Prion Strain- and Species-Dependent Effects of Antiprion Molecules in Primary Neuronal Cultures

Sabrina Cronier,1† Vincent Beringue,1 Anne Bellon,1,2‡ Jean-Michel Peyrin,1* and Hubert Laude1*

Unité de Virologie Immunologie Moléculaires, INRA, 78350 Jouy-en-Josas, France, 1 and Virology Department, Preclinical Research and Development, CSL Behring, Marburg, Germany2

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Transmissible spongiform encephalopathies (TSE) arise as a consequence of infection of the central nervous system by prions and are incurable. To date, most antiprion compounds identified by in vitro screening failed to exhibit therapeutic activity in animals, thus calling for new assays that could more accurately predict their in vivo potency. Primary nerve cell cultures are routinely used to assess neurotoxicity of chemical compounds. Here, we report that prion strains from different species can propagate in primary neuronal cultures derived from transgenic mouse lines overexpressing ovine, murine, hamster, or human prion protein. Using this newly developed cell system, the activity of three generic compounds known to cure prion-infected cell lines was evaluated. We show that the antiprion activity observed in neuronal cultures is species or strain dependent and recapitulates to some extent the activity reported in vivo in rodent models. Therefore, infected primary neuronal cultures may be a relevant system in which to investigate the efficacy and mode of action of antiprion drugs, including toward human transmissible spongiform encephalopathy agents.

Transmissible spongiform encephalopathies (TSE), which include Creutzfeldt-Jakob disease (CJD) in humans, bovine spongiform encephalopathy in cattle, and scrapie in sheep, are fatal, incurable neurodegenerative disorders caused by prions, a class of misfolded form of the cellular PrP (PrPC). Transmissibility is believed to stem from the ability of the prion isoform to promote the conformational transition from PrPC to PrPSc (39, 40). Biologically distinct prion strains can propagate in the same host (for a review, see reference 9), presumably through the perpetuation of different, specific PrPSc conformers (47).

The search for drugs able to impede infection or prion-induced neuropathology currently relies on various experimental models, including an acellular PrP transconformation assay (27, 46), yeast prion systems (2), PrPSc accumulation in chronically infected mammalian cell lines (28), and assay in TSE animal models (for a review, see reference 51). Among many compounds selected for their ability to prevent PrPSc accumulation in cultured cells, only some of the most potent inhibitors significantly delay disease onset in prion-infected rodents. A few of them showed a therapeutic activity sensu stricto, and none was effective in clinically affected human patients (56). The reasons for these discrepancies remain unclear but probably include pharmacokinetic limitations (reviewed in reference 51). However, compounds known to cross the blood-brain barrier such as quinacrine and chlorpromazine proved to be ineffective in vivo (4, 5, 21). It is conceivable that biological differences between the available permissive cell lines and postmitotic neurons, the primary target of prions, may account for the disparity between in vitro and in vivo results. In addition, there is evidence to suggest that drug efficacy may depend upon the infecting prion strain (18, 28). Thus, there is a need for in vitro screening systems able to replicate different strains in a congruent cellular context and to predict more accurately the in vivo potency of antiprion drugs.

Dissociated primary neurons can be explanted from various brain regions from a wide range of organisms, thus allowing the growth of highly differentiated neuronal subtypes. These systems have several advantages for in vitro studies. They make it possible to study the behavior of neurons in a more physiological way (in vivo one accessible for local application of pharmacological compounds or neurotropic infectious agents and allow morphological studies of, for example, neuronal connectivity and viability. As such, primary neuronal cultures are valuable tools routinely used for neurotrophic and antiapoptotic drug evaluation in neurodegenerative as well as infectious diseases (15, 35, 52, 57).

The propagation of sheep prions in primary nerve cell cultures derived from transgenic mice overexpressing ovine PrP has been recently reported (16). We show here that it is feasible to propagate rodent and human prions in cultures derived from transgenic mouse lines expressing the cognate PrPC. Using this cell system, we assayed the antiprion activity of three generic compounds that are known to cure prion-infected cell lines and for which efficacy has been evaluated in vivo (see Discussion). In primary cell culture, clear differences in the efficacy of these compounds were observed depending on the prion strain and/or species combination.

* Corresponding author. Mailing address: INRA, Virologie Immunologie Moléculaires, 78350 Jouy-en-Josas, France. Phone: 33 1 3465 2600. Fax: 33 1 3465 2621. E-mail for Jean-Michel Peyrin: jean-michel.peyrin@jouy.inra.fr. E-mail for Hubert Laude: hubert.laude@jouy.inra.fr.
† Present address: MRC Prion Unit and Department of Neurodegenerative Disease, Institute of Neurology, University College London, National Hospital for Neurology and Neurosurgery, Queen Square, London WC1N 3BG, United Kingdom.
‡ Present address: Department of Immunology, The Scripps Research Institute, La Jolla, CA 92037.
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Primary neuronal cultures transgenic for PrP were used to propagate prions from various species. As reported in our previous study (16), CGN primary cultures derived from transgenic mice expressing ovine PrP \((\text{PrP}^\text{Ov})\) and exposed to infectious inoculum at 2 to 3 days after plating are able to replicate the sheep scrapie agent. In such cultures, infection is effective at an inoculum dilution up to 0.0001% of brain homogenate, corresponding to a multiplicity of infection of \(~1\) infectious dose/500 cells (Fig. 1A). The permissiveness to infection of CGNexo cultures exposed at 8 days postplating, i.e., after phenotypical and functional differentiation of the neurons (38), was also examined. Such cultures were propagated at 85% MAP2- and b3 tubulin-positive cells and less than 1% nestin-positive cells (data not shown). As shown in Fig. 1B, PrPres accumulation at levels approaching those in cultures exposed at 2 days postplating (despite a 6-day-shorter incubation) is of interest as it suggests that prion replication may initiate and take place in postmitotic, differentiated neurons.

Results

Primary neuronal cultures transgenic for PrP allow the propagation of prions from various species. As reported in our previous study (16), CGN primary cultures derived from transgenic mice expressing ovine PrP \((\text{PrP}^\text{Ov})\) and exposed to infectious inoculum at 2 to 3 days after plating are able to replicate the sheep scrapie agent. In such cultures, infection is
ons affecting humans can be propagated. To examine whether CGN cells would give access to a system permissive to a human agent, cultures derived from tg650 transgenic mice expressing human PrP (CGN Hu) at 28 days postinfection with this TSE agent (unpublished data). As a result, tg650 mice develop a TSE disease within 150 days when inoculated with this TSE agent (unpublished data). In a first series of experiments, drug treatments were started 3 days after inoculation in chronically infected cell lines (4, 7, 29, 45). In a first experiment, CGN cells expressing human PrP (CGN Hu) at 28 days postinfection (Fig. 3A). Both Congo red and MS-8209 reproducibly inhibited PrPSc formation (Fig. 3B and C). Contrary to early postexposure treatments (chlorpromazine, 5 μM; Congo red, 7 μM; MS-8209, 7.5 μM; or solvent, 0.1%) were added 1 day or 3 days postexposure (d.p.e.) and was weak or absent in nonpermissive CGN0/0 cultures. Cell lysates were PK treated, and PrPSc was detected by immunoblotting using biotinylated monoclonal antibody ICSM18 (A and C) or Sha31 (B).

FIG. 2. Accumulation of PrPSc in CGN cultures upon exposure to rodent and human prions. CGN cultures were established from transgenic mice expressing mouse (A), hamster (B) or human (C) PrP and were exposed to brain homogenates (A and B) or purified PrPSc (C) from terminally ill mice infected with prions. CGN cultures established from PrP0/0 transgenic mice (CGN 0/0) were also exposed to infectious prions in parallel. (A) CGN0/0 cultures exposed to mouse strain 139A, Fukuoka-1 (FK), 22L, or ME7 at a final concentration of 0.1% (vol/vol). (B) CGN0/0 cultures exposed to hamster strain Sc237 at a final concentration of 0.002%. (C) CGN0/0 cultures exposed to type 1 CJD (T1 CJD) at a final concentration equivalent to 0.1% of brain homogenate. The data shown in panels B and C correspond to duplicate culture wells within a representative experiment. In all PrP-expressing cultures, PrPSc accumulation increased from 14 to 28 days postexposure (d.p.e.) and was weak or absent in nonpermissive CGN0/0 cultures. Cell lysates were PK treated, and PrPSc was detected by immunoblotting using biotinylated monoclonal antibody ICSM18 (A and C) or Sha31 (B).

FIG. 3. Effect of chlorpromazine, Congo red, and MS-8209 on sheep scrapie propagation in CGN0/0 cultures at an early or advanced stage of infection. (A) CGN0/0 cultures were infected on day 2, two treatments (chlorpromazine, 5 μM; Congo red, 7 μM; MS-8209, 7.5 μM; or solvent, 0.1%) were performed on days 5 and 8, and cells were lysed on day 12. Control wells of CGN0/0 and nonpermissive CGN0/0 cultures were infected similarly and left untreated (Control). (B and C) CGN0/0 cultures were infected on day 3, drugs (B, Congo red at 1.5 μM and 7 μM; C, 7.5 μM MS-8209 or 0.1% solvent) were added 1 day or 11 days after prion exposure and then twice a week; cells were lysed on day 21 (respectively, after 5 or 2 treatments). PrPSc was revealed by immunoblotting of PK-treated lysates using biotinylated monoclonal antibody ICSM18. d.p.e., days postexposure.

Drug efficiency in primary neuronal cultures can vary according to the infecting prion. The cell lines currently used for paired prion propagation, on the markedly lowered PrPSc accumulation observed in treated versus untreated cultures (inhibition ≥80% as quantified by densitometric image analysis; n = 3). In contrast, chlorpromazine treatment had no effect on PrPSc accumulation in infected CGN0/0 cells.

In the brain of prion-infected individuals, propagation of the infectious agent is likely to induce infection of new neurons while PrPSc accumulation continues in already infected neurons. Therefore, we questioned whether Congo red or MS-8209 would show a curative activity in infected CGN0/0 cultures once PrPSc had accumulated in substantial amounts. CGN0/0 cultures were treated 11 days after exposure to infectious inoculum (Fig. 3B and C). Contrary to early postexposure treatment, late treatment with Congo red was inefficient. However, a moderate inhibitory effect could be observed with MS-8209 (approximately 40% PrPSc decrease in comparison to untreated, infected cells).
antiprion drug screening propagate only mouse-adapted strains or sheep scrapie agent (28), and the data thus generated might not be fully transposable to prions infecting other species. Since primary cultured neurons are able to propagate prions from different species within a comparable environment, we sought to investigate whether any species-related effect of antiprion drugs would be observed. CGN<sub>M</sub>, CGN<sub>H</sub>, and CGN<sub>0/0</sub> cultures were exposed to diluted homogenates from brains infected with one of the above-mentioned prion strains and then submitted to early postexposure treatment with one each of the three antiprion compounds (Fig. 4). PrP<sup>Sc</sup><sup>sus</sup> accumulation levels reproducibly showed clear disparities of drug efficacy according to the host PrP species (<i>n</i> = 2). Congo red markedly inhibited PrP<sup>Sc</sup><sup>sus</sup> accumulation in CGN<sub>H</sub> cultures (Fig. 4B), as in CGN<sub>M</sub> cultures (Fig. 3A), but had little or no effect in CGN<sub>Mo</sub> and CGN<sub>0/0</sub> cultures compared to untreated, control cultures (Fig. 4A and C). Notably, the PrP<sup>Sc</sup><sup>sus</sup> levels in CJD-exposed CGN<sub>H</sub> and nonpermissive CGN<sub>0/0</sub> cultures were similar following Congo red treatment (Fig. 4C), indicating that input PrP<sup>Sc</sup> present in the inoculum was actually stabilized by the drug. Chlorpromazine presented a relatively modest antiprion efficacy overall, except in CGN<sub>Mo</sub> cultures. MS-8209 proved to be the only drug to be efficient across the range of prion species in these experiments, although with variable efficacy.

Earlier studies mainly in hamster TSE models have revealed a variable effect of amphotericin B and its analogue MS-8209 on the survival of the infected host according to the strain of prion (18, 33, 58). To see whether such a differential effect could also be visualized in cell culture, we compared the effects of MS-8209 in CGN<sub>H</sub> cultures infected in parallel with either Sc237 or 139H, another well-characterized Syrian hamster strain. Upon early postexposure treatment of these cultures, a weaker inhibitory effect on PrP<sup>Sc</sup><sup>sus</sup> accumulation was consistently found with 139H than with Sc237 prion (Fig. 5) (<i>n</i> = 3), in accordance with in vivo observations (see Discussion).

**DISCUSSION**

Despite the efforts of many research groups, only a few cell systems susceptible to prion infection have been developed, offering limited genetic diversity and susceptibility to a small number of strains. While several cell lines currently provide robust systems in which a few mouse-adapted strains (3, 48) or sheep scrapie (1, 54) and, recently, a deer TSE agent (42) can be cultivated, no cell system enabling routine propagation of prions affecting other species is available. Although a hamster TSE-infected hamster cell line and a CJD-infected human cell line were reported in earlier publications (30, 50), they have not been mentioned for more than a decade. More recently, there have been descriptions of alternative approaches utilizing mouse brain fetal stem cells or neurospheres that enabled the propagation of mouse-adapted prions (22, 34).

In this study, we report that primary cultured, differentiated neurons are susceptible to infection by a range of prion strains from four different species: sheep, mouse, hamster, and human. Modeling our previously described approach (16), we employed CGN cultures established from transgenic mice expressing PrP proteins of different species. Upon exposure, these cells were found to be susceptible to infection by prions propagated in the corresponding species, based on the accumulation of abnormal PrP. This newly introduced cell system thus offers a common, biologically relevant cellular environ-
ment in which prion agents from different species can be studied comparatively. While CGN cells may not provide a universal, susceptible system, one can expect that extending this approach to other neuron populations—as done successfully with cortical cells expressing ovine PrP (11)—might enlarge the spectrum of permissiveness. This would also pave the way for future studies of the determinism of the apparent, strain-dependent neuronal selectivity exhibited by these agents (17).

Primary nerve cell cultures have proved their usefulness for the evaluation of therapeutic compounds in neurodegenerative or infectious diseases (15, 52, 57). Here, we addressed the relevance of scrapie-infected primary nerve cell cultures as a potential model for the evaluation of antiprion molecules by using three different compounds that were previously described to clear PrP<sup>res</sup> in chronically infected cell lines but presented inconsistent prophylactic and therapeutic activities in vivo (51). As a main finding, these compounds markedly differed in their antiprion efficacy according to the species and/or prion strain, based on their effect on the accumulation of PrP<sup>res</sup>. In several instances, the differential effects observed in CGN cultures paralleled those documented in vivo for the same compound.

As a striking example, Congo red was shown here to exhibit a marked inhibitory activity toward ovine and hamster prions but not human and mouse prions. Such a result was not particularly expected in the case of the mouse prion, since this compound manifested clear antiprion activity when tested in the mouse cell lines ScN2a (12, 13) and ScSMB (45) infected by the Chandler strain, which is closely related to the 139A strain used in this study. The reason for these apparent discrepancies is uncertain but might reflect the limited effect of promazine was more toxic than in stable cell cultures, which is in keeping with previous in vivo observations showing an apparent strain-dependent effect of polyene antibiotics in the absence of PrP<sup>res</sup> sequence variation. In hamsters, amphotericin B could significantly delay clinical phase onset following inoculation with the prion strain 263K (equivalent to Sc237) but not with the strains 139H (58) and DY (33), which have a longer incubation period. MS-8209 also exhibited some specificity, extending the life spans of transgenic mice with neuron-restricted hamster PrP expression when infected with the 263K strain but not with the DY strain (18). Thus, the strain-specific effect observed in CGN cultures recapitulated to some extent those reported in vivo for this class of compounds. Such cells may therefore provide a relevant tissue culture system in which to investigate the mechanisms underlying the antiprion activity.

The cell system developed in this study made it possible for the first time to test the relative potency of several drugs toward a human prion. Thus far, intraventricular infusion of pentosan polysulfate in a variant CJD patient has been the only treatment that might slow the disease progression in human (41). Failure of compassionate treatments with other compounds was attributed to the late intervention and/or a poor penetration of the blood-brain barrier (19, 23, 32, 36). Another issue that is also supported by our findings is that screening in rodent TSE models might be inaccurate and select molecules that may not be particularly active against human agents. In this regard, this ex vivo assay against a CJD agent in primary nerve cells derived from transgenic mice expressing human PrP may provide new opportunities for the selection of compounds active against human prions.

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