A cell line infectible by prion strains from different species

M.-P. Courageot,† N. Daude,† R. Nonno,‡ S. Paquet,§ M. A. Di Bari,‡ A. Le Dur,† J. Chapuis,† A. F. Hill,§ U. Agrimi,‡ H. Laude† and D. Vilette†

1Virologie et Immunologie Moléculaires, INRA, 78350 Jouy-en-Josas, France
2Department of Food Safety and Veterinary Public Health, Istituto Superiore di Sanità, Rome, Italy
3Department of Biochemistry and Molecular Biology, Bio21, Molecular Science and Biotechnology Institute, University of Melbourne, Victoria, Australia

It has been shown previously that ovine prion protein (PrP<sup>C</sup>) renders rabbit epithelial RK13 cells permissive to the multiplication of ovine prions, thus providing evidence that species barriers can be crossed in cultured cells through the expression of a relevant PrP<sup>C</sup>. The present study significantly extended this observation by showing that mouse and bank vole prions can be propagated in RK13 cells that express the corresponding PrP<sup>C</sup>. Importantly, the respective molecular patterns of abnormal PrP (PrP<sup>SC</sup>) and, where examined, the neuropathological features of the infecting strains appeared to be maintained during the propagation in cell culture. These findings indicate that RK13 cells can be genetically engineered to replicate prion strains faithfully from different species. Such an approach may facilitate investigations of the molecular basis of strain identity and prion diversity.

INTRODUCTION

Whilst the host-encoded prion protein (PrP<sup>C</sup>) is essential for prion multiplication in cells (Daude et al., 2003) and tissues (Bueler et al., 1993), several lines of evidence indicate that PrP<sup>C</sup> expression is not sufficient to confer permissiveness to prion propagation (Enari et al., 2001; Raeber et al., 1999). Although several new permissive cell lines were recently identified, the number of cell models remains quite limited (for a review, see Vilette, 2007). More importantly, the ex vivo multiplication of a number of strains of particular interest has not yet been achieved. This suggests that prion multiplication may require strain-specific, PrP<sup>C</sup>-independent factors, the conjunction of which would be restricted to a few cell lines. Previous work has identified epithelial RK13 cells as an efficient and robust cell system for the multiplication of ovine scrapie, provided that these non-ovine cells express sheep PrP<sup>C</sup> (Rov cells; Vilette et al., 2001). Here, we have shown that RK13 cells genetically engineered to express rodent PrP<sup>C</sup>, either from the mouse or the bank vole, a species highly permissive to various prion isolates or strains (Cartoni et al., 2005; Chandler & Turfrey, 1972; Nonno et al., 2006), were readily infected by prions adapted to and propagated in these two species, thus extending the range of prion strains that can be multiplied in this cell-culture model.

METHODS

Reagents. Dextran sulfate 500 (DS 500; Sigma) was prepared at a concentration of 5 mg ml<sup>-1</sup> in PBS and stored at −20 °C. Aliquots were diluted in cell-culture medium and filter-sterilized before incubation with cells. Stock solutions of the amphotericin B derivative MS-8209 were dissolved at a concentration of 10 mg ml<sup>-1</sup> in DMSO.

Generation of RK13 cells expressing mouse and vole PrP<sup>C</sup>. The open reading frame (ORF) of vole PrP<sup>C</sup> was PCR amplified from bank vole (Myodes glareolus, formerly Clethrionomys glareolus) genomic DNA, cloned into pBluescript plasmid and verified by sequencing. The ORF encoding the vole PrP<sup>C</sup> with methionine at position 109 (Cartoni et al., 2005) was then subcloned into the pTRE plasmid (Clontech). The pTRE plasmid encoding the mouse PrP<sup>C</sup> (allele a) was provided by Dr S. Lehmann (Institut de Génétique Humaine, France). Each plasmid was introduced by transfection into RK13 cells as described previously (Vilette et al., 2001), and puromycin-resistant cell clones were selected and tested for doxycycline (dox)-inducible expression of PrP<sup>C</sup>. The data reported in this study with mouse (moRK13) and bank vole (voRK13) RK13 cells were obtained using clones #55 and #1/9, respectively, but similar findings were observed with other cell clones. The cultures were maintained at 37 °C in 6% CO<sub>2</sub> in Opti-MEM (Invitrogen/
Gibco) supplemented with 10% fetal bovine serum, 100 U penicillin ml⁻¹ and 10 μg streptomycin ml⁻¹. Cell lines were split by a 1:4 dilution every week. To induce expression of PrP⁺ in the cell clones, 1 μg dox ml⁻¹ was added to the culture medium.

**Prion strains.** The murine strains Fukuoka-1 (Tateishi et al., 1979, originating from the laboratory of S. Katamine, Nagasaki, Japan), Chandler, 22L and ME7 (Bruce & Fraser, 1991, originating from the laboratory of R. Carp, Staten Island, NY, USA) were maintained in C57BL/6 mice. Transmission and stabilization of the natural sheep scrapie isolate S3 and of sheep bovine spongiform encephalopathy (BSE) into bank voles have been described previously (Nonno et al., 2006; Piening et al., 2006). The survival times of bank voles serially infected with cattle BSE were similar to those of bank voles infected with sheep BSE (U. Agrimi, unpublished data). Infected brains were homogenized at 10% (w/v) in a sterile 5% glucose solution with a RibiLyser (Hybaid) and sonicated for 1–2 min before incubation with the cells.

**Infection of RK13 cultures.** Confluent cultures grown for 2 days in single wells of 12-well plates in the presence of 1 μg dox ml⁻¹ were incubated in culture medium containing 2.5% infected brain homogenates. After 2 days, the medium was removed and the cells rinsed with PBS and split into two 25 cm² flasks. Each week, one flask was used for subcultivation, whilst the other was used to prepare a cell lysate for PrP analysis.

**Isolation and Western blot analysis of PrP⁺es.** The isolation of cell-derived PrP⁺ has been described previously (Paquet et al., 2004; Vilette et al., 2001).Briefly, cells were solubilized in lysis buffer [50 mM Tris/HCl (pH 7.4), 0.5% Triton X-100, 0.5% sodium deoxycholate]. Lysates were clarified (2000 r.p.m. for 1 min in a microcentrifuge) and cellular proteins were quantified by bicinchoninic acid using a BCA Protein Assay kit (Pierce). Identical amounts of cellular protein (500 μg) were digested with 2 μg proteinase K (PK) for 2 h at 37 °C in the presence of 0.02% bromophenol blue for increased visualization of the pellets obtained after centrifugation. Pefabloc (4 mM) was added to stop the reaction and blue pellets containing aggregated PK-resistant PrP (PrP⁺es) were collected by centrifugation at 13 000 r.p.m. in a microcentrifuge for 30 min at room temperature and separated by 12% SDS-PAGE before transfer to nitrocellulose filters. In some experiments, pellets were treated with the endoglycosidase PNGaseF (New England Biolabs) prior to immunoblotting. For brain-derived PrP⁺ analysis, 150–250 μg brain tissue equivalent was solubilized in lysis buffer. Clarification, PK digestion and PrP⁺ recovery was as described for cell-derived PrP⁺es.

Monoclonal antibody (mAb) 4F2 (Krasemann et al., 1996) recognizing the N-terminal region of PrP⁺ was used to detect expression of the entire PrP⁺. As this antibody cannot react with N-terminally truncated PrP⁺es, immunoblot analysis of abnormal PrP in PK-digested cell lysates and brain homogenates was performed with mAb Sha31 (Feraudet et al., 2005). Blots were developed using an ECL+ reagent kit (Amersham).

**Pharmacological treatment of infected voRK13 cultures.** Infected voRK13 cultures were seeded in six-well plates in the presence of D500 (1 μg ml⁻¹), MS-8209 (50 μg ml⁻¹) or with vehicle only and incubated for 5 days with one medium change. The different treatments did not induce any obvious phenotypic effect, and after solubilization of the cultures, the protein concentration was similar in treated and untreated cultures. The same amount of cellular protein (250 μg) was digested with PK and analysed for PrP⁺ levels by immunoblotting.

**Bioassay.** Cultures were rinsed three times with PBS, scrapped into PBS and recovered by centrifugation. The cells were resuspended in a sterile 5% glucose solution, frozen (−80 °C) and thawed four times, sonicated and stored at −80 °C until inoculation. MoRK13 and voRK13 cultures (20 μl) were inoculated intracerebrally into ovine transgenic tga20 mice (Fischer et al., 1996) and bank voles, respectively. Animals showing neurological signs were monitored almost daily and euthanized in extremis.

**Histoblotting.** The procedure for histoblot analysis (Taraboulos et al., 1992) of tga20 mice was as described previously (Beringue et al., 2007).

**Histopathology and paraffin-embedded tissue (PET) blots.** Each bank vole brain was divided into two parts by a sagittal paramedian cut. One was frozen for immunoblot analysis and further passages into vole, and the remaining part was embedded in paraffin. Lesion profiles were established using the first and the second passage of bank voles inoculated with vole-adapted sheep BSE agent serially propagated in voRK13 cells. PET blots were performed with the second vole passage. The procedures for the construction of lesion profiles and PET blot analysis have been described previously (Nonno et al., 2006).

**RESULTS AND DISCUSSION**

**Heterologous PrP⁺ confers permissiveness in RK13 cells to various prion strains**

For stable expression of PrP⁺ from mouse and bank vole in RK13 cells, the ORF of PrP⁺ from the corresponding species was cloned in a dox-inducible expression vector. Expression of mouse and bank vole PrP⁺ in representative, stable RK13 transfectants is shown in Fig. 1. These data confirmed that RK13 cells did not express detectable levels of endogenous PrP⁺ and established that PrP⁺ from various species could be expressed efficiently in these cells.

Permissiveness to prion multiplication was tested with three murine strains (Fukuoka-1, Chandler and ME7) and three sources of vole prions. One source of vole prions was a sheep scrapie isolate (Ss3) adapted to bank vole (Cartoni et al., 2005; Piening et al., 2006), whilst the other two were the BSE agent (either from cattle or from sheep, see Methods) serially transmitted to bank voles (Piening et al., 2006). Prior to exposure of the cell cultures, abnormal PrP (PrP⁺es) in brain homogenates from the infected animals was analysed by immunoblotting. Fig. 2 confirmed that PrP⁺es can show strain-specific features. Brain-derived PrP⁺ from strain Fukuoka-1 migrated more slowly than PrP⁺ from strains Chandler and ME7 (Fig. 2a), as reported previously (Arima et al., 2005). PrP⁺es from the BSE agent in bank vole had a higher electrophoretic mobility compared with that from Ss3 sheep scrapie (Fig. 2b), consistent with the unique pattern of BSE PrP⁺es observed in different species (Berrie et al., 2006; Castilla et al., 2003; Eloit et al., 2005; Hill et al., 1997; Houston et al., 2000; Scott et al., 1997).

The permissiveness of RK13 cultures was determined by incubating each strain with cells expressing the relevant PrP⁺. The cultures were then grown for up to 10 weeks and the accumulation of PrP⁺es in the cultures was monitored regularly to assess successful and persistent infection.
Control cultures inoculated and grown in the absence of dox, and thus expressing no detectable levels of PrP<sup>C</sup>, were analysed in parallel. Immunoblot analysis of PK-digested cell lysates showed that RK13 cells expressing the mouse PrP<sup>C</sup> (moRK13) persistently accumulated PrPres following exposure to the Fukuoka-1 and 22L strains of mouse prions (Fig. 2c, lanes 1 and 3). Additional experiments indicated that PrPres was also readily detected in moRK13 cells exposed to the 22L strain of mouse prions (data not shown), whilst no PrPres was detectable in cultures exposed to ME7 (Fig. 2c, lane 2). Cell-derived PrPres was produced in voRK13 cells infected with the bank vole BSE agent originating from either cattle or sheep (Fig. 2d, lanes 2, 3, 6), whilst no PrPres was observed in voRK13 inoculated with the vole-adapted sheep scrapie Ss3 isolate (Fig. 2d, lane 1). In each case of successful transmission, no PrPres was observed when infections were carried out in the absence of dox. This demonstrated that no residual abnormal PrP from the inoculum was detected under these experimental conditions and that PrPres was produced de novo by the cells.

Our findings indicate that permissiveness of RK13 appears to be strain-specific. No PrPres was detected in voRK13 cells inoculated with Ss3 or in moRK13 cells exposed to the ME7 strain. This latter observation is reminiscent of previous observations with N2a and GT1, two cell lines permissive to the Chandler/RML strain but resistant to ME7 (Bosque & Prusiner, 2000; Klohn et al., 2003). However, successful multiplication of ME7 in other cell lines (e.g. SN56 (Baron et al., 2006), MG20 (Iwamaru et al., 2007) and L929 (Vorberg et al., 2004)) indicate that the cell tropism of prion multiplication observed in vivo can manifest in cultured cells.

To compare more accurately the electrophoretic mobility of abnormal PrP of these strains after propagation in culture, PrPres in moRK13 cells infected with strains Chandler and Fukuoka-1 and in voRK13 cells infected with vole-adapted BSE agent were deglycosylated by PNGaseF treatment and analysed by Western blotting. Fukuoka-1 PrPres retained a lower electrophoretic mobility compared with Chandler PrPres (Fig. 3, lanes 1 and 2), whilst PrPres generated in voRK13 cells infected with the vole-adapted BSE agent migrated faster (Fig. 3, lanes 3 and 4).

**Biological characterization of the prion strains propagated in RK13 cells**

Fukuoka-1 and vole-adapted BSE strains propagated for 2 months in moRK13 and voRK13 cells, respectively, were inoculated into relevant indicator animals (tga20 mice or bank voles, respectively). All inoculated animals died with typical neurological symptoms (Table 1). The survival time of tga20 mice inoculated with moRK13 cultures (3 x 10<sup>5</sup> cells per mouse) was 127 ± 3 days, and all had PrPres in their brain, the pattern of which was indistinguishable from that in the brain material used as inoculum (data not shown). Bank voles inoculated with infected voRK13 cultures (3 x 10<sup>6</sup> cells) had a survival time of 90 ± 5 days, an incubation period similar to that obtained when the strain was grown in the animal (Piening et al., 2006). No detectable infectivity was associated with inoculated cells expressing no bank vole PrP<sup>C</sup> (parental RK13 cells, Table 1). All of the diseased bank voles showed PrPres in the brain with an electrophoretic pattern indistinguishable from that in the original inoculum (data not shown). These results demonstrated that mouse and bank vole infectious prions are propagated efficiently in RK13 cells.
After infection by RK13- or mouse-passaged Fukuoka-1 strain, PrP\textsuperscript{res} deposits were diffuse and present in numerous areas of the brain such as the fimbria, corpus callosum, anterior medial thalamus nuclei and, to a lesser extent, the lateral hypothalamus (Fig. 4a and data not shown). 22L staining was more punctate and for both groups predominantly involved the septum, basal nuclei, anterior medial and ventral thalamic nuclei, lateral hypothalamus, habenular nuclei, inferior colliculus, raphe nuclei and aqueduct region (Fig. 4a and data not shown). voRK13- and bank vole-passaged sheep BSE caused indistinguishable PrP\textsuperscript{res} distribution, as assessed by PET blot analysis of bank vole brains (Fig. 4b). Accumulation of PrP\textsuperscript{res} was evident throughout the brain and in particular in the medulla, cerebellar nuclei, lateral geniculate nuclei, superior colliculus, red nucleus, thalamic nuclei, septum and cingulate, and parietal cortices. The lesion profile in the brains of bank voles inoculated with infected voRK13 cells was also determined (Fig. 4c). Bank vole- and voRK13-passaged sheep BSE produced identical patterns of vacuolar degeneration in bank voles, characterized by abundant grey-matter spongiform degeneration in all brain areas examined with the exception of the cerebellum. Collectively, these findings supported the view that the three strains studied were not modified through multiplication in RK13 cells.

A pharmacological assay for BSE-type agent

A limited number of compounds has been tested for anti-prion activity in animal models infected with BSE-derived agents (for a review, see Trevitt & Collinge, 2006). To determine whether the multiplication of bank vole-adapted BSE agent was sensitive to pharmacological treatment, infected cultures were incubated for 1 week in the presence of DS 500 and the amphotericin B derivative MS-8209, two
molecules known to delay the onset of disease in experimentally infected rodents (Adjou et al., 1995; Ehlers & Diringer, 1984). Fig. 5 shows that DS 500 and MS-8209 decreased PrP\textsuperscript{res} levels in infected, treated voRK13 cultures. As no cell system enabling replication of cattle BSE or human vCJD agents is available to date, voRK13 cells, together with a recently identified cell line permissive to mouse-adapted BSE (Iwamaru et al., 2007), may provide improved tools for the screening of drugs potentially effective against this human-affecting agent.

**Table 1. Bioassay of infected cultures**

<table>
<thead>
<tr>
<th>Inoculum*</th>
<th>No. cells inoculated</th>
<th>Recipient host</th>
<th>Survival time (n/n(_0))†</th>
</tr>
</thead>
<tbody>
<tr>
<td>moRK13/Fukuoka-1</td>
<td>3 × 10(^5)</td>
<td>tga20 mice</td>
<td>127 ± 3 (3/3)</td>
</tr>
<tr>
<td>(p9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>voRK13/sheep BSE</td>
<td>3 × 10(^4)</td>
<td>tga20 mice</td>
<td>150 ± 3 (5/5)</td>
</tr>
<tr>
<td>(p8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RK13/sheep BSE</td>
<td>3 × 10(^6)</td>
<td>Bank voles</td>
<td>90 ± 5 (9/9)</td>
</tr>
<tr>
<td>(p8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*moRK13 and voRK13 cultures infected with Fukuoka-1 mouse strain or vole-adapted sheep BSE were inoculated at the indicated passage (p) post-infection. Parental RK13 cells exposed to vole-adapted sheep BSE were assayed in parallel. Each animal was inoculated intracerebrally with 20 μL undiluted cell extract (3 × 10\(^6\) cells) or with 10- or 100-fold diluted cell extracts (3 × 10\(^5\) or 3 × 10\(^4\) cells, respectively), as indicated.

†Mean days to death ± SEM; n, number of terminally ill animals; n\(_0\), number of inoculated animals.

**Fig. 3.** Strain-specific electrophoretic mobility of PrP\textsuperscript{res} produced in RK13 cells. PrP\textsuperscript{res} from moRK13 cells infected with Chandler or Fukuoka-1 (lanes 1 and 2) or from voRK13 cells infected with cattle BSE or sheep BSE agent (lanes 3 and 4) was deglycosylated by PNGaseF treatment and analysed by immunoblotting with mAb Sha31. M, Biotinylated molecular mass marker proteins (kDa).

**Fig. 4.** Regional distribution of PrP\textsuperscript{res} and vacuoles in the brains of animals infected with RK13-passaged strains. (a) Histoblots of coronal sections (at the level of the basal nuclei) comparing the distribution of PrP\textsuperscript{res} deposits in the brains of tga20 transgenic mice infected with moRK13 cell- or mouse-passaged Fukuoka-1 and 22L strains. (b) Comparison of PrP\textsuperscript{res} distribution by PET blot analysis of coronal sections of bank voles inoculated with voRK13-passaged (second passage, upper panels) or bank vole-passaged (middle panels) sheep BSE agent. The lower panels show sections from a healthy control vole. (c) Lesion profiles in voles inoculated with voRK13 cells infected with the sheep BSE agent (▲, first passage; ■, second passage) or with the vole-passaged sheep BSE agent (▼). Brain-scoring positions are medulla (1), cerebellum (2), superior colliculus (3), hypothalamus (4), thalamus (5), hippocampus (6), septum (7), retrosplenial and adjacent motor cortex (8) and cingulate and adjacent motor cortex (9).
In conclusion, we have shown here that strain-specific features of PrP\textsuperscript{res} were maintained following serial multiplication in RK13 cells and that cell multiplication did not affect the disease phenotype when infected cultures were inoculated back into animals, suggesting that the RK13 cell line is able to propagate prion strains faithfully from different species. During preparation of this manuscript, the mouse-adapted M1000 prion strain was reported to multiply in RK13 cells constitutively expressing mouse PrP\textsuperscript{c} (Vella et al., 2007), confirming that RK13 represents an interesting biological cell system to study how distinct abnormal PrP from various prion strains and species can be generated in a single cell type. This cell model may provide new opportunities for investigating the basis of strain identity.

**ACKNOWLEDGEMENTS**

We thank S. Lehmann and R. Carp for kindly providing the mouse strain materials. We also acknowledge J. Grassi (CEA, Saclay, France) for 4F2, 12F10 and Sha31 mAbs; C. Weissmann for goat20 mice; and S. Lehmann for pTREmoPrP plasmid. We thank L. J. Vella (University of Melbourne, Australia), R. A. Sharples (University of Melbourne, Australia), P. Thebault (INRA) for their assistance and V. Beringue (INRA) for helpful discussions. A.F.H. is an Australian National Health and Medical Research Council R. D. Wright Fellow. S. P. was supported by a fellowship from INRA, the Ile de France region and by the Fondation pour la Recherche Médicale. N. D., M.-P. C. and this work were partially supported by a grant from the European Union (projects EuroVoTE QLRI-CT-2002-81333).

**REFERENCES**


