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Extended period of asymptomatic prion disease after low dose inoculation: Assessment of detection methods and implications for infection control

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We used quantal dose-titration of a mouse-adapted human transmissible spongiform encephalopathy strain (M470) to compare different analytical methods for their ability to detect asymptomatic brain prion infection after low dose inoculation. At a time point approximately 2.5-fold beyond the mean incubation period of high dose inocula, asymptomatic brain infection was commonly observed using histologic examination, Western blot, and "blind" bioassay following intracerebral inoculation with low titer inocula. At this time point, when a clinical end-point titration would usually be determined, evidence of infection was seen in all healthy animals inoculated with up to 100-fold lower inoculation doses than the lowest causing consistent clinical disease. For the assessment of the presence of asymptomatic infection, we compared different Western immunoblot and histopathological methods in relation to "blind" bioassay using transgenic Tga/20 mice overexpressing mouse prion protein (PrP). Sodium phosphotungstic acid (NaPTA) precipitation of protease-resistant PrP isoforms (PrP^{res}) prior to Western blotting was found to approach the sensitivity of the Tga/20 bioassay and was superior to conventional Western blot and histopathological methods, wherein infectivity was commonly found when both of the latter were negative. Re-scaling the original titer by incorporating "blind" transmission data from surviving asymptomatic mice revises the estimate two orders of magnitude higher than the value derived using the conventional clinical disease outcome approach. We also found that the sensitivity of the NaPTA Western blot technique, if used with a diluent such as PBS compared with 10% normal brain homogenate, is adversely affected by up to around 20-fold. We postulate that infectious titer estimates based on more sensitive detection systems such as we report provide a more accurate indication of ultimate transmission risk.

Keywords: Prions; Creutzfeldt–Jakob disease; Asymptomatic infection; Preclinical infection; Subclinical infection; Transmissible spongiform encephalopathy; Detection methods

Introduction

The transmissible spongiform encephalopathies (TSEs) constitute a group of neurodegenerative disorders, which includes Creutzfeldt–Jakob disease (CJD) and its variant form (vCJD) in humans, as well as scrapie in sheep and bovine spongiform encephalopathy (BSE) (Prusiner, 1997). After the onset of clinical disease, all are invariably fatal disorders sharing neuropathological hallmarks such as spongiform change, neuronal loss, gliosis, and accumulation of abnormal isoforms of the host encoded prion protein. An underlying feature of TSEs is the conversion of the normal cellular form of the prion protein (PrP^{c}), a glycosylphosphatidylinositol-anchored cell surface protein, into a protease-resistant conformer (PrP^{res}; considered hereafter as equivalent to PrP^{sc}, the abnormal protease-resistant conformers found in scrapie), a process intimately linked to infectivity and neurodegeneration (Prusiner, 1997). Much progress in clarifying the molecular details of this conversion process has been made, but a complete and detailed understanding remains to be achieved. Notwithstanding this, the detection of abnormal isoforms of PrP serves as a very useful and reliable marker of disease and infectivity, both for diagnostic and experimental purposes in humans and animals.

CJD is the most common human TSE, but remains rare, with an incidence of only 1–1.5/million/year in comprehensive national

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surveillance studies (Collins et al., 2002b). Only a small proportion (10–15%) of CJD can be accounted for by mutations in the prion protein gene (*PRNP*) or as a consequence of recognizable transmission events (1–2%), with the majority (approximately 85%) occurring without identifiable cause (sporadic). Although uncertainty persists, the prevailing concept is that sporadic CJD arises as a consequence of either rare somatic mutations in the neuronal *PRNP* pool or secondary to a spontaneous conformation change in PrP^{c} (Prusiner, 1997). An alternative hypothesis is that a proportion of apparently sporadic CJD may be due to unrecognized, low-level, contamination events. Large risk factor case-control studies lend some support to this postulate (Collins et al., 1999; Ward et al., 2002), but detailed analysis of spatiotemporal clustering of sporadic CJD has been unable to substantiate plausible transmission pathways (Collins et al., 2002a). Although convincing evidence for either of these two etiological explanations is lacking, additional circumstantial support for the covert transmission hypothesis is derived from a number of studies, including primate and mouse paradigms, showing evidence of TSE infection for lengthy periods prior to the onset of overt disease.

A common theme in murine models of prolonged asymptomatic TSE infection is that "inefficient" introduction of the infectious inoculum, by using peripheral (Collis and Kimberlin, 1985; Dickinson et al., 1975; Zlotnik, 1965) or low-titer (Thackray et al., 2002) inoculations, donor–host species barriers to the particular TSE strain (Asante et al., 2002; Hill et al., 2000; Race et al., 2001, 2002), or more resistant recipient transgenic mice (Büeler et al., 1994; Frigg et al., 1999), results in very prolonged incubation periods (preclinical infection) or complete failure to induce clinical disease during the normal lifespan of the host animal (subclinical infection). A possible additional intraspecies explanation for inefficient transmission is that despite development of TSE, the PrP^{res} produced may not be physically competent to subserve PrP^{c} conversion. Irrespective of the mechanism, for much of the extended asymptomatic phase, peripheral organs such as the spleen, as well as the brain, harbor levels of infectivity often comparable to those seen in terminally sick animals (Büeler et al., 1994; Collis and Kimberlin, 1985; Dickinson et al., 1975; Frigg et al., 1999; Thackray et al., 2002). Acknowledging that the extended incubation period may exceed the lifespan of the host (Dickinson et al., 1975), differentiation between preclinical and subclinical infection may be impossible, but does not detract from the fundamental importance of this phenomenon, especially as it relates to potential unrecognized transmission during the provision of health care. Recent reports of PrP^{res} detection in the skeletal muscles and spleens in some patients with sporadic CJD (Glatzel et al., 2003), as well as the likely secondary transmission through blood transfusion from a presymptomatic vCJD patient (Llewelyn et al., 2004), further add to the concerns generated by previous studies. Of these, the demonstration of PrP^{res} in peripheral lymphoreticular organs of living vCJD patients (Hill et al., 1999; Wadsworth et al., 2001) is of particular interest and supports the hypothesis that transmission is possible for a lengthy period before the onset of clinical disease (Hilton et al., 1998, 2002, 2004).

We undertook the present study to assess the prevalence of asymptomatic TSE infection in a model that in part simulates hypothesized neurosurgical contamination events in the health care setting, such as may occur through intracranial surgery performed unwittingly on a patient with early symptomatic sporadic CJD. Based on the premise that health care transmissions most likely involve very low infectious doses, we employed a standard quantal dose-titration paradigm using a human-derived TSE strain in non-transgenic host animals. A concomitant aim of the study was to carefully compare bioassay with the utility of standard biochemical and histopathological methods for detection of presymptomatic TSE infection, which is of relevance to screening for potential infectivity in at risk populations such as dura mater and human-derived pituitary hormone recipients. In comparative studies, we found that a high sensitivity Western blot technique utilizing a metal-enhanced (sodium phosphotungstic acid [NaPTA]) precipitation method nearly approximated the sensitivity of bioassay, as recently reported when used in a different in vitro technique (Safar et al., 2002; Wadsworth et al., 2001). Similar to others (Hill et al., 2000; Lee et al., 2000; Race et al., 2001), we determined that bioassay using highly susceptible recipient animals was clearly superior to conventional biochemical PrP^{res} detection methods and routine neuropathological examination. Finally, we also assessed factors affecting the sensitivity of the NaPTA Western blot technique as a screening method for potential asymptomatic TSE infection.

Methods

Mice and inoculations

The strain (M470) used for all experiments is a mouse-adapted form of human TSE derived from a patient dying from probable Gerstmann–Sträussler–Scheinker syndrome (GSS) (Tateishi et al., 1979). For initial calculation of infectious titer, a 10% homogenate (w/v) in phosphate-buffered saline (PBS) of pooled whole brains from mice dying from TSE was serially log diluted in PBS with concentrations spanning 10^{-1} to 10^{-9}. Prepared dilutions of inocula were immediately used for intracerebral inoculations of groups of six random outbred weanling female *Balb/c* mice (except the 10^{-9} dilution when only three mice were used). Approximately 30 μl of the appropriate dilution was injected into the left parietal region under methoxyflurane anesthesia. Unused inocula were stored at −80°C until subsequent use for Western immunoblot studies. Titer estimations were according to the method of Reed and Muench (1938).

Prior experiments employing an analogous human TSE strain (M1000) in *Balb/c* mice had demonstrated the earliest presence and highest amounts of PrP^{res} occurred in brain regions approximating the telencephalic subcortical gray nuclei and thalamus (authors' unpublished data). For bioassays assessing asymptomatic infection, 30-μl aliquots of 1% homogenates made from this brain region from individual *Balb/c* mice were inoculated intracerebrally into groups of 3–5 male and female weanling Tga/20 transgenic (PrP^{c} overexpressing) mice (Fischer et al., 1996). Brain tissues from *Balb/c* mice receiving the original 10^{-9} inoculum dilution were not subject to bioassay.

Inoculated *Balb/c* and Tga/20 mice were observed daily for signs of TSE. Animals were sacrificed under methoxyflurane anesthesia when persistent signs consistent with murine TSE were evident, such as reduced motor activity, weight loss, hunched posture, hind limb paresis, and ataxia. Mice were given food and water ad libitum, with all handling according to prescribed national guidelines, and ethical approval from the University of Melbourne Animal Ethics Committee.