Supporting Online Material for

Reciprocal Interference Between Specific CJD and Scrapie Agents in Neural Cell Cultures

Noriuki Nishida, Shigeru Katamine, Laura Manuelidis*

*To whom correspondence should be addressed. E-mail: laura.manuelidis@yale.edu

Published 21 October 2005, Science 310, 493 (2005)
DOI: 10.1126/science.1118155

This PDF file includes:

Materials and Methods
Supporting Online Material

Neural cell lines and in vitro challenge: GT cells were used because stable and persistent infection did not require individual cell cloning as it does in other neural lines (9, 10). For generation of target cells (GTneo), neomycin (neo) resistance was established by introducing the plasmid pEGFP-C1 (Clonetech) with Effectene transfection reagent (QIAGEN) according to manufacturer’s instructions. Cells were then selected with 500 µg/ml of G418 antibiotic (GIBCO BRL). Persistently infected GT+SY cells (10) that had been continuously subcultured more than 106 passages (>1 year) were transfected and selected with G418; bioassays reconfirmed the presence of SY infection in target cells at >110 passages (see below). Other uninfected GTneo cells were incubated with 0.2% mouse brain homogenates that were uninfected, 22L, or Ch infected as described (9) to produce the mock and scrapie infected GTneo target cells. Infected GTneo target cells were challenged only after stable infection was confirmed by immunoblotting for PrP-res at two sequential passages (p10 and p15) in vitro. Infected GT challenge (neo—) cells had been previously infected, passaged, and assayed for infectivity in mice. For example, GT+FU cells maintained for more than a year showed persistent high titers of infectivity (10). Similar high titers were also verified in mouse bioassays of GT+Ch and GT+22L cells as detailed below. Cell passage and maintenance was as previously described (9, 10).

For superinfection, GTneo target cells were co-cultured with GT challenge cells for 2 days. On the day before starting co-culture, 1 x 10⁶ of neo-resistant target cells were plated in a T25 flask, and on the next day overlayed with same number of the challenge cells. These co-cultures were incubated for 2 days at 37ºC 5% CO₂ in medium without G418 antibiotic. To eradicate challenge GT cells from cocultures, the cultures were split at a 1:3 dilution while being treated with fresh 500 µg/ml G418 at each passage. Some neo-sensitive cells started dying on the first treatment day, but the killing effect of G418 was gradual, with ~60% of GT the cells eliminated by 5 days of treatment.
(split 1). However, by 10 days of treatment (split 2), all infected challenge cells were eradicated since no residual PrP-res was detectable by immunoblotting. We also confirmed neo-sensitive GT cells were completely removed by day 10 in parallel culture-G418 studies. Thus immunoblot assays for superinfection at ≥5 passages (25 days of G418) ensured all PrP-res originated only from GTneo target cells.

**PrP-res assays:** After 5 passages, confluent GTneo cells in a T25 flask were washed twice with cold PBS- and then lysed with 500 µl of cell-lysis buffer (0.5% Triton X-100, 0.5% sodium deoxycholic acid, 150 mM NaCl, 50 mM TrisHCl pH7.5, 2 mM EDTA). Cell lysates were spun at 3,000 RPM for 5 min to remove nuclei and insoluble material. This also removed some large PrP aggregates as determined by Western blotting (data not shown). After normalizing the supernatant protein concentration to 1 mg/ml, 500 µl of replicate samples were treated with 10 µg of proteinase K (20 µg/mg protein, Boehringer) for 30 min at 37ºC, and the digestion stopped by adding 3 mM PMSF. All PK-digested samples were then centrifuged at 20,000g for 45 min at 4ºC, and the pellets were resuspended in 30 µl of 1 x Laemmli’s sample buffer. SDS-PAGE and Western blotting were done as described (9) with equal amounts of cell protein (50 µg) loaded per lane. For detection of PrP in undigested total cell lysates the membrane was incubated with the SAF32 monoclonal antibody that recognizes the octapeptide repeat region (supplied by Jacques Grassi, France), while the polyclonal M-20 antibody (Santa Cruz Biotech, CA) that recognizes the C-terminal 13kd region of PrP was used to detect PrP-res (10). The extra 13kd C terminal band of PrP-res in FU had not been appreciable in digested whole cell lysates previously (10), and the double centrifugation/fractionation steps here, and possibly the use of Triton-X 100 rather than NP-40, led to the relative enrichment of this small C-terminal PrP-res band. This fractionation procedure did not bring out the 13kd band with any of the other strains as shown in Fig. 2.

**Infectivity titers:** Bioassays of uninfected control cells and cells infected with SY and FU
were done by inoculating mice intracerebrally with cells as described (10). Repeat assays of SY infected cells at the time of co-culture challenges here (>110 passages) showed comparable incubation times (1 year) and the SY-CJD diagnostic scratching syndrome as shown in previous SY bioassays at p36 (10). Challenges of SY infected cultures were formidable in terms of infectious titers. SY target cells had titers that were <1/10,000 of GT cells infected with FU-CJD (~1 LD$_{50}$/30,000 cells for SY versus ~1 LD$_{50}$ per average cell for FU). Thus FU challenges here were of greater magnitude than in previous animal studies (10). Scrapie challenge cells also had titers that were ≤1,000 fold that of SY target cells; 22L infected cells contained ~1 LD$_{50}$/30 cells, and Ch scrapie had ~1LD$_{50}$/10 cells (K. Arima et al. J Virol. 79:7104-12).

**Cell curing and chamber separation studies:** Pentosan polysulfate (PPS, calcium salt from Bene Co, Ltd, Germany) was dissolved in distilled water at 20 mg/ml and stored at 4ºC. It was diluted to 20 µg/ml in GT growth medium and 0.2 µm filter sterilized just before adding to 22L+GTneo cells. Freshly prepared PPS was added at every split (every ~4 days). PrP-res signals disappeared after 3 splits, and treatment was continued for 4 more weeks. The cured cells were then challenged with FU+GT by co-culture as above. After 5 splits, the cells became positive for PrP-res.

For preventing cell to cell contact between co-cultures, inserts with 0.4u filters were purchased from Falcon, and challenge cells were grown on the membrane above target cells that were plated in the bottom chamber using recommended amounts of medium to ensure good supernatant exchange. We then split cells and passaged them until PrP-res was visible (>8 passages). We thank Laura Simarro, Nuria Banquero and Kayo Matsumoto for participating in some of these filter tests.