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A rapid accurate culture assay for infectivity in Transmissible Encephalopathies

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The molecular and structural features of infectious agents that cause CJD, scrapie and BSE remain controversial. A major impediment for agent resolution is the very long and expensive animal assays of infectivity. It is crucial to develop a rapid and broadly applicable cell culture assay to titer and compare different TSE agent strains. Because we found GT1 hypothalamic cells, unlike neuroblastoma N2a clones, were highly susceptible to a variety of TSE agents, and could stably produce high agent titers for >1 year, we studied the progressive display of abnormal prion protein (PrP-res) in GT1 cells following exposure to serially diluted 22L scrapie brain homogenates; PrP-res was used as a surrogate, but non-quantitative marker of GT1 infection. Even as early as the first cell split after 22L exposure, GT1 cells produced their own PrP-res bands that were clearly different than brain bands. Plots from passages 3–7 showed a good discrimination of 3 fold differences in titer over a range of >2 logs, with the same endpoint sensitivity (2×10^8 LD_{50}/gm) as animal assays. Interestingly, the rapid production of de novo PrP-res suggested that GT1 PrP-res might be induced by interaction with an early-intermediate form of a particle that was not fully infectious. The GT1 assay here was also invaluable for rapidly identifying cell cultures with variant titers, even after detergent lysis. Additionally, in-situ PrP amyloid staining provided an independent measure of the minimum infectious dose per cell. Standardized GT1 assays can be used for direct comparison of different agent strains, and will facilitate the rapid isolation of essential agent components. *Journal of NeuroVirology* (2008) **14,** 352–361.

Keywords: titer; scrapie; CJD; neurons; prion protein; amyloid; detergent

Introduction

Transmissible spongiform encephalopathies (TSEs) such as human Creutzfeldt-Jakob Disease (CJD), endemic sheep scrapie, epidemic bovine spongiform encephalopathy (BSE) and chronic wasting disease of cervids, are lethal neurodegenerative diseases caused by an infectious agent. It has been proposed that a normal host membrane protein, the ''prion'' protein (PrP), becomes infectious though its own spontaneous or self-seeded amyloid misfolding (Prusiner, 1998; Prusiner, 1999). This ''infectious form'' is diagnosed on Western blots by its relative resistance proteolysis (PrP-res). PrP-res is claimed to be free of nucleic acid, the defining molecule of all other infectious agents. Despite the current popular acceptance of an infectious prion protein, many different experimental TSE results are more parsimoniously explained by a conventional viral structure, i.e., a $\geq$1,000 nt core of nucleic acid that is protected by protein. In this case, host PrP acts as a required receptor or scaffold for the viral agent, and PrP amyloid starts from PrP interactions with the infectious TSE particle (Manuelidis, 1997; Manuelidis, 2003).

A large body of experimental data undermines the major prion assertions (reviewed in Manuelidis, 2006). These include, for example, 1) the failure of various forms of PrP to determine agent strains. As shown by mouse inoculation of several infected cell lines, agent strains, but not PrP-res bands, breed true despite major changes in PrP-res glycosylation and cleavage patterns that are cell type specific, e.g., (Arjona *et al*, 2004). 2) The reported germline

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inheritance of TSEs has also been overturned experimentally (Barron and Manson, 2003). 3) Most host PrP can be separated from infectivity during subcellular fractionations, and nucleic acids of >1,000nt have been detected in all ''purified'' prion preparations. 4) The claim that recombinant PrP itself, without other molecules, can be made into an infectious form has also been questioned by many investigators. 5) TSE agents display viruslike interference (Nishida *et al*, 2005). 6) Innate immune host responses are provoked early in infection, long before PrP changes are detected (Lu *et al*, 2004). These host responses are consistent with a foreign pathogen, but not host-encoded PrP. The epidemic outbreak of BSE also strongly implicates an exogenous infectious agent.

On the other hand, no TSE viral sequence has been published. Viruslike particles ~25nm in diameter have, however, been observed repeatedly in both experimental and naturally infected animals by electron microscopy (EM). Dense 25nm particles in brain also sediment with the infectious gradient peak and these particles have a homogeneous viral size of 10^6–10^7Da by HPLC (Sklaviadis *et al*, 1992; Manuelidis, 2006). More recently, murine cell cultures that produce high titers of mouse adapted CJD and scrapie agents have revealed comparable 25nm dense particles arranged in viruslike arrays (Manuelidis *et al*, 2007). These particles, unlike PrP amyloid fibrils, do not bind PrP antibodies. In summary, the structure and molecular components of the infectious TSE particle remain open. The generation of monotypic cells with high levels of infectivity provide new opportunities for the purification and molecular definition of essential agent particles and components. The major impediment to impartial resolution of agent components has involved extraordinarily long and expensive TSE animal infectivity assays. Accurate end-point titrations in mice can take over 300 days, and even incubation time assays of ~200 days are often required for strain verification.

We sought to develop a simple, inexpensive, accurate, and rapid cell culture assay of infectivity that can be applied to the many different TSE agent strains carried in mice. A live cell assay is biologically meaningful because it requires de novo infection of target indicator cells. In TSEs this can be followed by de novo and continuous production of PrP amyloid, an accepted surrogate marker of cellular infection. As indicator cells, we chose immortalized GT1–7 hypothalamic neurons (here abbreviated as GT1), rather than neuroblastoma N2a cells that were previously used for titer determinations (Klohn *et al*, 2003). First, the only a small proportion of cloned N2a cells are susceptible to permanent infection whereas GT1 cells are reproducibly and uniformly susceptible to infection, and do not need to be subcloned for stable agent replication (reviewed in Arjona *et al*, 2004). Second, GT1 cells, unlike established N2a scrapie clones, support high titers of agent for >50 passages or >1 year in culture (Arjona *et al*, 2004). Scrapie infected N2a subclones typically have relatively low titers (Arjona *et al*, 2004), and often lose PrP-res after only a few passages (Klohn *et al*, 2003; Mahal *et al*, 2007). Third, abnormal PrP in the first few passages after exposure can be due to retention of the original inoculum rather than to true infection of the target cells (Vorberg *et al*, 2004). Fourth, the preservation of an agent's phenotype in changeable N2a cells has not been documented. In contrast, the phenotype of several different TSE agents after long-term in GT1 passage show each of these agents breeds true when reinoculated in mice. Fifth, uncloned stock GT1 cells have been shown to be highly susceptible to a variety of very different TSE agents propagated in mice, and these include 3 scrapie strains (22L, Chandler-RML and 139a) as well as two CJD strains (SY and FU) (Arjona *et al*, 2004; Nishida *et al*, 2005); the SY strain of sporadic CJD could not be stably passaged in N2a58 cells. Two additional unique CJD strains also replicate in GT1 cells, and these also have never been passaged in any N2a or any other murine cultures (LM in preparation). In contrast infection of N2a subclones has been generally limited to the Chandler-RML scrapie agent. Moreover, the recent generation of many different N2a subclones was found necessary for propagating four different scrapie strains with varying success, and the agent phenotype in the target N2a clones was not verified by mouse inoculation (Mahal *et al*, 2007). The need for a panel of N2a subclones limits the ability to compare agent properties and titers in a single controlled and stable culture system.

We here show conditions for the reproducible, rapid and accurate titration of the 22L strain of scrapie in brain homogenates using GT1 cells as targets. Previous studies have verified preservation of the 22L agent phenotype in GT1 cells (Nishida *et al*, 2005) and the brain titers by GT1 assay here were the same as found by animal assay. We then used this standard titration to follow 22L agent titers in different batches N2a58 cells carried over a span of culture passages. Some batches of 22L infected cells with high PrP-res (i.e., C2a clones) developed different growth rates or PrP-res changes with passage and we assessed infectivity in these variant cells. In addition, the format of the assay allows for In-situ studies of C2a clones to independently verify cell titers. This assay system provides a rapid and powerful way to evaluate experimental treatments and derivative subcellular fractions for agent purification.

Results

As previously demonstrated, target murine GT1 cells (GT1–7 hypothalamic neuronal cells) can be

stably infected by many different mouse adapted CJD and scrapie agent strains. After infection, host prion protein converts into a pathological form (PrP-res) as assayed by its relative resistance to proteinase K (PK). PrP-res is a surrogate marker of GT1 cell infection that is specific for infection because mock-infected cells do not produce PrP-res (Arjona *et al*, 2004; Nishida *et al*, 2005). We here used the common 22L scrapie agent to develop an assay that can be applied to a variety of other TSE strains. To construct a standard reference curve for 22L titration using GT1 indicator cells, we serially diluted 22L infected mouse brain homogenates and applied these dilutions to duplicate culture wells. In TSE animal assays, high concentrations of agent cannot be discriminated from each other and intracerebral inoculation of 10% to 0.1% brain homogenates yield the same minimal incubation time, one that is characteristic for each TSE agent. However, with several serial dilutions, a linear relationship of infectivity to incubation time is found (Manuelidis *et al*, 1987). Further dilutions give highly variable incubation times. Typically, mice inoculated with more dilute suspensions are arbitrarily killed at 300–350 days to determine an endpoint LD_{50}, even though some infected mice will not show symptoms until $\geq$600 days. In this paper dilutions are calculated in terms of cell equivalents (CE) applied, a reference that facilitates comparison across a broad spectrum of diverse samples, including LD_{50} mouse assays (see Methods). When a mouse is injected intracerebrally with a 1% brain homogenate it receives 3×10^5 CE.

Animal bioassays indicated it would be most informative to examine both the incubation time and the amount of PrP-res produced by indicator GT1 target cells after exposure to serial brain homogenate dilutions. Low doses of 22L should take longer to be manifest than higher doses; the rise in PrP-res in GT1 target cells, insofar as it is linked to infectivity, should also have a lower slope, i.e., rise less rapidly. Moreover, at limiting infectious doses (ID), where few GT1 cells are likely to be infected, the%PrP-res should remain at a steady level if GT1 target cells maintain a monotypic rate of cell division. Unlike the agent doubling time defined in non-dividing neurons (Manuelidis and Fritch, 1996), low doses of TSE agent that infect only a few GT1 cells should be offset by the doubling of the uninfected GT1 cells. This would define the tissue culture end point. The experiments below support these predictions. We chose to analyze the% PrP-res made by GT1 cells by Western blotting for two major reasons. In contrast to Elisa spot, or dot blot assays, PrP-res bands can be clearly discriminated from background staining of unrelated proteins, as seen with some PK-antibody combinations. Moreover, PrP-res bands in 22L infected mouse brain and in C2a cells are markedly different than the PrP-res bands produced in GT1 cells (Arjona *et al*, 2004). Thus Western blot analyses allowed us to determine if PrP-res was carried over from the brain or C2a inoculum, or if it represented new PrP-res production by GT1 cells. Finally, because in-situ studies previously revealed that most GT1 target cells contain PrP-res when Western blots show 20% PrP-res, >20% PrP-res levels would likely indicate infection of essentially all target cells.

Figure 1A shows a representative experiment where a serial dilution of 22L infected mouse brain was applied to GT1 target cells and assayed for PrP-res at sequential passages. High concentrations of brain, as in mouse bioassays, could not be discriminated from each other because the conversion of PrP to PrP-res became maximal ($\sim$25% of total PrP in GT1 cells) after inoculation of 3×10^5 and 3×10^4 CE. Maximal PrP-res was maintained for the total 7 passages at these high CE inputs. A dramatic and sudden rise in de novo PrP-res production by GT1 cells was also verified by the lack of residual brain PrP-res at p1 (see Figure 2 and below). This indicated GT1 cells were an efficient system for 22L infection, one that takes place within the 7 days of initial exposure and cell growth during p0. A rapid rise in PrP-res at higher doses, e.g., 3×10^4 CE, where virtually all cells are infected, is also consistent with efficient cell to cell spread between GT1 cells as determined by previous experiments (Nishida *et al*, 2005). With more dilute inocula (3×10^3 and 300 CE), GT1 target cells produce PrP-res less rapidly. Efficiency with these lower inocula is also notable because they contain only 1%–0.1% CE relative to the plated GT1 cells. Repeat experiments were comparable to those shown, and there was an obvious and significant discrimination of samples over $\sim$3 logs of infectious input, i.e., 30 to 3×10^4 CE at p3 and thereafter.

To further simplify and standardize this assay, we tested smaller 2-fold dilutions at p3 and p5 when titration differences first became clear. This data suggested %PrP-res (of total GT1 PrP) limited to these 2 passages could be used to rapidly calculate tissue culture infectious doses (TC-ID). Figure 1B shows a scatter plot of these assay points from two different 22L infected brain samples. SEMs are shown for points with 6–8 determinations that included both brains; the other points are an average of 2–4 wells of one brain sample. At both p3 and p5, there was a 10 fold change in brain titer that corresponded to a linear $\sim$10% difference in the PrP-res made by GT1 cells (from 5–20%). Slopes at both passages were comparable, and even 2-fold changes fit a simple line as depicted ($R > 99$). In this range, a 3-fold change in titer (5% PrP-res difference) was clearly readable and comparable to the best discrimination of infectious titer by animal assay (Manuelidis *et al*, 1987). At PrP-res values of 0.5–5%, the slope of the best-fit line was steeper, and is, therefore, less precise for quantifying TC-ID

in an unknown sample. Because PrP-res is not detectable in mock cells low, but appreciable levels of PrP-res, i.e., 0.2–0.5%, were able to define the culture endpoint or TC-ID_{50}. In summary, GT1 cells can be used to assay infectious titers in a standard 2–3 log range. Although the reliable quantitative range is not as wide as seen in long animal assays, where 4 logs can be discriminated on the basis of incubation time (Manuelidis *et al*, 1987), the test of 3–4 dilutions of 1:3 permits a rapid and reproducible titer of an unknown 22L brain homogenate. The samples at p4 and p6, furthermore, provide back up for an additional confirmation of titer, and entail negligible extra work or expense. This is not possible with limited animals scored when clinical signs are rapidly progressing to death, or in cells that lose PrP-res with passage.

Western blot PrP band changes in GT1 indicator cells exposed to infected brain are also remarkably clear, even at the first passages after inoculation. Figure 2 shows a Western blot of PrP and PrP-res (PK+ lanes) of brain homogenate at different CE. The PrP profiles of the GT1 cells at p1 from duplicate group 1 wells (G1p1), and from parallel replicate group 2 wells at p2 (G2p2), are shown and are representative. We here evaluated the total brain PrP and PrP-res that would be visible if 100% of the applied homogenate were non-specifically absorbed by the GT1 target cells at p0 and not removed by washing. An input load of 3×10^4 CE (3µl of a 1% brain homogenate), but not lower amounts of brain, was detectable by Western blotting. However, these brain-specific bands were not visible in the GT1 target cells. Already at p1, the GT1 cells show only the PrP-res bands of GT1 cells. Moreover, even at the high 3×10^4 CE input, the GT1 profile is the same as at the 10-fold and lower CE inputs where brain PrPs would not be detectable, even with complete homogenate incorporation. Additionally, the PrP-res

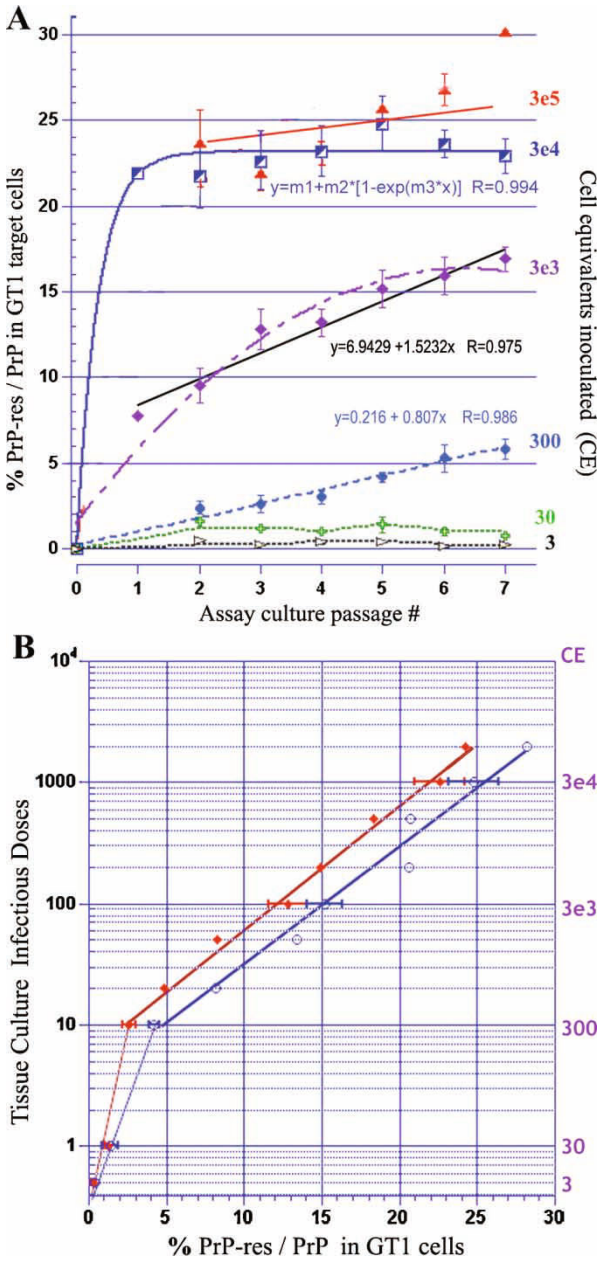

Figure 1 (*Continued*)

Figure 1 **A**: GT1 cells exposed to 22L infected brain homogenate dilutions and assayed for%PrP-res at sequential culture passages. There is a clear discrimination over ~3 logs of infectious input (30–30,000 CE) from p3 onwards. At doses $\geq 3 \times 10^4$ CE, the%PrP-res rapidly reaches its GT1 maximal level of 25%, and this maximal PrP-res is maintained in subsequent passages (exponential curve fit of the data with an R = 0.994 shown). A ten-fold input reduction to 3,000 CE converts PrP more slowly; from p1-p7 there is an excellent linear fit of the data (solid black line, y = 69429 + 1.523x; R = 0.975). These points could also fit a 2nd order polynomial when p0 is included (R = 0.98, dotted curve), but the acute initial rise in PrP-res from p0 to p1 should be considered separately (see Discussion). A linear but slower increase in PrP-res at the next lower dose of 300 CE is also apparent. At an input 30 CE and 3 CE, lower levels of PrP-res over 7 passages are maintained, indicating these GT1 cells are stably infected. However, the%PrP-res did not increase with these lower doses, suggesting an equilibrium is maintained between dividing uninfected cells and agent replication and/or spread. The lowest CE eliciting a low but constant PrP-res is defined as the tissue culture endpoint (here 3 CE) where equally loaded mock infected lanes were used as membrane background (PrP-res bands were not detected). Each plotted point with ±SEM bars represents the average of 2–4 independent experiments (6–12 wells assayed); two points at p1 and one at p7 are averages from one experiment. **B**: Standard 22L titration plot used as a reference in subsequent rapid assays of 22L infected samples with unknown titers. To make this plot, multiple dilutions of brain were assayed for%PrP-res at p3 (red) and p5 (blue). One tissue culture infectious dose (TC-ID) was experimentally defined as 1% PrP-res (by p3) and 2% PrP-res (by p5). These values were observed with application of 30 CE, and the other points plotted relative to this value, e.g., 3×10^4 CE will have 1,000x that TCID. There is a simple linear-log relationship between%PrP-res and infectivity from 10–2,000 TC-ID (R > 0.99); the slope is steeper between 2–10 TC-ID (R > 99), and unknowns in this range were confirmed by using a higher input inoculum. This GT1 assay yielded the same infectious dose per gram of 22L brain homogenate as the mouse assay (see text). Points shown with SEMs are from >3 independent experiments and the data include two different 22L infected brains.

bands were considerably more intense than seen in the highest brain homogenate loads, Thus there is no evidence of non-specific binding of the brain inoculum with our protocol, and new PrP-res is rapidly made by the exposed GT1 cells. Furthermore by p1, PrP-res already accounts for 22% of the total PrP (Figure 1A), a value close to the maximal amount that can be produced, and consistent with infection of all target cells. These Western blots also show a lower PrP-res production by GT1 cells after inoculation with fewer CEs. The continued high production of PrP-res in GT1 target cells is maintained (G2p7, bottom panel), and the same GT1 specific PrP band pattern is seen in all passages. Lower PrP-res amounts are also clearly visible with 22L brain inputs that range from 3,000–30 CE, the most accurate titration range for 22L infected brain.

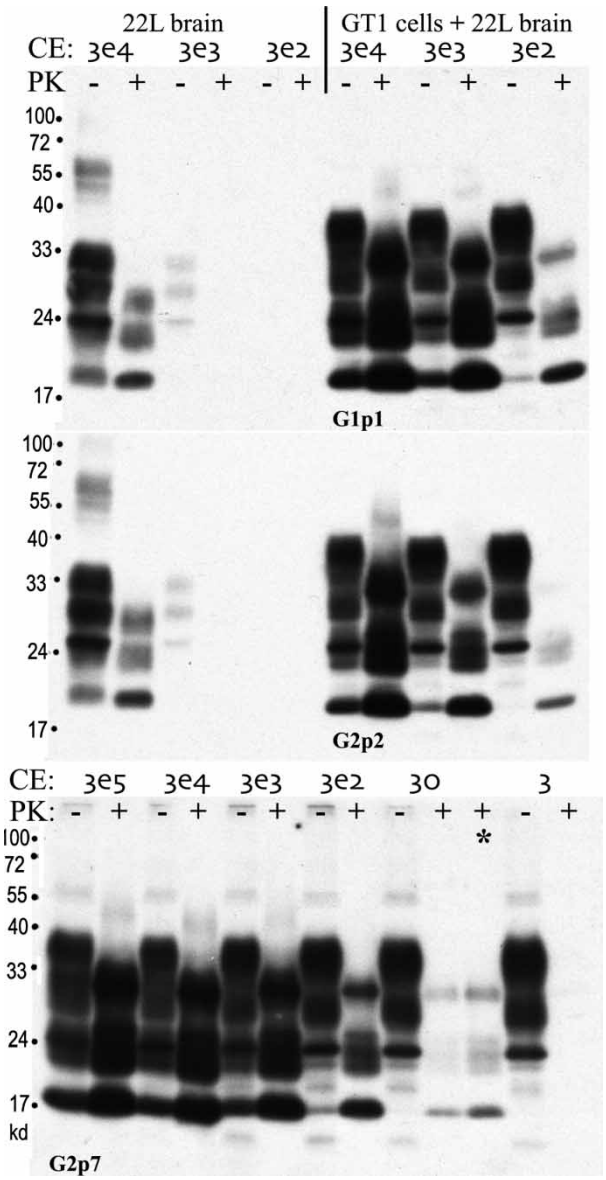

Figure 2 Western blot of PrP and PrP-res bands in a 22L brain homogenate used for infection and in the GT1 target cells exposed to this inoculum analyzed at early and later passages. The PrP-res band intensity in the infected GT1 cell lanes are considerably higher than in brain CE, even at the earliest passages (G1p1 and G2p2). These high levels are maintained for 7 passages (G2p7 panel). Moreover, at p1 and p2 post-infection the very different brain-specific PrP-res pattern is not visible in the GT1 target cells. Hence the inoculum has been effectively removed in p0, and the GT1 target cells at p1 and p2 are already producing substantial quantities of their own PrP amyloid. The PrP-res in the wells exposed to only 3 CE was not appreciable on film but did frequently show a faithful PrP-res signal of 0.3% with imaging equipment. A CE of 3 was therefore defined as the tissue culture endpoint (TC-ID_{50}) for 22L. PrP-res lanes (PK+) were loaded with 8x the PrP control, except for the duplicate 30 CE lane marked with an * in passage 7 (G2p7); this lane was loaded 19x to show the 1.1% signal on film.

We then used the culture assay to evaluate the titer of several batches of N2a58+22L cells that developed variable growth characteristics. We compared variations in GT1 infectivity from TC-ID values plotted for two different 22L brain homogenates and compared it to the mouse LD_{50} values. Figure 3A shows individual well values in the GT1 assay using two different 22L brain homogenates evaluated at p3 and p5 (columns 1 & 2 respectively). This shows the entire scatter of data with no replicate well averaging which narrowed the range of values considerably. The combined p3 and p5 points are also plotted (column 3). The average TC-ID for brain in each column shows <2 fold differences, and ranged from $0.9–1.3 \times 10^8$ TC-ID per 10^9 cells (gram). An additional GT1 assay of a third 22L infected brain using a dot blot format with no visible signal in the mock control (data not shown), also yielded a comparable end-point titer of 1.5×10^8 TC-ID/gram. These values are the same as extensive animal end-point LD_{50} assays for 22L infected mouse brain reported by others (Kim *et al*, 1987) that show an average of 2×10^8 LD_{50} (8.3 logs) per gram. These values are also in accord with our own incubation assays of 22L in CD-1 mice (3×10^8 LD_{50} per gram). Because the GT1 assay here is indistinguishable (<2 fold different) from the mouse assays of 22L, the GT1 culture assay revealed not only a relative equivalence of titer among different brain samples and experiments, but one that was numerically equal to and as accurate as the long end-point animal assay.

We then used the culture assay to evaluate the titer of several batches of N2a58+22L clones that developed variable growth characteristics. These titer determinations would have been prohibitive in cost and time using end-point mouse experiments. They also allowed us to immediately stop working with low titer cells. This was important because we found that some batches and passages of C2a cells carried by different individuals began to grow more rapidly. We wanted to find if rapid growth reduced 22L titers. Given the known instability of malignant murine neuroblastoma cells, it was also not surprising that other changes were also observed. One batch of cells showed a sudden change

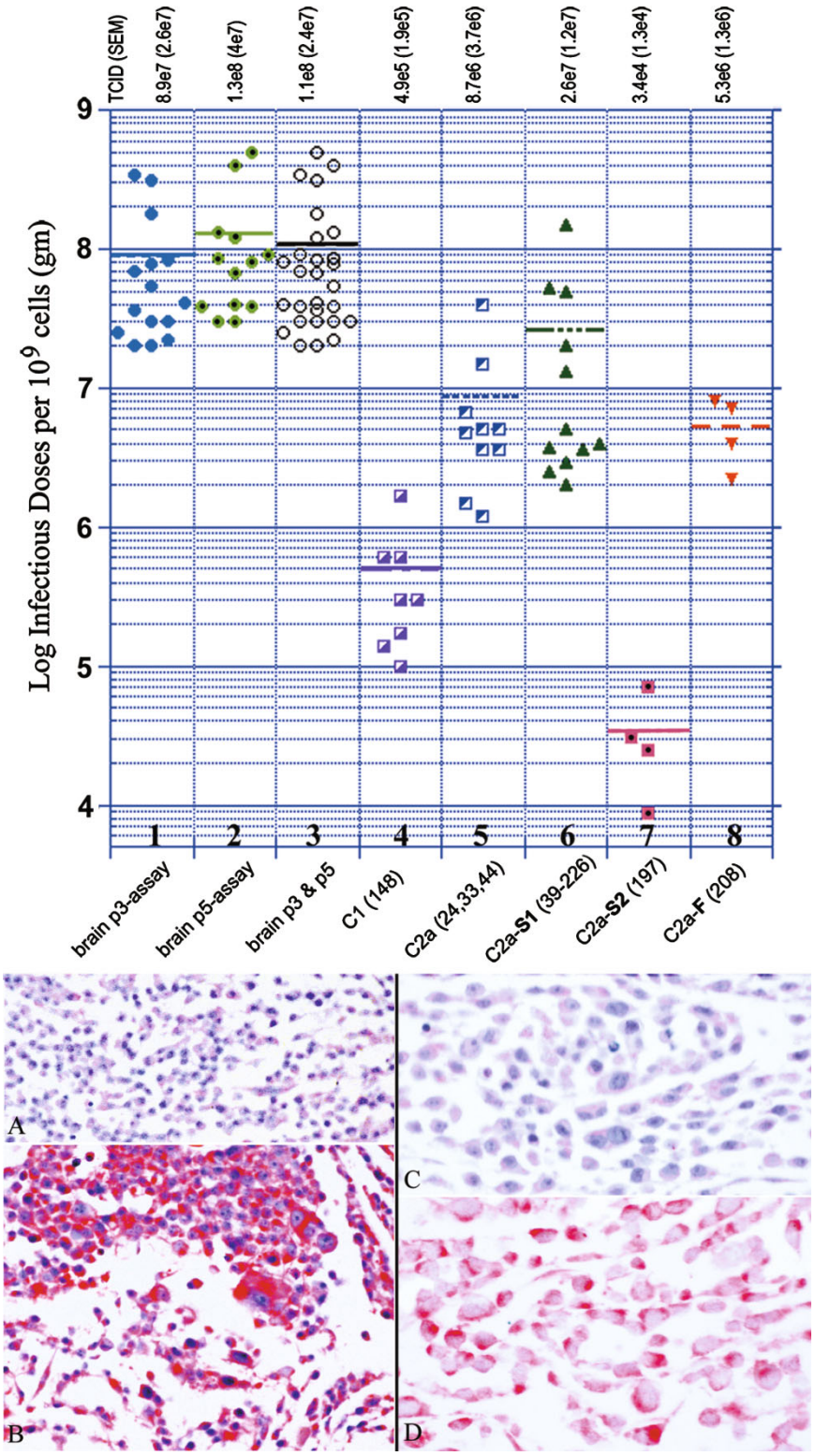

Figure 3 (*Continued*)

in%PrP-res, and we wanted to know if this might be linked in a quantitatively consistent way to infectious titer. The initial C1 parental N2a58 + 22L clone at p148 provided a good baseline reference because these p148 C1 cells were used to generate the high PrP-res C2a clone previously described (Manuelidis *et al*, 2007). This C2a clone produced 40–45% PrP-res, or 3–4x the amount of its C1 parent (~13% PrP-res). Column 4 in Figure 3A shows the infectivity of parental C1 cells and Column 5 shows the C2a cells titered at passages 24, 33 and 44 after cloning. The C2a cells showed ~1 log more infectivity than the parallel parental C1 cells. Even at much later passages these cells continued to grow at the same slow rate and were designated C2a-S1 cells. They yielded the same TC-ID ($\sim 1.7 \times 10^7$) as the original C2a cells with little variation in values over a range of passages up to p226 (column 6). However, C2a cells, carried in parallel by another investigator that retained the same slow growth rate (designated C2a-S2 cells), showed a decline of PrP-res to 18% by p197 (column 7). The TC-ID in these C2a-S2 cells was only 0.4% of the C2a-S1 cells. Moreover, these C2a-S2 cells had >1 log lower titer than the original C1 cells (column 4), even though they contained more PrP-res than those C1 cells. Thus a reduction in PrP-res indicated a loss in titer, but it failed to reflect major quantitative titer changes. Faster growth itself did not reduce titer in C2a cells. The faster C2a-F cells at p208 (column 8) that had to be split 1:10 versus 1:4 every 4 days for the ~20 preceding passages showed no significant difference in titer from their slower C2a-S1 counterparts (column 6, $t = 0.06$).

In contrast to the brain homogenates, the TC-ID of the C2a cells was 1 log lower than expected from a mouse incubation time assay of p34 cells. These C2a cells had ~1 infectious dose per 3 cells by mouse assay a very high titer for any scrapie strain in vitro, and equivalent to the titer of 22L infected brain (Manuelidis *et al*, 2007). The lower titer in the C2a (and C2a-S1 cells) in the GT1 assay might be due to the lysis of cells in detergents because detergents can increase mouse incubation times in TSEs. Although the small CE needed for titrating C2a cells allowed dilution of detergents to levels that were non-toxic for the GT1 target cells (typically ~10,000 fold), the addition of detergents could, nevertheless, affect agent uptake and/or its initial replication in GT1 cells. Alternatively, high detergent levels might also irreparably compromise some infectious particles in the C2a cells. A third possibility was that the mouse bioassay titer might be an overestimate.

To find if the mouse assay of C2a cells was more accurate than the GT1 assay, we evaluated PrP-res production in individual C2a cells. Any cell producing PrP-res should be infected by at least 1 infectious particle, especially since no mock infected cells show this change. We examined p29 and p31 C2a cells because they corresponded to the passages titered for infectivity both in mice and in GT1 cells. These studies show the vast majority of C2a cells were positive for PrP-res (red intracellular deposits in Figure 3B). Mock N2a58 controls from a comparable passage yielded no PrP-res deposits when processed on the same slide with the infected cells as shown. At higher power, positive cells revealed large, round, intensely red cytoplasmic PrP amyloid aggregates and could be counted with confidence; ~85% of the C2a cells shown are positive. A conservative in-situ estimate of only 33% positive C2a cells would indicate 1 infectious particle per 3 cells, as found with the mouse bioassay. Thus the TC-ID of detergent lysates can underestimate titer. More experiments are needed to evaluate the effect of different detergents and other additives on infectious particles in cultured cells and/or on the GT1 assay. Nevertheless, even with detergents, the current rapid GT1 assay gives a reliable estimate of relative titer among similarly exposed samples.

Discussion

The above results show that immortalized neuronal GT1 cells can be used to rapidly, inexpensively, and accurately titer a TSE agent. We used 22L scrapie as a model agent for these tests. Since a variety of other TSE agents also readily infect GT1 target cells, the methods for quantitation of other agent strains should be similar and provide a way to compare titers of new TSE agents in brain. Indeed, in preliminary studies several other strains, including mouse adapted human CJD agents, can be identically titered in GT1 cells with comparable accuracy to animal assays. Western blot assays also allowed us to verify de novo PrP-res production by GT1 cells as early as the first split after exposure. The PrP-res

Figure 3 GT1 assays of different 22L infected N2a58 clones and passages for infectivity by Western blot and in-situ PrP-res detection. **Top** shows scatter plot of p3 versus p5 brain TC-ID determinations (column 1 & 2) as well as the combined brain data (column 3). The average TC-ID is shown by a line and numerically at the top of each column (±SEM). Different batches of the starting 22L infected N2a58 C1 clone (Column 4) and its C2a derivatives are shown. Some of the C2a cells developed variant phenotypes of growth and PrP expression sampled at the passages indicated (see text). **Bottom** shows an independent assessment of PrP-res by in-situ immunodetection. Mock infected N2a58 cells (A&C) are compared p39 and p31 C2a cells (B&D) and show representative fields at low and higher magnifications respectively. The PrP amyloid (dark red bodies) are obvious in the 22L infected C2a cells and not present in the mock controls. At the higher magnification the number of cells with large PrP amyloid aggregates are easily counted, e.g., 30 of 36 cells in panel D are positive. Thus a high proportion of the C2a cells are infected; positive cells are also an underestimate because only a 4μM section of each cell is sampled.

bands were not due to non-specific binding of brain or C2a cell inocula, both of which have their own distinctive PrP-res band patterns that differ from GT1 cells. Additionally, the format described demonstrates an independent in-situ assessment of TC-ID in input C2a cells, as well as in target GT1 cells as previously depicted (Arjona *et al*, 2004). Because the GT1 PrP-res patterns were so clear and free of background, further simplification of the assay in a dot blot format was tested and found to be accurate; in this case more replicate samples at each passage can be easily accommodated. Other automated and individual cell analyses are also feasible for high throughput assays, although no special equipment is needed for the assay described here.

Because GT1 cells are ideal for assay of TSE agents propagated in mice, development of human immortalized neuronal cells could be very useful for directly testing human CJD infected tissues. A cell infectivity assay may be of most benefit for samples such as blood, where detection of any PrP-res has been problematic. As shown by brain dilution studies here, a sample with undetectable PrP-res can show appreciable infectivity in this culture assay. Unlike the original and very recent N2a assays where PrP-res spots can be very variable and inconstantly produced after only a few passages (Klohn *et al*, 2003; Mahal *et al*, 2007), the production of maximal PrP-res in GT1 cells was stable and constant with passage. Inoculation of lower input CE that do not rapidly elicit maximal PrP-res also showed a steady accumulation PrP-res with each passage, indicating bone fide GT1 infection. This accumulation was also consistent with the described cell-to-cell spread of infectious TSE particles within GT1 cultures (Nishida *et al*, 2005). We detailed multiple dilution points for the most rapid p3 and p5 infectivity determinations. Nevertheless, it is clear that p4 and p6–7 are also valid, and they provide additional back-up samples for verification of the tissue culture infectious dose (TC-ID). The progressive accumulation of PrP-res, as well as previous results showing that most GT1 cells develop PrP amyloid after FU-CJD infection (Arjona *et al*, 2004), also indicates that essentially all GT1 cells, unlike neuroblastoma cells, are susceptible to and productive for both the 22L and FU-CJD agents (Arjona *et al*, 2004; Nishida *et al*, 2005). The straightforward TC-ID calculation (per 10^9 cells/gram), defined by the de novo% PrP-res as well as its endpoint, is also directly comparable to the standard LD_{50} (per gram) of long animal assays.

The speed of the PrP-res response, although of no consequence for the validity of the TC-ID assay here, raises an intriguing question: does the GT1 PrP-res percent really reflect de novo production of complete infectious particles? We have previously shown by mouse bioassay that there was a >600 fold increase in infectivity in GT1–1 cells between p6 and p26 even though the%PrP-res remained the same. Increasing infectivity (>50 fold) was also found in GT1–7 cells with progressive passages even though the%PrP-res remained constant. Inoculation of infected GT1 cells at different passages in mice also showed that the infectious FU agent bred true, with no difference in the clinical signs and pathology as compared to FU-CJD infected brain (Arjona *et al*, 2004). It is therefore possible that the high PrP-res produced in GT1 target cells shortly after exposure to higher doses of 22L scrapie similarly reflects a disproportionate expression of PrP-res relative to infectious particles. As with many viruses, such a cellular response could be due to the production of some early-intermediate form of the TSE agent. This incomplete form could bind cellular PrP to induce PrP amyloid before complete and capable infectious particles assemble. It will be interesting to test early passages of 22L infected GT1 cell lysates to find 1) if maximal PrP-res can be produced in GT1 cells with low titers of agent, and 2) if 22L infectious titers increase with sequential passages in cells showing a constant maximal PrP-res, as was found with FU-CJD infections. The rapid TC-ID assay will be invaluable for this work. Analyzing proteins at early and sequential passages after infection, with parallel mock- infected GT1 cells, given their remarkable phenotypic stability, may also resolve new and essential pathways for TSE agent maturation.

The TC-ID of 22L infected brain homogenates was indistinguishable from the LD_{50} reported in the literature. However, detergent treated neuroblastoma C2a+22L infected cells, unlike mechanically disrupted brain homogenates, displayed a TC-ID that was $\sim$1 log lower than found by our mouse bioassay. In-situ analyses showed that most of these C2a cells contained large PrP amyloid bodies not seen in mock controls, indicating most of the cells must contain a minimum of 1 TSE agent particle. This observation independently validated the higher mouse assay titer. Detergents may compromise the infectious particle, and it remains to be determined if TSE particles in cell cultures are less resistant to detergent inactivation than particles in more complex brain tissue. Alternatively, even though detergents were highly diluted in the applied inoculum, small amounts of detergent might still affect agent uptake as well as other cellular processes necessary for efficient agent replication. In any case, the relative differences among different batches of C2a+22L cells lysed identically still revealed large differences in their titers. This assay saved a great deal of time that would have been wasted on less productive batches of cells. Further studies with these detergent treated samples were used to evaluate gradient fractions that could enrich infectious particles, All the applied infectivity was quantitatively recovered in the gradient fractions and these fractions could be made much more

simply and reproducibly than from degenerating brain tissue (in preparation). In short, the GT1 TC-ID assay is an efficient and accessible test that can uncover many fundamental but still unknown properties of infectious TSE particles along with their essential molecular components.

Materials & Methods

GT1 Infectivity assays: Cell exposure and propagation

To determine the sensitivity and accuracy of GT1 target cells for infectious titrations based on PrP-res conversion, and to compare these results with infectivity assays in mice, stock GT1 cells were maintained in Optimem medium with 10% serum as detailed (Arjona *et al*, 2004). For the infectious culture assay, 2 replicate wells of a 6 well dish were seeded with 2ml medium containing a total of 10^5 GT1 cells. After $\sim$36 hrs at 37°C (30–40% confluence), cells were washed with warm medium, and the medium replaced with 1ml of test material in medium. An additional 1ml of plain medium was added the next day, and the wells were incubated for 2 more days. The infectious medium was then removed by suction and the cells washed x3 with serum-free medium, x1 with complete medium, and then incubated with 2 ml of fresh medium for 4 more days (to 90–95% confluence). This treatment protocol was developed to maximize the time of exposure to infectious inocula, and to provide time for undisturbed growth after rigorous washing. This minimized non-specific retention of inoculum that would be carried over to the first passage (p1). For passage, cells in each duplicate well were split at 1:4 every 4 days (10^5 cells per well) and the left over cells were seeded into 25cm flasks for corresponding PrP assays. Flask cells were collected in parallel every 4 days for Western blotting. The culture requirements of this assay are minimal because only two 6-well plates are needed for duplicates of 6 test samples. The replicate 25cm flasks also provide a back up.

Dilution of brain and culture samples

We previously developed a culture reference system for infectivity based on cell counts where brain contains $\sim 10^9$ cells per gram and a 10% or 10^{-1} dilution contains 10^8 cells/ml (Arjona *et al*, 2004). Thus standard infectivity titrations of brain and other tissue samples in the literature can be easily compared to each other and to cultured cells on both a cell equivalent (CE) and gram basis. For example, a mouse injected intracerebrally with 30µl a brain homogenate diluted 10^{-4} (10^5 cells/ml) receives a dose of 3×10^3 CE. The number (n) of tissue culture infectious doses (TC-ID) measured in GT1 target cells at this input therefore is n*3.3×10^5 per 10^9 cells (gram of brain). Similarly, CE infected cultures (or of a subcellular fraction derived from the same number of cells), can also be converted into TC-ID per 10^9 cells (gram equivalents) for comparison. The total CE added to each well are calculated rather than dilutions that vary with the well format; this also reveals the ratio of input CE to target cells, an indication of target cell sensitivity. Control wells exposed to normal brain at high input ($\geq 3 \times 10^4$ CE) were included in each assay and were always negative for PrP-res. Serial homogenate dilutions from two different 22L infected brains taken at clinical stages of disease were used to determine the standard titration curve for 22L on GT1 indicator cells. Dilutions were made with DMEM containing 0.01% normal brain homogenate to ensure major sample components were the same. For cultures, counted 22L infected cells were frozen at −70°C, thawed, syringed ×10 through a 27g needle, vortexed, and then bath sonicated x10 for 10s, interrupted by 10s rests on ice. Each dilution contained brain carrier as above. Assays of infected culture tests always included a control 22L infected brain dilution, e.g., 3×10^3 CE, to verify that the indicator cells worked as expected. Cultures of highly passaged N2a58 (mock and 22L scrapie agent infected) and the derivative C2a +22L clone have been described in detail (Manuelidis *et al*, 2007). Cells grown in 150cm flasks ($>10^7$cells) were washed with PBS, detached, counted, and sedimented for 5 min at 1500rpm (450g) in an HS4 Sorvall rotor at 4°C. The pellet was suspended for 10min in standard lysis buffer [100mM NaCl, 10mM TrisCl pH 7.5, 8% sucrose, 0.5% Deoxycholate (DOC), 0.5% NP40] at 22°C, and became optically clear within 1–3min. Whole cell lysates, as well as nuclei-free cytosol (collected at1000g ×15min at 22°C) were frozen at −70°C, thawed, and diluted as above to load appropriate CE in each well. Aliquots were also evaluated in mice as previously described (Arjona *et al*, 2004).

PrP detection

For western blots, GT1 indicator cells in the 25cm flasks (90% confluent) were washed once with PBS at 4°C, and the supernatants collected as above by adding 10µl of the above lysis buffer without sucrose per 10^6 pelleted cells. Nuclei were then pelleted for 5min at 1,000g in a microfuge. Supernatants were typically used for assay to avoid nuclear viscosity problems. There was no loss of PrP-res, cytoplasmic proteins or infectivity with this nuclear removal step (ms in preparation). An aliquot of 4.5µl of the supernatant was mixed with 0.5µl PMSF (2mM final) to determine total cellular PrP. The remaining 36µl was digested with PK for PrP-res analysis. Before PK digestion, 4µl of a 2% normal brain suspension was added to the 36µl samples. We found this allowed quantitative sedimentation of PrP for digestion. It also equalized the total protein in each sample and normalized digestions. Samples

were vortexed and centrifuged at 18,000g × 45min at 4°C in a microfuge, and the pellet thoroughly resuspended with 10µl of 0.1% sarkosyl in PBS. Then 3.3µl of 4x standard PK solution (100µg/ml PK, 2% NP40, 2% DOC and 20mM EDTA in 10mM TrisCl pH 7.4) was added, and the mixture incubated for 30min at 37°C. The reaction was stopped with PMSF, samples boiled in standard SDS sample buffer and blotted as previously (Manuelidis *et al*, 1985). The commercial M20 PrP antibody as described (Arjona *et al*, 2004) was used to delineate chemiluminescent bands that were developed with Chemiglow (Alpha Innotech); bands were analyzed with a flat field lens equipped Alpha Innotech imager calibrated to discriminate PrP chemiluminescent signals over 3 orders of magnitude. Low PrP-res amounts were also verified by increasing gel loads for less intense signals, and showed that no special imaging equipment is required for these assays. In-situ detection of PrP-res was done on 4µm paraffin sections of cells fixed in 4% paraformaldehyde and the PrP amyloid unmasked by autoclaving followed by limited trypsin digestion as described in detail (Arjona *et al*, 2004). Control uninfected and infected cell sections were processed together on a single slide to insure equal unmasking and antibody reagent development.

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