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A rapid dual staining procedure for the quantitative discrimination of prion amyloid from tissues reveals how interactions between amyloid and lipids in tissue homogenates may hinder the detection of prions

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ABSTRACT

Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative diseases with no cure to this day, and are often associated with the accumulation of amyloid plaques in the brain and other tissues in affected individuals. The emergence of new variant Creutzfeldt–Jakob disease, an acquired TSE with a relatively long asymptomatic incubation period and unknown prevalence or incidence, which could potentially be iatrogenically transmitted, has prompted the need for sensitive and rapid methods of detection of the pathology indicator, the protease-resistant prion protein (PrP^{Sc}), in tissues and on surgical instruments. To discriminate between common tissue proteins and amyloid-rich aggregates such as those formed by abnormal prion, we developed a quantitative thioflavin T/SYPRO Ruby dual staining procedure, used in combination with episcopic differential interference contrast/epifluorescence (EDIC/EF) microscopy for rapid scanning of samples. The detection limit of this direct observation technique applied to brain homogenates was greatly enhanced by the addition of Tween 20, as demonstrated in double-blind studies using various proportions of ME7-infected brain mixed with normal brain homogenate. The characteristic thioflavin T signal correlated with the relative amount of prion amyloid and proved at least 2-log more sensitive than the classic Western blot using the same prepared samples. This new sensitive microscopy procedure, which can be easily applied in instrument decontamination surveys, is likely to be more sensitive that Western blot in practice since it does not rely on the elution of resilient PrP^{Sc} bound to the instrument surfaces. Our study also demonstrates how interactions between prion and lipid-rich tissue homogenates may reduce the sensitivity of such detection assays.

1. Introduction

Transmissible spongiform encephalopathies (TSEs) are rare, fatal neurodegenerative diseases which may occur in various animal species (see reviews by Brown, 2008; Collinge, 2001; Prusiner, 1998). TSEs may develop when the host-encoded constitutive prion protein (PrP^{C}) is mutated to an isoform richer in beta-pleated sheets, which is partly resistant to proteolytic digestion. In humans, identified TSEs include Creutzfeldt–Jakob disease (CJD), Kuru, Gerstmann–Sträussler–Scheinker syndrome (GSS) and fatal familial insomnia (FFI), and these have been further classified according to their etiology (Gambetti et al., 2003). The incidence of a genetic mutation leading to sporadic TSEs is believed to be low (about 1 per million annually for sporadic CJD). According to the "prion only" hypothesis, absorption of exogenous PrP^{Sc} is sufficient to trigger the post-translational refolding of endogenous PrP^{C} in healthy, genetically susceptible individuals, leading to an acquired form of the disease. The accumulation of protease-resistant PrP^{Sc} in the form of amyloid plaques in tissues, particularly in the lymphoreticular and central nervous systems, are a common pathological feature of TSEs.

The consumption of contaminated tissues has been identified as a route of transmission leading to acquired CJD. This horizontal transmission of the disease was described in the cannibalistic Fore tribe of Papua New Guinea in the middle of last century (see reviews by Ironside, 2003; Collinge et al., 2006), and there is strong evidence that the same route was implicated in the bovine spongiform encephalopathy (BSE) epidemic originating from the UK during the mid 1980s, in which case cattle were fed on reprocessed carcasses of slaughtered contaminated animals ("meat and bone meal"), leading to the contamination of an estimated 1 million animals destined to human consumption (Wells et al., 1991; Anderson et al., 1996). There has been increasing experimental evidence that various protease-resistant prion strains are capable of crossing the species barrier (Mishra et al., 2004; Priola and Vorberg, 2004; Foster et al., 1993), and that the ingestion of BSE-infected beef meat is the actual cause of the so-called variant form of CJD in humans (vCJD; Hill et al., 1997). Acquired TSEs are characterized by a relatively long and initially asymptomatic incubation period (up to 50 years for Kuru; Collinge et al., 2006) which

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varies depending on the nature of the infective strain, the route of transmission, and the genetic susceptibility of the recipient. This has led to the fear that an unknown proportion of the population who have been eating potentially contaminated meat in the 1980s might currently present as asymptomatic carriers of the vCJD infectious agent, despite current statistics which indicate that the number of deaths by vCJD peaked in 2000 and has been in decline since (Caramelli et al., 2006; Setbon et al., 2005).

Beside transmission of the infectious agent through the ingestion of contaminated products, bearing in mind the potential threat of vCJD incubating in the population, a number of medical procedures are now also considered as posing a potential risk of iatrogenic contamination with PrP^{Sc} (Pana and Jung, 2005; Lumley and Serious Hazards of Transfusion Committee, 2008). The procedures identified at risk include neurosurgery and eye surgery (Dinakaran and Kayarkar, 2002), and other invasive interventions such as blood transfusion (Ironside, 2003; Llewelyn et al., 2004; Wroe et al., 2006), endoscopy (Ponchon, 1997; Axon et al., 2001) and dentistry (Azarpazhooh and Leake, 2006; Whitworth, 2007). In the absence of a cure for the disease, avoidance and precautionary measures are the only ethical option currently available to public and private health services. PrP^{Sc} is known to be resistant to most inactivation and decontamination protocols, and current methods of neutralisation in the laboratory involve strong alkali compounds (Jung et al., 2005), which are themselves detrimental to most surfaces. In recent years great emphasis has been put on the decontamination of surgical instruments as a means of preventing iatrogenic transmission, and a number of cleaning products have been developed and marketed for the purpose of tackling the protease-resistant prion issue, though relatively little has been done to assess the efficiency of these cleaners in a clinical context, where the turnover of surgical instruments is high and the time to clean them is limited. We have already reported that common methods currently available to assess the cleanliness of surgical instruments, such as direct visual inspection and indirect Biuret or ninhydrin chromogenic assays, are far from being sensitive at picking up remaining contamination on surgical stainless steel surfaces (Lipscomb et al., 2006a, 2008). We have previously described a new microscopic technique, episcopic differential interference contrast/epifluorescence (EDIC/EF) for the rapid and sensitive *in situ* detection of general protein contamination on surgical instruments (Lipscomb et al., 2006b), and more recently we have modified this technique for the detection of amyloid-like proteins using fluorescent thiazole reagents (Lipscomb et al., 2007). At present no other molecule or microorganism than PrP^{Sc} have been clearly associated with TSEs, despite recent reports on infective tissues that appear PrP^{Sc}-free. The presence of amyloid aggregates as those which may be seen with PrP^{Sc} are therefore a useful, if not the only, preclinical indicator of a potential pathology. Since PrP^{Sc} appears to bind strongly to surfaces, the detection of amyloid aggregates among other proteinaceous contamination on surgical instruments might serve as a useful tool in the prevention of iatrogenic dissemination of TSEs. In addition, the detection of PrP^{Sc} or other amyloid aggregates in tissues remains the best approach for clinical diagnosis of TSEs and other amyloidoses, though this is most often performed *post-mortem* via Western blot or immunohistochemistry.

Although the presence of amyloid aggregates is admittedly only a pathological indicator and does not directly correlate with the potential infectivity of a particular sample, a sensitive method to ensure that reused surgical instruments are free of general protein soil, especially detectable amyloid-containing deposits, would be a major advance in the prevention of potential iatrogenic contamination during surgical procedures. Accordingly, we have developed a new dual staining procedure which allows the rapid and sensitive quantification of amyloid content in proteinaceous deposits. Here we show the potential of this technique applied to the detection of the pathological amyloid aggregates in sample tissues, and show how tissue lipids may be a factor contributing to reduced sensitivity of such direct detection assays. We also demonstrate how this method may be used to evaluate decontamination practices using test surgical instruments spiked with better defined test soils, in terms of protein and amyloid concentration.

2. Methods

Preparation of samples for the determination of the lowest limit of detection of amyloidogenic prion in homogenates.

Brain homogenates from ME7-infected mice and control normal brain homogenate (NBH; both from TSE Resource Centre, Institute for Animal Health, Newbury, UK) were assayed and normalized to a protein concentration of 1 mg ml^{-1} in deionised water with 0.1% (v/v) Tween 20 (Sigma) to improve homogenisation for calibration (see results), aliquoted and stored at −20 °C until use. On the day of each experiment, ME7-infected homogenate and NBH stock aliquots were mixed in various proportions (from 0 up to 100% ME7 in NBH), anonymised, and kept in ice until staining and observation (usually within 1 h). One sample containing NBH only was clearly labelled as "negative control". Surgical stainless steel tokens were thoroughly cleaned as described previously (Lipscomb et al., 2007), and a 1 µl drop of each anonymised sample and the negative control was applied and dried on the tokens surface for 30 min at 37 °C prior to staining.

2.1. Dual staining procedure

We developed a dual staining protocol based on our previously described methods for the rapid detection of general protein contamination (Lipscomb et al., 2006b) and the specific detection of amyloid-like formations on surgical instruments (Lipscomb et al., 2007), with slight modifications. All incubations were performed at room temperature, and samples were protected from direct light as much as possible to prevent fading of the fluorescent signal. A thioflavin T (ThT; Sigma) working solution (0.2% w/v) was prepared shortly before use in 0.01 M hydrochloric acid, and applied onto samples for 10 min. Samples were washed by gentle immersion (no flushing) and incubated for 10 min with 0.1% (v/v) acetic acid, followed by a wash in PBS and another wash in deionised water. SYPRO Ruby solution (SR; Invitrogen, UK) was then applied for 15 min, followed by 3 final washes in deionised water. Excess water was dried off and samples were microscopically examined immediately after staining under an EDIC/EF microscope.

2.2. Microscopy

EDIC/EF microscopy and its application for the detection of proteinaceous deposits on surfaces have been described previously (Keevil, 2003; Lipscomb et al., 2006b, 2008). Volunteers were asked to examine each anonymous sample in two steps. Firstly, a scan of the whole area of surgical stainless steel covered by each sample was performed at 100× magnification using the SR filter set (excitation: 470 nm; emission: 618 nm; Nikon) to assess the total amount of protein still present after the dual staining procedure. Secondly, observers were asked to repeat the observation under 1000× magnification and select 10 areas for sampling and analysis, excluding relatively big tissue aggregates if present. Images of these areas were then captured using a CCD camera for both the ThT (excitation: 450 nm; emission: 480 nm; Nikon) and SYPRO Ruby signals. For each sample 10 areas of interest were used for further analysis.

2.3. Image analysis

Images were analysed using the ImagePro plus software (Media Cybernetics, Bethesda, MD, USA). To ensure that the sampling was homogeneous and the same amount of tissue was observed in each sample, the total amount of protein observed was estimated from the SR staining. For each experiment, the minimum threshold was