JOURNAL OF VIROLOGY, May 1988, p. 1558–1564
0022-538X/88/051558-07$02.00/0

Vol. 62, No. 5

Scrapie-Infected Murine Neuroblastoma Cells Produce Protease-Resistant Prion Proteins

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Received 17 July 1987/Accepted 12 January 1988

Scrapie and Creutzfeldt-Jakob disease are transmissible, degenerative neurological diseases caused by prions. Considerable evidence argues that prions contain protease-resistant sialoglycoproteins, designated PrP^{Sc}, encoded by a cellular gene. The prion protein (PrP) gene also encodes a normal cellular protein designated PrP^{C}. We established clonal cell lines which support the replication of mouse scrapie or Creutzfeldt-Jakob disease prions. Mouse neuroblastoma N2a cells were exposed to mouse scrapie prions and subsequently cloned. After limited proteinase K digestion, three PrP-immunoreactive proteins with apparent molecular masses ranging between 20 and 30 kilodaltons were detected in extracts of scrapie-infected N2a cells by Western (immuno-) blotting. The authenticity of these PrP^{Sc} molecules was established by using monospecific antiserum raised against a synthetic peptide corresponding to a portion of the prion protein. Those clones synthesizing PrP^{Sc} molecules possessed scrapie prion infectivity as measured by bioassay; clones without PrP^{Sc} failed to demonstrate infectivity. Detection of PrP^{Sc} molecules in scrapie-infected N2a cells supports the contention that PrP^{Sc} is a component of the infectious scrapie particle and opens new approaches to the study of prion diseases.

Prions are slow infectious pathogens which cause several transmissible, degenerative neurological disorders, including scrapie of sheep and goats as well as Creutzfeldt-Jakob disease (CJD), kuru, and Gerstmann Sträussler syndrome of humans (16, 25, 32). Prions are unusual in that they display extreme resistance to inactivation by procedures which inactivate nucleic acids (5, 6, 32).

The precise molecular composition of prions has remained elusive. All attempts to identify a scrapie-specific nucleic acid have been unsuccessful (5, 6, 29). To date, only a single macromolecule has been shown to be required for infectivity; this macromolecule in hamsters is a protease-resistant sialoglycoprotein designated PrP 27-30 (26), for prion protein of M_r 27 to 30 kilodaltons (kDa). PrP 27-30 purifies with and is inseparable from scrapie infectivity (10, 11, 26, 33, 35, 36). Additionally, proteolytic digestion of PrP 27-30 proceeds with kinetics identical to proteolytic inactivation of prions (26). Recent studies show that PrP 27-30 is formed during purification from a larger protein in hamsters of 33 to 35 kDa and that normal, uninfected hamsters contain a related, protease-sensitive protein of 33 to 35 kDa (3, 29). Studies of scrapie in mice revealed similar protease-resistant proteins of similar molecular masses (11, 18). We designate the mouse scrapie prion proteins $MoPrP^{Sc}$ and the cellular isoform $MoPrP^{C}$.

Presently most studies involving prions are conducted in experimental models of scrapie in rodents. Although these systems have proven advantageous because of their comparatively short incubation period for prions, they are not optimal for detailed molecular analyses. Hence, several groups have attempted to develop cell cultures for scrapie infection and prion replication. In one attempt, mouse L cells were infected with a scrapie mouse brain homogenate (13, 14); however, these cells lost their infectivity with repeated passages. Since these cells were not cloned, it is possible that much of the observed infectivity was carried over from the original inoculum. Other groups have reported successful replication of the infectious agent in neuronal models such as N1E 115, a mouse neuroblastoma line (24), and PC12, a rat pheochromocytoma line (39). In these studies, clonal infected lines were not established, necessitating reinfection for each infectivity study and thereby jeopardizing consistency from experiment to experiment. The investigation with PC12 cells was further complicated by infecting these rat cells with mouse scrapie prions and measuring the infectivity associated with the rat cells in mice. Crossing species barriers is associated with prolonging the incubation period (30, 31). Species-specific epitopes have recently been demonstrated for prion proteins, reflecting their cellular origin (9, 29).

Herein, we report experimental studies with clonal cell lines which are chronically infected with either mouse scrapie prions or mouse CJD prions. These lines are derived from the mouse neuroblastoma line N2a. The initial phase of this work involved the identification of murine N2a cells as a suitable host for infection with partially purified fractions derived from murine brains infected with CJD prions and the demonstration that infected clonal cell lines could be derived (D. T. Kingsbury, D. Smeltzer, and J. Bockman, 6th Int. Cong. Virol. Abstr., 1984, no. W47-6, p. 70). For the same N2a cells, other investigators subsequently reported similar but more extensive infectivity studies with murine scrapie prions and demonstrated the reproducibility of productive infection in mouse neuroblastoma cell lines (37). Cloning of the infectious cell lines makes it unnecessary to infect cells for each study; the neuronal background for prion replication is advantageous for comparisons with scrapie- or CJD-infected brains. We demonstrated that a scrapie-infected cell line produces mouse (Mo) PrP^{Sc} and established the authenticity of the PrP molecules by using monospecific antiserum raised against a synthetic PrP peptide (D. A. Butler,

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MATERIALS AND METHODS

Cell lines. Neuro-2a (N2a) (R. J. Klebe and F. H. Ruddle, J. Cell Biol. 43:69a, 1969) cells were obtained from the American Type Culture Collection. HJC-15 (44) and p12-3 cells were a gift from D. Walker, University of Wisconsin. CHO cells were obtained from the University of California, San Francisco, Cell Culture Facility.

Prion preparations. Sucrose gradient preparations of Swiss mouse scrapie prions containing $10^{7.8}$ 50% infectious dose (ID_{50}) U/ml and of Syrian hamster scrapie prions containing $10^{8.6}$ ID_{50} U/ml were used for the scrapie infections. The CJD prions were derived from the Fukuoka-1 isolate of CJD, which has been adapted to mice (20, 41). CJD prions were purified by a procedure similar to that published for purification of scrapie prions (35), and the ammonium sulfate precipitate was used for the infections.

Infection of cell lines with scrapie prions. One day prior to infection, cells were plated at a density of 2×10^5 cells per 35-mm plastic petri dish in Eagle minimal essential medium supplemented with 10% heat-inactivated fetal calf serum. The following day, the old medium was removed and replaced with 0.5 ml of fresh medium. Sucrose gradient preparations of prions were diluted sequentially into phosphate-buffered saline (PBS) and medium and added to the cells at a specified multiplicity of infection (MOI), defined as the ratio of ID_{50} units (as determined by bioassay) to cells. After incubation at 37°C for 1 h in 95% O_2 and 5% CO_2, the cells were fed with 4.5 ml of medium and grown to confluency. Each sample of cells was then passaged at a 1:10 dilution into a single 75-cm^2 plastic flask, grown to confluency, and passaged at a 1:10 dilution into a single 75-cm^2 flask. Once the cells reached confluency, each sample was subcloned by limiting dilution, seeding 0.3 cells per well into 96-well plates. Six positive wells at each MOI were transferred sequentially into 24-well plates, 25-cm^2 flasks, and finally into 75-cm^2 flasks. Confluent 75-cm^2 flasks of each clone were split into two 75-cm^2 flasks, one of which was used to prepare the inoculum.

Preparation of cells for prion bioassay. Inocula from cells were prepared by growing clones to confluency in 75-cm^2 flasks ($\sim 1.5 \times 10^7$ cells per flask), washing once with cold PBS, scraping cells into PBS, and pelleting cells at 2,000 × *g*, 10 min, 4°C. All PBS was carefully removed. Cell pellets were then suspended in 100 μl of inoculum buffer consisting of PBS, 5% bovine serum albumin, 2.5 μg of fungizone, 0.5 U of penicillin, and 0.5 μg of streptomycin per ml. Cell suspensions from a given MOI were pooled, the volume of the inoculum was adjusted to 1 ml with the inoculum buffer, and the cells were sonicated with two 10-s pulses of a Branson model 350 cell disrupter at a setting of 4.

Inocula from the media were prepared by collecting the media and removing residual cells and debris by centrifugation at 1,500 × *g*, 5 min, 4°C. The proteins were then precipitated with 4 volumes of ice-cold methanol, suspended in 1 ml of inoculum buffer, and inoculated without sonication into animals.

Assay for scrapie prion infectivity. Inocula were injected intracerebrally into eight Swiss mice for each mouse sample (30 μl per mouse) or into eight Syrian hamsters for each hamster sample (50 μl per hamster). Positive controls from either a scrapie-infected mouse brain homogenate or a scrapie-infected hamster brain homogenate were done in parallel. Animals were diagnosed as having scrapie by clinical examination, and the diagnosis was confirmed by histopathological examination and comparison with scrapie-infected age-matched controls. Titers were determined by plotting the average time from inoculation of the animals to sickness on a dose-incubation time curve for the Chandler isolate in Swiss mice.

Infection of N2a cells with mouse CJD prions. N2a cells were grown in minimal essential medium containing 10% fetal calf serum and gentamicin (100 μg/ml). Subconfluent monolayers in 25-cm^2 flasks ($\sim 2.5 \times 10^6$ cells) were inoculated with a 1:10 dilution of the ammonium sulfate precipitate ($\sim 6.7 \times 10^4$ ID_{50} units) of murine CJD prions. Cells were subcultured 1:3 and then cloned into 96-well plates. Viable clones were subcultured 1:2, expanded into 24-well plates, subcultured 1:2, and then harvested at confluency. Groups of four wells were pooled, and the cell pellets were suspended in 150 mM NaCl. The suspensions were then subjected to three rounds of freeze-thawing. Supernatants from a low-speed centrifugation were inoculated intracerebrally into NAMRU mice. Passage and cloning of the cells would have resulted in each mouse receiving a maximum of 10^{-5} ID_{50} units of the original inoculum. Animals were observed for 1 year and scored positive for CJD by clinical symptoms. Diagnosis was verified histopathologically and by resistance of the agent to heat inactivation. Titers were determined by the incubation time assay as modified for CJD in NAMRU mice (42).

Preparation of cell lysates for Western blotting. Two procedures were used to prepare cell lysates for analysis. In the first procedure, two confluent 175-cm^2 flasks per sample were washed once with cold PBS, scraped into cold PBS, pelleted by centrifugation, and suspended in 5 ml of 320 mM sucrose. Cells were homogenized by four 5-s pulses with a polytron homogenizer. Homogenates were then cleared by centrifugation at 1,500 × *g*, 15 min, 4°C. The supernatants were centrifuged at 200,000 × *g*, 30 min, 4°C, in a 50 Ti rotor. The resulting pellets constituted enriched membrane fractions and were suspended in 20 mM Tris hydrochloride (pH 7.4)–150 mM NaCl–0.2% Sarkosyl (sodium dodecyl sarcosinate). Suspensions were split into two equal portions, and one was digested with proteinase K (5 μg/ml, 37°C, 30 min) and the other was left undigested. Phenylmethylsulfonyl fluoride was then added to a final concentration of 5 mM to all samples on ice. Proteins were precipitated by addition of 10% trichloroacetic acid on ice and centrifuged in a microfuge for 15 min. Pellets were washed four times with cold acetone and then suspended in 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (62.5 mM Tris hydrochloride, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue). Samples were boiled for 5 min, and 60 μl was loaded onto an SDS–12.5% polyacrylamide gel.

Subsequently, a second protocol was developed which was used for extraction of both mouse brain and N2a cells. A 20% (wt/vol) homogenate of normal or scrapie-infected mouse brain was prepared by dounce homogenization in 0.05 M Tris hydrochloride (pH 7.5)–0.15 M NaCl–5 mM EDTA at 4°C, followed by detergent extraction with 1% Triton X-100 and 1% deoxycholate. Nuclei and insoluble debri were removed by centrifugation at 3,000 × *g* for 5 min. The supernatants were then precipitated with 4 volumes of methanol at −20°C for at least 30 min. Detergent extracts of normal and scrapie-infected N2a cells were prepared in an identical manner except that dounce homogenization was not necessary. Extracts of both brains and cells were sus-

TABLE 1. Bioassay of cell lines infected with species-specific scrapie prions[a]

Cell line	Cell type	MOI	No. of clones[b]	Titer (ID_{50} U/10^7 cells)
N2a	Mouse neuroblastoma	100	5	$10^{3.6}$
		10	4	<1
		1	1	<1
		0	N.C.	<1
p12-3	Hamster neuroblastoma	10	4	<1
		1	3	<1
		0	N.C.	<1
HJC-15	Hamster glioma	10	5	<1
		1	6	<1
		0	N.C.	<1
CHO	Hamster fibroblast	10	6	<1
		1	6	<1
		0	N.C.	<1

[a] The experiment was terminated at day 223 after inoculation of the animals.
[b] The individual clones are characterized in Table 4 and Fig. 4. N.C., Not cloned.

pended in 0.05 M Tris hydrochloride (pH 7.5)–0.15 M NaCl–0.2% Sarkosyl. Portions of each were then digested with proteinase K (5 μg/ml) for 30 min at 37°C; the relative concentration of substrate to enzyme in each sample was identical. Digestions were terminated by addition of 5 mM phenylmethylsulfonyl fluoride. Samples were then methanol precipitated, centrifuged, suspended in 1× sample buffer (62.5 mM Tris hydrochloride [pH 6.8], 2% SDS, 25% sucrose, 0.75 M β-mercaptoethanol, 0.03% bromphenol blue), and boiled for 3 to 5 min prior to SDS-PAGE. The Laemmli buffer system with 12% polyacrylamide gels was used for SDS-PAGE (25).

PrP synthetic peptides and antisera. Rabbit polyclonal antisera (R003 and R017) were raised against PrP 27-30 purified from scrapie-infected hamster brain (2, 7). Peptide antigens were synthesized on an Applied Biosystems (Foster City, Calif.) 430A peptide synthesizer (19). The amino acid sequences of the PrP peptides are as follows, with some residue numbers (4) shown: P3, *Cys-Gly-Gly*-Lys-220-Glu-Ser-Gln-Ala-Tyr-Tyr-Asp-Gly-Arg-Arg-Ser-Ser-Ala-233; P5, *Cys-Gly-Gly*-Gly-142-Asn-Asp-Trp-Glu-Asp-Arg-Tyr-Tyr-Arg-Glu-Asn-Met-Asn-Arg-Tyr-Pro-Asn-Gln-Asn-Asn-174. The glycine and cysteine residues shown in italics were added to facilitate coupling of the synthetic peptide P3 to carrier proteins as described previously (1).

Immunoblotting. Immunoblotting was performed essentially as described before (29). Rabbit PrP 27-30 antisera (R003 and R017) were diluted 1:500, 1:750, or 1:1,000; synthetic peptide P3 antiserum was diluted 1:1,000. Goat anti-rabbit immunoglobulin sera coupled to alkaline phosphatase was obtained from Promega and diluted 1:7,500. Electrotransfer of protein from SDS-polyacrylamide gels to nitrocellulose was accomplished with standard buffers containing either 0.01 or 0.05% (wt/vol) SDS.

RESULTS

Replication of prions in cultured cells. To establish cell culture models for prion diseases, we attempted to infect several different cell lines with scrapie prions. Four cell lines were used: N2a, a mouse neuroblastoma line; p12-3, a Syrian hamster neuroblastoma line; HJC-15, a Syrian hamster glioma line; and CHO, a Chinese hamster fibroblast (ovary) line. These cell lines were infected at several MOIs, defined as the number of ID_{50} units per cell, with (iso)species-specific, sucrose gradient-purified preparations of scrapie prions. After incubation with the prions, the cells were serially passaged for several generations. The cells were then subcloned at limiting dilution. The number of clones analyzed for infectivity at each MOI varied in the viability of the clones (Table 1). Throughout the experiment, the cells were examined by phase-contrast microscopy. No cytopathology was observed which could be unambiguously attributed to prion infection.

As shown in Table 1, only the N2a cells which had been infected at an MOI of 100 were infective. The average incubation period for mice inoculated with this sample was 154 days (Fig. 1). Passaging and subcloning the cells resulted in a dilution of the original inoculum by at least a factor of 10^9. At the highest MOI (100), a dilution of 10^9 would result in a titer of 0.02 ID_{50} U/10^7 cells if the infectivity was simply due to the presence of the original inoculum. In the infectious sample, we detected a titer of $\sim 10^4$ ID_{50} U/10^7 cells (Fig. 1), which cannot be accounted for by residual inoculum; clearly, replication of prions must have occurred. Three of the four clinically ill mice were sacrificed, and the diagnosis of scrapie was confirmed by histopathology.

Similar experiments were done with mouse CJD prions in N2a cells. Semiconfluent monolayers of N2a cells in 25-cm^2 culture flasks were infected with a partially purified ammonium sulfate precipitate of CJD prions. This dilution of inoculum resulted in the addition of approximately 6.7×10^4 ID_{50} units to the cells. Control cultures were mock infected with saline. Clonal lines were established in a manner analogous to that described above, and several samples derived from four clones each were inoculated intracerebrally into NAMRU mice. No cytopathology or alteration of growth rate or cloning efficiency was observed with the CJD-infected N2a cells.

Mice inoculated intracerebrally with preparations of the CJD-infected N2a cells developed characteristic symptoms of CJD ~6 months later (Table 2). Five sick mice which had been inoculated with one of four different samples were sacrificed and their brains were removed. To confirm the clinical diagnosis of CJD, half of each brain was prepared for histopathological examination, while the remaining half was used for serial passage into mice and to test for heat inactivation of the CJD prions. Each of the five brains analyzed by histopathology demonstrated frank vacuolation of neurons, pathology characteristic of CJD, compared with age-matched normal mice. Mice inoculated with either heated or untreated preparations of these brains all developed symptoms of CJD after approximately 5 months and subsequently died of their progressive illness (Table 3). The subcloned CJD-infected N2a lines were serially propagated for more than 10 transfers with no apparent loss in infectivity.

Detection of $MoPrP^{Sc}$ in cloned cell lines. Given the association of $MoPrP^{Sc}$ with murine scrapie infectivity (11), we

TABLE 2. Bioassay of N2a cells infected with mouse CJD prions

Sample[a]	Mean incubation time (days) ± SD	Titer (ID_{50} U/10^7 cells)
1	198 ± 16	$10^{3.9}$
2	189 ± 8	$10^{4.5}$
3	188 ± 18	$10^{4.5}$
4	196 ± 16	$10^{4.1}$
5	187 ± 15	$10^{4.6}$

[a] Each sample represents a pool of four cloned lines.

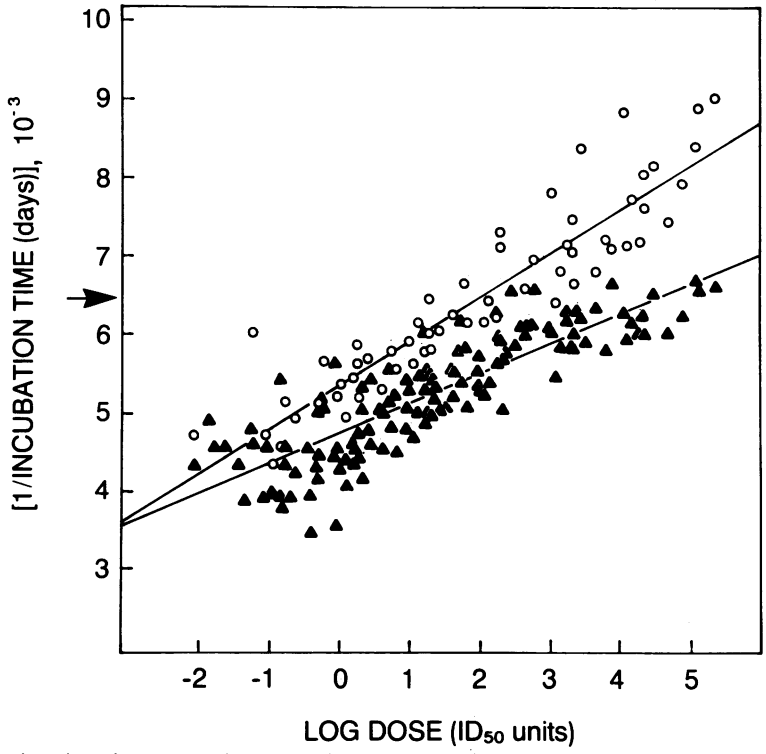

FIG. 1. Replication of scrapie prions in N2a murine neuroblastoma cells. Calibration curve for the Chandler isolate of mouse scrapie prions in Swiss mice plotted as the reciprocal of the incubation time versus the average dose given each animal. This curve is derived from endpoint titration of 29 independent preparations (18 spleen samples, 10 sucrose gradient fractions, and 1 brain homogenate). The arrow depicts the infectivity associated with the scrapie-infected N2a cells (Table 1). Total ID_{50} units were calculated by dividing the dose as given above by the volume of the inoculum given to each mouse (30 μl). Titers, when given, are expressed as total ID_{50} units per confluent 75-cm^2 flask (~10^7 cells per flask). The experiment was terminated at 226 days after inoculation of the mice. Symbols: ○, sick dates; ▲, death dates.

used Western blotting analysis to determine whether we could detect $MoPrP^{Sc}$ in any of the five clones which composed the infectious inoculum (Table 1). Normal mouse brain contains PrP^C, but no proteinase K-resistant proteins were detected by immunoblotting (Fig. 2, lanes 1 and 2). PrP^C was not readily detected in extracts of uninfected N2a cells, and no immunostaining was seen after limited proteinase K digestion (Fig. 2, lanes 3 and 4). PrP^{Sc} was also not readily detectable in infected N2a cells by immunostaining prior to purification. Digesting detergent extracts of infected cells with proteinase K provided the purification needed for PrP^{Sc} detection on Western blotting (Fig. 2, lanes 7 and 8). The apparent molecular masses of these proteinase K-resistant proteins were between 20 and 30 kDa, as determined by SDS-PAGE. These proteins were absent from the uninfected N2a cells (Fig. 2, lane 4). The PrP-immunoreactive proteins aligned well with the proteinase K-resistant proteins from a scrapie mouse brain homogenate (Fig. 2, lanes 6 and 8). We detected $MoPrP^{Sc}$ in this cell line by preparation of membrane fractions, by lysis of the cells with a variety of detergents, and with two different polyclonal PrP 27-30 antisera. The production of $MoPrP^{Sc}$ in this clone was stable for at least 30 passages.

The authenticity of the proteinase K-resistant, PrP-immunoreactive proteins in scrapie-infected N2a cells was established by using monospecific rabbit antisera raised against a synthetic peptide (P3) corresponding to amino acid residues 220 to 233 of hamster PrP (4). The corresponding sequence in mouse is identical except for the first and last residues (23, 43). As shown in Fig. 3, the preimmune serum failed to react with $MoPrP^{Sc}$, while the immune serum strongly bound to the protein (lanes 1 and 2). Immunostaining was abolished by absorbing the antisera with the peptide P3 (lanes 3 and 4), while absorption with an unrelated synthetic peptide corresponding to hamster PrP residues 142 to 174 did not alter immunostaining (lane 5). The Western blot pattern of scrapie-infected N2a cells with synthetic peptide antisera was identical to that observed with polyclonal rabbit antisera raised against purified hamster PrP 27-30 (lane 6). Likewise, the PrP^{Sc} immunoblots from scrapie N2a cells were almost indistinguishable from those obtained with scrapie-infected brain with either the synthetic PrP peptide antiserum (Fig. 3, lanes 2 and 7) or the polyclonal PrP 27-30 antiserum (Fig. 2).

Infected cells produce $MoPrP^{Sc}$. To determine whether the clone 3, which produced $MoPrP^{Sc}$, was infectious by itself,

TABLE 3. Serial propagation and heat resistance of CJD prions from mice inoculated with CJD-infected N2a cells[a]

Sample	Titer (ID_{50} U/ml)	
	Control	Heated
1	$10^{5.6}$	$10^{5.3}$
2	$10^{6.1}$	$10^{6.0}$
3	$10^{6.0}$	$10^{5.8}$
4	$10^{6.1}$	$10^{5.9}$
5	$10^{5.8}$	$10^{5.6}$

[a] One-half of a 10% (wt/vol) homogenate of mouse brain in 320 mM sucrose was heated at 80°C for 45 min. A 30-μl amount of either the heated or unheated homogenate was inoculated intracerebrally into each of seven NAMRU mice.

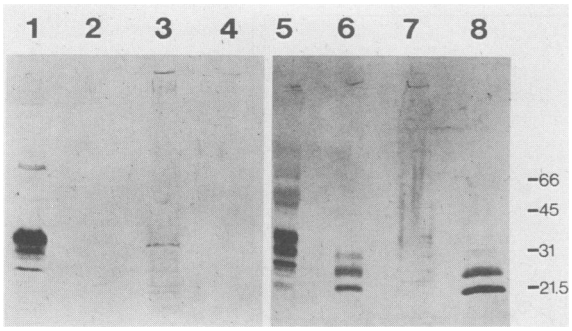

FIG. 2. Detection of MoPrP^{Sc} in scrapie-infected N2a cells by immunoblotting. Detergent extracts were prepared by the second protocol outlined in Materials and Methods. Lanes contained uninfected mouse brain (lanes 1 and 2) and N2a cells (lanes 3 and 4) or scrapie-infected mouse brain (lanes 5 and 6) and clone 3 N2a cells (lanes 7 and 8). Brain extract or extract of ~10^7 N2a cells (40 μl) was loaded onto each lane. Portions were digested with proteinase K (5 μg/ml) for 30 min at 37°C (even-numbered lanes) prior to SDS denaturation and electrophoresis. A rabbit antiserum (R017) raised against PrP 27-30 purified from scrapie-infected hamster brains was used at a dilution of 1:750. Apparent molecular masses are given (in kilodaltons).

we bioassayed each of the clones separately. Our results indicate that the clone which produced MoPrP^{Sc} as shown by Western blotting was indeed infectious by itself and was the only one of the original five clones (Table 1) that was infectious (Table 4). To assess whether we could detect secretion of prions from the cells, the medium in which each clone had been grown overnight was collected and bioassayed. We found trace infectivity in the medium of clone 3 which may have resulted from the release of prions by cell lysis. The lack of appreciable infectivity in the medium suggests that the scrapie-infected N2a cells were not actively secreting prions. It is possible, though, that higher titers might be found in the medium if the medium was harvested after a longer period of time.

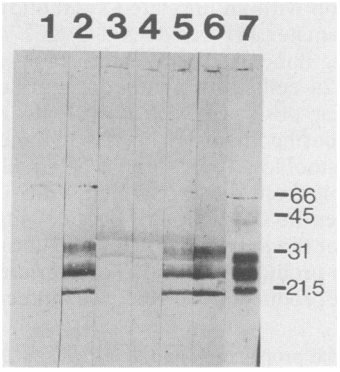

FIG. 3. Detection of MoPrP^{Sc} by using a monospecific antiserum raised against a synthetic PrP peptide. Detergent extracts were prepared by the second protocol described in Materials and Methods. Lanes 1 to 6 each contain scrapie-infected N2a cells (clone 3) from one plate (10-cm diameter) grown to confluency (~10^7 cells). Lane 7 contains 50 μl of a detergent-extracted scrapie-infected mouse brain homogenate. Lanes 2 to 5 and 7 were stained with rabbit anti-P3 peptide serum diluted 1:1,000. Lane 1 was stained with the corresponding preimmune serum; lane 6 was stained with rabbit anti-PrP 27-30 serum diluted 1:1,000. P3 antiserum was preabsorbed with either the P3 peptide at 2.5 μg/ml (lane 3) and 250 μg/ml (lane 4) or the unrelated P5 peptide at 250 μg/ml (lane 5). All samples were digested with proteinase K prior to denaturation and SDS-PAGE. Apparent molecular masses are given (in kilodaltons).

TABLE 4. Bioassay and immunoblotting of five neuroblastoma (N2a) cell clones

Clone	MoPrP^{Sc}[a]	Titer[b] (ID_{50} U/10^7 cells)	
		Cells	Medium
1	−	<1	<1
2	−	<1	<1
3	+	$10^{4.3}$	$10^{1.3}$
4	−	<1	<1
5	−	<1	<1

[a] Western blots with rabbit PrP 27-30 antiserum to detect MoPrP^{Sc} were performed on clones 1 to 5 prior to bioassays.

[b] Titers were determined by using the calibration curve shown in Fig. 1. The experiment was terminated 266 days after inoculation of the mice.

In Fig. 4, Western blots of N2a cells from clones 3, 4, and 5 (Table 4) are shown. Clones 4 and 5 did not contain infectious prions, and like clones 1 and 2 (not shown), they did not contain proteinase K-resistant, PrP-immunoreactive proteins. In contrast, infectious clone 3 did contain PrP^{Sc} molecules (lane 5). A proteinase K-resistant, PrP-immunoreactive protein with an apparent mass of ~16 kDa was detected sometimes if the clone 3 cells were lysed with detergents; presumably, endogenous enzymes partially degrade PrP^{Sc} to yield this polypeptide after proteinase K digestion.

The residual population of scrapie-infected N2a cells from which clone 3 was isolated (Table 1) were frozen at −70°C and thawed after 1.5 years. Six additional clones were isolated and tested for the presence of MoPrP^{Sc} by Western blotting. Two of the six clones produced proteinase K-resistant, PrP-immunoreactive proteins with apparent molecular masses identical to those found in clone 3 (data not shown). Bioassay of these clones is in progress.

DISCUSSION

We have successfully established clonal cell lines which are chronically infected with either mouse scrapie or CJD

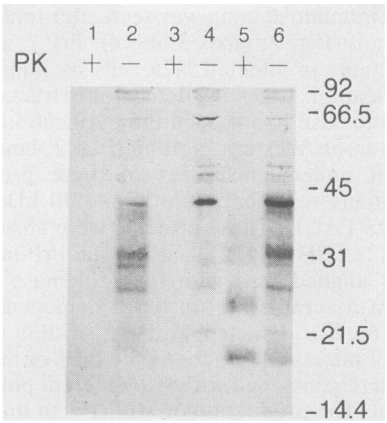

FIG. 4. Immunoblots of uninfected and scrapie-infected N2a cell clones. Detergent extracts were prepared by the first protocol described in Materials and Methods. Lanes 1 and 2, extracts of clone 5 cells (Table 4); lanes 3 and 4, clone 4; lanes 5 and 6, clone 3. The samples in odd-numbered lanes were digested with proteinase K (PK) prior to denaturation and SDS-PAGE. Rabbit polyclonal antiserum (R003) raised against PrP 27-30 purified from scrapie-infected hamster brain was used at a dilution of 1:500. Apparent molecular masses are given (in kilodaltons).

prions. These cell lines were isolated and subcloned from a population of mouse neuroblastoma (N2a) cells which were infected with either a sucrose gradient preparation of mouse scrapie prions or an ammonium sulfate precipitate of mouse CJD prions.

We found that the scrapie-infected cell line produced three major protease-resistant, PrP-immunoreactive prion proteins with apparent molecular masses ranging between 20 and 30 kDa. This correlation between scrapie infectivity and MoPrP^{Sc} extends earlier studies indicating that hamster PrP 27-30 is inseparable from scrapie infectivity (26). The argument that PrP^{Sc} is an integral component of the infectious scrapie prion is now supported by seven distinct experimental observations: (i) PrP 27-30 and scrapie prions copurify; PrP 27-30 is the most abundant molecule in purified prion preparations (10, 36); (ii) PrP 27-30 concentration is proportional to prion titer (26); (iii) hydrolysis, denaturation, or selective modification of PrP 27-30 results in a diminution of prion titer (26); (iv) the PrP gene is linked and perhaps identical to the *Prn-i* gene which controls scrapie incubation periods (12); (v) PrP 27-30 and prion infectivity partition together in membranes, rods, spheres, detergent-lipid-protein complexes, and liposomes (2, 15, 27, 28, 34–36); (vi) PrP^{Sc} is specific for prion diseases (8, 17, 21, 38); and (vii) scrapie-infected cultured cells produce MoPrP^{Sc} (this work). The fact that the results presented here were obtained in cell culture and not animals or humans makes this argument all the more compelling.

The lack of clear immunoreactive bands indicative of PrP in the undigested extracts of normal or scrapie-infected N2a cells may be due to proteins which compete with PrP^{C} or PrP^{Sc} for binding sites on the nitrocellulose membrane. Proteinase K digestion of scrapie-infected N2a cell extracts resulted in the partial purification of PrP^{Sc}, which presumably increases the efficacy of electrotransfer after SDS-PAGE. Recent studies with PrP^{C} support this interpretation. PrP^{C} partially purified from N2a cells by digestion with phosphatidylinositol phospholipase C was readily detected by Western blotting. Purified PrP^{C} added to extracts of either infected or uninfected N2a cells was poorly detected by immunoblotting (D. Borchelt and A. Taraboulos, unpublished observations). These observations suggest that one or several comigrating proteins may interfere with the electrotransfer of prion proteins in extracts of N2a cells. This problem does not seem to be encountered with detergent extracts of mouse brain, because of either a fortuitous lack of interfering proteins or structural differences between brain prion proteins and those found in cultured N2a cells. Because of the problems with immunoblotting described above and the low titers of scrapie prions found in infected N2a cells, no attempt was made to correlate the concentration of PrP^{Sc} with the prion titer as was done previously for purified prions from hamster brain (26, 34).

Factors governing the infectability of cultured cells by prions are unknown. From the results of animal studies (9, 30, 31), we presume that prions from the same species as the host-cultured cells will be most infectious. Crossing species in animal studies clearly results in prolongation of the incubation period. Molecular cloning studies show that ~10% of amino acid residues vary among mouse, hamster, and human PrP sequences. Inbred strains of mice having short or long scrapie incubation periods possess prion proteins differing by two residues (43). Whether this difference of two amino acids is significant with respect to infection of cultured cells is unknown. No data are available about the initial events necessary to establish a prion infection. Mechanisms governing the release of prions from cells and their subsequent binding to neighboring uninfected cells are unknown. PrP^{C} has been localized to the surface of mouse N2a cells, rat PC-12 cells, and primary cultures of neonatal hamster brain (40); the topology of PrP^{Sc} is being investigated. Whether specific receptors exist for prions or PrP^{C} figures in transmission from cell to cell remains unknown. Of note, immunofluorescence studies with scrapie N2a cells failed to demonstrate a significant difference in the pattern of staining compared with uninfected controls (data not shown).

Our discovery of PrP^{Sc} molecules in cultured cells harboring scrapie prion infectivity provides a readily detectable marker for future experimental studies. Cultured cells infected with scrapie prions should facilitate the structural analysis of PrP^{C} and PrP^{Sc} molecules. The profound differences in the properties of these two molecules are thought to arise from a posttranslational event, since both PrP isoforms probably have the same amino acid sequence (4). The use of radiolabeled precursors to investigate the posttranslational modifications of the two PrP isoforms may prove plausible if isotope incorporation is sufficiently great; however, it is likely that much higher levels of PrP^{C} and PrP^{Sc} in cell culture will be needed before many studies can be performed. Hopefully, cloned PrP genes inserted into expression vectors (M. Scott, D. Butler, D. Bredesen, M. Wälchli, K. Hsiao, and S. B. Prusiner, Protein Eng., in press) will be useful in raising the levels of prion proteins in cultured cells.

ACKNOWLEDGMENTS

We thank Sherrie Coleman and Marilyn Peterson for expert technical assistance and Lorrie Gallagher for document production assistance.

D.A.B. was supported by a San Francisco Foundation/University of California San Francisco grant for medical students. D.R.B. was supported by a training grant (NS07219) from the National Institutes of Health. A.T. was supported by a John Douglas French Foundation fellowship. K.K.H. was supported by a postdoctoral fellowship (GM11354) from the National Institutes of Health. This work was supported by Public Health Service research grants (AG02132 and NS14069) from the National Institutes of Health and a Senator Jacob Javits Center of Excellence in Neuroscience grant (NS22786) as well as by gifts from Sherman Fairchild Foundation and R.J.R.-Nabisco, Inc.

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