and developed a standard curve which allowed various cultures to be compared. Since a proteinase K (PK)-resistant form of a protein designated prion protein (PrP) has been found in partially purified preparations of scrapie agent from infected animal spleens and brains, we sought to identify this protein in cell cultures. No PK-resistant PrP was found in infected or uninfected cultures, although the PK-sensitive PrP was readily detected. These results suggested that PK-resistant PrP may not be an essential component of the infectious scrapie agent.

Scrapie agent derived from animal tissues has not been purified to sufficient homogeneity to allow adequate biochemical characterization. However, several properties, particularly unusual resistance to inactivation by radiation, have been attributed to the agent and distinguish it from most (2, 8, 14), but not all, viruses (24). The only macromolecular structures consistently detected in partially purified preparations from brains of infected animals are fibrils known as scrapie-associated fibrils (9, 17) or prion rods (22) which consist primarily of a proteinase K (PK)-resistant protein called prion protein (PrP) (3, 11, 16) or scrapie-associated fibril protein (9, 11). Samples that contain the protein also have infectivity but no (2) or little detectable nucleic acid (19a). These associations have led several investigators to propose that infectivity is transmitted by the fibrils (9, 18, 27) or the PK-resistant form of PrP itself (3, 20). In contrast, other investigators suggest that the protein may accumulate as a by-product of central nervous system degeneration (4).

Tissue cultures supporting scrapie agent replication should be useful in characterizing the agent and distinguishing it from associated pathogenic by-products present in in vivo tissue sources. However, the low and unpredictable levels of infectivity present in previously described scrapie-infected cultures have hampered the usefulness of tissue cultures in this regard (6, 7, 15, 25, 29). We have previously described in vitro infection of mouse neuroblastoma cells with scrapie agent and determined that the frequency of infected cells in these cultures was approximately 1% (23). In the present study, infected cells were cloned and expanded into cultures in which most of the cells replicated scrapie agent. These cultures had infectivity titers 100-fold higher than those of uncloned cultures available before and were used to study the biosynthesis and relationship of PrP to infectivity.

* Corresponding author.

MATERIALS AND METHODS

Cells, cloning, and assay for infectivity. Cloning of individual scrapie-infected mouse neuroblastoma cells was done from in vitro passage 18 of previously described cultures infected with a mouse scrapie spleen homogenate (23). Briefly, cells were counted and then diluted in media at a concentration of 1 cell per ml. An aliquot (1 ml) was added to each of 200 to 400 TC-24 Linbro tissue culture wells, and an additional 1 ml of medium was added. TC-24 wells in which single-colony cell growth became visible (after 14 to 21 days) were expanded by transferring their cells to a TC-25 Corning tissue culture flask. When confluent, the neuroblastoma cells were trypsinized, centrifuged, suspended in medium, and divided into two aliquots. One aliquot of cells was kept for future expansion by freezing by standard techniques. The cells in the other aliquot were counted and then frozen until analysis in mice. For some experiments, cells were counted and dilutions were accomplished before freezing. Cell aliquots for mouse inoculations were thawed and sonicated, and cellular debris was pelleted (1,000 × *g* for 5 min). Supernatant suspension (50 μl) containing known numbers of cell equivalents was inoculated intracerebrally into Rocky Mountain Laboratory random-bred Swiss mice (RML mice). Mice were observed periodically for 12 months, and all those dying of scrapie were counted.

Metabolic labeling and radioimmunoprecipitation of PrP. Nearly confluent cells in 25-cm^2 flasks were preincubated for 30 min at 37°C with methionine-free minimal essential medium (Gibco Laboratories, Grand Island, N.Y.) and labeled for 2 h with 100 μCi of [^{35}S]methionine (New England Nuclear Corp., Boston, Mass.) per ml in 2 ml of the same medium per flask. After a rinse with cold phosphate-buffered balanced salt solution, the cells were lysed with 1 ml of lysing buffer containing 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, 0.15 M NaCl, 1 mM EDTA, 0.1% gelatin, 0.1% sodium azide, and 0.05 M Tris (pH 8.0) per flask at 4°C. The lysates were spun in a Microfuge (Beckman Instruments, Inc., Fullerton, Calif.) for 5 min at 11,000 × *g*. About 0.5 flask equivalent of supernatant was incubated overnight at 4°C with 2 μl of

either anti-PrP peptide 783 or anti-PrP peptide 783 preincubated for 30 min at room temperature with 0.005 μg of the synthetic PrP peptide antigen per μl of antiserum to block PrP-specific immunoprecipitation. The anti-PrP peptide 783 was raised against the synthetic PrP peptide fragment conjugated to keyhole limpet hemocyanin as described previously (28) and was kindly provided by M. J. Buchmeier (Scripps Clinic, La Jolla, Calif.). A 15-μl portion of a 10% (wt/vol) suspension of protein A-Sepharose beads (Pharmacia) was added per μl of antiserum, and the suspension was mixed for 30 min at 4°C. The beads were washed two times in lysing buffer and three times with 0.5 ml of LiCl–0.1 M Tris (pH 9)–1% 2-mercaptoethanol at 4°C. Bound proteins were eluted by boiling in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (13). Labeled proteins were analyzed by SDS-PAGE by using a 10% acrylamide gel and 0.5 flask equivalent of immunoprecipitate per lane. The gels were fluorographed by using Entensify (New England Nuclear) according to manufacturer instructions.

PK treatment of metabolically labeled PrP. Cells in two 25-cm^2 flasks were labeled with [^{35}S]methionine, as described above, for 4 h. The cells were rinsed once and then detached with phosphate-buffered saline containing 2 mM EDTA. The cells were pelleted by centrifugation and lysed by suspension in 5 pellet volumes of 1 mM $NaHCO_3$–1 mM $MgCl_2$–0.5 mM KCl–0.32 M sucrose–1% sarcosyl as described previously (26). After the viscosity of the lysates was reduced by passage through 18- and 26-g needles, equal volumes of the lysates were left on ice or treated for 30 min at 37°C with 5 or 50 μg of PK per ml. Proteolysis was terminated by adding 0.02 volume of 0.1 M phenylmethylsulfonyl fluoride (PMSF) in isopropanol and incubating for 10 min at room temperature. The lysates were diluted with 5 volumes of lysing buffer, immunoprecipitated, and analyzed by SDS-PAGE as described above, with 0.33 flask equivalent per lane. Control experiments in which 50 μg of PK per ml was added to lysates after the addition of PMSF indicated that the inhibitor prevented any interference by PK in subsequent immunoprecipitation steps.

Preparation of brain, spleen, and neuroblastoma cells for Western blotting (immunoblotting). The technique used to prepare PrP from spleen was modified from one previously described (M. Shinagawa, S. Doi, M. Ito, H. Goto, and G. Sato, Proc. 7th Int. Conf. Virol., p. 148, 1987). Briefly, several mouse spleens were weighed and then dissociated in 7 volumes of 0.01 M Tris hydrochloride (pH 7.5)–5 mM $MgCl_2$ by forcing them through a fine-mesh stainless steel screen. The suspension that resulted, including spleen capsules, was frozen at −70°C for a few hours or up to several weeks. DNase I (40 μg/100 mg of original tissue) was added to thawed suspensions for 1 h at 37°C. An equal volume of 20% sarcosyl in 0.01 M Tris hydrochloride (pH 7.5) was added for 30 min at room temperature. The suspension was centrifuged at 18,000 × *g* for 5 min at room temperature. The supernatant was recovered, and a weight of NaCl equal to the original weight of the tissue was added. This suspension was left overnight at 4°C and then was centrifuged at 27,000 × *g* at 4°C for 1 h. The pellet was suspended in a small volume of 0.01 M Tris hydrochloride (pH 7.5) and then treated with PK (0.1 μg/100 mg of original tissue) for 30 min at 37°C. The suspension was made 2 mM PMSF and left for 15 min on ice to inactivate the PK. Ice-cold ethanol (2.5 volumes) was added for 2 h at −20°C followed by centrifugation at 5,000 × *g* for 10 min at 4°C. Proteins were dissolved by boiling for 5 min in 4% SDS. An equal volume of 2× sample buffer (13) was added prior to electrophoresis and Western blotting. Neuroblastoma cells were prepared as follows. Confluent cells in 150-cm^2 Corning tissue culture flasks were rinsed once with cold 0.32 M sucrose and then scraped into 0.32 M sucrose by using a rubber policeman. Cells were then pelleted, suspended in 0.01 M Tris hydrochloride (pH 7.5)–5 mM $MgCl_2$, and processed as for spleen. Brain was processed as previously described (12).

Western blotting. Western blots were done as follows. Proteins were separated on 12.5% polyacrylamide gels (13) and transferred to nitrocellulose by using a Trans-blot apparatus (Bio-Rad) at 35 V for 2 h in phosphate buffer (1). The nitrocellulose filter was blocked overnight in Blotto (1) and then incubated for 2 h at room temperature with anti-PrP peptide 783 serum at a 1:1,000 dilution and washed three times for 10 min in Blotto. Filters were incubated with ^{125}I-protein A at 10^6 cpm/ml for 30 min, washed three times for 10 min, and then autoradiographed.

RESULTS

Development and analysis of scrapie-infected cultures derived from single cells. We determined previously that only 1 of 144 cells within an uncloned scrapie-infected mouse neuroblastoma cell culture contained agent (23). To obtain homogeneously infected neuroblastoma cell cultures, single cell clones were established from this parent culture. Of 116 clones that were analyzed, 2 contained scrapie agent. One of these, designated 29-161, was studied further. Mice were inoculated with various numbers of cells from 2×10^1 to 2×10^6. There was an inverse correlation between the number of cells inoculated and the duration of disease (Fig. 1). Over the linear portion of the graph, a 10-fold difference in the number of cells inoculated corresponded to a change in the interval to death of 12 to 14 days. A similar relationship has been shown with scrapie-infected hamster brain homogenates and is related to the titer of agent present (21). Furthermore, when the method of Spearman and Karber (10) was used, a specific infectivity of 1 mouse 50% lethal dose (LD_{50}) per 158 cells was calculated. This contrasts with earlier experiments with uncloned cells where a specific infectivity of 1 LD_{50} per 4,134 cells was found (23). When equivalent numbers of cells were inoculated, clone 29-161 killed mice 31 days sooner than the parent uncloned culture (Fig. 1).

Frequency of infected cells in cultures derived from clones. Because clone 29-161 killed mice significantly sooner than the parent culture from which it was derived, we concluded that either the percentage of infected cells in the clone was increased approximately 100-fold or the number of infectious particles was approximately 100-fold greater per infected cell. To distinguish between these possibilities, the frequency of infected cells within clone 29-161 was determined by an additional cycle of cloning. Of the subcultures derived from single cells of clone 29-161, 23 of 26 (88%) caused scrapie in mice. This increased frequency of infected cells in the clone compared with the parent culture correlated well with the approximate 100-fold increase in infectivity predicted by the data shown in Fig. 1. The positive subclones appeared to be identical to each other and to the parent 29-161 clone, because when equivalent numbers of cells were inoculated into mice, all of them gave essentially identical intervals to death. The level of infection of clone 29-161 has remained stable for over 40 in vitro passages.

PrP biosynthesis. Because aberrant PrP metabolism may play a role in scrapie agent replication or the pathogenesis of the disease, we compared PrP biosynthesis in infected and uninfected neuroblastoma cells (Fig. 2). A discrete PrP band

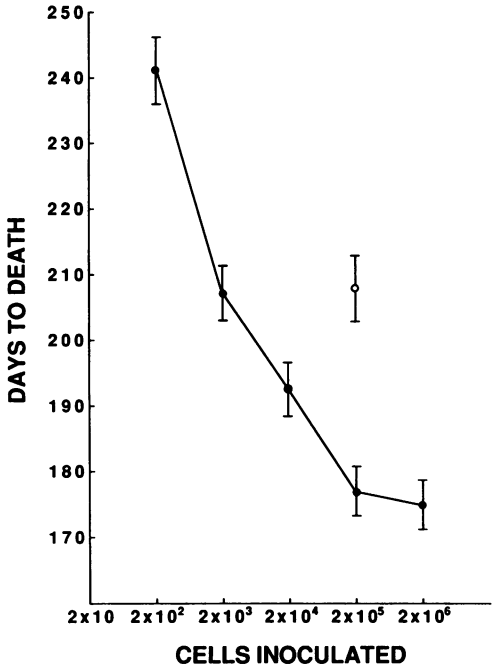

FIG. 1. Relationship between the number of neuroblastoma cell equivalents inoculated and the interval to death. Cells from infected neuroblastoma cell cultures were trypsinized, washed once, suspended in media, frozen, thawed, sonicated, and diluted for intracerebral inoculation of RML mice with various numbers of cell equivalents. The ordinate shows the number of neuroblastoma cell equivalents that were inoculated. The abscissa shows the number of days from inoculation to death of assay mice. ●, Clone 29-161; ○, parent culture from which 29-161 was derived. Each datum point and the brackets represent the mean and standard error of 7 to 10 RML Swiss mice inoculated intracerebrally with 50 μl of suspension containing the designated number of neuroblastoma cell equivalents. All mice inoculated with $>2 \times 10^2$ neuroblastoma cell equivalents died of scrapie, 78% (7 of 9) of the mice inoculated with 2×10^2 neuroblastoma cell equivalents died of scrapie, and none of those inoculated with 2×10^1 neuroblastoma cell equivalents died of scrapie.

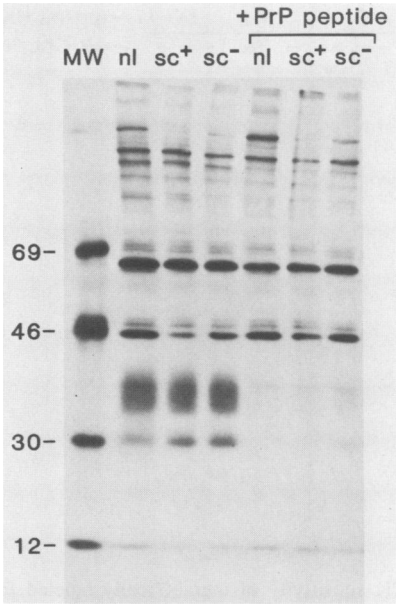

FIG. 2. Metabolic labeling and immunoprecipitation of PrP from normal neuroblastoma (nl), scrapie-infected neuroblastoma clone 29-161 (sc^+), and a scrapie-negative subclone of 29-161 (sc^-) designated 29-321. The PrP bands were those removed from the immunoprecipitate by pretreatment of the anti-PrP serum with unlabeled synthetic PrP peptide antigen used to raise the antiserum. ^{14}C-labeled molecular mass markers (MW) are labeled in kilodaltons.

migrating at 30 kilodaltons (kDa) and a diffuse band spanning 33 to 41 kDa were immunoprecipitated from both scrapie-infected and uninfected clones derived from infected cultures and from normal neuroblastoma cells. These bands were shown to contain a specific PrP peptide antigenic determinant by competitive inhibition of immunoprecipitation by the unlabeled PrP peptide. Identical PrP banding patterns were also observed in four scrapie-positive subclones of clone 29-161 (data not shown). Studies on the scrapie-infected cells indicated that varying the labeling period between 1 and 6 h or chasing the label with unlabeled methionine for up to 3 h did not change the PrP banding pattern observed (data not shown). This suggested that the bands observed were final products of PrP biosynthesis. This is consistent with the fact that a 35- to 41-kDa protein was a secreted product of PrP biosynthesis in C127 cells expressing the PrP gene cloned from scrapie-infected mouse brain (B. Caughey, R. Race, M. Vogel, M. Buchmeier, and B. Chesebro, Proc. Natl. Acad. Sci. USA, in press). These experiments indicated that the presence of scrapie agent did not influence the rate of PrP synthesis, as indicated by the incorporation of [^{35}S]methionine, or the electrophoretic mobilites of the major PrP products.

PK sensitivity of PrP. A characteristic feature which distinguishes most of the PrP isolated from scrapie-infected brain from that of normal brain is its partial resistance to PK digestion under certain conditions (19). To test whether metabolically labeled PrP in the scrapie-infected neuroblastoma cells shared this characteristic, detergent-treated homogenates were prepared and treated with PK under conditions similar to those used to detect PK-resistant PrP in whole scrapie-infected brain homogenates by Western blot (26). Although PrP bands were clearly visible in untreated samples, none remained after treatment with PK at concentrations equal to and 10-fold lower than the concentration (50 μg/ml) to which scrapie-infected brain PrP was partially resistant (Fig. 3). Although not visible in the figure, a weak band at 27 kDa was generated by the 5-μg/ml PK treatment. However, this band was also observed in PK-treated preparations of normal neuroblastoma cells and scrapie-negative subclones, and its immunoprecipitation was not inhibited by the unlabeled PrP peptide. Thus, this band was neither scrapie nor PrP specific. Similar results were also obtained when PrP-containing membranes were partially purified from metabolically labeled, scrapie-infected cells before PK treatment. Clone 29-301 (a scrapie-positive subclone of 29-161) cells were homogenized, fractionated by differential centrifugation into pellets at $10,000 \times g$ and $100,000 \times g$, and treated with PK by methods described for the detection of PrP in brain tissue by Western blot (19). PrP bands identical to those shown in Fig. 2 and 3 were immunoprecipitated from both the pellets, but again no PK-resistant PrP bands remained after treatment with either the prescribed PK concentration (100 μg/ml) or with a 20-fold-lower concentration (data not shown). Thus, under a variety of conditions similar to those appropriate for observing PK-resistant PrP from scrapie-infected brain tissue, the metabolically labeled

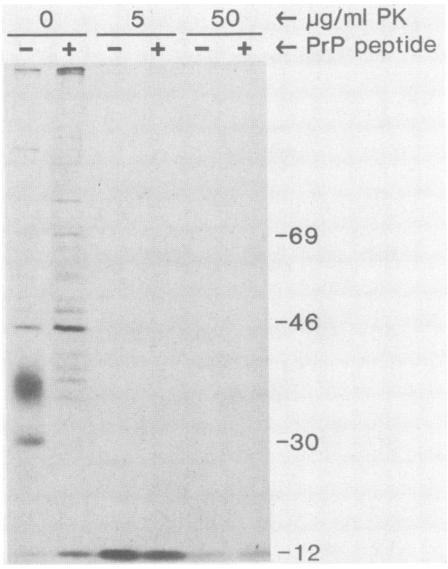

FIG. 3. PK sensitivity of metabolically labeled PrP in scrapie-infected clone 29-161. Detergent lysates of the cells were treated with PK prior to radioimmunoprecipitation as described in Materials and Methods. As described in the legend to Fig. 2, unlabeled synthetic PrP peptide antigen was used as a specificity control for the anti-PrP serum. Molecular mass markers are designated in kilodaltons.

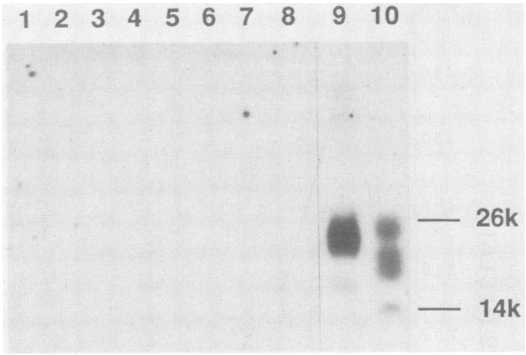

FIG. 4. Search for PK-resistant PrP in infected and uninfected mouse neuroblastoma cell clones by Western blot. Lanes 1, 2, 3, 4, and 5 contain 40×10^6 neuroblastoma cell equivalents (2.5×10^5 LD_{50}s), 125×10^6 cell equivalents (7.9×10^5 LD_{50}s), 25×10^6 cell equivalents (1.5×10^5 LD_{50}s), 32×10^6 cell equivalents (2.0×10^5 LD_{50}s), and 25.1×10^6 cell equivalents (1.6×10^5 LD_{50}s) of scrapie-infected clones 302, 302, 303, 304, and 306, respectively. Lanes 6, 7, and 8 contain 17×10^6 to 30×10^6 cell equivalents of scrapie-negative clones 300, 320, and 321, respectively. All clones are subclones of 29-161. Lane 9 contains 3×10^8 spleen cell equivalents ($\sim 2 \times 10^6$ LD_{50}s), and lane 10 contains 50-mg equivalents of mouse brain derived from clinical scrapie-infected mice ($\sim 10^8$ LD_{50}s). Equivalent samples not treated with PK were also analyzed and were negative (data not shown).

PrP in scrapie-infected neuroblastoma cells appeared completely sensitive to PK. The possibility remains, however, that a small portion of the total PrP in these cells was PK resistant but below the level of detection.

Analysis of scrapie-infected and uninfected clones by Western blotting. Since we were unable to detect a PK-resistant form of PrP by immunoprecipitation, we sought to do so by using Western blotting. Aliquots of four infected and three uninfected clones prepared by using techniques appropriate for detecting the protein in spleen and brain were analyzed. No cell-associated PrP was detected either before (data not shown) or after PK digestion (Fig. 4). We also analyzed spleen and neuroblastoma cell clone extracts prepared by the procedure of Kascsak (12) and obtained similar results (data not shown). An additional method has been described recently (5). We used the second method in this report and a broadly reactive polyclonal rabbit antiserum made against purified hamster brain PrP to analyze scrapie-positive clones. PrP was readily detected in scrapie-positive mouse brain but not in any of five scrapie-positive clones.

We considered the possibility that other proteins in the samples may have blocked the transfer of PK-resistant PrP to the nitrocellulose or may have masked PrP after transfer. However, when identical samples (Shinagawa procedure) were run on gels and stained with Coomassie blue rather than being transferred to nitrocellulose, virtually no protein was observed, indicating the effectiveness of the PK treatment and making blocking an unlikely explanation. However, in samples where no PK was used, a large amount of protein was present throughout the lane and may account for our inability to detect the PK-sensitive form of PrP with this technique. In addition, since the purification procedures we used were designed to detect PK-resistant PrP, the normal protease-sensitive PrP may have fractionated differently or may have been digested by endogenous proteases.

DISCUSSION

Scrapie has been recognized as a disease of sheep for over 200 years and as a transmissible infectious agent for several decades. Apart from the unusual resistance of the agent to antiviral treatments, little has been unequivocally established about its biochemical nature. Clearly, sources of agent other than infected animal tissues and new approaches toward agent purification are needed if we are to understand the scrapie agent. Toward this goal, we established a protocol that reliably results in infection of mouse neuroblastoma cells with scrapie agent (23). However, more highly titered cultures seemed necessary. In this report, we describe cloning of scrapie-infected neuroblastoma cells which gave cultures with markedly improved scrapie titers and high frequencies of infected cells. We also established a cell-dose infectivity curve that allows the relative infectivities of various clones to be compared.

Since the relationship of PrP to infectivity is a significant and controversial issue, we used the infected neuroblastoma cell clones to study this problem. Two forms of PrP are thought to exist, a form found in both normal and infected tissues and a different form found only in infected tissues. The sole criterion for distinguishing between the two forms is the PK resistance of the PrP found in sick animals. It is not known how the disease process itself influences the formation of the PK-resistant form. Some investigators have proposed that PK-resistant PrP could merely be a consequence of the lesions of scrapie (4) rather than a necessary component of the infectious agent (3, 9, 18, 20). If PK-resistant PrP arises as a consequence of tissue lesions and the pathogenesis of the disease, there would be little reason to believe that PK-resistant PrP would accumulate in in vitro-infected cells. Although PrP was readily detected in scrapie-infected and uninfected neuroblastoma cells by radioimmunoprecipitation, none was PK resistant. Furthermore, there was no quantitative or qualitative difference in the PrP that would distinguish infected from uninfected

clones. By Western blot, neither form of PrP was detected. Thus, our results are consistent with the hypothesis that a PK-resistant form of PrP is not necessary for infectivity.

If the infectious agent of scrapie does not directly induce the formation of the PK-resistant PrP, how does it arise? The PK-resistant form of PrP could arise in vivo because of alteration of the endogenous PK-sensitive PrP by changes in physiological conditions such as pH, salt concentration, and the action of cellular enzymes released as cells degenerate. Its accumulation to detectable levels in vivo could occur over a period of several weeks. In addition, the protein in diseased animals might contribute to its own formation by forming insoluble complexes under the influence of abnormal physiological conditions much as immunoglobulin and other proteins do in certain amyloid diseases (G. G. Glenner, review, N. Engl. J. Med. **302**:1283–1292, 1333–1343, 1980). Thus the correlation of PrP with infectious agent may simply represent incidental copurification of agent and PrP. The evidence for the inseparability of scrapie infectivity and PrP, though tantalizing, is still circumstantial (3, 9, 18, 20). Additional studies will be necessary to prove an association of PK-resistant PrP and infectivity if such an association actually exists.

ACKNOWLEDGMENTS

We thank Irene Cook Rodriguez for preparation of the manuscript, John Portis for critical evaluation of it, Gary Hettrick and Bob Evans for preparing the figures, and Missi Langley for technical assistance.

LITERATURE CITED

1. **Batteiger, B., W. J. Newhall, and R. B. Jones.** 1982. The use of Tween 20 as a blocking agent in the immunological detection of proteins transferred to nitrocellulose membranes. J. Immunol. Methods **55**:297–307.
2. **Bellinger-Kawahara, C., J. E. Cleaver, T. O. Diener, and S. B. Prusiner.** 1987. Purified scrapie prions resist inactivation by UV irradiation. J. Virol. **61**:159–166.
3. **Bolton, D. C., M. P. McKinley, and S. B. Prusiner.** 1982. Identification of a protein that purifies with the scrapie prion. Science **218**:1309–1311.
4. **Braig, H. R., and H. Diringer.** 1985. Scrapie: concept of a virus-induced amyloidosis of the brain. EMBO J. **4**:2309–2312.
5. **Butler, D. A., M. R. D. Scott, J. M. Bockman, D. R. Borchelt, A. Taraboulos, K. K. Hsiao, D. T. Kingsbury, and S. B. Prusiner.** 1988. Scrapie-infected murine neuroblastoma cells produce protease-resistant prion proteins. J. Virol. **62**:1558–1564.
6. **Cherednichenko, Y. N., G. R. Mikhailova, J. Rajcani, and V. M. Zhdanov.** 1985. In vitro studies with the scrapie agent. Acta Virol. **29**:285–293.
7. **Clarke, M. C., and D. A. Haig.** 1970. Evidence for the multiplication of scrapie agent in cell culture. Nature (London) **225**:100–101.
8. **Dees, C., W. F. Wade, T. L. German, and R. F. Marsh.** 1985. Inactivation of the scrapie agent by ultraviolet irradiation in the presence of chlorpromazine. J. Gen. Virol. **66**:845–849.
9. **Diringer, H., H. Gelderblom, H. Hilmert, M. Ozel, C. Edelbluth, and R. H. Kimberlin.** 1983. Scrapie infectivity, fibrils and low molecular weight protein. Nature (London) **306**:476–478.
10. **Dougherty, R. M.** 1964. Animal virus titration techniques, p. 183–186. *In* R. J. C. Harris (ed.), Techniques in experimental virology. Academic Press, Inc., New York.
11. **Hope, J., L. J. D. Morton, C. F. Farquhar, G. Multhaup, K. Beyreuther, and R. H. Kimberlin.** 1986. The major polypeptide of scrapie-associated fibrils (SAF) has the same size, charge distribution and N-terminal protein sequence as predicted for the normal brain protein (PrP). EMBO J. **5**:2591–2597.
12. **Kascsak, R. J., R. Rubenstein, P. A. Merz, R. I. Carp, N. K. Robakis, H. M. Wisniewski, and H. Diringer.** 1986. Immunological comparison of scrapie-associated fibrils isolated from animals infected with four different scrapie strains. J. Virol. **59**: 676–683.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
14. **Latarjet, R.** 1979. Inactivation of the agents of scrapie, Creutzfeldt-Jakob disease, and Kuru by radiations, p. 387–407. *In* S. B. Prusiner and W. J. Hadlow (ed.), Slow transmissible diseases of the nervous system, vol. 2. Academic Press, Inc., New York.
15. **Markovits, P., D. Dormont, B. Delpeck, L. Court, and R. Latarjet.** 1981. Essais de propagation in vitro de l'agent scrapie dans des cellules nerveuses de souris. C. R. Acad. Sci. **293**:413–417.
16. **McKinley, M. P., D. C. Bolton, and S. B. Prusiner.** 1983. A protease-resistant protein is a structural component of the scrapie prion. Cell **35**:57–62.
17. **Merz, P. A., R. A. Somerville, H. M. Wisniewski, and K. Iqbal.** 1981. Abnormal fibrils from scrapie-infected brain. Acta Neuropathol. **54**:63–74.
18. **Merz, P. A., R. A. Somerville, H. M. Wisniewski, L. Manuelidis, and E. E. Manuelidis.** 1983. Scrapie-associated fibrils in Creutzfeldt-Jakob disease. Nature (London) **306**:474–476.
19. **Meyer, R. K., M. P. McKinley, K. A. Bowman, M. B. Braunfeld, R. A. Barry, and S. B. Prusiner.** 1986. Separation and properties of cellular and scrapie prion proteins. Proc. Natl. Acad. Sci. USA **83**:2310–2314.

19a. **Oesch, B., D. F. Groth, S. B. Prusiner, and C. Weissmann.** 1988. Search for a scrapie-specific nucleic acid: a progress report, p. 209–217. *In* J. Marsh (ed.), Novel infectious agents and the central nervous system. John Wiley & Sons Ltd., Chichester, United Kingdom.

20. **Prusiner, S. B.** 1982. Novel proteinaceous infectious particles cause scrapie. Science **216**:136–144.
21. **Prusiner, S. B., D. F. Groth, S. P. Cochran, F. R. Masiarz, M. P. McKinley, and H. M. Martinez.** 1980. Molecular properties, partial purification and assay by incubation period measurements of the hamster scrapie agent. Biochemistry **19**:4883–4891.
22. **Prusiner, S. B., M. P. McKinley, K. A. Bowman, D. C. Bolton, D. F. Groth, and G. G. Glenner.** 1983. Scrapie prions aggregate to form amyloid-like birefringent rods. Cell **35**:349–358.
23. **Race, R. E., L. H. Fadness, and B. Chesebro.** 1987. Characterization of scrapie infection in mouse neuroblastoma cells. J. Gen. Virol. **68**:1391–1399.
24. **Rohwer, R. G.** 1984. Scrapie infectious agent is virus-like in size and susceptibility to inactivation. Nature (London) **308**:658–661.
25. **Rubenstein, R., R. I. Carp, and S. M. Callahan.** 1984. In vitro replication of scrapie agent in a neuronal model: infection of PC12 cells. J. Gen. Virol. **65**:2191–2198.
26. **Rubenstein, R., R. J. Kascsak, P. A. Merz, M. C. Papini, R. I. Carp, N. K. Robakis, and H. M. Wisniewski.** 1986. Detection of scrapie-associated fibril (SAF) proteins using anti-SAF antibody in non-purified tissue preparations. J. Gen. Virol. **67**:671–681.
27. **Somerville, R. A., P. A. Merz, and R. I. Carp.** 1986. Partial copurification of scrapie-associated fibrils and scrapie infectivity. Intervirology **25**:48–55.
28. **Wiley, C. A., P. G. Burrola, M. J. Buchmeier, M. K. Wooddell, R. A. Barry, S. B. Prusiner, and P. W. Lampert.** 1987. Immuno-Gold localization of prion filaments in scrapie-infected hamster brains. Lab. Invest. **57**:646–656.
29. **Yanagihara, R. I., D. M. Asher, C. J. Gibb, Jr., and D. C. Gajdusek.** 1980. Attempts to establish cell cultures infected with the viruses of subacute spongiform encephalopathies. Proc. Soc. Exp. Biol. Med. **165**:298–305.