*Sveriges lantbruksuniversitet*
Fakulteten för Veterinärmedicin och husdjursvetenskap
Institutionen för biomedicin och veterinär folkhälsovetenskap

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Viktor Ahlberg

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Viktor Ahlberg

*Handledare: Tommy Linné, Institutionen för biomedicin och veterinär folkhälsovetenskap*

*Examinator: Caroline Fossum, Institutionen för biomedicin och veterinär folkhälsovetenskap*

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## SUMMARY

Scrapie is an infectious neurologic disease in sheep caused by prions, corresponding to transmissible spongiform encephalopathies (TSEs) in other species. The prion is presumably constituted of $PrP^{Sc}$, the misfolded form of the normal endogenous prion protein, $PrP^{C}$, which is found in practically all cells in the body. $PrP^{Sc}$ can induce a conformational change in $PrP^{C}$ and misfold it as an imprint of itself; this is how prions amplify and spread. The process of conformational change is poorly understood and there might exist intermediate forms between $PrP^{C}$ and $PrP^{Sc}$. Bioassay using mice has traditionally been the golden standard in prion studies but is limited by cost and time. Cell culture studies are now used but often rely on using so called rodent-adapted strains and/or cells overexpressing the prion protein.

In this study we made an attempt to infect FLK-BLV cells (ovine cells persistently infected with bovine leukemia virus) with brain material from a Swedish scrapie case in 1986. We have also developed a sensitive western blot assay that detects prion formation but also conformational changes in the prion protein. Digestion with proteinase K for different periods of time is used to establish conformation. No $PrP^{Sc}$ was formed in the inoculated cells but there were indications of increased resistance to proteinase K, perhaps reflecting an intermediate form of the prion protein in the cells. Further studies on this cell system are needed before substantial conclusions can be made.

## SAMMANFATTNING

Scrapie är en infektiös neurologisk sjukdom hos får orsakad av prioner, motsvarande transmissibla spongiforma encefalopatier (TSE) hos andra arter. Prioner består av $PrP^{Sc}$ vilket är den felveckade formen av det cellegna prionproteinet, $PrP^{C}$, som finns i praktiskt taget alla celler i kroppen. $PrP^{Sc}$ kan inducera en konformationsändring i $PrP^{C}$ i vilken den senare felveckas att likna $PrP^{Sc}$; det är på detta sätt som prioner mångfaldigas och sprider sig. Processen bakom konformationsändringen är i stort okänd och det kan finnas intermediära former mellan $PrP^{C}$ och $PrP^{Sc}$. Traditionellt har djurmodeller med möss använts inom prionforskningen men detta är kostsamt och tidskrävande. Studier i cellkulturer används numer men dessa är ofta beroende av musadapterade stammar och/eller överuttryck av prionproteinet i cellerna.

I denna studie har vi försökt infektera FLK-BLV-celler (ovina celler persistent infekterade med bovint leukemivirus) med hjärnmaterial från det svenska scrapiefallet 1986. Vi har även utvecklat en känslig western blot-analysmetod som undersöker förekomst av prioner men även konformationsändring. Detta görs genom att digerera prionproteinet med proteinas K under olika lång tid. I studien fann vi inget $PrP^{Sc}$ men det finns indikationer på en ökad resistens mot proteinas K vilket kan tyda på intermediära former av prionproteinet i cellerna. Fortsatta studier på detta cellsystem behövs för att kunna dra några betydande slutsatser.

# INTRODUCTION

## Scrapie

### *History*

Scrapie is a disease in sheep and goats that has been known for more than two hundreds years; the earliest descriptions of the disease are from the mid 18$^{th}$ century. The neuronal vacuolization in the brains of scrapie-sick sheep, typical of all TSEs, were discovered as early as in the late 19$^{th}$ century. In the 1920s it was suspected that scrapie was a contagious disease and in 1939 the first successful experimental transmission of scrapie was reported (reviewed in Aguzzi & Polymenidou, 2004). Despite this it was not until quite recently that the agent behind the disease, and other TSEs, was found. In 1982 Prusiner presented his hypothesis that scrapie was caused by an agent, partially or fully, constituted of proteins and at the same time introduced the concept *prion* (Prusiner, 1982). This hypothesis has been refined and is now generally accepted although not yet proved (Weissmann, 2004). Of the TSEs, scrapie has been known for the longest period of time and is also the most widespread (Bulgin & Melson, 2007) and has thus been studied the most (Hunter, 1998).

### *Pathogenesis*

The disease mainly affects blackface sheep breeds, for example Suffolk and Hampshire. It is also linked to polymorphisms at three codons, amino acids 136, 154 and 171 (Bulgin & Melson, 2007). The alleles VRQ and ARQ predispose for disease whereas ARR is associated with resistance to disease (the letters represent the amino acids of the three codons where V stands for valine, R for arginine, Q for glutamine and A for alanine) (Jeffrey & Gonzalez, 2007).

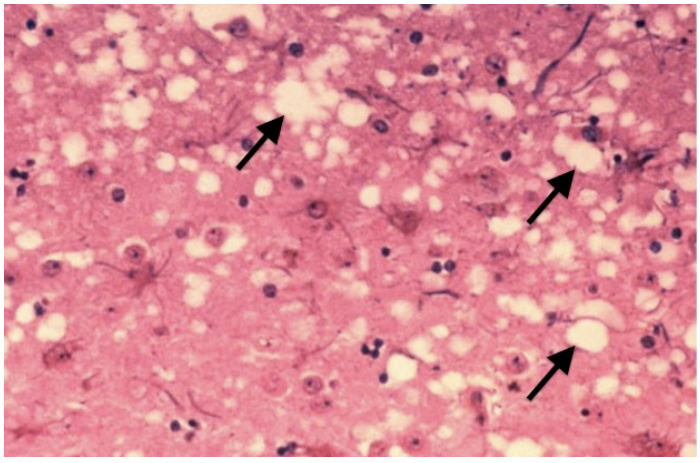

*Figure 1. Histologic view of brain with prion disease showing the characteristic vacuolation (arrows). Picture is public domain and modified by author.*

The disease is characterized by vacuolation, astrocytosis and neuronal loss in the brain (see figure 1), although all these changes are not always seen. It is also associated with accumulation of abnormal forms of the prion protein, $PrP^{Sc}$ (prion protein *scrapie*), in the brain. The neuroanatomical distribution differs by different scrapie strains and sources (Jeffrey & Gonzalez, 2007). $PrP^{Sc}$ deposits in the brain can be seen as amyloid fibers, diffuse deposits or plaques (Riesner, 2003). The pathogenesis doesn't include any inflammatory response (Hunter, 1998). Accumulation of $PrP^{Sc}$ occurs initially in central nerves that have connection with

the enteric nervous system (ENS), for example the vagus nerve. It is assumed that neuroinvasion follows infection of the ENS after acquiring the prion orally (Jeffrey & Gonzalez, 2007).

In animals with clinical disease $PrP^{Sc}$ can be found in the peripheral nervous system, blood as well as in all lymphoid tissue except thymus, in addition to the CNS (Terry *et al.*, 2009; Jeffrey & Gonzalez, 2007). The cause of clinical disease in scrapie is probably not due to the morphological signs of vacuolation, astrocytosis and neuronal loss. There are at least no relation between area of neuronal loss and the nature of mental changes. Because of vague early symptoms it is hard to find a correlation between morphological changes and the clinical deficits (Jeffrey & Gonzalez, 2007).

### *Clinical manifestation*

Scrapie is a long-term progressive, invariably fatal, neurologic illness. Signs usually appear 18 months to 5 years after exposure. It generally starts with impaired social behavior followed by progressive ataxia, fine head tremors and cutaneous hypersensitivity. Animals often show progressive weight loss, which can become severe, without loss of appetite. Affected animals can also show behavior changes such as assuming a fixed stare or becoming aggressive. Hypersensitivity often shows as rubbing or scratching on the back or throwing its head back biting at the limbs but also chewing motions and licking air. Ataxia is first shown when running; hind- and forelimbs become uncoordinated resulting in a "bunny-hopping gait" and when worsened the hind limbs may sway when the animal is standing (Bulgin & Melson, 2007).

Clinical signs last for 1 to 3 months, sometimes longer. In final stages animals generally become recumbent and death follows within 1 to 2 weeks after the animal is able to right itself (Bulgin & Melson, 2007). In some cases animals die after a very short incubation time and/or duration of illness. There are even records of animals dying without obvious prior signs (Jeffrey & Gonzalez, 2007). These variations are caused by different genotype and strain of scrapie (Bulgin & Melson, 2007).

### *Diagnosis*

Sheep do not mount an immune response to $PrP^{Sc}$ and histological changes can be unapparent and all diagnosis is thus based on detection of $PrP^{Sc}$ in tissue. The golden standard is immunohistochemical testing of post mortem biopsy material from brain or lymphoid tissue. Detection of $PrP^{Sc}$ prior clinical signs has been done in blood, lymph nodes, third eye lid, tongue, retina, rectal mucosa and spinal fluid (Bulgin & Melson, 2007). Diagnosis can also be done on brain homogenate using western blot (SVA, 2005). The authority responsible for scrapie testing in Sweden, the Swedish National Veterinary Institute (SVA), uses an ELISA test as screening and western blot for confirmation. The latter is enough to differentiate scrapie from Nor98 but immunohistochemistry is still needed to exclude BSE (Renström, 2009).

### *Disease distribution, prevention and control*

There has only been one known outbreak of scrapie in Sweden when two ewes was diagnosed with the disease in 1986 (Elvander *et al.*, 1988). Scrapie exists in

most European countries and both Denmark, Norway and Finland (neighbors of Sweden) have had cases reported in the last few years (OIE, 2009). In the United States the prevalence is between 0.1% and 0.3% and it was presumed to be 0.5% before their eradication program (Bulgin & Melson, 2007). In Sweden surveillance testing is done on clinical suspicion and on animals dying without prior clinical signs. Routine testing in slaughterhouses was done before but this program is terminated (Renström, 2009). In the United States, where scrapie is endemic, there are extensive eradication programs based on susceptible and resistant genotypes and all sheep that die unexpected is advised for a complete autopsy with testing of the brain for scrapie (Bulgin & Melson, 2007).

## Other prion diseases

### *Nor98*

Nor98 is an atypical form of scrapie, affecting sheep, which was first diagnosed in Norway in 1998 (thus the name). Since then it has been found in most European countries (Benestad *et al.*, 2008) as well as in the United States (Bulgin & Melson, 2007). It is associated with $PrP^{Sc}$ accumulation, just as in scrapie and BSE, but shows different patterns on western blot as well as on histopathology and immunohistochemistry. It can be experimentally transmitted to both sheep and mice but epidemiology and biological features suggests it being non-contagious. It is speculated that this disease is spontaneous, much like sporadic Creutzfeldt-Jakob disease in humans (Benestad *et al.*, 2008).

### *Bovine spongiform encephalopathy*

The bovine spongiform encephalopathy (BSE) created a massive epidemic in Europe, predominantly in Great Britain, from when first reported in 1987 and the following ten years (Bradley, 2002). The total number of infected animals is estimated to one million and in 1992 40,000 cattle died of BSE in Great Britain, which was the largest annual number of cases (Prusiner, 1997). Since then the disease has been decreasing and is now practically extinct (Bradley, 2002).

It is assumed that the outbreak was caused by modifying the rendering process of meat and bone meal (MBM). The change made it possible for prions to survive, which created a magnifying cycle process when fed to cattle (Prusiner, 1997). Feeding MBM to mammals, and also fish, was later banned. Because of the long incubation time, a mean of five years, the cases still accumulated for some time before declining (Bradley, 2002). BSE can infect a wide selection of mammals including humans where it cause variant Creutzfeldt–Jakob disease. Spreading of the disease to humans was the main concern in the BSE crisis (Prusiner, 1997).

### *Creutzfeldt–Jakob disease*

Creutzfeldt–Jakob disease (CJD) is a term for several human prion diseases that can be sporadic, genetic or infectious. All of them cause progressive dementia and ataxia and have typical spongiform appearance in microscopic examination of the brain. Sporadic CJD is the most common but it is still considered idiopathic, i.e. the cause is unknown. Suggested causes are somatic mutation, horizontal transmission and spontaneous conversion. Familial CJD is caused by different hereditary mutations in the prion protein gene that predisposes for misfolding. Variant CJD is caused by ingestion of bovine prions from BSE cases. This disease

differs from the two previous mentioned by mainly affecting younger people, most cases found are people below 40 years. Iatrogenic CJD is caused by infection with prion-contaminated tissue, for example some cases of medication with growth hormone (Prusiner, 1997).

## Cellular biology of prions

### *Protein-only hypothesis*

Prusiner formulated the protein-only hypothesis about 25 years ago, arguing that the scrapie agent is mainly or solely constituted of proteins. He showed that the agent is unaffected by methods that modify or destroy nucleic acid but methods that affect proteins inactivates its infectivity. This was done after noticing that the scrapie agent was unusually resistant to radiation and UV lightning (Prusiner, 1982). He named the agent *prion* and later defined that "[a] prion is a proteinaceous infectious particle that lacks nucleic acid" (Prusiner, 1998).

*Table 1. Some arguments for the protein-only hypothesis, modified from Prusiner (1998).*

| | |
|---|---|
| 1. | PrPSc and scrapie infectivity copurify when biochemical and immunologic procedures are used. |
| 2. | The unusual properties of $PrP^{Sc}$ mimic those of prions. Many different procedures that modify or hydrolyze $PrP^{Sc}$ inactivate prions. |
| 3. | Levels of $PrP^{Sc}$ are directly proportional to prion titers. |
| 4. | No evidence exists for a virus-like particle or nucleic acid genome. |
| 5. | Accumulation of $PrP^{Sc}$ is invariably associated with the pathology of prion diseases, including PrP amyloid plaques that are pathognomonic. |
| 6. | PrP gene mutations are genetically linked to inherited prion disease ad cause formation of $PrP^{Sc}$. |
| 7. | Knock out of the PrP gene eliminates the substrate necessary for $PrP^{Sc}$ formation and prevents both prion disease and prion replication. |

Final proof of the protein-only hypothesis is still lacking (Caughey & Baron, 2006) but several features make it evident that the prion is actually the agent for scrapie and other TSE's as seen in table 1 (Prusiner, 1998). The strong, but not complete, resistance to degradation with proteinase K (PK) supports the prion hypothesis. The idea of different isoforms of the protein was developed after the finding of non-resistant forms of the same protein in non-infected (Riesner, 2003). Maybe the strongest support for the protein-only hypothesis is that knockout mice without the normal prion protein $PrP^{C}$, so called $Prnp^{0/0}$ mice, are resistant to prion disease (Weissmann, 2004). Many attempts have been done to falsify the protein-only hypothesis, mainly focusing on finding foreign nucleic acid, but all have been unsuccessful (Safar et al., 2005b).

### *Endogenous $PrP^{C}$*

The normal, endogenous, prion protein $PrP^{C}$ (prion protein *cellular*) can be found in many different cell types in both mammals and other species (Ford *et al.*, 2002). Its presence in neuronal cells in the brain is of most concern, at least when considering prion diseases. The chemical properties of the prion protein are well known but its biological function is far from clear (Riesner, 2003).

The prion protein is a N-linked glycoprotein bound to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor. Depending on species it is approximately 200 amino acids in length and has a N-terminal region (residues 23-125) and a C-terminal region (residues 126-231) as seen in figure 2. The N-terminal is an unstructured and flexible tail whereas the C-terminal forms three alpha helices and one short beta sheet (Ronga *et al.*, 2006). The N-terminal includes two hexarepeats and five octarepeats (Riesner, 2003). The protein is translated in the endoplasmatic reticulum where residues 1 to 22 act as a signal peptide and is therefore cleaved. After that it gets glycosylated (mono- or di-) in the Golgi apparatus before transportation to the cell membrane (Riesner, 2003; Caughey & Baron, 2006). Residues 232 to 254 are replaced with the GPI-anchor needed for cell membrane attachment (Riesner, 2003).

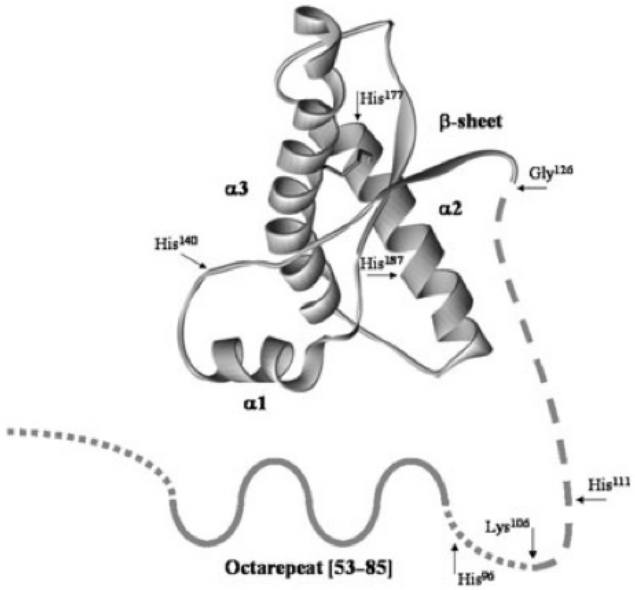


*Figure 2. The tertiary structure of the prion protein (human). Shows the three alpha helices, the beta sheet and the octarepeat. Glycosylation or GPI-anchor is not shown. From Ronga et al. (2006).*

In the cell membrane $PrP^C$ is located in so called lipid rafts which are cholesterol rich areas with generally a lot of surface receptors and cytoplasmic signaling molecules. Some propose that $PrP^C$ binds to some of these molecules and that it might have a function in cell signaling (Taylor & Hooper, 2006). It has also been shown that $PrP^C$ binds to many other different structures such as sulphated glycosaminoglycans (GAGs) and extracellular-matrix proteins. It also binds copper in the form of $Cu^{2+}$ within the octarepeats. The reason to this is unknown but it doesn't happen with other divalent cations (Ronga *et al.*, 2006).

### *Formation and structure of $PrP^{Sc}$*

$PrP^C$ and $PrP^{Sc}$ have the same primary structure but differ in their secondary and tertiary structure. Where $PrP^C$ has about 40% of alpha helices and very little beta sheets, $PrP^{Sc}$ consists of about 20% alpha helices and 34% beta sheets. The exact tertiary structure of $PrP^{Sc}$ is unknown as it is insoluble which makes analysis with X-ray or nuclear magnetic resonance difficult (Riesner, 2003). After moderate digestion with proteinase K a fragment of about 27-30 kDa will remain, which for example can be detected using western blot. This fragment is called $PrP^{27\text{-}30}$ and

shares many characteristics with $PrP^{Sc}$, for example its infectivity. Depending on strain the cleavage is done at amino acids 87 – 91 (Weissmann, 2004).

Formation of beta sheet rich $PrP^{Sc}$ always leads to oligomerization. Larger such structures can be seen in electron microscopy as fibrillar rods, being amyloid fibers (Riesner, 2003). The alpha2-helix of amino acids 173-195 has strong preference of conversion to beta sheet, and this could be a key area for conversion of $PrP^{C}$ to $PrP^{Sc}$. This area has also been shown to be an oligomerization site (Ronga *et al.*, 2006). $PrP^{Sc}$ is membrane bound just as $PrP^{C}$ unless accumulated as amyloid fibers (Riesner, 2003). Knockout mice lacking the GPI anchor (with the PrP not membrane bound) don't get clinical disease, showing that anchoring is a prerequisite for toxicity (Ronga *et al.*, 2006). If the prion protein is located out of the lipid raft, by experimentally changing the membrane anchor, it can also not be converted to $PrP^{Sc}$ (Taylor & Hooper, 2006).

### *Propagation of $PrP^{Sc}$*

Studies with cell cultures have shown that conversion of $PrP^{C}$ to $PrP^{Sc}$ occurs on the cell surface. $PrP^{Sc}$ induces a conformational change in $PrP^{C}$ to adopt its structure and probably adding to a $PrP^{Sc}$ oligomer. $PrP^{Sc}$ can be accumulated on cell surface, in intracellular vesicles or in extracellular deposits. The most infectious unit of $PrP^{Sc}$ has shown to be non-fibrillar oligomers of 14 to 28 PrP-molecules (Caughey & Baron, 2006). It is possible, or even probable, that another substance is needed for propagation. This substance has earlier been designated "Protein X" but could perhaps be glycosaminoglycans or host-derived nucleic acid (Weissmann, 2004; Caughey & Baron, 2006). The fact that localization to lipid rafts is necessary for conversion supports the hypothesis of a co-factor. There are two explanation models for $PrP^{Sc}$ propagation, the template-directed refolding model and the seeded nucleation model, as shown in figure 3.

#### *Template-directed refolding*

The template-directed refolding model, also called the refolding model, propose that $PrP^{C}$ cannot convert spontaneously to $PrP^{Sc}$ within detectable levels. $PrP^{Sc}$ works as a template and causes a conformational change in $PrP^{C}$. This could be facilitated by some other structure. Spontaneous formation can occur as a rare event or secondary to a mutation that makes $PrP^{C}$ instable (Weissmann, 2004).

#### *Seeded nucleation*

The seeded nucleation model argues that $PrP^{C}$ and $PrP^{Sc}$ are in equilibrium, although highly shifted towards $PrP^{C}$. "Seeds" of $PrP^{Sc}$ oligomers serve as foci for stabilization and accumulation of $PrP^{Sc}$ that lead to further propagation. Seed formation is assumed to be slow whereas subsequent addition of single $PrP^{Sc}$ molecules occurs rapidly. Larger aggregates have to be fragmented to create more numerous seeds for the infection to spread (Weissmann, 2004).

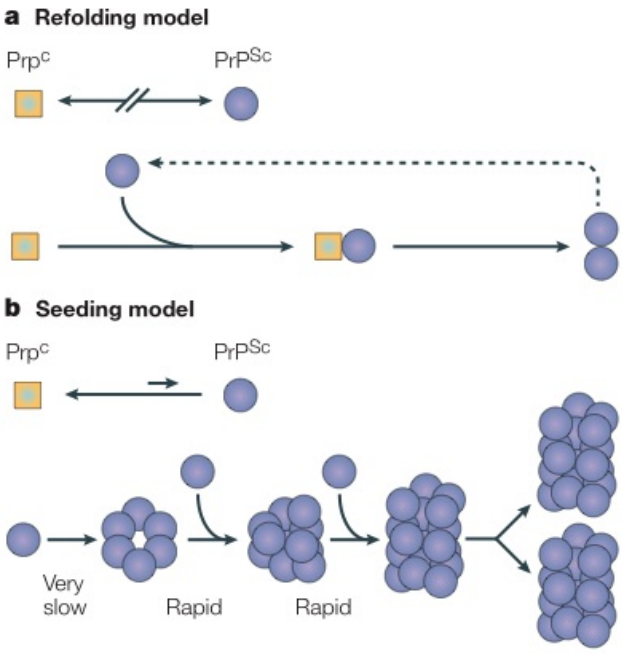


*Figure 3. Brief schematics of the two main prion propagation models. From Weissmann (2004).*

### *Species barrier*

Transmission between mammalian species is far less effective than within species and this is called the species barrier. Generally, in an initial passage from species A to B not all animals get infected and the incubation time is longer (than within the same species). For subsequent transmissions from B to B, parameters resemble intra-species transmission. Even though prions propagate easier if the primary structure is identical (same host species) some characteristics are kept even if passaged through another species. For example does vCJD infect mice whereas classical CJD doesn't despite both of them having the same primary structure (Collinge & Clarke, 2007).

### *Prion strains*

A concept somewhat related to the species barrier is prion strains. These strains, constituted of different prion agents, were originally characterized by incubation time and neuropathology. Nowadays we also see differences in resistant to proteinase K-digestion, cleavage sites (forming different fragment sizes), stability to denaturation agents and the ratio of un-, mono- and diglycosylation (Weissmann, 2004). Strains cannot be defined from the protein's primary structure because characteristics are kept if passaged through another species (Collinge & Clarke, 2007) and even if passaged in cell culture and then inoculated back to an animal (Vilette, 2008). Strains passaged through another species is said to be *adapted* and for example are rodent-adapted strains widely used (Collinge & Clarke, 2007).

It is not clear how a polypeptide or protein can encode multiple disease phenotypes. Different isoforms of the PrP are assumed to be the cause and in that case these have to be able to imprint their conformation on $PrP^C$. The fact that strains have different fragment sizes (when digested with proteinase K) supports this idea (Collinge & Clarke, 2007). However, this hypothesis demands a vast number of

conformations of the prion protein, although they could to some part be constituted of poly- and oligomers (Weissmann, 2004). Glycosylation could also be a part to this concept as glycosylation ratio is a strain characteristic. The prion strains can mutate, mainly if transferred to a new host or if the primary structure is different (as in polymorphism) but even among animals with the same primary structure. Some strains are more prone to mutate than others (Collinge & Clarke, 2007).

#### *Other prion hypothesis*

Some still advocate that TSE's are probably not caused by prions but instead a small virus or virion. Manuelidis (2007), for example, argues that prions do not fulfill Koch's postulate for infection, pointing out that no recombinant or artificial prions have ever been created. There are also claims of a 25-30 nm large virus-like particle associated with infectious homogenates. The virus hypothesis is also supported by the fact that $PrP^{Sc}$ concentration is not proportional to infectious titers (Manuelidis, 2007).

### Assay techniques

Most assays for prions rely on detecting proteinase K-digested $PrP^{Sc}$ since it is easily separated from endogenous $PrP^{C}$. Type of sample and method differs; those mainly used are Western blotting, enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry. Animal bioassay, inoculation of test animals, is also used extensively and has until for a few years ago been the golden standard. Today other techniques match the reliability and accuracy of the bioassay. However, all other methods assume that $PrP^{Sc}$ is identical with the prion, so for infectivity analysis bioassay is still needed (Sakudo *et al.*, 2007).

There is no method that can detect proteins as well as there are for nucleic acid (Galbraith, 2002). Quite recently, though, a protein misfolding cyclic amplification (PMCA) method was described that can amplify infectious titers of $PrP^{Sc}$. This can enhance the sensitivity for many of the assays. There exist both antibodies and aptamers that can discriminate between $PrP^{C}$ and $PrP^{Sc}$ although these have not yet been sufficiently evaluated and are not used in practice. Aptamers are nucleic acid or peptides that specifically binds to a target protein, much like an antibody (Sakudo *et al.*, 2007).

#### *Immunohistochemistry*

Immunohistochemistry is done on formalin fixed or frozen tissue samples. The method detects $PrP^{Sc}$ in situ, which means both detection for diagnosis and the distribution in tissue (Grathwohl *et al.*, 1997). It can be used for many different tissues and organs. It is considered to be the golden standard for prion diagnosis in the US and is also confirmatory test at some of the World Health Organization's laboratories (Bulgin & Melson, 2007).

#### *Western blot*

Western blot, or protein immunoblot, is a method to detect proteins in a given sample. It consists of a gel electrophoresis, which separate the proteins depending on size or structure, after which the proteins are transferred to a membrane. Proteins are then visualized using primary antibodies, directed towards the sought

protein, and a marked secondary antibody. Marker can be based on chemiluminescent or radioactivity, for example (Abbas *et al.*, 2010).

As you can also see glycosylation of proteins on Western blot, the prion protein will show itself as three bands representing the un-, mono- and diglycosylated form. With diglycosylation treatment, for example with PNGase F, you will only see the full-length protein (and the C1 fragment). With proteinase K digestion, $PrP^{C}$ will disappear but $PrP^{Sc}$ will remain. $PrP^{Sc}$ will be somewhat cleaved and shifted downwards and show as a 27 - 30 kDa fragment, $PrP^{27\text{-}30}$ (or its glycosylated forms) (Weissmann, 2004).

Different prion strains have different glycosylation pattern depending on the strain. Thus, with Western blot you can differentiate between strains, and also between BSE, scrapie and the different forms of CJD (Sakudo *et al.*, 2007).

### *ELISA*

Enzyme-linked immunosorbent assay (ELISA) is used to identify antigen or antibodies. When detecting the agent, an antibody bound to an enzyme is added to the sample. After this a substance is added that the linked enzyme can convert to a detectable signal. Most new ELISAs use fluorescence for detection (Abbas *et al.*, 2010).

For some ten years, ELISAs for prion diagnosis has been available that match the sensitivity of Western blot. Since this method is fast this has reduced the analysis time, although it still needs time-consuming sample preparations (Grathwohl *et al.*, 1997). Today there is at least four ELISAs used in the EU for surveillance on scrapie although none of these has been validated in the US (Bulgin & Melson, 2007).

### *Bioassay*

Intracerebral inoculation on sheep and goats as bioassay was done as early as in the 30s and later on scientists began to work with rodents (Galbraith, 2002). Initially mice were used and about 60 mice were needed for the titer of one sample and it took more than 12 month. With the “incubation time bioassay” and using Syrian hamsters only four animals are needed. This reduced incubation time to 70 days speeding up research almost 100-fold (Prusiner, 1998).

Today, animal studies are the golden standard for detection of prion infectivity (which not necessarily has to be the same as $PrP^{Sc}$). Recently, transgenic mice that overexpress $PrP^{C}$ have led to even shorter incubation times (Kocisko & Caughey, 2006). Mice that express species-specific $PrP^{C}$ without any background (no endogenous $PrP^{C}$) are generally used as these are very sensitive to infection (Grathwohl *et al.*, 1997). Incubation time in mice is shorter and less variable with higher doses of agent. However this also reduces the possibility to test anti-prion substances, which in one major reason for bioassays. For an incubation time of about 70 days in the Tg7 mouse it is needed 50 µl of 0.001% infectious brain homogenate, but higher dosage can reduce the time to 44 days (Kocisko & Caughey, 2006).

## Prions in cell culture

Cell cultures can propagate prions with maintained infectivity. This allows for easier research on the cell biology of $PrP^{C}$ as well as $PrP^{Sc}$, the pathophysiology of prion disease and to determine strain characteristics. Cell cultures can also be used in diagnosis (Solassol *et al.*, 2003) and is of value when looking at the genetic basis for prion infection (Vilette, 2008).

Several cell lines are permissive to rodent-adapted strains of prions. Cells of neuronal origin are most common but for example fibroblasts, muscle cells and microglial cells are also used. The most used cell lines are the N2a (mouse neuroblastoma) and the RK13 (rabbit kidney epithelia) cell lines. In 2001 the first successful attempt to infect a cell culture with natural TSE agent was done. As of now there are three known cell models for natural scrapie agent, two of these are from ovine transgenic mice and one are rabbit cells expressing the ovine prion protein (see table 2). No cell model for Creutzfeldt–Jakob disease or bovine spongiform encephalopathy exists yet. Generally each cell line is only susceptible to one or a few scrapie strains and the polymorphism in the sheep prion protein plays an important role (Vilette, 2008).

*Table 2. A few of the available cell lines susceptible to prion infection. Modified from Vilette (2008).*

| Cell line | Original cell type | Species | Comment |
|---|---|---|---|
| *Rodent-adapted strain* | | | |
| N2a | Neuroblastoma | Mouse | |
| GT1 | Hypothalamic cells | Mouse | |
| moRK13 | Kidney epithelial cells | Rabbit | RK13 with mouse $PrP^{C}$ |
| PC12 | Pheochromocytoma | Rat | |
| *Natural scrapie agent* | | | |
| ovRK13 | Kidney pithelial cells | Rabbit | RK13 with ovine $PrP^{C}$ |
| MovS | Schwann-like from dorsal root ganglia | Transgenic mice | Mice with ovine $PrP^{C}$ |
| $CGN^{ov}$ | Neuronal cells | Transgenic mice | Mice with ovine $PrP^{C}$ |

Basically, infection of cell cultures with prions is done by incubating the cells with a prion preparation, e.g. brain homogenate. Some studies show that the infection is much more efficient if using already infected living cells as inoculum (Kanu *et al.*, 2002). On the other hand, when using brain homogenate, prior sonication and treatment in 80°C reduce the risk of bacterial and viral contamination and reduce toxicity to cells (Solassol *et al.*, 2003). Overexpression of $PrP^{C}$ is not required but a similarity in the primary sequence of the cell line and the inoculum is thought to be essential (Weissmann, 2004). Infecting the cell cultures with some retroviruses has proven to increase both the susceptibility and the accumulation of prions (Leblanc *et al.*, 2006; Stanton *et al.*, 2008). Table 3 shows a short summary of some protocols used when infecting cell cultures with prions.

To create a permanently infected cell culture the propagation of the prion needs to be within certain limits. Splitting cells to often in relation to propagation will dilute the prions and they will disappear, splitting them to seldom and you will

reach an upper level of toxicity leading to cell death. This has been called the "dynamic susceptibility model" (Weissmann, 2004). It has also been shown that cell cultures can degrade $PrP^{Sc}$, which further supports the idea that infection is more about balance than simply an on/off-state (Vilette, 2008).

*Table 3. Different protocols used for cell infection with prions. In some protocols more medium is added after a while and cells are incubated further, thus "initial" and "secondary" incubation. "Rest phase" refers to the time from washing the cells to splitting.*

| Reference | Homogenate preparation | Homogenate concentration | Initial incubation | Secondary incubation | Rest phase |
|---|---|---|---|---|---|
| Bosque & Prusiner, 2000 | None | 0.33% | 4 days | None | None |
| Vilette *et al.*, 2001 | Heating and sonication | 2.5% | 6 h | 48 h | 60 h |
| Kanu *et al.*, 2002 | Sonication | 2% | 5 h | 18 h | Until confluent |
| Archer *et al.*, 2004 | None | 2.5% | 4 days | None | 24 – 48 h |
| Lehmann, 2005 | None | 2% / 0.2% | 5 h | 14 – 16 h | 24 h |
| Vella *et al.*, 2007 | None | 1% / 0.1% | 5 h | 72 h | None |
| Courageot *et al.*, 2008 | Sonication | 2.5% | 48 h | None | None |
| Lawson *et al.*, 2008 | None | 1% / 0.1% | 5 h | 72 h | None |

## Aim of project

The aim of this project was to infect FLK-BLV cell cultures with prions from a scrapie-infected brain from one of the Swedish cases in 1986. As a part of this we looked for a new more sensitive assay to detect infection of prions in cell culture. This cell line could create new possibilities to investigate prion protein conformational changes as well as a basis for therapeutic research.

## MATERIAL AND METHODS

### Cell line

Fetal Lamb Kidney cells permanently infected with Bovine Leukemia Virus (cell line 44/2), denoted FLK-BLV, (Van Der Maaten & Miller, 1975) were acquired from Svanova Biotech, Uppsala. All cell cultures were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Lonza) supplemented with 100 IU/ml penicillin, 200 μg/ml streptomycin, 2 mM L-glutamine and 5% Fetal Calf Serum (Invitrogen) and this solution is further denoted DMEMcomplete. Cell cultures were kept at 37°C in a humidified atmosphere with 5% $CO_2$ in a HERAcell incubator (Heraeus).

### Brain material

Frozen brain material from one of the two Swedish scrapie cases in 1986, journal number 311/86, was a gift from SVA (Marianne Elvander). Preparation of brain homogenate was done according to Lawson *et al.* (2008). 0.18225 g of brain material was added to 9 volumes, 1.64 ml, of PBS. This was passed through 18, 23 and 26 gauge needles. When needed, gross cellular debris was removed with a clearing spin at 100 *g* for 1 min. Aliquots of 200 μl homogenate were stored in 1.5 ml microtubes at -20°C.

### Infection of FLK-BLV cell cultures

Brain homogenate was sonicated three times 15 seconds with 45-second breaks, heated to 80°C for 20 minutes and then centrifuged at 8,000 *g* for 10 seconds and cooled to room temperature. The homogenate was diluted with 4 volumes of DMEMcomplete to a brain homogenate concentration of 2%. 2 ml of this solution was added to a 25 $cm^2$ flask with FLK-BLV cells at 70% confluence and inoculated for 5 hours at 37°C. After initial incubation, 2 ml DMEMcomplete was added and the cell culture was incubated for 67 hours at 37°C. Brain homogenate was then removed by rinsing the cell culture twice with PBS before incubating for 72 hours in DMEMcomplete.

Day 6 post infection the cell culture was transferred from the 25 $cm^2$ flask to a 75 $cm^2$ flask. Day 13, 20 and 27 after infection one cell culture flask was split 1 to 3. Splits were performed by rinsing cells with PBS before incubating with trypsin at 37° until cells loosened from the flask, approximately 10 minutes. Trypsinized cells were transferred to new flasks and maintained in DMEMcomplete.

### Cell extraction

Cell cultures, both normal and those incubated with scrapie material, were lysed in lysis buffer (0.5% Triton X-100, 0.5% Sodium deoxycholate, 5 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA) and incubated for 10 minutes at 0°C. 1 ml lysis buffer was used for one 75 $cm^2$ flask and confluent cell cultures were used. Lysates were then clarified by centrifugation at 3,000 *g* for 5 minutes at 5°C and supernatants were transferred and stored at -20°C until analysis.

Cell lysates of the infection trial cultures were made day 20, 27 and 34 post infection. Two flasks were lysed each time. Cell lysates from normal FLK-BLV cell cultures were made continuously.

## Proteinase K digestion

In the assay evaluation prestudy, cell lysates and brain homogenate were either left undigested or digested with proteinase K (PK; Sigma) at a concentration of either 50 μg/ml or 10 μg/ml for 30 minutes at 37°C. Undigested samples were corrected with lysis buffer to make the same total volume. Digestion was terminated by addition of 5 mM phenylmethylsulphonyl fluoride (PMSF) and incubation for 10 minutes at room temperature.

To test the efficiency of proteinase K, cell lysates from uninfected FLK-BLV cells were subject to digestion with PK at a concentration of 2 μg/ml for 1, 2, 4, 8, 15, 30 and 60 minutes at 37°C. One undigested sample was used as reference. PK digestion was stopped as described above.

In the infection trial analysis, cell lysates were digested with PK at a concentration of 2 μg/ml for 2, 10 and 50 minutes as well as having one sample left undigested as reference. PK digestion was stopped as described above.

## Deglycosylation and protein precipitation

Denaturation of all samples was done by boiling with 10% Denaturation Buffer (New England BioLabs) for 10 minutes at 100°C. For deglycosylation, PNGase F (500,000 U/ml; New England BioLabs) was used in combination with 10% G7 buffer (New England BioLabs) and 10% NP40 (New England BioLabs) for 120 minutes at 37°C. Samples were then precipitated using trichloroacetic acid (TCA) for 30 minutes at 4°C and centrifugation at 18,000 *g* for 10 minutes at 4°C. Pellets were washed by suspension in a solution of 50% methanol and 50% acetic acid and centrifugation at 18,000 *g* for 5 minutes at 4°C after which the supernatant was removed and pellets were subjected to short evaporation by holding them close to a lamp.

## Western blot analysis

### *SDS-PAGE and transfer to PVDF-membranes*

Protein pellets were suspended in 30 μl 1x NuPAGE LDS Sample Buffer (Invitrogen) with addition of 3 μl 0,1 M dithiothreitol (DTT) and incubated at 100°C for 10 minutes before loading onto gels. Two markers were used on each gel, one with 0,5 μl MagicMark XP (Invitrogen) and 29,5 μl 1x LDS Sample Buffer and one with 10 μl SeeBlue Plus2 (Invitrogen), 0,5 μl MagicMark and 19,5 μl 1x LDS Sample Buffer. These were loaded onto gels without any prior treatment.

SDS-PAGE gel electrophoresis was run with 12% NuPAGE Novex Bis-Tris Gels (Invitrogen) with 1x NuPAGE MOPS SDS Running Buffer (Invitrogen) with 200 μl NuPAGE Antioxidant (Invitrogen) added to the load buffer (70 ml MOPS). Electrophoresis was run until the Coomassie G250 stain in the sample buffer reached the lower edge. Transfer was done using Hybond–P polyvinylidene difluoride (PVDF) membrane filters (GE Healthcare). Filters were first activated in methanol and as transfer buffer a solution of 20% methanol, 25 mM Tris and 192 mM glycine was used.

### *Immunoblotting and visualization*

Non-specific binding sites on the membrane filters were blocked using 5% milk powder (Semper) in PBS-Tween (PBST) incubated at room temperature for 60 minutes and then washed by incubation for 15 minutes in PBST. The monoclonal antibody 6H4 of mouse origin (0.56 μg/μl; Prionics AG) was used at a 1:30,000 dilution in PBST with 1% milk powder. This antibody binds to sheep prion protein amino acid residues 147–156 and thus recognizes full-length prion protein as well as both C1 and C2 fragments (Korth *et al.*, 1997). The antibody was incubated with membrane filters for 60 minutes at room temperature followed by four 5-minute washes with PBST. Anti-mouse ECL antibody, HRP linked, (GE Healthcare) was then added at a 1:15,000 dilution in PBST with 1% milk powder to the membrane filters for 60 minutes at room temperature. Finally, the filters were again washed with PBST four times, 5 minutes each.

For visualization, Immobilon Western chemiluminescent HRP substrate (Millipore) was used following immediate detection by exposure to X-ray films (Hyperfilm; GE Healthcare). Exposures were done for different periods of time and subsequently developed.

## RESULTS

### Assay evaluation prestudy

Initially a prestudy was carried out to evaluate the proposed assay. The expression of cellular prion protein, $PrP^C$, in the FLK-BLV cell culture was investigated. This cell culture express detectable amount of $PrP^C$ (Tommy Linné, unpublished data) and it has been shown in other cell lines that retroviruses can enhance the susceptibility to, and accumulation of, $PrP^{Sc}$ (Leblanc *et al.*, 2006). The brain homogenate from the scrapie-infected sheep was analyzed for the presence of both $PrP^C$ and $PrP^{Sc}$. Also the first passage of a previous infection trial (not mentioned further in the report) was analyzed for the presence of $PrP^C$ and $PrP^{Sc}$. These samples were analyzed with two different concentrations of proteinase K and two different cell lysis sample volumes. In this study the western blot detects the normal and abnormal forms of the prion protein as well as the size of the protein, as indicated by the markers.

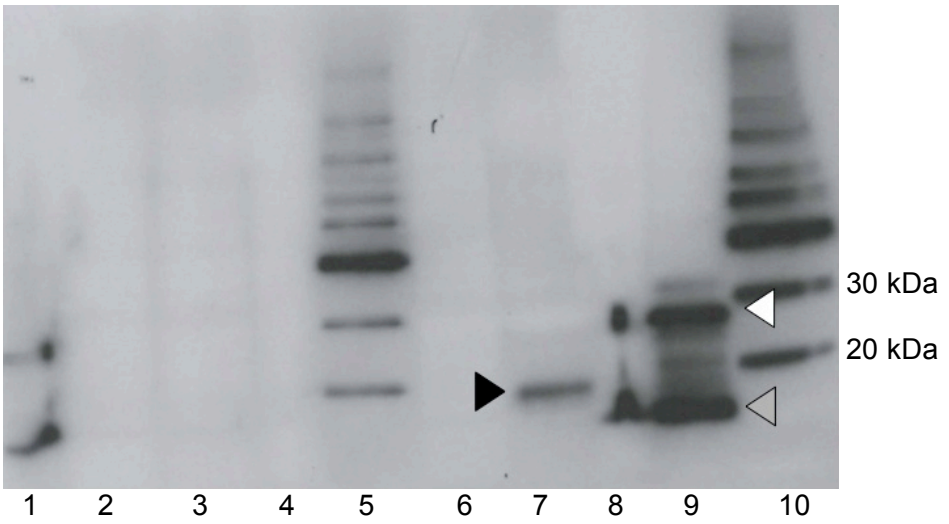


*Figure 4. Western blot assay prestudy using the 6H4 antiprion antibody. Lanes 1 to 4 show the first infection trial: undigested (lane 1), treated with 10 µg/ml PK (lane 2), with 50 µg/ml PK (lane 3) and the double sample volume treated with 50 µg/ml PK (lane 4). Lanes 6 and 8 show FLK-BLV cell lysates with and without 50 µg/ml PK treatment, respectively. Lanes 7 and 9 show scrapie brain homogenate with and without 50 µg/ml PK treatment, respectively. White arrowhead marks full-length prion protein, grey arrowhead marks the C1 fragment and black arrowhead marks the C2 fragment. All samples were deglycosylated and proteinase K treatments were done for 30 minutes. Lane 5 and 10 are markers showing the protein size.*

The western blot from the prestudy is shown in figure 4. The FLK-BLV cell line expresses well above detectable levels of $PrP^C$, as both the full-length prion protein and the C1 fragment can be seen (lane 8). This is fully digested after treatment with 50 µg/ml proteinase K for 30 minutes. The scrapie brain homogenate show both presence of full-length and the C1 fragment when undigested. After treatment with 50 µg/ml proteinase K for 30 minutes the C2 fragment is revealed in the scrapie brain, showing that there are prions in this sample. The cells from this infection trial express $PrP^C$ but when treated with proteinase K there is no C2 fragment indicative of prion infection (lanes 1 – 4). This is also valid for the lower concentration of proteinase K (10 µg/ml for 30 minutes), where the prion protein is still fully digested.

## Proteinase K kinetics study

To increase the sensitivity of the assay there was a need to find a more appropriate proteinase K activity instead of the 50 µg/ml (and 10 µg/ml) for 30 minutes used in the prestudy. This is the amount used in practical prion disease diagnosis and might not be suitable when using cell culture. A kinetics study was made with the aim to find the levels where the full-length and the whole protein (the C1 fragment) were degraded, respectively. The level of activity was based on literature and practical experience.

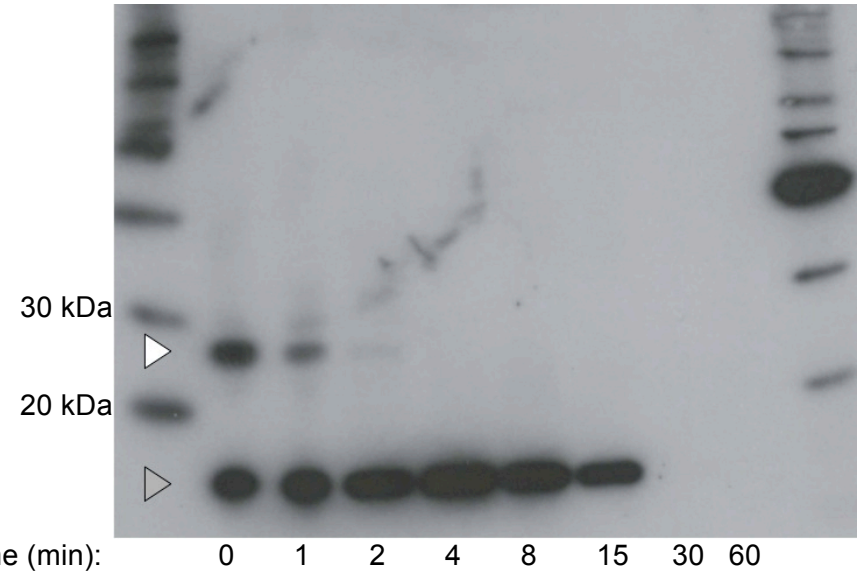


*Figure 5. Western blot showing proteinase K kinetics study on FLK-BLV cells. Samples was digested with 2 µg/ml proteinase K for the stated times. White arrowhead marks full-length prion protein, grey arrowhead marks the C1 fragment. All samples were deglycosylated. The antiprion 6H4 antibody was used. The first and last lanes are markers showing the protein size.*

Figure 5 shows that a treatment with 2 µg/ml proteinase K for 30 minutes is sufficient to digest all endogenous $PrP^C$ in BLV/FLV cell lysates. Also, the full-length prion protein is digested after only 2 minutes of treatment. The initial increase of the C1 fragment after 2 to 4 minutes indicate that the full-length protein is degraded to the C1 fragment.

## Infection trial analysis

The first infection trial proved ineffectual (data not shown) and is excluded. A second infection attempt was done with a different protocol. Cells at three passages after infection were compared to a negative control of normal FLK-BLV cells. This experiment used a kinetics-based assay for increased sensitivity on proteinase K resistance, using four time-points. The concentration of proteinase K was set to 2 µg/ml according to the findings in the previous experiment.

The prion protein in the normal FLK-BLV cells shows the same pattern as the previous experiment, as seen in figure 6; the full-length prion protein is digested after 2 minutes and the C1 fragment after 50 minutes. In the supposedly infected cells a band representing the full-length prion protein is still visible after 2 minutes in all three passages. In passage 2 of these cells there is also a C1

fragment still undigested after 50 minutes. These findings could represent an increase in proteinase K resistance in the inoculated cells, or reflect a bias as either an increased sample size or increased protein content in these samples.

No band corresponding to the C2 fragment, that would represent the existence of prions in the cells, can be seen in the inoculated cells or the negative controls.

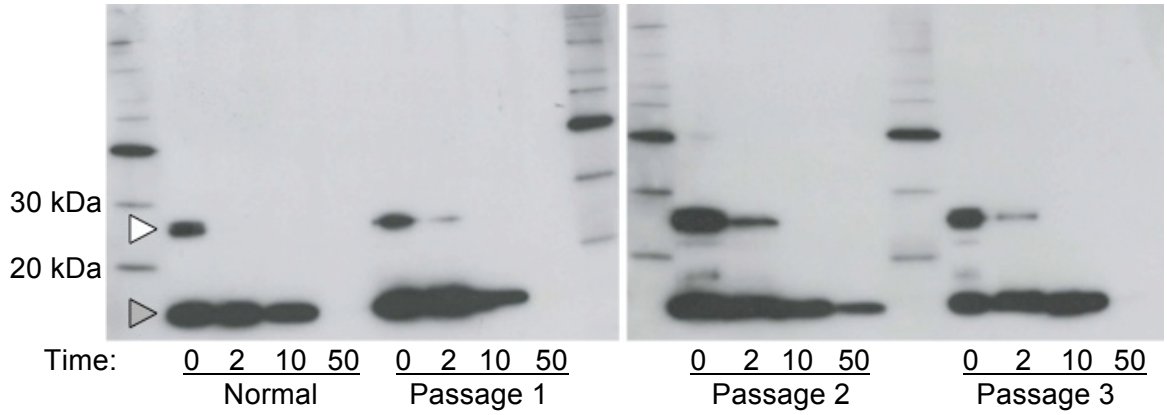


*Figure 6. Western blot of FLK-BLV cells inoculated with prions (passages 1 to 3), as well as a negative control (Normal). Every set of samples are treated with 2 µg/ml proteinase K for the stated times. White arrowhead marks full-length prion protein, grey arrowhead marks the C1 fragment. All samples were deglycosylated. The antiprion 6H4 antibody was used. Unmarked lanes are markers showing the protein size.*

## DISCUSSION

### Sensitive prion protein assay

The traditional way to detect prions in brains of diseased animals using Western blot is to digest the sample with a strong treatment of proteinase K prior to analysis. If the sample contains the fully misfolded form of the prion protein, $PrP^{Sc}$, this will show itself as the truncated partially PK-resistant fragment C2. The C2 fragment is easily differentiated from the full-length prion protein and the C1 fragment, as well as being the only prion protein band still remaining after strong digestion with proteinase K. Instead of the traditional method, this study use increased resistance to proteinase K of the normal full-length and the C1 fragment as an assay for conformational change. Increase in resistance can indicate the existence of prion proteins in one or more intermediate conformations not equal to either $PrP^{Sc}$ or $PrP^{C}$, but still potentially infectious.

The first experiment shows three important things. The FLK-BLV cells do express $PrP^{C}$ and is a suitable candidate for infection trials. Also, the brain homogenate from the scrapie-infected brain do contain $PrP^{Sc}$ and can be used as an inoculum. Last, no remnant $PrP^{Sc}$ from the brain homogenate is seen in the inoculated cells, something that could otherwise bias further studies. These findings together validate the assay used in the later experiment. The second experiment establishes a level of proteinase K activity much more sensitive than the traditional method. We determine the breakpoint for when the normal full-length and the C1 fragment, respectively, is fully digested. These two events occur at a proteinase K activity level much lower than the one used in traditional scrapie diagnostics and also in some cell culture studies (Vella et al., 2007; Lawson et al., 2008). The proteinase K activity for scrapie diagnosis (C2 fragment detection) is 50 μg/ml for 30 minutes and we have shown that the full-length cellular prion protein is degraded (in the FLK-BLV cells) after 2 minutes at 2 μg/ml proteinase K; this combined lower time and concentration correspond to an almost 400-fold reduction in activity. An assay using a proteinase K activity at this level should be able to detect very slight changes in proteinase K resistance of the prion protein.

### Intermediate conformations

There are indications that the inoculated cells in this study display increased proteinase K resistance of the prion protein. Digestion times in these cells are slightly longer than the normal cells; this finding is obtained with the sensitive assay previously mentioned.

This finding could also be due to bias, that the samples were either larger in volume or that they had an increased protein content relative to the normal cells. If it actually does reflect an increase in resistance, it is most probably due to conformational changes corresponding to intermediate forms of the prion protein. These intermediate forms have previously been given the name $sPrP^{Sc}$ (*sensitive* $PrP^{Sc}$) and they might constitute a major part of the infectious prion (Safar *et al.*, 2005a). Whether these intermediates, if existing, will increase in amount in the cell culture after several passages cannot be answered with this study. Two scenarios seem plausible: 1) there is an intermediate form of PrP in the cells that will slowly fold the $PrP^{C}$ into a copy of itself and thus increase the resistance yet

more although not creating fully resistant $PrP^{Sc}$, or 2) there is a low undetectable amount of $PrP^{Sc}$ in the cells creating the intermediate form seen now, but given time the $PrP^{Sc}$ will eventually propagate above detection limit. Only the second alternative would today be considered as a true infection. But if alternative one is true and these cells are in fact infectious it would surely shed new light on what constitutes the "prion".

### Therapeutic research

Given that it exists an intermediate form of $PrP^{Sc}$ in the FLK-BLV cells of this study, this cell line could be a good candidate for therapeutic research. New knowledge suggests that it might not be the amyloid fibrils or $PrP^{Sc}$, but instead the intermediate forms, that are the most neurotoxic and are causing the neuronal loss characteristic of prion diseases (Ronga *et al.*, 2006). When searching for therapeutics in such case, one should focus on finding agents that decrease the proteinase K resistance of the prion protein instead of just lowering the titer of formed $PrP^{Sc}$.

### Genotype effect

The genotype of the cells and the scrapie-infected brain has not been considered in this study. As mentioned, polymorphism at three codons in the sheep prion protein is a critical factor for scrapie susceptibility (Jeffrey & Gonzalez, 2007). This is also valid for cell cultures; in ovRK13 cells there is a $10^4$-fold difference in susceptibility between the VRQ and the ARR alleles (Sabuncu *et al.*, 2003). Assuming there is a difference in genotype in cells and inoculum in this study there might be other results from a similar infection trial with the FLK-BLV cells if they have the same allele. On the other hand, the formation of intermediate prion protein conformations might be a result of having a mismatch in genotype. Anyhow, genotyping and possibly repeating this experiment with cell cultures of different genotypes can give valuable information on the relation between susceptibility, polymorphism and conformational change of the prion protein.

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