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Proteins as markers of TSE infection in sheep blood

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ABSTRACT
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PROTEINS AS MARKERS OF TSE INFECTION IN SHEEP BLOOD

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Transmissible spongiform encephalopathies (TSEs) are a group of fatal infectious neurodegenerative diseases affecting both humans and agricultural animals. TSE transmission *via* blood transfusion has been demonstrated experimentally in rodent, primate and sheep models. Additionally, in humans, four variant Creutzfeld-Jakob disease (vCJD) cases have been reported which probably resulted from infected blood transfusions. Although TSEs can be transmitted *via* blood transfusion, little is known about which blood cells are involved in the replication of the TSE agent and how infectivity is spread throughout the body prior to neuroinvasion. There are no currently validated diagnostic tests for TSE infection in blood.

Detection of PK-resistant PrP^{Sc} has been extensively used as a biochemical marker for TSE infectivity. However, when this project was started it was not known if PK-resistant PrP^{Sc} was present in TSE-infected sheep blood in sufficient quantities to explain the infectivity levels shown by bioassay. Following the development of an optimised Western blot method, this project has demonstrated that the pattern of protein detected with novel anti-PrP monoclonal antibodies is very different from the conventional triple banded pattern of PK-resistant PrP^{Sc}. High molecular weight bands were apparent in phosphotungstic acid (NaPTA) concentrated scrapie-infected and uninfected blood and may represent a novel form of blood-specific PrP. PK-resistant PrP^{Sc} is not therefore a suitable marker for TSE infection in blood.

Other proteins in TSE infected blood were also investigated. Using a proteomics approach three protein markers, lactate dehydrogenase, elongation factor 1 and annexin 1 had altered expression patterns in scrapie infected blood. These proteins, in addition to the novel forms of PrP found in blood, may provide new information on the mechanisms of pathogenesis in scrapie-infected sheep and might prove to be useful molecular indicators of diagnostic value.

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Abbreviations

A1	Annexin 1
ANOVA	Analysis of variance
APS	Ammonium Persulphate
AS-ELISA	Aggregate Specific ELISA
BASE	Bovine Amyloidotic Spongiform Encephalopathy
BSA	Bovine Serum Albumin
BSE	Bovine Spongiform Encephalopathy
CDI	Conformation-Dependent Immunoassay
CHAPS	3- [(3-cholamidopropyl) dimethylammonio] - 1 - propanesulfonate
CJD	Creutzfeldt-Jakob Disease
CNS	Central Nervous System
CO_2	Carbon Dioxide
CPD-1	Citrate Phosphate Dextrose
^{ctm} PrP	Transmembrane PrP (C-terminus on the extracellular side)
CWD	Chronic Wasting Disease
DC	Dendritic Cell
DELFIA	Dissociation Enhanced Lanthanide Fluoroimmunoassay
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	Dithiothreitol
ECL	Enhanced Chemiluminesence
EDRF	Erythroid Differentiation-Related Factor
EDTA	Ethylenediaminetetraacetic Acid
EF-1	Elongation Factor 1
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
ERAF	Erythroid Associated Factor
ESI	Electrospray Ionisation

FACS	Fluorescence Activated Cell Sorting
FDC	Follicular Dendritic Cell
FFI	Fatal Familial Insomnia
Fg	Fetagram
FITC	Fluorescein Isothiocyanate
FSE	Feline Spongiform Encephalopathy
FTIR	Fourier-Transformed Infrared Spectroscopy
g	Gravity
GALT	Gut-Associated Lymphoid Tissue
GAGs	Glycosaminoglycans
GC	Germinal Centre
GFP	Green Fluorescent Protein
GPI	Glycosylphosphatidylinositol
Grb 2	Growth factor receptor-bound protein 2
GSS	Gerstmann-Straussler-Scheinker
HaB	Hamster Brain Cell Line
HIV	Human Immunodeficiency Virus
HRP	Horseradish Peroxidase
IAH	Institute For Animal Health
ic	Intracerebral
ICE	Immunocapillary Electrophoresis
IEF	Isoelectric Focussing
IgG	Immunoglobulin G
IHC	Immunohistochemistry
im	Intra-muscular
ip	Intraperitoneal
IPG	Immobilised pH Gradient
IU	Infectious Unit
iv	Intravenous
kDa	Kilo Dalton
LDH	Lactate Dehydrogenase
LDH-H	Tetrameric isoenzyme of LDH with H subunit

LDH-M	Tetrameric isoenzymes of LDH with M subunit
LDS	Lithium Dodecyl Sulphate
LTα/β	Lymphotoxin α/β
MACS	Magnetic Activated Cell Sorting
$MgCl_2$	Magnesium Chloride
MHC	Major Histocompatibility Complex
mRNA	Messenger RNA
MVBs	Multi-Vesicular Bodies
NBS	National Blood Service
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NaPTA	Sodium Phosphotungstic Acid
NMR	Nuclear Magnetic Resonance
^{ntm} PrP	Transmembrane PrP (N-terminus on the extracellular side)
PBL	Peripheral Blood Leukocytes
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline With Tween 20 (0.05 %)
PE	Phycoerythrin
Pint 1	Protein interactor 1
PK	Proteinase K
PMCA	Protein Misfolding Cyclic Amplification
PMSF	Phenylmethylsulphonyl Fluoride
PNGase F	Peptide-N-Glycosidase F
POM	Polyoxometalates
PrP	Prion Protein
PrP^C	Cellular Form Of PrP
Prnp $^{0/0}$	PrP Null Mice
PrP^{Sc}	Disease Associated Form Of PrP
P 27-30	PK Resistant Form Of PrP
Q-TOF-MS	Quadrupole Time-of-Flight mass spectrometry
RAG-1	Recombinase Activating Gene

RNA	Ribonucleic Acid
rPrP	Recombinant PrP
RT	Room Temperature
sc	Subcutaneous
SCID	Severe Combined Immunodeficient Mice
sCJD	Sporadic Creutzfeldt-Jakob Disease
ScMNB	Scrapie-Infected Mouse Neuroblastoma-Derived Cells
ScN2a	Scrapie-Infected Neuroblastoma N2a
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
^{sec} PrP	Secreted PrP
SMB	Scrapie-Infected Mouse Brain Cells
SMB-PS	Scrapie-Infected Mouse Brain Pentosan Sulphate Cells
SOD	Superoxide Dismutase
SSBP/1	Scrapie Sheep Brain Pool 1
STI1	Stress Inducible Protein 1
TNF-α	Tumour Necrosis Factor
Tris-HCL	Tris-Hydrochloric Acid
TME	Transmissible Mink Encephalopathy
TSEs	Transmissible Spongiform Encephalopathies
vCJD	Variant Creutzfeldt-Jakob Disease

Chapter 1

General Introduction

1.1. Transmissible spongiform encephalopathies

Transmissible spongiform encephalopathies (TSEs) or prion diseases are fatal infectious neurodegenerative diseases of humans and animals. They have long incubation times with clinical signs taking months, years or even decades to develop, followed by a short clinical phase, usually measured in months. These diseases are biologically unique, as they are believed by some to be transmitted by an infectious agent comprised only of protein, with no nucleic acid component (Prusiner, 1982). Clinically, these diseases present with motor disturbances and behavioural changes. There is currently no effective treatment or cure and a definitive diagnosis is only possible after death upon histopathological examination of the brain. The major pathological changes seen are neuronal loss, vacuolation (spongiform change), proliferation and branching of glial cells, astrocytic proliferation and accumulation of the prion protein PrP^{Sc}, which can form amyloid plaques (Prusiner, 1998).

The prototypic TSE is scrapie, a naturally occurring disease affecting sheep and goats, first identified over 200 years ago and now endemic in many countries. An increasing number of animal and human TSEs have since been recognised (Table 1.1). In humans the most common TSE is Creutzfeldt-Jakob disease (CJD) of which three forms have been recognised: sporadic, acquired and inherited/familial (Collinge, 2001). Although these disorders are rare, interest has increased in recent years with the emergence of bovine spongiform encephalopathy (BSE) which affects cattle (Wells *et al*., 1987). It is believed that consumption of meat infected with BSE has led to the appearance of a new variant form of Creutzfeldt-Jakob disease (vCJD) (Will *et al*., 1996). This first emerged in the UK, and identification of this new variant form and the risk it poses to humans has renewed interest in TSE research.

Clinical signs of TSEs differ depending on the species and reflect progressive neurological dysfunction. In sheep symptoms include excitability, rubbing/scratching (pruritus) and ataxia. Animals also may show weight loss, hindlimb weakness, and in some cases develop impaired vision (Hadlow *et al*., 1982).

Disease	Host	Year first described
Scrapie	Sheep and goats	1750
Creutzfeldt-Jakob disease (CJD) sporadic, familial, and iatrogenic forms	Humans	1920
Gerstmann-Straussler-Scheinker syndrome (GSS)	Humans	1928
Kuru	Humans	1957
Transmissible mink encephalopathy (TME)	Mink	1965
Chronic wasting disease (CWD)	Whitetail deer, mule deer and elk	1967
Fatal familial insomnia (FFI)	Humans	1986
Bovine spongiform encephalopathy (BSE)	Cattle	1987
Feline spongiform encephalopathy (FSE)	Domestic cats	1990
Exotic ungulate encephalopathy	Oryx, nyala and greater kudu	1990
New variant CJD (vCJD)	Humans	1996

Table 1.1: TSE diseases of animals and humans.

1.1.1. The prion protein (PrP)

The prion protein was identified in 1982, being the major component in the infectious fraction of scrapie-infected hamster brains (Prusiner, 1982). PrP consists of approximately 250 amino acids and exists in two alternate forms; a normal cellular form (PrP^{c}) and a disease-associated form (PrP^{Sc}). PrP^{c} is a 30-35 kDa glycoprotein with two N-glycosylation sites and is attached to the outside of the cell membrane by a glycosylphosphatidylinositol (GPI) anchor at amino acid 231 (Hope, 1993; Stahl *et al*., 1987). The normal and pathological forms of prion protein have identical amino acid sequences and differ only in their folded tertiary structure and biochemical properties. PrP^{C} is non-infectious and exists as a monomer, consisting of 43 % α-helix and 3 % β-sheet (Pan *et al*., 1993). It is found in many tissues and cell types where it is rapidly turned over and has a half life of approximately 6 hours (Anderson *et al*., 1992). PrP^{C}

is also sensitive to digestion with PK (Meyer *et al*., 1986). PrP^{Sc} is the infection associated variant of the protein and forms aggregates and amyloid fibrils which bind to histological dyes such as Congo red, giving a green birefringence when viewed under polarized light (Divry, 1927). Abnormal PrP consists of 34 % α-helix and 43 % β-sheet (Pan *et al*., 1993). PrP^{Sc} turn over is slower and has a half life of years while accumulation occurs in TSE-infected brain. Unlike PrP^{C}, PrP^{Sc} is partially resistant to treatment with proteinase K (PK) leaving a 27-30 KDa protein fragment. The two conformers also have differing solubilities in detergents and guanidinium hydrochloride (Safar *et al*., 1998). Table 1.2 summarises the structural and biochemical differences between PrP^{C} and PrP^{Sc}.

PrP^{C}	**PrP^{Sc}**
Normal, cellular form	Disease-associated form
43 % α-helical	34 % α-helical
3 % β-sheet	43 % β-sheet
Monomeric	Forms aggregates/ amyloid fibrils
Soluble in mild detergents	Insoluble in mild detergents
PK sensitive	Partially PK resistant
Non-infectious	Infectious
Present in many tissues and cells	Accumulates in CNS and lymphoid tissue

Table 1.2: Structural and biochemical differences between PrP^{C} and PrP^{Sc}

1.1.2. Animal TSEs

Scrapie is a disease of sheep and goats that has been known in Europe for over 200 years (McGowan, 1922) and is present worldwide, with the exception of New Zealand and Australia. Scrapie has been reported in the majority of sheep breeds, and resistance or susceptibility to scrapie infection has been shown to be associated with several polymorphic codons in the sheep prion protein (PrP) gene. In particular, an arginine at amino acid codon 171 is associated with scrapie resistance and a valine at amino acid codon 136 is associated with scrapie susceptibility (Hunter, 1997).

BSE first emerged in UK cattle in 1986 (Wells *et al*., 1987) and rapidly evolved into a major epidemic with more than 180,000 cases confirmed. Contaminated protein feed supplements prepared from rendered ruminant carcasses were found to be the most likely source of infection (Wilesmith *et al*., 1988). The rendering process of abattoir waste was altered during the 1970s which is thought to have led to a less efficient system for inactivating scrapie-infected sheep offal. An alternative hypothesis is that BSE resulted from recycling of rare sporadic BSE cases, as cattle were also rendered to produce cattle feed. Following the emergence of BSE, many new species developed spongiform encephalopathies, including nyala (Jeffrey and Wells, 1988) greater kudu (Cunningham *et al*., 1993, Kirkwood *et al*., 1992) oryx (Kirkwood *et al*., 1990) eland (Fleetwood and Furley, 1990) cheetah (Peet and Curran, 1992) puma (Willoughby *et al*., 1992) and the domestic cat (Pearson *et al*., 1992). Several of these cases have been confirmed to be caused by a BSE-like prion strain and contaminated feed is suspected (Bruce *et al*., 1994, Fraser *et al*., 1988). In 1988 the ruminant feed ban was introduced and since then the number of BSE-infected cattle in the UK has declined, although cases have since been reported in other countries, with significant epidemics reported in Switzerland (Doherr *et al*., 1999) Ireland, France and Portugal.

Chronic wasting disease (CWD) is a TSE of North American cervid ruminants (Williams and Young, 1980). CWD has been present at low levels in free-ranging mule deer, white-tailed deer and Rocky Mountain elk in Wyoming, Nebraska and Colorado for over 30 years. CWD also occurs in farm-raised animals. The number of

clinically diagnosed CWD infected animals has increased (Knight, 2002) and cases are being identified in new locations in America and Canada. This may be due to improved diagnostic techniques and an increased awareness of TSE diseases. Alternatively, this may be attributed to the increase in the movement of animals since the expansion of the elk industry, (which raises animals for meat, the commercial value of their antlers, or game parks) in the 1990s. It is not known if CWD can be transmitted to other species, including humans who hunt and eat these animals.

Transmissible mink encephalopathy (TME) (Marsh and Kimberlin, 1975) was at first recognised as a food-borne disease of ranch-raised mink in the USA. Infrequent epidemics have since occurred.

Active surveillance of TSEs in ruminants has allowed for identification of a number of unusual isolates in cattle, sheep and goats. An unusual presentation of scrapie in sheep was first recognised and described in Norway in 1998 (Nor98) (Benestad *et al*., 2003). In this novel scrapie type, PrP^{Sc}, although never present at high levels, was found to be more abundant in the cerebellum and the cortex than in the brain stem, in contrast with classicial scrapie or ovine BSE. Also Western blot analyses showed a complex electrophoretic pattern, including an unusual band of low apparent molecular mass (10-12 kDa) while in classical scrapie PrP^{Sc} is detected as three bands between 18 and 30 kDa corresponding to the un-, mono, and di-glycosylated PrP^{Sc} forms (Gavier-Widen *et al*., 2004). A number of Nor98 isolates have been identified in sheep with genotypes of the prion gene associated with resistance to classical scrapie or BSE (Buschmann *et al*., 2004). The possibility that these cases were not genuine TSEs was ruled out following studies where atypical isolates were transmitted successfully to transgenic mice (Le Dur *et al*., 2005). The origin of such atypical scrapie cases is unclear, as is their potential transmissibility within the affected species and to other species.

Until 2004, BSE in cattle was considered to be a disease with unique features, in relation with its origin as a food borne epidemic. However, evidence of a second cattle TSE was reported in March 2004 (Casalone *et al*., 2004). During a routine screening program of cattle in Italy, pathological differences (such as the nature and distribution

of brain lesions) to classical BSE were observed. This led to the term bovine amyloidotic spongiform encephalopathy (BASE). The molecular signature of BASE is not characteristic of classical BSE, but shows similarities to a subtype of human sporadic CJD (sCJD). Molecular characterisation of PrP^{Sc} shows a lower apparent molecular mass of unglycosylated PrP^{Sc}, although the most striking difference is that of strongly decreased levels of diglycosylated PrP^{Sc}, which is prominent in typical BSE (Casalone *et al*., 2004). The origin of such atypical BSE cases remains unknown, and it is unclear whether BASE is a sporadic form of cattle TSE or is linked to human sporadic TSE. Interestingly, in transmission studies where transgenic mice expressing human PrP are inoculated with BSE material, the glycoform pattern on gels is consistent with sporadic CJD (sCJD) (Collinge *et al*., 1996).

In some atypical BSE cases, the unglycosylated PrP^{Sc} band has a higher molecular mass relative to that observed in classical BSE. Such cases are referred to as H-type BSE. Recently, the first case of BSE in the UK showing an H-type molecular profile was documented (Terry *et al*., 2007).

1.1.3. Human TSEs

Creutzfeldt-Jakob disease (CJD) was first identified in 1921 (Jakob, 1921). CJD can be divided into three forms; sporadic, inherited and acquired. The majority of CJD cases (85 %) are sporadic, and occur in all countries with a random case distribution and an annual incidence of one per million (Masters and Richardson, 1978). Around 15 % of CJD cases are inherited, and are associated with coding mutations in the prion protein gene, of which over 20 distinct types are recognised (Masters and Richardson, 1978; Masters *et al*., 1981; Goldfarb *et al*., 1991). Acquired disease includes iatrogenic CJD which occurs through accidental exposure to TSE agent through medical procedures such as surgical operations, dura mater implants, corneal transplants, blood transfusion and human pituitary-derived growth hormone.

Fatal familial insomnia (FFI) (Lugaresi *et al*., 1986) is a very rare, autosomal dominant inherited disorder. FFI is associated with a dual mutation in the PrP gene, where an

aspartic acid to asparagine mutation occurs at PrP amino acid codon 178, and methionine is present at PrP amino acid codon 129.

Kuru is seen only among the Fore Highlanders of Papua New Guinea (Gajdusek and Zigas, 1957). The Fore tribe reportedly honoured the dead by eating their brains, and it is thought that affected individuals acquired kuru through this ritual cannibalism. Kuru became a major cause of death, especially in women and children who consumed the brains during these rituals and the recycling of the infectious agent in this isolated population caused an epidemic. Cannibalism ceased in the 1950s and kuru has virtually disappeared (Collinge, 2001).

Gerstmann-Straussler syndrome (GSS) (Gerstmann *et al*., 1936) is a rare, autosomal dominant disorder. Initially GSS was associated with a proline to leucine mutation at PrP amino acid codon 102 (Doh-ura *et al*., 1989) however GSS is now known to be associated with several different mutations in the PrP encoding gene (Hsiao *et al*., 1989, 1991, and 1992; Goldfarb *et al*., 1992).

In 1995, a novel human prion disease characterised by florid plaques and early age at onset (Wills *et al*., 1986) called variant CJD (vCJD) appeared in the UK. This disease has been shown to be caused by a TSE agent with identical strain characteristics to that isolated from BSE-infected cattle (Bruce *et al*., 1997) and probably occurred as a result of dietary exposure to BSE contaminated meat. A large proportion of the population in the UK were exposed to BSE, as by 1995 almost one million BSE-infected carcasses had entered the food chain. At the time of writing (June 2008) 164 definite cases of vCJD (of which 3 remain alive and are classed as probable cases) have been reported in the UK, 23 cases in France and 18 elsewhere in the world (www.cjd.ed.ac.uk). Although the number of vCJD cases per year has fallen, it is unclear if numbers will remain small. Mathematical models have been developed to predict numbers of future cases (Ghani *et al*., 2000) but early models do not take into account levels of asymptomatic infection, secondary transmissions from human to human, or whether BSE has entered the sheep population. Mathematical modelling has indicated that vCJD could become a self-sustaining disease in the UK population *via* secondary

transmission by blood transfusion or surgical instruments (Clarke and Ghani, 2005). However, a more recent study suggests that a self sustaining epidemic although possible, is unlikely due to the interventions made by the UK Department of Health to prevent vCJD spread *via* secondary transmissions (Clarke *et al*., 2007). The National Blood Service (NBS), for example, have put in place a number of control measures, such as leucodepletion, to reduce the risk of vCJD transmission by blood products. These factors may influence the number of future vCJD cases (Ferguson *et al*., 2002). A study in 1994 of 13,000 appendix and tonsil samples revealed that PrP^{Sc} (assumed to be associated with vCJD as in other human TSE diseases PrP^{Sc} is not detected in lymphoid tissues) was found in three of the samples, indicating that as many as 4000 individuals may be infected (Hilton *et al*., 2004). However, statistical evidence acquired by the National CJD surveillance unit in Edinburgh, UK suggests that the epidemic may have reached its peak (www.cjd.ed.ac.uk). It is possible that a significant number of people are asymptomatic carriers of disease which may account for the differences seen in these estimates.

Asante and colleagues (2002) showed in transgenic mouse experiments that individuals homozygous for methionine at PrP amino acid codon 129, may be at risk through iatrogenic vCJD spread *via* surgical procedures or blood transfusion (Asante *et al*., 2002). Until 2004 all recorded vCJD cases had occurred in people homozygous for methionine, however an elderly patient, heterozygous at PrP amino acid codon 129 who died from unrelated causes five years after receiving an infected blood transfusion from an asymptomatic donor, had PrP^{Sc} deposits in the lymphoid tissues following post mortem examination (Peden *et al*., 2004). Additionally, two of the tonsil/appendix samples that were positive for PrP^{Sc} in Hiltons study were genotyped as homozygous for valine at PrP amino acid codon 129 (Ironside, 2006) and a possible clinical case of vCJD in an individual homozygous for valine has also been reported (Mead *et al*., 2007). Transgenic mouse lines have recently been produced that express human PrP with the codon 129 homozygous for methionine (MM), heterozygous for methionine and valine (MV) and homozygous for valine (VV). All individuals, irrespective of codon-129 genotype, were susceptible to vCJD infection (Bishop *et al*., 2006). These studies suggest that susceptibility to vCJD is not confined to the methionine

homozygous PrP genotype. The nature of the transmissible agent following transmission from human to human *via* blood has also been studied using the transgenic mouse lines that express human PrP. Brain material from the first case of blood transfusion associated vCJD (reported by Llewelyn *et al*., 2004) was inoculated into wild-type and transgenic mice. The agent was transmitted with the same efficiency in both murine models, suggesting that there is no increased virulence of the agent following human to human transmission of blood transfusion associated vCJD (Bishop *et al*., 2008).

1.2. Nature of the infectious agent

In the absence of a specific TSE agent directed immune response and/or inflammatory cell infiltrate in the brain, it was thought that the infectious agent was a slow virus. However ionizing and UV irradiation experiments that normally destroy nucleic acids, performed on mouse brain homogenates showed that the agent was resistant to such treatment (Alper *et al*., 1966; Alper *et al*., 1967; Latarjet *et al*., 1970). This then excluded viruses and gave rise to an alterative hypothesis, that the agent could be a small piece of nucleic acid surrounded by a protein coat- a virino (Kimberlin, 1982). Several different approaches have been taken to try to identify a scrapie-specific nucleic acid. Numerous procedures that hydrolyse or modify nucleic acid without altering protein have been employed but none reported a reduction in the titer of TSE agent (Diener *et al*., 1982; Prusiner, 1991). Additionally, molecular cloning and differential hybridization studies could not identify an agent-specific polynucleotide (Aiken *et al*., 1990). More recently, in case a nucleic acid molecule had been overlooked, techniques increasing overall sensitivity of electrophoretic separations were applied to purified brain fractions (highly enriched for agent infectivity) from scrapie-infected hamsters. However, no TSE agent-specific polynucleotide was identified (Safar *et al*., 2005). In over 30 years of research no specific nucleic acid has been found associated with a TSE infectious particle.

Griffith (1967) suggested that a protein, which could replicate in the body, may be responsible for TSE disease progression. This was supported in experiments which

showed that scrapie infectivity could be reduced by procedures that hydrolyze or modify proteins (Diener *et al*., 1982). Prusiner and colleagues then isolated an unusual protease- resistant protein (PrP^{Sc}) in the brains of scrapie-infected hamsters that was not present in healthy animals. Using biochemical and immunological methods they showed that PrP^{Sc} and infectivity copurified. These experiments led to Prusiner coining the term "prion" (derived from proteinaceous and infectious) for the infectious particle (Bolton *et al*., 1982). The "protein-only" hypothesis proposes that the prion is a conformational isoform of the normal host protein PrP^{c} and is exclusively the causative agent of TSE disease.

Primary support for the protein-only hypothesis is based on the finding that PrP knockout mice ($Prnp^{o/o}$) appear completely protected against scrapie disease and fail to propagate infectious agent (Bueler *et al*., 1993). If murine PrP transgenes are re-introduced into these mice susceptibility is restored (Fischer *et al*., 1996). Additionally, procedures that inactivate PrP^{Sc} markedly reduce or eliminate infectivity (Prusiner *et al*., 1993) and transgenic mice overexpressing the PrP gene spontaneously develop neuropathological and clinical features of TSEs (Hsiao *et al*., 1990).

Further evidence supporting the protein-only hypothesis emerged after it was discovered that the pathological protein catalyzes the cell-free conversion of PrP^{c} into PrP^{Sc} (Kocisko *et al*., 1994). In these experiments purified PrP^{c} was mixed with stoichiometeric amounts of purified PrP^{Sc}, producing a low yield of newly formed PrP^{Sc}. More recently a new *in vitro* conversion system has been developed where large quantities of PrP^{c} are transformed using minute amounts of PrP^{Sc}. This system called protein misfolding cyclic amplification (PMCA) supports the idea that PrP^{Sc} replication may be a cyclical process with newly produced PrP^{Sc} triggering further misfolding to maintain abnormal protein propagation (Saborio *et al*., 2001; Castilla *et al*., 2005). PrP^{Sc} generated in a PMCA reaction was recently shown to be infectious following inoculation into hamsters, where animals developed a disease with scrapie characteristics (Castilla *et al*., 2005). In these experiments, the source of the PrP^{C} substrate was not purified but from whole brain homogenate from healthy hamsters, so

the possibility that other molecules may be involved in agent infectivity cannot be ruled out.

Experiments where PrP^{Sc} is synthesized *in vitro* and shown that it can, by itself, produce an infectious disease in healthy animals are required to finally prove the "protein-only" hypothesis. Successful efforts in yeast (Sparrer *et al*., 2000) have been achieved and more recently "synthetic prions" consisting of part mouse PrP were generated in *Escherichia coli* and then polymerized into misfolded fibrils akin to PrP^{Sc}. Following injection into the brains of mice (transgenic mice overexpressing PrP), neurodegenerative disease was triggered that could be transmitted to other animals (Legname *et al*., 2004, 2005). However, the "synthetic prions" were not given to normal mice and to guarantee that lab contamination didn't influence the infections, other labs would have to replicate the experiments. Therefore these studies don't conclusively prove the "protein-only" hypothesis.

There are characteristics of TSEs that challenge the "protein-only" hypothesis. For instance, scrapie and other TSEs occur in multiple strains, which have markedly different incubation times, clinical features and neuropathology when passaged in laboratory rodents (Bruce *et al*., 1991). In other infectious micro-organisms, different strains arise as a result of mutations or polymorphisms in the genetic makeup of the agent. To explain this for an agent composed exclusively of protein it has been proposed that PrP^{Sc} has slightly different conformation states that replicate faithfully at the expense of the host PrP^{c} (Prusiner, 1998; Safar *et al*., 1998). In cell-free conversion assays where PrP^{C} is incubated with PrP^{Sc} from two different hamster TSE strains (called hyper or drowsy) PrP^{C} appears to fold and exhibit the biochemical properties of the PrP^{Sc} in each respective strain (Bessen *et al*., 1995). The conformational stability of PrP^{Sc} molecules can be studied using guanidine hydrochloride (GdnHCL) and different TSE strains have been distinguished based on the concentration of GdnHCL required to denature different conformations of PrP^{Sc} (Safar *et* al., 1998; Peretz *et al*., 2001). Using this technique, more recent studies also show that distinct TSE strains can be discriminated by their distinct conformers of PrP^{Sc} (Khalili-Shirazi *et al*., 2005; Thackray *et al*., 2007).

There are also reports of TSE transmission to rodents where protease resistant prion protein is undetectable (Lasmezas *et al*., 1997; Manson *et al*., 1999; Barron *et al*., 2001). Supporters of the "protein-only" hypothesis suggest that protease resistance does not necessarily equate with infectivity (Safar *et al*., 1998). Also, TSE infectivity can be associated with a wide range of PrP^{Sc} aggregates (Caughey *et al*., 1997) and it may be that detection methods for disease-associated PrP are currently unable to detect the infectious form of the PrP.

Some researchers believe that TSE diseases are caused by slow acting viruses although this idea is not well supported. In a recent study, infection with a slow-acting Creutzfeldt Jakob strain was shown to protect mouse cells from infection with a faster one and this may point to an immune defence reaction and thus a virus (Nishida *et al*., 2005). Dickinson proposed another explanation which he termed "the scrapie replication-site hypothesis" to explain a similar phenomenon of strain interference. Although hypothetical, he suggested that the agent can only replicate in a limited number of sites in peripheral tissues and if these are occupied by a slowly replicating strain, a faster strain could be blocked (Dickinson and Outram, 1979).

There is also evidence for virus-like particles in TSEs. Such particles were first described in experimental mouse scrapie in 1968 (David-Ferreira *et al*., 1968) in natural sheep scrapie in 1971 (Bignami and Parry, 1971) and more recently in primate and human CJD brain samples (Liberski *et al*., 2004). Interestingly, recent studies of neuroblastoma cells infected with the 22L strain of scrapie produced intracellular 25-nm virus-like particles very similar to the ones described in 1968 (Manuelidis *et al*., 2007).

Sheep pathogenesis studies also question whether prion protein alone causes disease. Using surgically modified sheep, Jeffery and co-workers loaded a small area of the gut with scrapie-infected brain homogenate. They observed that PrP^{Sc} was taken up in villi and passed into the lymph. Interestingly it was not taken up by the Peyer's patches, where amplification of the TSE agent is thought to take place. It is possible that the PrP^{Sc} may have been amplified in other lymphoid tissues for instance the mesenteric

lymph nodes, although this result may also imply that the PrP^{Sc} alone does not cause the infection (Jeffery *et al*., 2006). In the same study, the researchers pre-digested a mixture containing high levels of PrP^{Sc} with standard stomach contents, and injected the resultant mixture into the gut. Western blot analysis indicated that only a very small amount of PrP^{Sc} had survived. In the natural environment, grazing sheep are not naturally exposed to highly infective brain material, but are more likely to pick up small amounts of PrP^{Sc} from discarded placental tissue, or faecal/urinary contamination of pasture. It is possible therefore that sheep in the field may be exposed to very little abnormal prion protein in the gut as the stomach would dispose of the small amount ingested. This suggests that infectious molecules other than PrP^{Sc} may be involved in disease pathogenesis. This study however, does not rule out the possibility that PrP^{Sc} could cause disease if absorbed in sufficient enough amounts or if the animals were exposed to small doses in the environment cumulatively over time. It may also be possible that PrP^{Sc} may be absorbed higher in the gut, before the abnormal proteins are digested, for instance in the mouth.

Finally, although no nucleic acid consistently associated with a TSE molecule has been identified, there is evidence to suggest that nucleic acids may help catalyze the conversion of PrP^{c} into PrP^{Sc}. Deleault and colleagues (2003) looked at the effect of nucleic acids on the propagation of PrP^{Sc} in brain homogenates. They found that PK resistant protein was amplified sixfold after mixing infected and normal brain homogenates. When the homogenates were depleted of single-stranded RNA, no amplification was detected. In contrast when exogenous RNA (extracted from uninfected hamster or mouse tissue) was added PrP^{Sc} amplification was boosted twenty-four fold (Deleault *et al*., 2003). These results suggest that single-stranded RNA is required for PrP^{Sc} amplification, although do not necessarily disprove the "protein-only" hypothesis. The authors also showed that active RNA was present in extracts of normal mammalian tissue indicating that these molecules are not specific to TSE infections. Also RNA may be a host-cell co-factor needed for the conversion of PrP^{C} rather than a component of the infectious agent. Other molecules such as glycosaminoglycans (GAGs) and GAG-containing proteoglycans have also been shown to stimulate prion conversion *in vitro* and may be candidate co-factors in the prion

protein conversion process (Wong *et al*., 2001). Additionally, biochemical analysis of purified PrP^{Sc} preparations have provided no evidence that a nucleic acid component is an essential part of the infectious unit (Meyer *et al*., 1991).

In conclusion, there is evidence that both supports and challenges the "protein-only" hypothesis and although PrP^{Sc} is commonly used as a disease specific marker of TSE infection, the precise nature of the infectious agent remains ill-defined.

1.3. The structure of the prion protein (PrP)

The structure of the ubiquitous cellular form of the PrP protein consists of a flexible N-terminal tail at amino acids 23-90 and a globular C-terminal domain (amino acids 90-250) with a structure made of three α-helices and a short β-sheet (Pan *et al*., 1993). PrP^{C} also has an intramolecular disulfide bond (Cys179-Cys214) which provides structural stability to the C-terminus of the protein (Bosques *et al*., 2003), an alanine rich (AGAAAAGAVVGGVG) hydrophobic region between amino acid 113-128, and a series of 5 or more repeats of an eight amino acid sequence (PHGGGWGQ), known as the octapeptide repeat region (Figure 1.1). Studies using synthetic peptides corresponding to this sequence showed the octapeptide repeat region to be a substrate of the proteolytic PK enzyme (Georgieva *et al*., 2004). The generation of transgenic mice expressing an anchorless, secreted version of PrP^{C}, has shown that membrane anchoring is a crucial prerequisite for agent toxicity resulting in clinical TSE (Chesebro *et al*., 2005).

Due to low abundance, natural PrP^{C} can not be used to apply nuclear magnetic resonance (NMR) spectroscopy or x-ray analysis to determine the exact three-dimensional structure. Structural analysis of PrP^{Sc} has also proved challenging due to the insoluble, non-crystalline nature of this protein. However, the structure of refolded recombinant PrP (rPrP) in solution is known from spectroscopic studies and with high resolutions from NMR analysis (Zahn *et al*., 2000; Riek *et al*., 1997; James *et al*., 1997). Because rPrP is soluble, monomeric and α-helical in structure it is thought to be representative of PrP^{C}, and x-ray crystal structures of rPrP have been published (Knaus

et al., 2001; Haire *et al.*, 2004; Eghiaion *et al.*, 2004). A final description of the structure of PrP^{Sc} has yet to be made, although hypothetical models exist. Wille and colleagues (2002) were able to prepare two-dimensional crystalline-like arrays of PrP^{Sc}-like molecules of N-terminally truncated PrP (PrP^{27-30}) and a miniprion composed of amino acids 23-88 and 141-176 (PrP106), both from Syrian hamsters. This model takes into account both electron microscopic and spectroscopic data as well as the intermolecular stabilization of the PrP^{Sc} structure (Wille *et al.*, 2002).

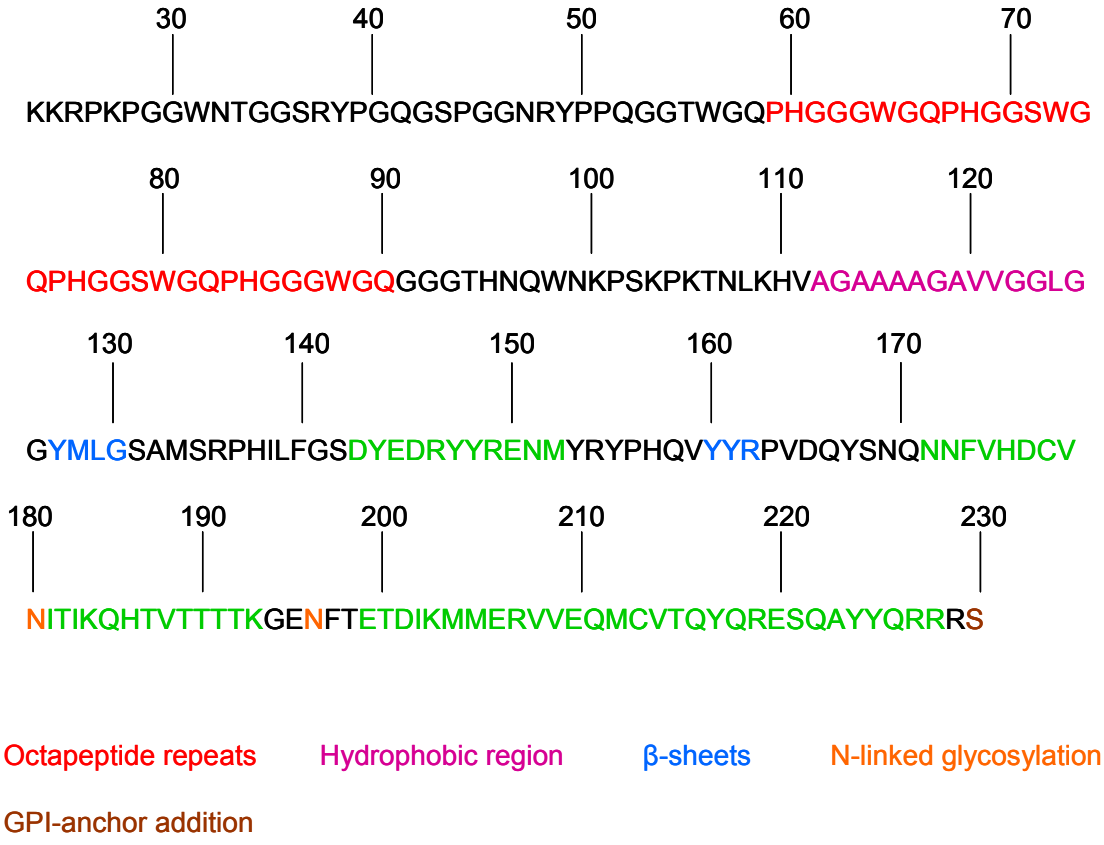


Figure 1.1: Primary amino acid sequence of PrP. Sheep PrP sequence from amino acids 23-230 without the N -terminal and C-terminal signal peptides. The features of PrP are highlighted in colour.

1.3.1. Biosynthesis of PrP^C

Like other membrane proteins, PrP^C is synthesized in the rough endoplasmic reticulum (ER). Two signal peptides are present in the molecule, one at the N-terminus which is cleaved during biosynthesis in the ER and the other at the C-terminus which is replaced with a GPI anchor (Stahl *et al*., 1987). The GPI anchor may play a role in stabilising the conformation of PrP^C to prevent conversion to PrP^{Sc} (Eberl *et al*., 2004; Hicks *et al*., 2006). Two glycosylation sites are also mapped to the C-terminus and the formation of a disulfide bond occurs between two cysteine residues at amino acid codons 179 and 214 (Endo *et al*., 1989). PrP^C trafficking through the Golgi complex occurs before the mature protein is transported to the cell surface. On the cell surface, PrP^C is continuously recycled between the plasma membrane and endocytic compartments (Harris *et al*., 2003). PrP^C is enriched in membrane rafts both at the transgolgi network, plasma membrane and in interconnecting chains of endocytic caveolae-like domains (Vey *et al*., 1996; Peters *et al*., 2003). Whilst still on the cell surface, PrP^C leaves its raft environment to cross the non-raft membrane and then enter coated pits where it is endocytosed. Endocytosed PrP^C-containing caveolae are not directed to the ER and Golgi complex, but return to the surface (Sunyach *et al*., 2003). Recent evidence from neuronal cell studies also suggest that cholesterol is essential for the transport of PrP^C to the cell surface. Treatment with the cholesterol inhibitor mevinolin appears to reduce the amount of cell surface PrP^C, resulting in PrP^C accumulation in the Golgi complex (Gilch *et al*., 2006). Cycling of PrP^C between the plasma membrane and endocytic compartments is though to occur *via* a clathrin-dependent mechanism, and it appears that the N-terminal domain of PrP^C is essential for endocytosis *via* clathrin-coated pits (Shyng *et al*., 1994; Sunyach *et al*., 2003; Taylor and Hooper, 2006).

The prion protein can adopt multiple membrane topologies including a fully secreted form (^{Sec}PrP) and two transmembrane forms (^{ntm}PrP and ^{ctm}PrP) (Hegde *et al*., 1998; Hedge *et al*., 1999). These forms span the lipid bilayer once *via* a highly conversed hydrophobic region in the centre of the molecule (amino acids 111-134), with either the N or C terminus, respectively on the extracytoplasmic side of the membrane. It has

been shown that these species are generated in small amounts (less than 10 % of total PrP) as part of the normal biosynthesis of wild type PrP in the ER.

1.3.2. The function of PrP^C

The function of PrP^c remains unclear and the first observations of PrP^c gene-ablated mice ($Prnp^{0/0}$) surprisingly suggested that the animals without the protein develop normally and do not show major behavioural abnormalities (Bueler *et al*., 1992). However, since then several minor differences were noticed in some strains of PrP^c null mice when compared to their wild-type controls. For instance, defects in synaptic function were observed (Collinge *et al*., 1994), increased vulnerability of neurones to oxidative stress (Brown *et al*., 1997), reduced Cu^{2+}/Zn^{2+} dependent superoxide dismutase (SOD) activity (Brown *et al*., 1999) and changes in membrane localisation of nitric oxide synthase (Ovadia *et al*., 1996). It is possible that a loss of PrP^C may contribute to neurodegeneration, as all of these features are associated with neuronal cell damage and death, and are observed in scrapie-infected animals. Based on these observations, several different roles for PrP^c in the central nervous system have been suggested, ranging from regulation of oxidative stress in neurones to influencing neurotransmission (Brown and Besinger. 1998; Collinge *et al*., 1994). PrP^C may also play a role in neurogenesis (Kanaani *et al*., 2005), differentiation of neural precursor cells (Steele *et al*., 2006) and release of the neurotransmitter acetylcholine at neuromuscular junctions (Re *et al*., 2006).

It is well known that the prion protein binds copper *in vivo* (Brown *et al*., 1997). The N-terminal domain of PrP^C contains octapeptide repeat motifs encompassing five Cu^{2+} ion binding sites. PrP^C may have a functional role in copper metabolism. It may be a principal copper-binding protein in brain membrane fractions and could control the activity of other membrane-associated copper binding proteins. It has been shown that the octapeptide repeat region of the prion protein is involved in copper reduction from Cu^{2+} to Cu^+ (Miura *et al*., 2005). Prion protein expression alters copper uptake into cells and enhances copper incorporation into SOD (Brown *et al*., 1999; Brown *et al*., 2001). More recently, copper-bound prion peptide fragments have been shown to

catalyse the formation of superoxide (Kawano, 2006) causing irreversible damage. However, in contrast to these findings, two other studies suggest that recombinant PrP (Hutter *et al*., 2003) and cellular PrP^C *in vivo* (Sakudo *et al*., 2008) may not possess any SOD-like activity.

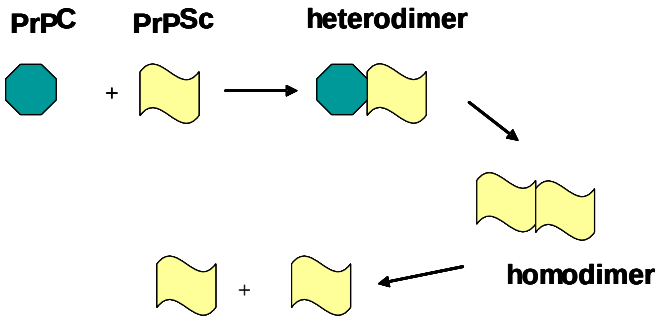
PrP^C interacts with various macromolecules at the cell membrane, in endocytic compartments and in the secretory pathway. Identification of PrP^C ligands may aid in establishing biological functions in the cell. PrP^C has been shown to interact with the transmembrane protein laminin receptor precursor (Rieger *et al*., 1997). Laminin mediates internalisation of PrP^C and directs the complex through clathrin coated pits (Gauczynski *et al*., 2001). Additionally, PrP^C has also been reported as a high affinity receptor for laminin (Graner *et al*., 2000). The interaction of these two proteins may be important in cell signalling pathways. Other specific PrP^C ligands include stress inducible protein 1 (STI1) which promotes neuroprotection of retinal neurones (Zanata *et al*., 2002), the adapter protein Grb2 and the prion interactor Pint1 (Petrakis and Sklavladis, 2006). PrP^C may also interact with hemin (iron protoporphyrin IX) which plays a role in hemin homeostasis. Hemin is a component of haemoglobin, a molecule essential for oxygen transport in red blood cells (Lee *et al*., 2007).

PrP^C may also have a specific role in the immune system. It is expressed on haematopoietic stem cells and may be important for their self-renewal (Zhang *et al*., 2006). Based on work in PrP null mice or cells derived from them, PrP^C has also been proposed to down-regulate phagocytosis by macrophages (De Almeida *et al*., 2005). Additionally, PrP^C may play a role in T cell activation and proliferation (Bainbridge and Walker, 2005) and could induce signalling cascades in monocytes (Krebs *et al*., 2006).

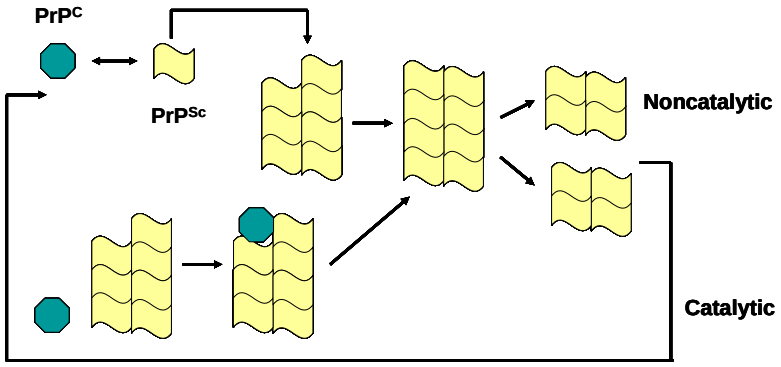
1.3.3. Models of PrP^{C}/PrP^{Sc} conversion

A key molecular event in TSE diseases is the conversion of PrP^{c} to PrP^{Sc}, however the exact mechanism is unknown. Prusiner *et al* (1982) have suggested that the interaction between exogenously introduced PrP^{Sc} and endogenous PrP^{c} may induce refolding of the native molecule, inducing a structural change from alpha helix to beta-sheet (Figure 1.2, A). Alternatively, in the nucleation-polymerisation model it has been suggested that unstable monomeric PrP^{Sc} molecules could slowly form an infectious stable seed. After further PrP^{Sc} recruitment, the oligomeric seed fragments into several infectious seeds which incorporate and convert PrP^{c} (Xiong *et al*., 2001; Griffith, 1967) (Figure 1.2, B). It has recently been reported that smaller PrP^{Sc}-containing aggregates with masses equivalent to 14-28 PrP molecules may be more toxic than larger amyloid fibrils (Silveira *et al*., 2005). It has also been considered that there may be an intermediate step in which another protein or molecule (often termed protein X) acts as a molecular chaperone and interacts with PrP^{Sc} to facilitate conversion (Saborio *et al*., 1999) (Figure 1.2, C). Evidence also suggests that nucleic acids may be host-cell co-factors needed for conversion of PrP^{C} (Deleault *et al*., 2003).

A)



B)



C)

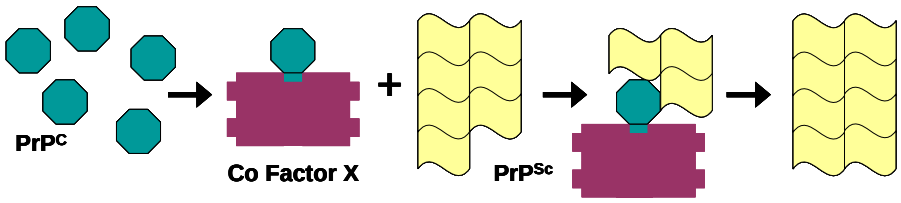


Figure 1.2: Models of PrP^{Sc} replication. (A) In the heterodimer model PrP^{Sc} exists as a monomer that binds to PrP^{C}, forming a heterodimer and catalysing the conversion of PrP^{C} to PrP^{Sc}. The homodimer then splits to give two PrP^{Sc} seeds for further conversion of PrP^{C}. (B) In the nucleated polymerisation model the conversion of PrP^{C} to PrP^{Sc} is reversible and PrP^{Sc} is stabilised by aggregation. In the noncatalytic model the monomer of PrP^{C} may interchange between the two conformations, with the β-form stabilised by binding to preformed PrP^{Sc}. In the catalytic model the α to β conformational change takes place on binding to PrP^{S}. (C) There may be an intermediate step in which another protein or molecule (often termed protein X) acts as a molecular chaperone and interacts with PrP^{Sc} to facilitate conversion of PrP^{C} to PrP^{Sc}.

1.4. Sheep PrP genetics

Studies of natural and experimental scrapie confirm the importance of 3 polymorphic codons in the sheep PrP gene 136, 154 and 171, that are associated with either resistance and susceptibility to TSE infection following experimental challenge with isolates of scrapie (e.g scrapie sheep brain pool 1 or SSBP/1) and BSE (see table 1.3 Hunter 1997).

Codon code	Amino acid alternatives	Single-letter
136	Valine	V
	Alanine	A
154	Arginine	R
	Histidine	H
171	Arginine	R
	Glutamine	Q
	Histidine	H

Table 1.3: The three most important disease-related polymorphisms of the sheep PrP gene. Taken from Hunter (1997).

The current commonly accepted designation for sheep genotypes is a 3 letter code for the amino acids at 136, 154 and 171 for each allele in turn, for instance VRQ or ARR. Polymorphisms of the PrP gene were found to be associated with differences in susceptibility and length of incubation period in sheep with natural scrapie and experimentally infected animals (Goldmann *et al*., 1994; Clouscard *et al*., 1995). Sheep genotypes with VRQ and ARQ are linked to a higher susceptibility to scrapie, whereas those with AHQ and ARR alleles are linked with increased resistance to scrapie. Scrapie susceptibility is also influenced by sheep breed. In Suffolk sheep the genotype

ARQ/ARQ is susceptible to natural scrapie, whereas in some flocks of Cheviots this genotype is relatively resistant (Hunter 1997).

In sheep, PrP genotype can influence the disease pathogenesis. Sheep with the genotype VRQ/ARR are relatively resistant to natural scrapie, and in the occasional cases that occur, minimal PrP^{Sc} deposits are found in their lymphoid tissues (Andreoletti *et* al., 2000). In contrast, highly susceptible VRQ/VRQ animals exposed to natural scrapie show PrP^{Sc} deposition in the tonsils one year before the onset of clinical disease (Schreuder *et al*., 1998). Therefore disease resistance associated with the VRQ/ARR genotype may result from the lack of lymphoid involvement in the peripheral pathogenesis of scrapie in these sheep.

1.5. Peripheral pathogenesis

1.5.1. Initial infection routes

Although the initial route of TSE infection is unclear, there is evidence in sheep to suggest that disease can be acquired orally (Hadlow *et al*., 1982; Andreoletti *et al*., 2000). Maternal or vertical transmission from mother to lamb is highly suspected, although contamination of foetuses *in utero* has not been shown. PrP^{C} is present in human and ruminant milk (Franscini *et al*., 2006), and recently transmission of scrapie infectivity was reported using milk from sheep infected with classical scrapie (Konold *et al*., 2008). Scrapie and mastitis infection may lead to accumulation of PrP^{Sc}/infectivity in inflamed ectopic lymphoid follicles of mammary glands, also indicating that milk may play a role in direct transmission (Ligios *et al*., 2005).

Rodent studies indicate that skin scarification is an effective means of scrapie transmission (Taylor *et al*., 1996) and it may be that natural scrapie in sheep could be transmitted through skin lesions in the mouth (Bartz *et al*., 2003). Additionally, traces of scrapie infectivity have been reported in the salivary glands of scrapie-infected goats (Hadlow *et al*., 1974) and more recently detection of abnormal PrP was observed in salivary glands of clinical scrapie sheep. Transmission of CWD *via* saliva has also been demonstrated. (Mathiason *et al*., 2006; Vascellari *et al*., 2007).

Infected faeces and urine have been suggested as a potential source of contamination and transmission of TSE disease in the environment. In murine models PrP^{Sc} is apparent in stools after oral ingestion of TSE agent (Maluquer de Motes *et al*., 2008) and PMCA amplification methods have demonstrated that PrP^{Sc} is present in the urine of some scrapie-infected hamsters during the clinical stage of disease (Murayama *et al*., 2007). Also, in hamster models, TSE transmission has been shown *via* inoculation of scrapie-infected urine into healthy animals (Kariv-Inbal *et al*., 2006). Additionally, animals suffering from inflammatory kidney disorders appear to accumulate TSE infectivity in urine, highlighting that general health may be important in disease pathogenesis (Seeger *et al*., 2005). TSE agents can persist in the environment for a number of years in soil, which may also be a source of infection for sheep grazing on pastures where infected sheep have been kept before (Seidel *et al*., 2007). Evidence also suggests animals may eat TSE infected discarded placental tissues (Pattison *et al*., 1972, 1974) which accumulate abnormal prion protein (Andreoletti *et al*., 2002). Although not formally demonstrated other possible sources of infection may include open wounds, ectoparasites, and colostrum.

1.5.2. Infectious agent uptake

Once ingested, TSE agents must replicate and reach the central nervous system (CNS). Research using rodent models suggest that ingested infectious particles (following either experimental intragastric or oral exposure to scrapie) initially gain entry to gut–associated lymphoid tissues (GALT) in the small intestinal mucosa (Kimberlin and Walker, 1989). This has also been demonstrated in sheep naturally exposed to scrapie (Hadlow *et al*., 1982; Andreoletti *et al*., 2000). GALT consists of highly organized Peyer's patches present in the mucosa of the small intestine. At the surface of Peyer's patches are specialised M cells which are able to transport proteins and pathogens from the gut lumen through the cytoplasm to the basal surface (Heppner *et al*., 2001). It is possible that the scrapie agent could be taken up in the same way. TSE agents could also pass across the gut epithelium independently of M-cell transport. Evidence suggests that digestive enzymes break down the infectious agent into smaller molecules which form complexes with other proteins such as ferritin. Particles are then endocytosed in vesicular structures by a ferritin dependent mechanism (Mishra *et al*.,

2004). Alternatively, TSE agents may be directly taken up by dendritic cells (DCs). DCs open up the tight junctions between epithelial cells and the captured antigen by inserting dendritic processes into the gut lumen. This process has yet to be demonstrated with TSE agents, but has been shown using microbial cells (Rescigno *et al*., 2001). Possible spread of scrapie infectivity from the gut to the nervous system following oral inoculation is illustrated in Figure 1.3.

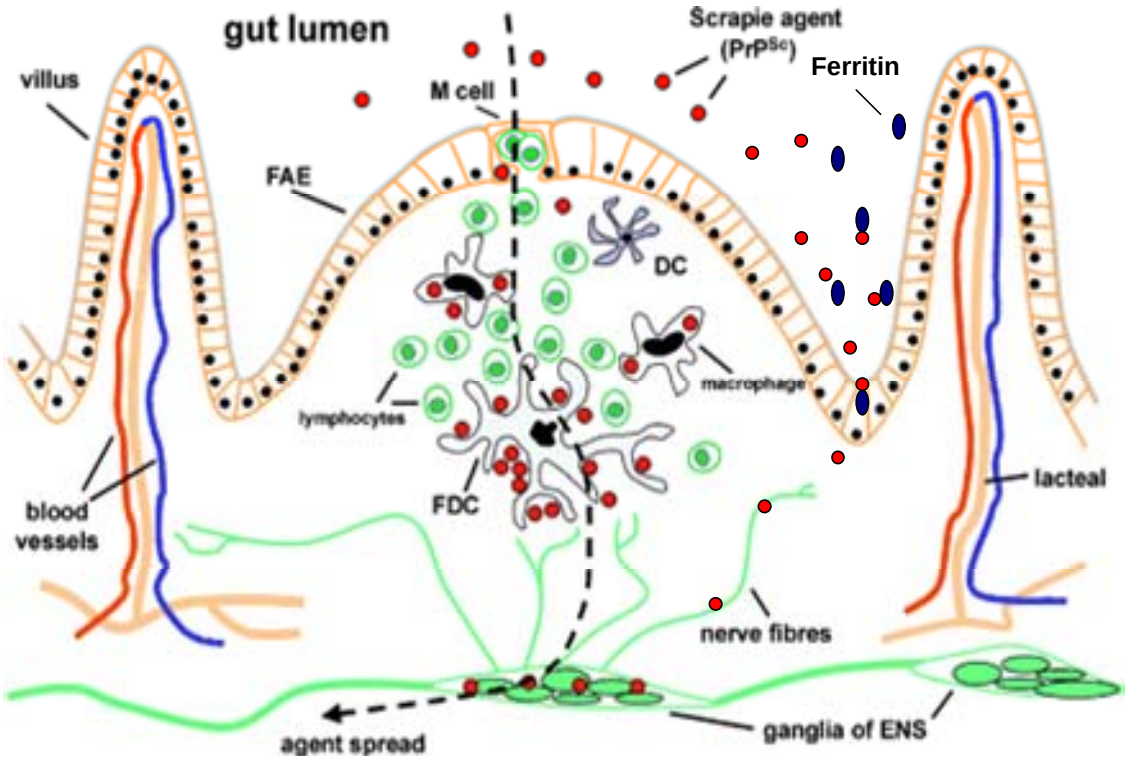


Figure 1.3: Possible spread of scrapie infectivity from the gut to the nervous system following oral inoculation. Figure 1.3 is adapted from Mabbott and Bruce, (2001). Key: FAE, follicle-associated epithelium, FDC, follicular dendritic cell, DC, dendritic cell, ENS, enteric nervous system, dotted line, route of infection. Following ingestion PrP^{Sc} is detected within Peyer's patches upon FDCs, within macrophages, within M cells and within ganglia of the ENS. Infectious agent may also be broken down into smaller molecules which form complexes with other proteins such as ferritin. These observations suggest that following uptake of scrapie infectivity from the gut lumen, infectivity accumulates upon FDCs in Peyer's patches and spreads *via* the ENS to the central nervous system.

Some TSE agents replicate in lymphoid tissues before neuroinvasion occurs. In sheep, research has shown the early appearance of scrapie agent in tonsil, retropharyngeal and mesenteric lymph nodes and intestine (Hadlow *et al*., 1982). Mice fed with scrapie or BSE agent show initial PrP^{Sc} deposition in Peyer's patches and mesenteric lymph nodes prior to infection of other lymphoid tissues, including the spleen (Maignien *et al*., 1999). Splenectomized mice (whether the spleen was removed surgically or genetically induced) were shown to have increased disease incubation periods when compared to mice with intact spleens after intra-peritoneal (i/p) inoculation (Fraser and Dickinson., 1970, 1978). Additionally, severe combined immunodeficient (SCID) mice which lack B and T lymphocytes are relatively resistant to experimental infection with scrapie (Fraser *et* al., 1996; Lasmezas *et* al., 1996). Susceptibility was restored following allogenic bone marrow transplantation from normal mice (Fraser *et* al., 1996). Exposing animals to ionising radiation does not appear to delay neuroinvasion (Fraser and Farquhar, 1987). Such treatment destroys all rapidly dividing cells, including bone marrow precursors of most of the cells that populate the immune system. However, it does not destroy follicular dendritic cells (FDCs). As neuroinvasion is not impaired by the effects of radiation this indicates that FDCs and other cells of the immune system may play an important role in TSE pathogenesis.

1.5.3. Follicular dendritic cells, macrophages, T cells, B cells and dendritic cells

Follicular dendritic cells (FDCs) are present within Peyers patches and all germinal centres (GCs) including those of the spleen, lymph nodes and tonsil (Montrasio *et al*., 2000). FDCs are thought to be derived from stromal precursor cells, and are non-phagocytic and non-migratory (Maeda *et al*., 1995). The majority of studies investigating the role of FDCs in TSE agent uptake have been carried out using rodent models. However, in sheep, PrP^{Sc} is also found on FDCs in addition to other cell types in lymphoid nodules in the gut (van Keulen *et al*., 2002). Mouse studies show early TSE agent accumulation on FDCs in the Peyer's patches and immature isolated lymphoid follicles of the intestine (Glaysher and Mabbott, 2007). Chimeric mouse models show that FDCs themselves express PrP^{c} rather than acquiring it from other PrP-expressing cells. In these models PrP^{C} can be expressed on FDCs, but not

lymphocytes and vice versa (Blattler *et al*., 1997; Klein *et al*., 1998; Brown *et al*., 1999). When chimeric mice are challenged with the ME7 scrapie stain, high levels of infectivity are observed in the spleen only in the presence of PrP-expressing FDCs, highlighting again that it is in these cells that early accumulation and replication of TSE agents occurs. Additionally, when FDCs are eliminated by disrupting cytokine signalling pathways required to maintain FDCs in their differentiated state, infectious agent does not accumulate in the spleen or lymph nodes and neuroinvasion is delayed after scrapie inoculation (Mabbott *et* al., 2000, Prinz *et al*., 2002).

Transgenic mice with specific immune system defects show that B cells also play a role in disease pathogenesis, as B cell deficient or knockout mice are also relatively resistant to scrapie (Frigg *et al*., 1999; Weissmann *et al*., 1994). It appears that PrP^{c} expression on B lymphocytes is not required for neuroinvasion (Klein *et* al., 1998) suggesting that rather than being targets for infection their role in pathogenesis may be indirect. B lymphocytes secrete the cytokines lymphotoxin α/β (LTα/β) and tumour necrosis factor (TNF-α) which are essential for the formation and maturation of FDC networks in GCs. Knockout mice with defective TNFα or LTα/β and/or their receptors lack mature FDCs and are less susceptible to disease when challenged with scrapie (Mabbott *et al*., 2000; Montrasio *et al*., 2000). The same effect is achieved when FDCs are temporarily depleted by blocking the LTβ pathway with an inhibitor (Mohan *et al*., 2004). Therefore, the importance of B cells in pathogenesis may be due to their role in maintenance of FDC networks. They may also deliver infectious agent to FDCs or even transfer infection from FDCs to other cell types (Raeber *et* al., 2001).

FDCs are immobile, so it is unclear how infection first reaches these cells and is transported to other tissues. It may be that FDCs directly trap cell-free PrP^{Sc} or other molecules associated with the agent in complement-bound complexes (Klein *et al*., 2001; Mabbot *et al*., 2001). In mice, once an antigen enters the body it is bound by antibody and complement components such as C3 fragments (C3b, C3d, C1q). FDCs express the complement receptors CR1 (CD35) and CR2 (CD21) which are important for trapping immune complexes *via* activated C3 fragments. C3 knockout mice show impaired PrP^{Sc} accumulation as do mice with transient C3 depletion, indicating that

such fragments may bind the infectious agent to FDCs (Klein *et al*., 2001; Mabbott *et al*., 2001). Two distinct pathways are involved in C3 activation. C1q can interact with antibody bound to antigen or can interact directly in an antibody-independent manner to cell surface molecules on pathogens. This is termed the classical pathway. In contrast, the alternative pathway is completely antibody-independent, and is initiated by direct binding of C3 to a range of pathogen cell surface components. Recently both recombinant PrP and PrP^{Sc} from diseased mouse brain has been shown to directly activate and fix complement *via* the classical but not the alterative pathway (Mitchell *et al*., 2007).

TSE agents could also be transferred to FDCs *via* mobile cells such as migratory bone marrow derived dendritic cells (DCs) which specifically migrate to lymphoid tissues once activated by tissue injury or infection. Human DCs express PrP^{c} (Burthem *et al*., 2001) and these cells are associated with PrP^{Sc} in intestinal lymph following experimental oral infection of rats (Huang *et al*., 2002). Other groups have shown that the neurotoxic prion protein fragment PrP106-126 is a chemoattractant for monocyte-derived DCs. These experiments were performed *in vitro* however and as yet there is no evidence that this PrP fragment is generated *in vivo* (Kaneider *et al*., 2003). It has also been demonstrated that DCs from scrapie-infected mice induce disease when injected intravenously into RAG-$1^{0/0}$ mice (recombinase activating gene null mice, deficient in B and T cells). The recipient mice developed scrapie without accumulation of agent in the spleen, which suggests that DCs alone may be able to mediate neuroinvasion without the involvement of other components of the lymphoid system (Aucouturier *et al*., 2001). Recently transgenic mice deficient in $CD11c^{+}$ DCs showed a lack of scrapie accumulation in the GALT and spleen following oral scrapie challenge. Additionally, $CD11c^{+}$ DC depletion increased resistance to oral scrapie challenge, indicating that migratory $CD11c^{+}$ DCs may play a role in TSE agent transfer from the gut lumen to the GALT which precedes neuroinvasion (Raymond *et al*., 2007). DCs have also been shown to express complement receptors (Castellano *et al*., 2004) and it is conceivable that DCs may utilize the complement system in the targeting and transport of TSE agents although this has not formally been demonstrated.

PrP^{Sc} has also been detected in GC macrophages suggesting that these cells may take up PrP^{Sc} from FDCs (Jeffrey *et al*., 2000). Macrophages may help clear infection by digesting or phagocytosing infectious agent (Beringue *et al*., 2000). Competition may then occur between destruction of infectivity by macrophages and accumulation by FDCs.

T lymphocytes are not thought to be involved in scrapie pathogenesis. Thymectomy has no effect on the disease incubation period after peripheral scrapie infection of mice (McFarlin *et al*., 1971; Fraser and Dickinson, 1978). Since this first observation, additional studies from Klein and colleagues have shown that T cell deficiencies in mice ($CD4^{-/-}$, $CD8^{-/-}$, $TCR\alpha^{-/-}$) have no effect on the accumulation of TSE agent in the spleen or on disease susceptibility (Klein *et al*., 1997, 1998).

1.5.4. Neuroinvasion

Before the onset of clinical signs, infection spreads from the lymphoid tissues to the CNS. As lymphocytes recirculate between lymphoid tissues and blood it is possible that infectivity and/or PrP^{Sc} may be spread to the CNS *via* blood. Also TSE infectivity has been demonstrated during the preclinical and clinical stages of incubation in the blood of sheep infected with scrapie and BSE (Houston *et al*., 2000; Hunter *et al*., 2002). Additionally, low levels of infectivity are detected in blood in mouse TSE models (Brown *et al*., 1998, 1999). Studies by Blattler and colleagues suggest however that neuroinvasion does not occur *via* blood. In these experiments neurografts (PrP^{+}) were introduced into PrP null mice. Following intracerebral inoculation with scrapie, the graft but not the surrounding tissue showed scrapie pathology. However, when animals were inoculated with scrapie agent *via* the intravenous route, the grafts were not infected (Blattler *et al*., 1997). This is a highly artificial situation and it is not clear if it relates to natural infection or not. It is not surprising that the grafts become infected following intracerebral inoculation with scrapie and it is possible that infectivity following intravenous inoculation may reach the blood brain barrier but not necessarily the graft site, which would prevent the graft becoming infected. Other evidence suggesting that neuroinvasion does not occur *via* blood, is presented in immunohistochemical studies which show that disease pathology appears to begin in

areas of the brain such as the dorsal nucleus of the vagus nerve that are not associated with blood vessels (Jeffrey *et al*., 2001).

In contrast, more recent evidence suggests that infectivity and/or PrP^{Sc} may be spread to the CNS *via* blood. Experiments in sheep show that factors such as the breed of sheep, PrP genotype, TSE source and the route of infection appear to have no effect on the location of PrP^{Sc} deposition in the brain, which was the same as if infection had been acquired orally (Siso *et al*., in press). PrP^{Sc} was found in capillary rich areas near the base of the brain that lack the usual blood-brain barrier. It is possible that infection, when present in the blood, may be transferred to the brain *via* such capillaries, thus representing an alternative pathway of neuroinvasion (Siso *et al*., in press).

Studies in sheep suggest that the enteric nervous system, at the level of the duodenum and ileum are the first neural tissues to be involved in neuroinvasion (van Keulen *et al*., 2000; van Keulen *et al*., 2002). Recent evidence suggests that it is the somatostatin-expressing enteric neurones, with fibre projections near Peyer's patches that are early targets (Schneider *et al*., 2008). From the enteric nervous system, infectivity and/or PrP^{Sc} may travel along sympathetic nerves (Chiocchetti *et al*., 2008) *via* the celiac-mesenteric ganglion to the spinal cord, or along parasympathetic neuronal pathways directly to the brain stem. Experiments in rodents have also shown that the sympathetic and parasympathetic branches of the autonomic nervous system may be involved in neuroinvasion (Kimberlin and Walker, 1989; McBride and Beekes, 1999). Infection may spread from FDCs directly or *via* other cell types to the vagus nerve which terminates in the ganglia of the enteric nervous system or from splanchnic nerve endings in lymphoid tissues such as the spleen and then be transported to the CNS along nerve fibres (Kimberlin and Walker, 1989; Baldauf *et al*., 1997; McBride and Beekes, 1999; van Keulen *et al*., 2000). The distance between FDCs and nerve endings may influence the rate of agent transfer to the nervous system, such that neuroinvasion could occur more rapidly from sites where FDCs are closely associated with nerve fibres (Prinz *et al*., 2003).

1.5.5. Summary

The mechanisms which underpin TSE agent accumulation and replication in lymphoid tissue and neuroinvasion may vary depending on factors including the strain of agent, the infectious dose, host species and route of infection. The general health of the animal may also be important, as it has been shown that animals suffering inflammatory conditions such as nephritis and pancreatitis appear to accumulate TSE agent in organs that are not normally affected in healthy mice (Heikenwalder *et al*., 2005). PrP^{Sc} is also present in ovine mammary glands, if animals are suffering from both scrapie and mastitis (Ligios *et al*., 2005). PrP genotype may also influence the distribution of infectious agent, with infectivity being replicated more efficiently in the periphery of susceptible sheep and less frequently in animals with resistant genotypes (Ersdal *et al*., 2005; Jeffrey *et al*., 2006).

1.6. Role of blood in pathogenesis

1.6.1. Evidence of infectivity in blood

Lymphocytes recirculate between lymphoid tissues and blood and in scrapie-infected animals it is possible that infectivity is spread in this manner, although it is not believed significant for neuroinvasion (Blattler *et al*., 1997). Scrapie and experimental BSE can be transmitted between sheep by transfusion of whole blood and buffy coat (Hunter *et al*., 2002; Houston *et al*., 2000). Experimental rodent TSE models have demonstrated low levels of infectivity in blood during preclinical and clinical stages of disease (Brown *et al*., 1998, 1999; Cervenakova *et al*., 2003). Taylor and co-workers found only a small proportion (four from a total group of forty eight) of healthy mice showed clinical signs of disease after intracerebral (i/c) inoculation with plasma infected with experimental BSE (Taylor *et al*., 2000). Transmission of infection may be limited by effects such as the species barrier (which occurs due to the difficulty to infect animals e.g mice with infectious agent from animals of a different species e.g cattle). Additionally, only small volumes of plasma can be assayed by i/c inoculation. Other rodent studies have shown that TSE infected whole blood does not cause infection

following transmission to healthy rodents (Eklund *et al*., 1967). It is possible that levels of infectivity in blood may be low and larger volumes are required to transmit disease.

More recently, infectivity in blood has been demonstrated in sheep and primate models (Houston *et al*., 2000; Hunter *et al*., 2002; Bons *et al*., 2002) after transfusion of TSE infected whole blood and buffy coat. Additionally, CWD transmission has been shown *via* blood transfusion in white tailed deer fawns (Mathiason *et al*., 2006). Blood from scrapie-infected sheep without accumulation of abnormal PrP in lymphoid tissues can also transmit infection and cause disease in healthy animals. This highlights the fact that animals early in the incubation period can transmit disease *via* blood (Siso *et al*., 2006). In humans four vCJD cases have also been reported which probably resulted from an infected blood transfusion (Llewelyn *et al*., 2004; Peden *et al*., 2004; Wroe *et al*., 2006; Health Protection Agency., 2007). The first case was identified in December 2003, where one recipient developed symptoms of vCJD six and a half years after receiving a transfusion of red blood cells donated by an individual three and a half years before the donor developed vCJD. The recipient was homozygous for methionine at PrP amino acid codon 129 (Llewelyn *et al*., 2004). Until 2004 all recorded vCJD cases had occurred in people homozygous for methionine, however a preclinical vCJD case was reported in a PrP codon 129 heterozygous (methionine/valine) patient, who died of a non-neurological disorder five years after receiving blood from a donor who developed vCJD eighteen months after donation. Although no pathological features of vCJD were seen, post mortem investigations revealed that PrP^{Sc} was present in the spleen and a cervical lymph node, but was absent in the brain (Peden *et al*., 2004). In 2006, the third case of vCJD infection associated with blood transfusion was reported (Wroe *et al*., 2006). The recipient developed symptoms of vCJD six years and died eight years, eight months after receiving blood from a donor who developed vCJD about twenty months after the blood was donated. The recipient was also homozygous for methionine at PrP amino acid codon 129 (Health Protection Agency., 2007). At the time of writing (August 2008) a fourth case of vCJD transmitted *via* blood transfusion has been reported. In this latest case, the recipient developed symptoms of vCJD eight and a half years after receiving blood

from a donor who died of vCJD about seventeen months after this blood was donated. The donor in this latest case also donated the vCJD-implicated blood that was transfused to the third case patient (Health Protection Agency., 2007). All the four blood transfusion transmissions of vCJD occurred following transfusion of nonleukodepleted red blood cells collected between 1996 and 1999 (Health Protection Agency., 2007). It is still unclear though which blood components carry infection.

Table 1.4 summarises studies showing TSE transmission *via* blood or blood components.

Table 1.4: Summary of the studies showing TSE transmission *via* blood and blood components. This table has been adapted from Brown, 2005. Key: BSE, bovine spongiform encephalopathy; CJD, Creutzfeldt-Jakob disease; CWD, chronic wasting disease; GSS, Gerstmann-Straussler-Scheinker syndrome; vCJD, variant Creutzfeldt-Jakob disease; i.c, intra-cerebral; i.p, intra-peritoneal; i.v., intra-venous; i.m, intra-muscular; s.c, subcutaneous.

Donor Animal	**Assay Animal**	**Inoculum**	**Route Of Inoculation**	**Positive/total donors**	**Reference**
Scrapie (natural)					
Goat	Mouse	Blood clot/serum	i.c.	0/3	Hadlow *et al*., 1980
Sheep	Mouse	Blood clot/serum	i.c.	0/18	Hadlow *et al*., 1982
Sheep	Sheep	Whole blood/buffy coat	i.v.	4/21	Hunter *et al*., 2002
Sheep	Sheep	Whole blood/buffy coat	i.v.	9/21	Houston *et al*., 2008 (follow up to the Hunter study in 2002)

Table 1.4: Summary of the studies showing TSE transmission *via* blood and blood components. This table has been adapted from Brown, 2005. Key: BSE, bovine spongiform encephalopathy; CJD, Creutzfeldt-Jakob disease; CWD, chronic wasting disease; GSS, Gerstmann-Straussler-Scheinker syndrome; vCJD, variant Creutzfeldt-Jakob disease; i.c, intra-cerebral; i.p, intra-peritoneal; i.v., intra-venous; i.m, intra-muscular; s.c, subcutaneous.

Donor Animal	**Assay Animal**	**Inoculum**	**Route Of Inoculation**	**Positive/total donors**	**Reference**
CJD (experimental)					
Guinea pig	Guinea pig	Buffy coat	i.c., s.c., i.m., i.p.	10/28	Manuelidis, 1978
Mouse	Mouse	Buffy coat	i.p.	4/7	Kuroda, 1983
Mouse	Mouse	All components	i.c.		Brown *et al*., 1998
Mouse	Mouse	All components	i.c.	22/235	Brown *et al*., 1999
CWD (natural)	Deer	Whole blood	i.v.	3/3	Mathiason *et al*., 2006
vCJD (experimental)	Mouse	Buffy coat	i.c.	2/2	Cervenakova *et al*., 2003
			i.v.	2/2	
		Plasma	i.c.	2/2	
			i.v.	2/2	

Table 1.4: Summary of the studies showing TSE transmission *via* blood and blood components. This table has been adapted from Brown, 2005. Key: BSE, bovine spongiform encephalopathy; CJD, Creutzfeldt-Jakob disease; CWD, chronic wasting disease; GSS, Gerstmann-Straussler-Scheinker syndrome; vCJD, variant Creutzfeldt-Jakob disease; i.c, intra-cerebral; i.p, intra-peritoneal; i.v., intra-venous; i.m, intra-muscular; s.c, subcutaneous.

Donor Animal	**Assay Animal**	**Inoculum**	**Route Of Inoculation**	**Positive/total donors**	**Reference**
Scrapie (experimental)					
Goat	Goat	Whole blood	i.c.	0/14	Pattison and Millson 1962
Sheep	Mouse	Serum	i.c.	1/1	Gibbs *et al*., 1965
Mouse	Mouse	Whole blood	i.c.	0/39	Eklund *et al*., 1967
Mouse	Mouse	Serum	i.c.	1/1	Clarke and Haig, 1967
Rat	Rat	Serum	i.c.	1/1	Clarke and Haig,1967
Mouse	Mouse	Whole blood	i.c.	3/13	Dickinson *et al*., 1969
Goat	Mouse	Blood clot	i.c or s.c.	0/20	Hadlow, 1974
Hamster	Hamster	Whole blood	i.c.	0/9	Diringer, 1984
Hamster	Hamster	Blood extract	i.c.	5/5	Diringer, 1984
Hamster	Hamster	Blood extract	i.c.	10/11	Casaccia *et al*., 1989

Table 1.4: Summary of the studies showing TSE transmission *via* blood and blood components. This table has been adapted from Brown, 2005. Key: BSE, bovine spongiform encephalopathy; CJD, Creutzfeldt-Jakob disease; CWD, chronic wasting disease; GSS, Gerstmann-Straussler-Scheinker syndrome; vCJD, variant Creutzfeldt-Jakob disease; i.c, intra-cerebral; i.p, intra-peritoneal; i.v., intra-venous; i.m, intra-muscular; s.c, subcutaneous.

Donor Animal	**Assay Animal**	**Inoculum**	**Route Of Inoculation**	**Positive/total donors**	**Reference**
BSE (experimental)					
Mouse	Mouse	Plasma	i.c.	4/48	Taylor *et al*., 2000
Cow	Mouse	Buffy coat	i.c. and i.p.	0/11	Wells *et al*., 2000
Sheep	Sheep	Whole blood/buffy coat	i.v.	2/17	Hunter *et al*., 2002
Sheep	Sheep	Whole blood/buffy coat	i.v.	5/22	Houston *et al*., 2008 (follow up to the Hunter study in 2002)
Primate (Microcebus murinus)	Primate (Microcebus murinus)	Buffy coat	i.c.	1/1	Bons *et al*., 2002
BSE (natural)					
Cow	Mouse	Blood clot/serum/buffy coat	i.c. and i.p.	0/4	Fraser *et al*., 1994

Table 1.4: Summary of the studies showing TSE transmission *via* blood and blood components. This table has been adapted from Brown, 2005. Key: BSE, bovine spongiform encephalopathy; CJD, Creutzfeldt-Jakob disease; CWD, chronic wasting disease; GSS, Gerstmann-Straussler-Scheinker syndrome; vCJD, variant Creutzfeldt-Jakob disease; i.c, intra-cerebral; i.p, intra-peritoneal; i.v., intra-venous; i.m, intra-muscular; s.c, subcutaneous.

Donor Animal	**Assay Animal**	**Inoculum**	**Route Of Inoculation**	**Positive/total donors**	**Reference**
Mink encephalopathy (experimental)					
Mink	Mink	Serum	i.c.	0/1	Marsh *et al*., 1969
Mink	Mink	Whole blood or components	i.c.	0/8	Marsh *et al*., 1973
GSS (experimental)					
Mouse	Mouse	Buffy coat	i.p.	4/7	Kuroda, 1983
Mouse	Mouse	Buffy coat/plasma	i.c.	5/5	Brown *et al*., 1999
Mouse	Mouse	Buffy coat/plasma	i.v.	2/2	Brown *et al*., 1999

1.6.2. Distribution of infectivity in blood

Evidence suggests that the highest levels of infectivity are found in the buffy coat fraction of blood. In one study, blood from mice infected with either a human-derived strain of GSS (Gerstmann-Straussler-Scheinker) disease or vCJD contained around 20-30 infectious units of agent infectivity per ml of buffy coat (containing platelets and leukocytes or white blood cells) while erythrocytes showed no infectivity (Cervenakova *et al*., 2003). Brown *et al* (1998) carried out experiments where scrapie-infected hamster brain homogenate was "spiked" into normal human blood and found that the highest levels were also in the buffy coat fraction with slightly lower levels in plasma. In follow up work these researchers used the same GSS model described by Cervenakova and co-workers to demonstrate that endogenous infectivity could be transmitted *via* the intravenous route, although larger volumes (seven times more plasma and five times more buffy coat) of plasma and buffy coat were needed to transmit disease by the intravenous route than by the intracerebral route, suggesting that transmission by the intravenous route is more inefficient (Brown *et al*., 1999). BSE infectivity was also transmitted *via* buffy coat after the primate species *Microcebus murinus* was inoculated intracerebrally (Bons *et al*., 2002). However, in a purified platelet population from a hamster model little infectivity was found (Holada *et al*., 2002). In sheep models, TSE infectivity is also found in the buffy coat fraction of blood (Houston *et al*., 2000; Hunter *et al*., 2002) and recent evidence suggest it is mainly linked to the monocyte subset of leukocytes (Andreoletti *et al*., 2007). This suggests that infection may be associated mainly with leukocytes in addition to plasma, and is the basis for blood transfusion services using leucodepletion as a precaution to prevent the spread of vCJD *via* blood in humans.

The significant levels of TSE infectivity observed in plasma may be associated with exosomes. Exosomes were first reported from culture of sheep reticulocytes (Johnstone *et al*., 1987). They are small membrane vesicles formed by invagination of the membrane of multi-vesicular bodies (MVBs) (Stoorvogel *et al*., 2002). Exosome secretion into the extracellular environment occurs upon fusion of MVBs with the cell

membrane and was suggested to be a mechanism of releasing unnecessary proteins during the maturation of reticulocytes (Johnstone *et al*., 1987). Exosomes have been shown to be associated with FDCs (Denzer *et al*., 2000), urine (Pisitkun *et al*., 2004), malignant tumour effusions (Andre *et al*., 2002), blood (Caby *et al*., 2005) and platelets (Robertson *et al*., 2006). In addition to removing unwanted cellular proteins, a role for exosomes has been described in the intercellular trafficking of human immunodeficiency virus (HIV-1) infectivity (Wiley *et al*., 2006) suggesting exosomes may play a role in distributing pathogens in the body. Endogenous PrP^{C} is associated with exosomes from epididymal fluid (Gatti *et al*., 2002), platelets (Robertson *et al*., 2006), primary cultured cortical neurones (Faure *et al*., 2006) and ovine cerebral spinal fluid (Vella *et al*., 2008). Recently, several groups have shown that PrP^{Sc} is associated with exosomes released from non-neuronal and neuronal cells in culture (Fevrier *et al*., 2004; Vella *et al*., 2007). The exosomes released from TSE-infected neuronal cells were efficient initiators of PrP^{Sc} propagation in uninfected recipient cells and to non-neuronal cells. Additionally, neuronal cells were susceptible to infection by non-neuronal cell derived exosome PrP^{Sc} (Vella *et al*., 2007). This provides evidence that exosomes may play a role in PrP^{Sc} spread *via* blood.

Soluble, oligomeric forms of PrP could also be present in blood (Kayed *et al*., 2003) and leucodepletion only removes 42 % of infectivity (Gregori *et al*., 2004). The effectiveness of this measure has recently been increased to 72 % by passing leucoreduced blood through filters containing resin designed to remove TSE infectivity (Gregori *et al*., 2006). These experiment provide further evidence for soluble infectivity in blood

1.6.3. PrP^{Sc} detection in blood

The detection of proteinase K resistant PrP^{Sc} is used as a biochemical marker for the presence of infectivity. So far, most attempts to detect PrP^{Sc} in blood have been unsuccessful. Methods used to date include immunohistochemistry (IHC) (Herrmann *et al*., 2002), immunocapillary electrophoresis (Schmerr *et al*., 1999) and Western blotting (Wadsworth *et al*., 2001). Immunocapillary electrophoresis (ICE) uses labelled synthetic peptides in a competitive binding assay. This test has been used to detect abnormal prions in scrapie infected sheep blood and elk blood infected with chronic wasting disease but with limited success. As a result this test has not been validated as a diagnostic method. An attempt has been made to adapt immunohistochemical methods to detect PrP^{Sc} in blood, specifically in sheep peripheral blood leukocytes (PBLs). By titrating PBLs with dissociated retropharyngeal lymph node cells known to express PrP^{Sc}, the researchers demonstrated that a minimum of 0.000205% or 60 PrP^{Sc} positive cells could be detected in $3x10^6$ PBLs (Herrmann *et al*., 2002). However, they failed to show any positive staining for PrP^{Sc} in PBL isolated from scrapie-affected sheep. Wadsworth et al (2001) developed a highly sensitive Western blot, but failed to detect PrP^{Sc} in the buffy coat fraction from a single vCJD case. These workers estimated that if PrP^{Sc} was present in the 150µl buffy coat sample, then the concentration in blood would be 30,000 times less than the maximum detection limit found in brain.

One possible explanation for the difficulty in detecting PrP^{Sc} in blood is that infectivity is in a form in blood which is dissimilar to that which is detected in brain. Experimental rodent models show that the detection of PrP^{Sc} does not always correlate with infectivity (Manson *et al*., 1999; Barron *et al*., 2001). It may be in blood that titres of infectivity are present in the absence of detectable PK-resistant PrP^{Sc}. Sensitive methods for PrP^{Sc} detection that have been applied to blood have so far produced negative results suggesting that if PK-resistant PrP^{Sc} is present it is so in very small amounts. It is possible that not all disease-associated PrP^{Sc} in blood is PK-resistant. Evidence suggests that PK sensitive forms of PrP^{Sc} do exist and therefore will not be detected by current methods which rely on treatment with PK (Tremblay *et al*., 2004).

Blood infectivity also appears reduced following PK treatment (Yakovleva *et al*., 2004).

Recently PK resistant PrP^{Sc} has been detected in the blood of sick hamsters (infected with the 263K scrapie strain) by means of the protein misfolding cyclic amplification (PMCA) technology (Saborio *et al*., 2001). By incubating infected buffy coat fractions with uninfected hamster brain homogenate, in a cyclical process the minute amounts of PrP^{Sc} in the buffy coat convert the larger quantities of PrP^{C} in the uninfected brain into more of the misfolded form of the protein, thus amplifying the quantity of PrP^{Sc} in the blood. This method is very sensitive and has been used to detect PrP^{Sc} in the blood of both clinical and preclinical animals although the technology has yet to detect abnormal prion protein in species other than the hamster 263K model (Castilla *et al*., 2005; Saá *et al*., 2006). Currently, PMCA technology is not generally available, is expensive and requires a high level of skill. Additionally, this technology has only recently been developed and was not available at the beginning of this project. Therefore PMCA was not pursed as a method for PrP^{Sc} detection in blood. Western blotting is a widely available method which can be very sensitive for detection of PrP^{Sc} (Lee *et al*., 2000) and was therefore developed in this project for detection of abnormal prion protein in blood.

1.6.4. Distribution of cellular PrP in blood

In the absence of a reliable method for detection of PrP^{Sc} in blood, the distribution of PrP^{C} expression on cellular blood components has been used to define potential targets for infection. This is despite the fact that the function of PrP^{C} in blood is unknown. Comparisons of healthy animals of different species (hamster, mouse, human, sheep and cattle) have revealed differences in the expression and distribution of PrP^{c} on blood cells (Barclay *et al*., 2002; Holada and Vostal, 2000). In humans, the highest PrP^{c} expression levels were seen on platelets and PBMCs and lower levels were detected on neutrophils and erythrocytes (Barclay *et al*., 2002; Herrmann *et al*., 2001). PrP mRNA and cell surface PrP^{C} have been found in peripheral blood mononuclear cells (PBMCs) of normal and scrapie-infected sheep. The PrP was found to be proteinase K sensitive

(Herrmann *et al*., 2001). Using FACS analysis, Barclay *et* al. (2002) also showed PrP^c expression on PBMC but were unable to detect PrP^c on the cell surface of platelets, erythrocytes and granulocytes prepared from uninfected sheep blood. However, using Western blot analysis, it has been shown that sheep platelets do express PrP^c internally rather than on the surface (Halliday *et al*., 2005). This difference in observation is likely due to the way in which the samples were prepared in Western blot assays (where cells were lysed) representing intracellular PrP^c, whereas FACS analysis represents PrP expression only on the cell surface.

In sheep PrP^C is expressed on all major subsets of PBMCs, with the highest levels on the $CD21^+$ subset of B cells. In some scrapie-infected animals PrP^C expression on $CD21^+$ B cells appears dramatically up-regulated (Halliday *et al*., 2005; Eaton *et al*., 2007). $CD21^+$ B cells have been highlighted as being important in preclinical disease in deer calves orally infected with CWD. Using confocal microscopy the authors demonstrated that PrP^{Sc} appeared to co-localise on membranes associated with FDCs and CD21 expressing B lymphocytes (Sigurdson *et al*., 2002). $CD21^+$ B cells may also play a role in scrapie pathogenesis in mice peripherally inoculated with scrapie (Frigg *et al*., 1999; Mabbott *et al*., 2001) which suggests these cells may be potential carriers of the infection.

Variation of cellular PrP levels in blood may occur depending on the TSE disease as PrP^c concentration differs in variant and sporadic CJD cases after detection using FACS and DELFIA techniques (dissociation enhanced lanthanide fluoroimmunoassay, Fagge *et al*., 2005). Levels of PrP^C in blood were reduced in vCJD cases. This may be because in vCJD replication of PrP^{Sc} occurs in the periphery before neuroinvasion and whole blood PrP^C may be converted to PrP^{Sc} as part of the replication process involved in disease pathogenesis. Reduction of PrP^C in whole blood was not seen in sporadic CJD cases and this may be due to PrP^{Sc} being largely confined to the central nervous system and replication of PrP^{Sc} in the periphery before neuroinvasion is not a feature of this disease.

Variation of PrP^C levels in blood may also occur depending on the PrP genotype of the animal. PrP^C was highly expressed on PBMC subsets of scrapie-susceptible VRQ/VRQ sheep, whereas lower levels were observed in scrapie-resistant (ARR/ARR) animals (Halliday *et al*., 2005). Surprisingly, although hamsters PBMCs do not express PrP^c, they are associated with infectivity (Holada *et al*., 2002). This may imply that PrP^C expression is not an absolute requirement for susceptibility to infection. In this case, hamster PBMCs may act as carriers, passing the infection to other cell types, without replicating the infectious agent themselves.

1.6.5. Protein markers of disease other than PrP^{Sc}

A major goal in TSE research is the development of methods which are sensitive enough to detect infection and/or PrP^{Sc} in blood. However, if PrP^{Sc} can not be detected in blood, it is possible that other molecules may be identified as surrogate markers for TSE infection. In blood, TSE associated biochemical changes in metabolites and hormones have been described. TSE infected hamsters show elevated levels of plasma noradrenalin after comparison with control animals (Pollera *et al*., 2007). Moorby and co-workers (2000) sampled blood from 47 healthy and BSE-infected dairy cows over a 28 week period, and observed increased levels of plasma β-hydroxybutyrate in diseased animals. In a follow up study, increased concentrations of lactic acid and amino acids were observed in pre-clinical and clinical BSE-infected animals (Moorby *et al*., 2002). However, changes in metabolite concentrations can be attributed to other conditions such as ketosis (Lindner, 1959).

Other novel molecular biomarkers in blood have also been described. Miele *et al*., (2001) found that transcription of the gene encoding erythroid differentiation related factor (EDRF) now known as erythroid-associated factor (ERAF) was down-regulated in TSE infected mice. However, ERAF transcription in healthy individuals was found to occur with a high degree of variation which questions the suitability of using ERAF as a marker for TSE disease. Alternatively PrP^{Sc} binding proteins could be sought such as plasminogen which has been shown to discriminate between PrP^{Sc} and PrP^C, selectively binding to the PK-resistant form of the protein (Fischer *et al*., 2000). If

PrP^{Sc} can not be detected in blood, other markers for TSE infection may form the basis of TSE diagnostic tests.

1.7. Methods of detecting and measuring PrP^{c}/PrP^{Sc}

Whilst the detection of proteinase K resistant PrP^{Sc} is used as a biochemical marker for the presence of infectivity, the only available method which formally demonstrates infectivity is the bioassay. Here the infectious agent or a particular strain is inoculated into another animal (usually mice) to see if the animals develop the same disease and the strain characteristics are maintained in primary and secondary passages. These assays are limited by effects such as the species barrier and can also take many months or years to obtain results. The development of transgenic mice lines is providing ways to overcome such limitations (Scott *et al*., 1997; Yokoyama *et al*., 2007).

1.7.1. Immunohistochemistry

Traditional methods used to confirm TSE infection include detection of PrP^{Sc} using immunohistochemistry (IHC). These techniques help give an idea of disease pathogenesis and their main advantage is that they allow identification of the cellular location of PrP^{Sc} in tissues. Using such methods infection can often be detected before neuroinvasion or the onset of clinical signs (Schreuder *et* al., 1998). IHC is one of the most sensitive methods of detection for PrP^{Sc} (Grassi, 2002). PrP^{Sc} can be distinguished from PrP^{c} by PK treating the tissue section (although PK treatment is not used in all methods) and using an antibody that recognises an epitope beyond the PK cleavage point. However IHC is not a rapid method and the amount of PrP^{Sc} deposited in tissues cannot be reliably measured.

1.7.2. Western blotting

Western blotting for detection of PrP^{Sc} relies on proteinase K digestion to distinguish the abnormal protein from PrP^{C}. Extracts of PrP^{Sc} from infected tissues are treated with proteinase K, run on an SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel

electrophoresis) gel, transferred to nitrocellulose membrane and then probed using specific antibodies to detect undigested PrP (Bolton *et al*., 1982). After digestion with proteinase K, PrP^{Sc} gives a distinct pattern on Western blots where three distinct bands can be identified and resolve between 27-30 kDa. Hence, the PK resistant core of PrP^{Sc} is called p27-30. The three distinct bands represent differential glycosylation as PrP has two sites at which carbohydrates can be attached. The diglycosylated form exists when both sites are occupied, monoglycosylated forms occur when either one of the two sites are occupied, and unglycosylated forms occur when no sites are occupied (Collinge *et al*., 1996).

Efforts made to enhance Western blot sensitivity include concentrating PrP^{Sc} by precipitation with sodium phosphotungstic acid (NaPTA). In a recent study adopting this step sensitivity was increased up to three-fold, allowing for the identification of extraneural abnormal PrP in spleen and muscle from sporadic CJD victims (Glatzel *et al*., 2003). Despite these advances, PrP^{Sc} has not to date been detected in blood by Western blot. However this method can be very sensitive for PrP^{Sc} detection (Lee *et al*., 2000) and is a relatively rapid method compared to the bioassay.

1.7.3. Sandwich immunoassays

More recently, immunoassays have been developed using the sandwich principle in which monoclonal antibodies are used for antigen (PrP) capture and detection e.g. an enzyme-linked immunosorbent assay (ELISA, Korth *et al*., 1999), dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA, Barnard *et al*., 2000) or conformation-dependent immunoassay (CDI, Safar *et al*., 1998; McCutcheon *et al*., 2005). In ELISAs an enzymatic reaction measures PrP, whereas in DELFIA systems the detection is based on formation of a fluorescent chelate (Safar *et al*., 1998). DELFIA and CDI use denaturation with guanidinium hydrochloride, rather than PK-resistance to distinguish between cellular and disease associated PrP. The basis of the CDI is that the detector antibody recognises an epitope that is always exposed in PrP^{c}, but only becomes exposed in PrP^{Sc} following denaturation. As a result of this PrP^{Sc} can

be identified and measured by comparing antibody binding to denatured and native forms of the prion protein.

1.7.4. PrP^{Sc} specific antibodies

One main focus in TSE research is the development of monoclonal antibodies that can specifically bind to PrP^{Sc} and not PrP^{c}. However despite early reports it has proved difficult to discriminate between the two isoforms using antibodies (Korth *et al*., 1999). Antibodies directed against tyrosine-tyrosine-arginine (YYR) motifs have been reported as being capable of selectively detecting PrP^{Sc} (Paramithiotis *et al*., 2003). However, YYR motifs are not unique to pathological prion proteins. Antibodies binding epitopes comprising residues 96-104 and 133-158 of cellular PrP have been shown to inhibit conversion of PrP^{c} to PrP^{Sc}. Moroncini and co-workers grafted PrP sequence motifs corresponding to these regions into a recipient antibody scaffold. This resulted in the production of motif-grafted antibodies specific for disease-associated PrP (Moroncini *et al*., 2004). PrP^{Sc} specific antibodies (which are not generally available) are tested by methods not dependent on PK digestion or denaturation of the protein such as immunoprecipitation. If conformational antibodies can be produced the sensitivity of methods may be increased and more direct assays for PrP^{Sc} detection may emerge.

1.7.5. Cell lines

Cultured cells persistently infected with scrapie offer a convenient system to study the biogenesis of the prion protein isoforms (Taraboulos *et al*., 1990). Only a few cell lines have been established *in vitro* that continuously produce low levels of PrP^{Sc}. These include the scrapie-infected mouse neuroblastoma-derived ScMNB and ScN2a cells (Race *et al*., 1987; Butler *et al*., 1988) and the HaB line, derived from the culture of a Syrian hamster brain (Taraboulos *et al*., 1990). The SMB cell line is also well established (Clarke and Haig, 1970, 1971; Birkett *et al*., 2001). Methods utilising such models have enabled the development of highly sensitive cell-based infectivity assays comparable to mouse bioassays (Klohn *et al*., 2003). In such assays uninfected

susceptible cell lines can be incubated with TSE infected brain and the transmission of infectious agent to the cells measured. Infected cells can be quantified by visualizing single PrP^{Sc} positive cells by an ELISA based method. Alternatively, infected cells can be cultured and the number of PrP^{Sc} positive colonies determined using cell blotting techniques. The infectivity titres measured in tissue culture and in the mouse are similar and if the method can be applied to more than one mouse scrapie strain, cellular components, in addition to tissue homogenates, could be used to assess the sensitivity of other methods (for instance Western blots) (Klohn *et al*., 2003).

1.8. Progress of TSE diagnostic tools

Accurate pre-mortem tests using IHC on lymphoid tissues (tonsil, 3rd eyelid and rectal mucosa) exist, although such tests do not always have the required sensitivity for detection (Gonzalez *et al*., 2008). Therefore, a definitive TSE diagnosis can only be made post-mortem. Following the BSE epidemic in the UK in the 1980s, the European Commission implemented a system to evaluate and validate biochemical tests that could rapidly detect infected animals. Currently, five post-mortem BSE detection tests have been approved, which all rely on immunodetection of PrP^{Sc} in brain tissue. The principle behind each test, the advantages and disadvantages and the detection limits of each are outlined in table 1.5.

Based on estimated sensitivities (table 1.5) the ability of these current tests to detect individuals during pre-clinical stages of infection is unlikely. Although all these current tests detect PrP^{Sc} in the brain, they do not have the required sensitivity of detection for use with other tissues or fluids, including blood.

Technique	Principle	Detection limit	Advantages	Disadvantages
Prionics-Check Western test	Gel electrophoresis and Western blot	5-20 pmol	Good reproducibility, low rate of false-positives	Low throughput, low sensitivity
Enfer test	Simple ELISA	1-10 pmol	Rapid and simple	Potential for false-positives
CEA/Biorad test	Sandwich ELISA using two different antibodies	0.5-2 pmol	High sensitivity	Longer and more laborious, potential for false-positives
Prionics-Check LIA test	Sandwich ELISA using two different antibodies	1-5 pmol	Rapid, automated, simple	Variability depending on sample preparation, potential for false-positives
Conformational-dependent immunoassay (CDI)	Differential antibody binding to native and denatured PrP^{Sc}	0.5-5 pmol	Independent of protease digestion, high sensitivity	Complicated and involves more steps than other tests
IDEXX BSE-Scrapie EIA	Seprion-capture technology applied to a microtiter plate format.	Detects classical scrapie brain diluted 1:4000	A single test kit can be used to test cattle, sheep, goat samples.	Relatively expensive.
Prionics –Check LIA	ELISA based	-	Suitable for processing sample volumes of several hundred assays or more per day.	Low sensitivity.

Table 1.5: Summary of the tests currently approved for post-mortem detection of PrP^{Sc} in cattle brain. This table has been taken from Soto, 2004. The detection limits are based on the estimates reported in previous publications, (Brown *et al*., 2001; Ingrosso *et al*., 2002).

Technique	Principle	Detection limit	Advantages	Disadvantages
Prionics-Check Prio-Strip	Lateral flow technology	-	Cost effective. Unique 8-strip format.	Low sensitivity.
HerdChek BSE Test BSE	No PK digestion and uses an aggregate specific capture ligand on a dextran polymer of PrPSc which after denaturation is detected using an anti-PrP antibody.	-	New technology with potential for greater sensitivity.	Relatively expensive.

Table 1.5: Summary of the tests currently approved for post-mortem detection of PrP^{Sc} in cattle brain. This table has been taken from Soto, 2004. The detection limits are based on the estimates reported in previous publications, (Brown *et al*., 2001; Ingrosso *et al*., 2002).

1.8.1. Current status of TSE blood tests

Infectivity studies in rodents show that high concentrations of infectivity reside in the buffy coat fraction of blood, where a level of 5-10 infectious units (IU) ml^{-1} can be present during the incubation period and up to 100 IU ml^{-1} at the onset of clinical signs (Brown *et al*., 1998; Brown, 2001; Cervenakova *et al*., 2003; Holada *et al*., 2002). Levels of infectivity in plasma and red cells are estimated to be a least 5-10 times lower than in buffy coat (Brown *et al*., 1998). This means that blood tests using PrP^{Sc} as a marker for infectivity would have to detect the protein in the femtomolar concentration range. The detection tests currently available are still approximately 2-4 orders of magnitude away from reaching the sensitivities required for clinical and pre-clinical blood detection (table 1.5). However, recently many companies and research groups have used novel strategies to try to overcome this problem.

1.8.2. Specific ligands

Ligands that are able to bind specificity to PrP^{Sc} could be used to concentrate abnormal PrP by immunoprecipitation techniques. Such ligands include plasminogen (Fischer *et al*., 2000), RNA (Weiss *et al*., 1997), the 15B3 antibody produced by Prionics (Korth *et al*., 1997) and the Seprion ligand developed by Microsens Biotechnologies (Oliver *et al*., 2006). PrP^{Sc} concentration using specific ligands could be combined with an ELISA-based immunoassay or Western blotting to increase overall sensitivity of these methods. However, not all ligands may be suitable for TSE diagnosis, for example it has been reported that plasminogen has a high affinity for PrP^{C} (Shaked *et al*., 2002; Kornblatt *et al*., 2003) and therefore may not bind specifically to PrP^{Sc}.

1.8.3. AS-ELISA

Aggregate specific ELISAs, termed AS-ELISAs, are immunoassays where the same monoclonal antibody is used for capture and detection, and which have been developed to detect polymerised PrP (Pan *et al*., 2005). Overall sensitivity is increased using a combination of signal amplification (fluorescence) and amplification of PrP^{Sc} using a simplified PMCA like procedure. AS-ELISA methods have enabled detection of PrP^{Sc} aggregates in clinical plasma from mice or deer infected with scrapie or CWD (Chang *et al*., 2007). However, to allow licensing as a diagnostic/screening test, these methods need to be successfully applied to pre-clinical blood samples to establish if the necessary levels of sensitivity and specificity can be achieved.

1.8.4. Conformationally sensitive peptides

Recently, fluorescence labelled palindromic PrP peptides have been developed and used to detect misfolded prion protein in clinical TSE-infected plasma. In this approach, when the labelled peptide is in contact with PrP^{Sc}, it undergoes a large coil to a β-sheet conformational change which largely modifies the properties of the fluorescent label. Fluorescence is then measured which allows for discrimination between TSE infected and uninfected animals (Pan *et al*., 2007). Similar to AS-ELISA, further testing is required on pre-clinical samples to assess suitability as an effective TSE diagnostic test.

1.8.5 Other approaches

Several other techniques may be used to increase the sensitivity of PrP^{Sc} for detection in blood. These include the use of NaPTA to concentrate PrP prior to Western blotting (described in Chapter 3) and PMCA technology which has be used to detect PrP^{Sc} in pre-clinical blood samples (Castilla *et al*., 2005; Saá *et al*., 2006). Additionally, more complicated methods requiring a high level of skill to measure and interpret results have also been described such as fourier-transformed infrared spectroscopy (FTIR) (Lasch *et al*., 2003).

1.8.6. Summary

Although current diagnostic tests do not have the required sensitivity to be applied to blood samples, some of the newer strategies remain promising for the early detection of pre-clinical TSE-infected individuals. Blood diagnostic tests are of particular importance for ensuring the safety of blood transfusion in countries that have experienced large BSE epidemics (UK and Western Europe). Therefore, the development of a TSE blood test remains a major goal in the field of TSE research. In order to achieve this goal, further knowledge of the mechanisms of TSE disease pathogenesis is required. For instance, it is not yet known which blood cells may be involved in replication and spread of the TSE agent within the body prior to neuroinvasion. If the blood cells that contribute to spread of infection can be identified, these findings may aid in the development of pre-mortem blood diagnostic tests.

1.9. Thesis Aims

TSE transmission *via* blood transfusion has been demonstrated experimentally in rodent, primate and sheep models. More concerning however, is the reporting of four human vCJD cases occurring as a result of infected blood transfusions. Despite a wealth of research within this area, little is known about which cells are involved in the replication of the TSE agent and once "infected", how infectivity is spread throughout the body prior to neuroinvasion.

Detection of PK-resistant PrP^{Sc} is used as a biochemical marker for the presence of infectivity. The aim of this thesis was to use PrP^{Sc} as a marker for TSE infection in blood, and to determine which blood cells may be responsible for harbouring/ spreading infection throughout the body. The difficulty here lies with the fact that conventional forms of PK-resistant PrP^{Sc} may not be detectable in blood and also that if they are, then levels may be very low (against a high background of PrP^{C}).

Preliminary aims were to give a definitive answer to the question of whether conventional PK-resistant PrP^{Sc} is present in TSE-infected sheep blood in sufficient quantities to explain the infectivity levels shown by bioassay. It was first therefore necessary to develop and optimise a sensitive Western blot immunoassay for conventional PK-resistant PrP^{Sc} using scrapie-infected sheep brain homogenates.

Secondary aims were to apply this immunoassay to blood cell subsets. By determining which cells are associated with infectivity and/or PrP^{Sc} it may be that underlying mechanisms of pathogenesis would be revealed. Of course, it may be the case that PrP^{Sc} is simply not a suitable marker for TSE infection, as evidence would suggest that this protein doesn't always correlate with TSE infection. Therefore, another aim of the project was to investigate the role of other protein markers in TSE pathogenesis within the blood system.

By taking these combined approaches, it is hoped that the nature of conventional PK-resistant PrP^{Sc} in blood may be better characterised, and it will be determined whether

conventional PrP^{Sc} correlates with infectivity. Additionally, mechanisms of TSE disease pathogenesis may be revealed, particularly which blood cells contribute to spread of infection within the body. Finally, such findings may aid in the development of pre-mortem blood diagnostic tests and the effectiveness of control measures currently used by the National Blood Service to safeguard the UK blood supply could also be assessed.

Chapter 2
Materials And Methods

2.1. Materials

2.1.1. Animal tissues

Brains from Cheviot sheep (genotype VRQ/VRQ) infected with natural scrapie, SSBP/1 scrapie or uninfected (controls) were obtained from Dr F. Houston (Glasgow University). Mouse brains infected with 139 A scrapie strain were obtained from R. Hennion (IAH, Compton). All brain samples were stored at – 80 ºC.

Starting blood volumes of 50 ml from sixteen different uninfected and fourteen different clinical SSBP/1 infected Cheviot sheep (genotypes VRQ/VRQ, VRQ/ARR) were obtained from Dr F. Houston (Glasgow University). A 50 ml sample of pre-clinical BSE infected blood from one Cheviot sheep (ARQ/ARQ) was also obtained from Dr S. McCutcheon (Roslin Institute). Control animals were inoculated with 2 ml of normal 10% (w/v) sheep brain homogenate (1 ml injected subcutaneously into each medial thigh). Experimentally infected sheep were inoculated subcutaneously with 2 ml scrapie sheep brain pool 1 (SSBP/1) 10% (w/v) brain homogenate in the same way. BSE infected sheep were orally inoculated with 5 g (w/v) of bovine BSE brain.

The 50 ml samples of uninfected and SSBP/1 scrapie infected blood were collected in Falcon tubes containing 60 mM ethylenediaminetetraacetic acid (EDTA). The 50 ml sample of pre-clinical BSE infected blood was obtained at 10-11 months post challenge and collected from a bag containing 12 % (w/v) citrate phosphate dextrose (CPD-1) anticoagulant. All blood samples were used immediately or stored at – 80 º C or – 20 º C. The number of buffy coat or PBMC cells obtained from 50 ml of blood fluctuated between different sheep but ranged from between 1 x 10^6 to 1 x 10^8 cells. All animals were housed in accordance with Home Office guidelines, and were killed after the development of clinical signs of scrapie.

2.1.2. Antibodies

The panel of novel PrP specific monoclonal antibodies described in this thesis were generously donated by Dr S. McCutcheon (Roslin Institute) (paper in preparation). Further information on antibodies, including the immunogen they were raised against, dilutions used and suppliers are tabulated in Appendix 1.

2.1.3. Chemical reagents

All chemicals were analytical grade and supplied by Sigma Aldrich Co. UK; BDH Ltd UK; Gibco, UK; Invitrogen, UK; Amersham Biosciences UK; GE Healthcare, UK, except where stated.

2.1.4. Enzymes

Porcine trypsin containing 0.02 % (w/v) EDTA and proteinase K (PK) enzymes were obtained from Sigma Aldrich Co. UK. Porcine trypsin was also obtained from Promega, USA. Benzonase (Benzon nuclease, purity 1) was obtained from Merck, Germany and deoxyribonuclease (DNase) type I from Sigma Aldrich Co. UK.

2.1.5. Magnetic beads

Magnetic beads conjugated to immunoglobulin isotype-specific rat anti-mouse antibodies (Miltenyi Biotech, USA) were used to sort leucocyte cell populations.

2.1.6. Monoclonal antibody purification

BC6 antibody was obtained from Dr S. McCutcheon (Roslin Institute) or purified as below. BC6 culture fluid was centrifuged at 1000 g for 5 min and adjusted to pH 8.0 by the addition of one tenth volume of neutralisation buffer (see section 2.2.6). A HiTrap protein G column was pre-equilibrated with wash buffer (see section 2.2.6) before loading with neutralised BC6 culture fluid. The column was washed with 50 ml

wash buffer and purified antibody was eluted with 7- 10 ml elution buffer (see section 2.2.6). Purified antibody was neutralised by the addition of one tenth volume of neutralisation buffer. After determining concentration using a Beckman DU 650 spectrophotometer, purified antibody was dialysed overnight in PBS (see section 2.2.3) using pre-prepared dialysis tubing with a 15,000 molecular weight cut off. PBS was changed twice after which purified antibody was stored at a concentration of 1 µg/ml at 4 º C.

2.1.7. Recombinant protein

Full length ARR genotype α-conformation recombinant ovine PrP (residues 25-233) at a concentration of 0.82 µg/ml was kindly provided by Dr A.C. Gill (Roslin Institute) for use in BC6 antibody blocking studies. BC6 (1 µg/ml) was incubated with 830 µl of full length ARR alpha recombinant ovine PrP protein (0.82 µg/ml) for 1 hr with agitation. After further incubation for 1 hr in antibody binding buffer (used in Western blotting, section 2.2.15) this mixture was added to nitrocellulose immobilon-P transfer membranes (Millpore, USA) containing protein from uninfected PBMCs and brain with and without NaPTA precipitation. Membranes were incubated overnight before Western blotting as described in chapter 2, section 2.3.5.5.

2.1.8. Scrapie mouse brain (SMB) cell lines

The SMBs15 (scrapie-infected) and SMB-PS (cured of infection with pentosan sulphate) cell lines (Clarke and Haig, 1970) were obtained from the TSE Resource Centre at IAH, Compton. All cells were cultured at 37 º C, used immediately or stored in liquid nitrogen or – 20 º C.

2.2. Solutions, buffers and media

All solutions were made using sterile double distilled Milli-Q water, except where stated otherwise.

2.2.1. Coomassie staining solutions (for mass spectrometry)

Coomassie fixative: 45 % (v/v) methanol, 1 % (v/v) glacial acetic acid.

Coomassie stain: 34 % (v/v) methanol, 17 % (w/v) ammonium sulphate, 0.5 % (v/v) glacial acetic acid, 0.1 % (w/v) coomassie brilliant blue R250.

2.2.2. Coomassie staining solutions (for 1-D SDS-PAGE)

Coomassie stain: 40 % (v/v) methanol, 10 % (v/v) glacial acetic acid, 0.1 % (w/v) coomassie brilliant blue R250.

Destain: 20 % (v/v) methanol, 5 % (v/v) glacial acetic acid.

2.2.3. Fluorescence activated cell sorting (FACS) solutions and buffers

FACS fixative: 1 % (w/v) paraformaldehyde in phosphate buffered saline (PBS).

PBS: 80 mM disodium hydrogen orthophosphate, 26 mM potassium chloride, 14 mM monopotassium phosphate, 1.4 mM sodium chloride.

FACS wash buffer: 1 % (w/v) bovine serum albumin (BSA) 0.1 % (w/v) sodium azide in PBS.

2.2.4. Lysis buffers

Ammonium chloride lysis buffer: 155 mM ammonium chloride, 10 mM potassium hydrogen carbonate, 0.1 mM EDTA in PBS. Adjust to pH 7.2 with 1M sodium hydroxide.

Brain lysis buffer: 1 % (v/v) Igepal, 1 % (w/v) sodium-deoxycholic monohydrate.

Cell lysis buffer: 100 mM sodium chloride, 10 mM Tris-HCL pH 7.8, 10 mM EDTA, 0.5 % (v/v) igepal, 0.5 % (v/v) sodium deoxycholate, 1 complete EDTA-free protease inhibitor tablet (Roche, UK) for each 50 ml cell lysis buffer.

2.2.5. Magnetic activated cell sorting (MACS) buffers

MACS wash buffer: 1 % (w/v) BSA in PBS.

2.2.6. Monoclonal antibody purification buffers

Elution buffer: 0.75 % (w/v) glycine. Adjust to pH 2.3 with 1 M HCL.

Neutralisation buffer: 12.13 % (w/v) Tris (free base). Adjust to pH 8 with 1 M HCL.

Wash buffer: 1.2 % (w/v) Tris (free base). Adjust to pH 8 with 1 M HCL.

2.2.7. Protein digestion and peptide extraction buffers

Digestion buffer 1: 50 mM ammonium bicarbonate, 5 mM calcium chloride, 0.013 % (w/v) porcine trypsin (Promega, USA).

Digestion buffer 2: 50 mM ammonium bicarbonate, 5 mM calcium chloride.

Reduction buffer 1: 100 mM ammonium bicarbonate, 10 mM dithiothreitol.

Reduction buffer 2: 100 mM ammonium bicarbonate, 55 mM iodoacetamide.

2.2.8. Sodium phosphotungstic acid (NaPTA) buffers

Benzonase/DNase buffer: 1 mM magnesium chloride, 50 units/ml benzonase, 0.5 % (w/v) DNase in PBS.

Magnesium chloride buffer: 1 mM magnesium chloride, 50 units/ml Benzonase in PBS.

Sodium *N*-lauroylsarcosinate buffer : 4 % (v/v) sodium *N*-lauroylsarcosinate in PBS.

NaPTA stock buffer: 170 mM magnesium chloride, 4 % (w/v) NaPTA in PBS.

NaPTA wash buffer 1: 12.5 mM EDTA, 0.1 % (v/v) sarcosyl in PBS.

NaPTA wash buffer 2: 0.1 % (v/v) sarcosyl in PBS.

2.2.9. Sodium dodecyl sulphate – polyacrylamide gel electrophoresis buffers (1D SDS-PAGE)

NuPAGE running buffer (20 X): 50 mM Tris-HCL pH 8.3, 0.5 mM glycine, 0.1 % (w/v) SDS.

NuPAGE sample buffer (4 X): 8 M urea, 65 mM Tris-HCL pH 6.8, 5 % (w/v) SDS, 5 % (v/v) glycerol, 5 % (v/v) β – mercaptoethanol, 0.01 % (w/v) bromophenol blue.

2.2.10. Sodium dodecyl sulphate – polyacrylamide gel electrophoresis solutions and buffers (2D SDS-PAGE)

Agarose sealing solution: 192 mM glycine, 25 mM Tris base, 0.1 % (w/v) SDS.

DeStreak rehydration buffer: Manufactured by GE Healthcare but contains optimized concentrations of urea, thiourea, 3-[(3-cholamidopropyl) dimethylammonio] - 1 - propanesulfonate (CHAPS) and DeStreak Reagent.

SDS equilibration buffer: 6 M urea, 75 mM Tris-HCL pH 8.8, 29.3 % (w/v) glycerol, 2 % (w/v) SDS, 0.002 % (w/v) bromophenol blue.

Running buffer (10 X): 1.92 M glycine, 250 mM Tris base, 1 % (w/v) SDS.

Sample preparation solution: 7 M urea, 2 M thiourea, 30 mM Tris-HCL pH 8.5, 4 % (w/v) CHAPS, one complete EDTA-free protease inhibitor tablet (Roche, UK).

Wash buffer: 30 mM Tris-HCL pH 8.5.

2.2.11. Silver staining solutions (1D PAGE)

Fixative: 50 % (v/v) ethanol, 10 % (v/v) glacial acetic acid.

Pre - sensitising: 5 % (v/v) ethanol, 1 % (v/v) glacial acetic acid.

Sensitising: 0.02 % sodium thiosulphate pentahydrate.

Silver reaction: 0.2 % (w/v) silver nitrate, 0.029 % (w/v) formaldehyde (Fluka, Switzerland).

Developing: 6 % (w/v) sodium carbonate, 0.029 % (w/v) formaldehyde (Fluka, Switzerland).

Stopping: 5 % (v/v) glacial acetic acid.

2.2.12. Silver staining solutions (2D PAGE)

Fixative: 30 % (v/v) ethanol, 10 % glacial acetic acid.

Sensitising: 30 % (v/v) ethanol, 0.5 % (v/v) glutaraldehyde, 0.2 % (w/v) sodium thiosulphate, 8.8 % (w/v) sodium acetate.

Silver reaction: 0.25 % (w/v) silver nitrate, 0.058 % (w/v) formaldehyde (Fluka, Switzerland).

Developing: 2.5 % (w/v) sodium carbonate, 0.12 % (w/v) formaldehyde (Fluka, Switzerland).

Stopping: 1.46 % (w/v) EDTA sodium salt.

2.2.13. Scrapie mouse brain (SMB) cell culture solutions

SMBs15 and SMB-PS cell growth media: Medium 199 with Earle's salts, 10 mM L-glutamine, 10 % (v/v) newborn calf serum, 5 % (v/v) foetal calf serum (PAA laboratories, UK) 0.1 % (w/v) streptomycin, 0.1 % (w/v) penicillin.

Trypsin/EDTA Solution: 0.05 % (w/v) porcine trypsin, 0.02 % (w/v) EDTA. Made in PBS.

Freezing media: 90 % (v/v) foetal calf serum (PAA laboratories, UK) 10 % (v/v) dimethylsulphoxide (DMSO).

2.2.14. SYPRO® orange staining solutions

Fixative: 40 % (v/v) ethanol, 2 % (v/v) glacial acetic acid, 0.0005 % (w/v) SDS.

Pre-stain wash: 2 % (v/v) glacial acetic acid, 0.0005 % (w/v) SDS.

SYPRO® orange reaction: 0.05 % (w/v) SYPRO® orange (Molecular probes, UK).

2.2.15. Western blotting buffers

Antibody binding buffer: 0.5 % (w/v) dried skimmed milk (Marvel, Premier Brands, UK) in PBS-Tween.

Blocking buffer: 5 % (w/v) Marvel in PBS-Tween.

PBS-Tween: 0.05 % (v/v) Tween-20 in PBS.

Transfer buffer (1 X): 25 mM Bicine, 25 mM Bis-Tris, 1 mM EDTA, 20 % (v/v) methanol.

2.3. Methods

All steps were performed at room temperature unless otherwise stated.

2.3.1. Brain tissue methods

2.3.1.1. Brain homogenisation

Scrapie-infected and uninfected half brains were chopped into small pieces and homogenised in phosphate buffered saline (PBS) (see section 2.2.3) (20 % w/v) using an electronic homogeniser (Camlab, UK). Uninfected brain homogenates were pooled, aliquotted into 5 ml volumes and stored at – 80 º C. Scrapie brain homogenates were aliquotted into 1 ml volumes (without pooling) and stored at – 80 º C.

2.3.1.2. Serial dilutions of brain homogenate

Brain homogenate pellets containing 200 mg wet brain tissue were diluted in PBS (see section 2.2.3) to give wet tissue equivalents of 2500 µg, 1250 µg, 625 µg, 313 µg, 156 µg, 78 µg, 19 µg, 9 µg, and 4.5 µg. For Western blotting of this standard dilution series see Appendix 2.

To prepare scrapie brain spikes (where small volumes of infected brain homogenate are diluted into larger volumes of uninfected brain homogenate) 20 % (w/v) scrapie brain homogenate was spiked into a 200 µl final volume, using 10 % (w/v) normal brain homogenate as diluent, to give final concentrations of 10 %, 1 %, 0.1 %, 0.01 % and 0.001 % scrapie brain. The respective wet tissue equivalents were 20 mg (10 %), 2 mg (1 %), 0.2 mg (0.1 %), 0.02 mg (0.01 %) and 0.002 mg (0.001 %). In each case the total amount of wet tissue (scrapie brain spike and normal brain homogenate combined) was made to 20 mg in a volume of 200 µl. Scrapie brain spikes described above were added to 300 µl 10 % uninfected brain homogenate (30 mg wet tissue) giving in each case a total sample volume of 500µl containing 50 mg total wet tissue. After sonication samples were proteinase K (PK) digested, sodium phosphotungstic acid (NaPTA)

precipitated and analysed by Western blotting after sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

2.3.1.3. Storage conditions of brain homogenate

Brain homogenates were stored at – 80 º C or – 20 º C unless otherwise stated. The efficiency of storing brain homogenates under these conditions was tested by Western blotting as shown in Appendix 3.

2.3.2. Preparation of scrapie mouse brain (SMB) cells

2.3.2.1. SMB cell culture

SMBs15 and SMB-PS cells were grown in T75 flasks (Costar,UK) in SMB cell growth media (see section 2.2.13) under 5 % (v/v) CO_2 in air, 95 % relative humidity at 37 º C until 70 % confluent. To remove cells from flasks 2 ml of trypsin/EDTA solution (see section 2.2.13) was added for 3-5 min before the addition of 10 ml of SMB cell growth media. Cells were centrifuged for 5 min at 100 g, counted and seeded at a density of 1.2×10^4 cells/cm^2.

2.3.2.2. SMB cell thawing and freezing

Freezing SMBs15 and SMB-PS cells: Cells grown to 70 % confluency were removed from T75 flasks (Costar, UK) after incubation with 2 ml of trypsin/EDTA solution (see section 2.2.13). After centrifugation for 5 min at 100 g, cells were resuspended in 2 ml freezing media (see section 2.2.13) transferred to 2 cryovials and frozen for 24 h at – 80 º C, followed by storage in liquid nitrogen.

Thawing SMBs15 and SMB-PS cells: Cells were thawed by submerging the cryovials in a 37 º C water bath for 3 min. Cells were then added to 12 ml of pre-warmed SMB cell growth media (see section 2.2.13). After centrifugation at 100 g for 5 min, cells

resuspended in 5 ml SMB cell growth media were seeded into a single T75 flask containing 15 ml SMB cell growth media.

2.3.3. Preparation of leukocytes

2.3.3.1. Buffy coat isolation from blood

Blood (50 ml) was centrifuged for 15 min, 500 g at 4 º C. Three distinct layers form corresponding to plasma, buffy coat interface and red cells. Buffy coat at the gradient interface was collected by gentle pipetting. To remove contaminating red cells from the buffy coat fraction 50 ml of ammonium chloride lysis buffer (see section 2.2.4) was added for 10 min at RT, followed by centrifugation at 300 g for 10 min at 4 º C. Buffy coat cells (mainly leucocytes including mononuclear cells and granulocytes) were washed twice by resuspending in 50 ml PBS (see section 2.2.3) and centrifugation for 5 min, 300 g at 4 º C. Cell pellets at a concentration of 1 x 10^8 or 1 x 10^7 cells were frozen at – 80 º C after snap freezing in liquid nitrogen.

2.3.3.2. Peripheral blood mononuclear cell (PBMC) isolation from whole blood

Whole blood was diluted with an equal volume of PBS (see section 2.2.3) underlaid with Histopaque (density 1.083 g ml^{-1}) and centrifuged (with no brake) at 1000 g for 30 min. PBMCs, at the gradient interface, were collected, incubated in ammonium chloride lysis buffer (see section 2.2.4) for 10 min, to lyse contaminating red cells and centrifuged at 300 g for 10 min at 4 º C. PBMCs were washed three times in 50 ml PBS by centrifugation at 100 g for 30 min. PBMC pellets were stored at – 80 º C.

2.3.3.3. Cell counting

After positioning a clean coverslip on a haemocytometer (Neubauer, Germany) 50 µl of cell suspension (in PBS) was pipetted into the haemocytometer chamber. If the concentration of cells covered approximately 50 % microscope field (at 40x magnification) the cells were counted, if not the sample was centrifuged for 5 min at

300 g and resuspended in a more appropriate volume. Once cells were well distributed all those in squares within the 1 mm^2 ruled area in the centre of the chamber were counted. Cells touching or lying on the top and right boundary lines were not counted. Cells touching or lying on the bottom or left boundary lines were included in the counts.

Cell viability/ trypan blue staining: Before counting 50 µl of cell suspension (in PBS) was added to 50 µl undiluted trypan blue to assess cell viability.
After 2 separate counts cell suspensions were centrifuged for 5 min at 300 g, cell pellets frozen at – 80 º C or – 20 º C, or used immediately for SDS-PAGE analysis after cell lysis.

2.3.3.4. Leukocyte cell sorting

Cells ($5 \times 10^7 – 3 \times 10^8$) were incubated in primary antibody (tabulated in Appendix 1) diluted in magnetic activated cell sorting (MACS) wash buffer (see section 2.2.5) (2 ml) for 10 min. Cells were washed once in MACS wash buffer before centrifugation at 100 g for 5 min. Cell pellets were then resuspended in 20 µl of immunoglobulin isotype-specific rat anti-mouse antibodies conjugated with magnetic beads (Miltenyi Biotec, USA) per 10^7 cells for an additional 10 min. After washing as above, cells were separated using a magnetic cell sorter with MACS mini columns and a MACS mini separator (MACS Magnetic Cell Sorting Systems; Miltenyi Biotec, USA). Prior to cell sorting, MACS mini columns were washed with 3 ml MACS wash buffer. Cells were then passed through the column, washed three times with 3 ml MACS wash buffer, before the column was removed from the MACS mini separator and cells eluted into 5 ml of MACS wash buffer. Unlabelled and labelled cells were counted, centrifuged at 100 g for 5 min and cell pellets were frozen at – 80 °C.

2.3.3.5. Flow cytometry staining and acquisition

Cells (1 x 10^5 – 1 x 10^6 per well) were transferred to 96 well U-shaped plates and incubated in primary antibody diluted in fluorescence activated cell sorting (FACS) wash buffer (see section 2.2.3) (25 µl) for 10 min. Information on antibodies and working dilutions are tabulated in Appendix 1. Following incubation, cells were washed three times in FACS wash buffer, with a centrifugation step of 2 min at 100 g after each wash. Cells were incubated for 10 min with isotype-specific secondary antibodies, then washed twice as above. Cells were resuspended in 100 µl FACS fixative (see section 2.2.3) and stored overnight at 4 ° C before analysis. Samples were analysed as described by Barclay *et al*., (2002) on a flow cytometer (FACSCalibur; Becton Dickinson) using CELLQuest software (Becton Dickinson). Software used for analysis was FACS Express version 3. For each sample studied a minimum of 10,000 events was acquired by appropriate gating based on light-scattering characteristics and/or expression of cell specific markers.

2.3.3.6. Leukocyte freezing and thawing

Freezing leukocytes: Buffy coat cells at a concentration of 1 x 10^8 cells / ml or 1 x 10^7 cells / ml, PBMCs and T cells at a concentration of 1 x 10^7 cells / ml, and B cells at a concentration of 1 x 10^6 cells /ml were resuspended in 1 ml of freezing media (see section 2.2.13) before storage at – 80 º C for 24 hr in cryovials (Nalgene, UK). Cryovials were transferred to liquid nitrogen for long term storage. Cell pellets at a concentration of 1 x 10^8, 1 x 10^7 and 1 x 10^6 were also frozen at – 80 º C or – 20 º C.

Thawing leukocytes: Cells were thawed by submerging the cryovials in a 37 º C water bath for 3 min.

2.3.4. Sample preparation for protein analysis on Western blots

2.3.4.1. Lysis of brain homogenates

Brain homogenates (10 and 20 %) (w/v) were incubated with an equal volume of brain lysis buffer (see section 2.2.4) for 10 min at 37 º C. After centrifugation at 1500 g for 5 min supernatant was transferred to a new eppendorf tube and stored at – 20 º C.

2.3.4.2. Lysis of cells

Leukocytes: Cell pellets at a concentration of 1 x 10^8, 1 x 10^7 and 1 x 10^6 cells were incubated with an equal volume of pre-chilled cell lysis buffer (see section 2.2.4) (with or without an EDTA-free protease inhibitor tablet, Roche, UK) for 5 min at 4 º C. After centrifugation at 100 g for 5 min, supernatants were analysed immediately by SDS-PAGE after PK digestion and NaPTA precipitation or stored at – 20 º C.

SMBs15 and SMB-PS cells: Cells growing in 60 mm culture plates (Corning, UK) were rinsed twice with ice cold PBS (see section 2.2.3) scraped from the plate in 1 ml of pre-chilled cell lysis buffer (see section 2.2.4) and centrifuged at 100 g for 5 min at 4 º C. Supernatants were frozen at – 80 º C or treated with PK, NaPTA precipitated and analysed by SDS-PAGE.

2.3.4.3. Proteinase K (PK) treatment of brain homogenates

Brain homogenates (10 and 20 %) (w/v) were lysed as described in 2.3.4.1. PK was added to a final concentration of 50 μg/ml and samples incubated for 1 hr 15 min at 37 º C with constant agitation. The reaction was stopped by the addition of 1 mg/ml phenylmethylsulphonyl fluoride (PMSF). Samples were snap frozen in liquid nitrogen before storage at – 20 º C.

2.3.4.4. PK treatment of cell lysates

Cells were lysed as described in 2.3.4.2. Leukocyte cell lysates were digested with a final concentration of 50 µg/ml PK, while 20, 50 or 75 µg/ml PK was added to SMB cell lysates. Samples were incubated and stored as described for brain homogenates in 2.3.4.3.

2.3.4.5. Sodium phosphotungstic acid (NaPTA) precipitation of brain homogenate : Method 1

Methods for NaPTA precipitation were adapted from Wadsworth *et al*. (2001) and McCutcheon *et al*. (2005). 10 % (w/v) brain homogenates were incubated with magnesium chloride buffer (see section 2.2.8) for 30 min at 37 º C with constant agitation. An equal volume of sodium *N*-lauroylsarcosinate buffer (see section 2.2.8) was added and samples incubated as above for a further 10 min. NaPTA stock buffer (see section 2.2.8) (pre-warmed to 37 º C) was added to give a final concentration of 0.3 % (w/v) NaPTA. Samples were incubated overnight with constant shaking at 37 º C. Samples were centrifuged for 30 min at 15 800 g. The pellet was resuspended in 200 µl of NaPTA wash buffer 1 (see section 2.2.8) before centrifugation at 15 800 g for 30 min. The pellet was washed in 200 µl of NaPTA wash buffer 2 (see section 2.2.8) before centrifugation as above for 15 min. After sonication (Mixsonics Ultrasonic Processor XL, USA) in a final volume of 20 µl wash buffer 2 samples were snap frozen in liquid nitrogen before storage at – 20 º C.

2.3.4.6. NaPTA precipitation of brain homogenate : Method 2

Brain homogenates (10 %) (w/v) were incubated with 500 µl of pre-chilled brain lysis buffer (see section 2.2.4) for 5 min at 4 º C. Gross cellular debris was removed by centrifugation at 500 g for 5 min at 4 º C, and the supernatant transferred to a new eppendorf tube. Samples were incubated in NaPTA stock buffer (see section 2.2.8) at a final concentration of 0.3 % (w/v) NaPTA, overnight at 37 º C with agitation. After centrifugation for 30 min at 15 800 g, the pellet was resuspended by sonication in a

final volume of 100 µl PBS (see section 2.2.3). Samples were snap frozen in liquid nitrogen before storage at – 20 º C.

2.3.4.7. NaPTA precipitation of leukocytes and SMB cell lysates : Method 1

Leukocyte and SMB cell lysates were resuspended in 500 µl pre-chilled cell lysis buffer (see section 2.2.4) and incubated in Benzonase/DNase buffer (see section 2.2.8) for 30 min at 37 º C with agitation. Lysates were then NaPTA precipitated as described for brain homogenates in 2.3.4.5.

2.3.4.8. NaPTA precipitation of leukocyte cell lysates : Method 2

Leukocyte cell lysates were NaPTA precipitated as described for brain homogenates in 2.3.4.6.

2.3.5. Protein analysis by SDS-PAGE and Western blotting

2.3.5.1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (1D)

SDS-PAGE is a technique used to separate proteins according to their molecular weight. Sodium dodecyl sulphate (SDS) is used to denature protein, and binds to polypeptides producing an even negative charge on the protein. Under the influence of an electric field and bound to SDS, proteins resolve through the gel. Large proteins resolve slowly, while smaller proteins resolve more quickly. Marker proteins of known molecular weight are also resolved in order to determine the molecular weights of unknown proteins within the test sample.

SDS-PAGE was carried out according to the method of Laemmli (1970) using pre-cast gels and the Novex X-Cell SureLock^{TM} Mini-cell (Invitrogen). 10 % and 4-12 %, 1 mm thick, 15 and 10 well, Bis-Tris gels were assembled in the gel rig according to the manufacturer's instructions and 1 X NuPAGE running buffer (see section 2.2.9) placed

in the buffer chambers. Protein samples were heated in an equal volume of 2 X NuPAGE sample buffer (see section 2.2.9) at 100 º C for 10 min. Samples were centrifuged for 1 min at 15 800 g, before being loaded into wells. 6 µl of Kaleidoscope prestained molecular weight standards (Bio-Rad) were loaded alongside samples. Gels were electrophoresed at 200 V, 130 mA for 45 min – 1 hr, until the dye front reached the bottom of the gel. Gels were removed from plates and analysed either by Commassie staining, silver staining or Western blotting.

2.3.5.2. Coomassie staining

Following SDS-PAGE gels were placed in Coomassie stain (see section 2.2.2) for 2 hr or overnight with gentle agitation. Coomassie stain was removed and replaced with destain (see section 2.2.2). Gels were destained with gentle agitation, replacing the destain 3-4 times, until protein bands could be visualised. Gels were scanned using a Umax Powerlook III scanner (Amersham Pharmacia Biotech) then stored at 4 º C in distilled water.

Coomassie staining for mass spectrometry: Gels were incubated for 1 hr in coomassie fixative (see section 2.2.1) with gentle agitation. Coomassie fixative was removed and gels incubated in Coomassie stain (see section 2.2.1) overnight with gentle agitation. Coomassie stain was removed and gels were incubated in distilled water, replacing the water eight times until protein bands could be visualised. Protein bands were excised from gels using disposable sterile scalpel blades (Swann-Morton, UK) and stored at 4 º C in distilled water.

2.3.5.3. Silver staining (1D PAGE)

Immediately after SDS-PAGE, gels were removed from plates and immersed in fixative (see section 2.2.11) overnight with gentle agitation. Fixative was removed and pre-sensitising solution (see section 2.2.11) added for 30 min with gentle agitation. Following three, 15 min washes with distilled water, sensitising solution (see section 2.2.11) was added and gels incubated for 1 min with gentle agitation. A further three,

30 sec washes with distilled water were carried out, before incubation in silver reaction solution (see section 2.2.11) for 1 hr with gentle agitation. After two, 30 sec washes in distilled water, developing solution (see section 2.2.11) was added for approximately 5-15 min, after which gels were transferred into stop solution (see section 2.2.11). Gels were scanned using a Umax Powerlook III scanner (Amersham Pharmacia Biotech) then stored at 4 º C in distilled water.

2.3.5.4. SYPRO® orange staining

After electrophoresis and removal from plates, gels were incubated overnight in fixative (see section 2.2.14) with gentle agitation. Fixative was removed and three washes for 1 hr each in pre-stain wash (see section 2.2.14) were carried out with gentle agitation. After washing gels were incubated in SYPRO® orange reaction solution (see section 2.2.14) for 4 hr, with gentle agitation, in the dark. Gels were scanned using a Typhoon Trio Imager laser scanner (GE Healthcare, UK) then stored at 4 º C in distilled water.

2.3.5.5. Western blotting

Immediately after SDS-PAGE proteins were transferred on to nitrocellulose immobilon-P transfer membrane (Millipore, USA) that had been methanol activated and equilibrated in transfer buffer (see section 2.2.15) by the use of a Transfer X-Cell II blot module (Invitrogen) at 430 V, 170 mA for 2 hr. Following transfer the surface of the membrane was blocked with blocking buffer (see section 2.2.15) for 1 hr with gentle agitation. The blocked membrane was incubated with primary antibody, diluted in antibody binding buffer (see section 2.2.15) overnight at 4 º C. For information on antibodies used and dilutions see Appendix 1. Following eight, 5 min washes in PBS-tween (see section 2.2.15) the membrane was incubated with secondary antibody conjugated to horse radish peroxidase (HRP) diluted in antibody binding buffer, for 1 hr with gentle agitation. The membrane was washed as described above, and the bound HRP conjugate visualised using SuperSignal West Pico chemiluminescent reagents (Pierce, UK) according to the manufacturer's instructions. Briefly equal volumes of

West Pico enhancer and peroxide solutions were mixed and added to the membrane for 1 min. The solution was drained from the membrane and the membrane wrapped in clear plastic food wrap and exposed to Hyperfilm (Amersham Pharmacia Biotech, UK). Film was developed using the Xograph Automatic Compact X4 processor.

2.3.6. Two-dimensional (2D) PAGE protein methods

Since protein bands on 1D PAGE will in most cases contain more than one protein species, 1D separation methods such as SDS-PAGE can resolve only a relatively small number of proteins. In contrast two-dimensional (2D) PAGE allows separation of proteins by isoelectric point and mass, allowing proteins to be more effectively separated, and so increasing the number of proteins visible. Furthermore each protein spot is more likely to be composed of a single protein, therefore allowing variations within individual proteins to be assessed.

2.3.6.1. Sample preparation for 2D PAGE

Method 1: Buffy coat cell pellets at a concentration of 1 x 10^7 were incubated in 500 µl pre-chilled cell lysis buffer (see section 2.2.4) before NaPTA precipitation as described in 2.3.4.5 and 2.3.4.7.

Method 2: Buffy coat cell pellets at a concentration of 1 x 10^7 were washed twice in wash buffer (see section 2.2.10) and centrifuged at 15 800 g for 10 min at 4 º C. Pellets were resuspended by sonication on ice for eight, 15 sec pulses in 500 µl sample preparation solution (see section 2.2.10). Following a further 45 min with agitation, cell lysates were centrifuged at 15 800 g for 45 min. The supernatant was transferred to a clean eppendorf tube and the protein concentration determined by a 2-D Quant kit (G Biosciences, UK) according to the manufacturers instructions. Supernatant was frozen at a final concentration of 50 µg protein at – 80 º C.

2.3.6.2. Sample rehydration and isoelectric focussing (IEF)

Protein samples were combined with DeStreak rehydration buffer (see section 2.2.10) containing 10 % immobilised pH gradient (IPG) buffers of pH 3-11 range, incubated for 45 min with agitation, and centrifuged at 15 800 g for a further 45 min. Supernatants were removed to a clean tube and samples were loaded into 18 cm immobilised pH gradients (IPG) strips by passive in-gel rehydration overnight covered in mineral oil (Amersham Biosciences, UK). IPG strips were subjected to IEF on a Phast 12 IEF unit (Nextgen Science Plc, UK) for a total of 40 kV hr. A typical IEF profile is shown in Appendix 4. Following IEF, IPG strips were stored at – 80 º C. Focussing profiles were determined empirically for each sample type used.

2.3.6.3. 2D SDS-PAGE Gel preparation

Large format SDS PAGE gels for use in 2D PAGE were prepared using a 2DeOptimizer (Nextgen Sciences plc). This instrument enables automated pouring of both homogeneous and gradient gels. All reagents (acrylamide, glycerol, Tris/SDS, TEMED and ammonium persulphate (APS) were supplied by Nextgen Sciences plc and with the exception of APS come ready to use. Prior to gel preparation, all reagents are allowed to equilibrate to RT and APS is reconstituted to 1 % (w/v) in distilled water and allowed to agitate for 30 min. Gel reagents were loaded on to a 2DeOptimizer for automated gel casting. 12 % homogenous gels were pre-cast into an autocast 12 large format gel caster and allowed to polymerize for 4 – 5 hr.

2.3.6.4. Second dimension separation

IPG strips were incubated in SDS equilibration buffer (see section 2.2.10) containing 1 % (w/v) dithiothreitol (DTT) for 15 min with agitation, followed by incubation in SDS equilibration buffer containing 2.5 % (w/v) iodoacetamide in the same way. IPG strips were added to 12 % homogenous gels and sealed with agarose sealing solution (see section 2.2.10). Gels were transferred to a Ettan DALT II gel tank containing 1 X

running buffer (see section 2.2.10) and electrophoresis performed at 12 W overnight at 30 º C.

2.3.6.5. Silver staining (2D PAGE)

Immediately after 2D SDS-PAGE, gels were removed from plates and immersed in fixative (see section 2.2.12) overnight with gentle agitation. Fixative was decanted and sensitising solution (see section 2.2.12) added for 1 hr with gentle agitation. Following four, 15 min washes with distilled water, silver reaction solution (see section 2.2.12) was added and gels incubated for 1 hr with gentle agitation. After two, 1 min washes in distilled water, developing solution (see section 2.2.12) was added for approximately 5-15 min, after which gels were transferred into stop solution (see section 2.2.12). Gels were scanned using a Umax Powerlook III scanner (Amersham Pharmacia Biotech) sealed in clear polythene and stored at 4 º C.

2.3.6.6. In-gel protein digestion and peptide extraction

Gel slices of approximately 1 cm^2 were excised from gels, washed in distilled water before centrifugation at 100 g for 2 min. After resuspension in 50 % (v/v) acetonitrile (AnaChem) for 15 min, gel slices were centrifuged as above. Gel slices were incubated in reduction buffer 1 (see section 2.2.7) for 1 hr at 37 º C before centrifugation as described above, and resuspension in 50 % (v/v) acetonitrile. Following centrifugation as before the supernatant was removed, pellets resuspended in reduction buffer 2 (see section 2.2.7) and incubated in the dark for 20 min. The acetonitrile step was repeated and the pellet incubated in digestion buffer 1 (see section 2.2.7) for 45 min at 4 º C. Following centrifugation at 100 g for 2 min, pellets were incubated in digestion buffer 2 (see section 2.2.7) overnight at 37 º C. 25 mM ammonium bicarbonate was added followed by incubation for 15 min at 37 º C. After centrifugation for 2 min at 100 g the supernatant was incubated in 5 % (v/v) formic acid as described above. After the addition of 50 % (v/v) acetonitrile and incubation as described above, pellets were dried using a Savant speed vac. Dried pellets were stored at 4 º C.

2.3.6.7. Mass spectrometry

Protein peptides were analysed by electrospray ionisation (ESI) mass spectrometry, using a Q Tof Premier instrument (Waters Ltd) equipped with an atmosphere pressure electrospray ionisation source and calibrated using human glutamate fibrinopeptide. Mass spectrometry was performed by Dr A.C. Gill (Roslin Institute).

Mass spectrometry was also performed by Dr P. Skipp (Southampton University) where protein peptides were separated by nano-reverse phase liquid chromatography, using a Dionex PepMap C18, 3 µm, 100 (150 mm x 75 µm, i.d.) column, and electrosprayed into a quadrupole time of flight tandem mass spectrometer (Waters Ltd). All data were acquired using a Q-tof Glabal Ultima (Waters Ltd) fitted with a nanoLockSpray^{TM} source.

Chapter 3

Development And Optimisation Of A Sensitive Western Blot Immunoassay For PrP^{Sc} Detection

3.1. Introduction

Transmissible spongiform encephalopathies (TSEs) or prion diseases are rare and fatal neurodegenerative disorders, characterized by the accumulation of an abnormal isoform of prion protein (PrP^{Sc}) in the central nervous system (Prusiner *et al*., 1998). The appearance of variant Creutzfeldt-Jakob disease (vCJD) which is caused by the same TSE agent as bovine spongiform encephalopathy (BSE) in cattle (Will *et al*., 1996; Bruce *et al*., 1997) has raised concerns worldwide. This is because the total number of vCJD infected people is unknown, there is a long pre-clinical phase of disease and affected individuals are able to potentially transmit infectious agent to others, possibly *via* blood and blood products or by contaminated surgical instruments. The ability to detect the infectious agent in blood is therefore of major importance to understanding the pathogenesis of the disease, estimating the risk of infection associated with blood products and the development of non-invasive diagnostic tests to identify TSE-infected animals and humans before the onset of clinical symptoms.

The only method which formally demonstrates the presence of infectious TSE agents is the bioassay, where infectious material is inoculated into animals of the same or different species. These experiments, however, are difficult to perform, expensive and take months or even years to obtain results. An alternative to bioassay is the detection of PrP^{Sc} which co-purifies with infectivity (Bolton *et al*., 1982) and is used as a biochemical marker for TSE disease. Traditionally, immunohistochemistry (IHC) is used to detect PrP^{Sc} in tissues following post mortem, although it can also be used to screen samples from pre-clinical individuals (Schreuder *et al*., 1998). PrP^{Sc} is distinguished from PrP^{C} by treating with proteinase K (PK), although this treatment is not routinely used for IHC. Other pre-treatments commonly used such as trypsin and autoclaving, may destroy much of the PrP^{C}. The effectiveness of IHC depends greatly on sample preparation and the nature of the antibodies used. Although excellent for confirmation of infection, IHC is not a rapid method and the amount of PrP^{Sc} present cannot reliably be measured.

A relatively rapid, widely validated, well established method is Western blotting, which can be very sensitive for PrP^{Sc} detection (Lee *et al*., 2000). Western blotting relies on proteinase K digestion to distinguish the abnormal protein from PrP^{C}. Extracts of PrP^{Sc} from infected tissues are treated with PK, run on an SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) gel, transferred to nitrocellulose membrane and then probed using specific antibodies to detect undigested PrP (Bolton *et al*., 1982). After digestion with PK, PrP^{Sc} gives a conventional pattern on Western blots where three distinct bands can be identified and resolve between 27-30 kDa. Hence the PK resistant core of PrP^{Sc} is called p27-30. The three bands represent differential glycosylation as PrP contains two *N*-linked glycosylation sites at codon 180 (N180) and codon 196 (N196) in mice at which carbohydrates can be attached (Oesch *et al*., 1985). These *N*-linked glycosylation sites are highly conserved among mammalian species (van Rheede *et al.*, 2003). The diglycosylated form exists when both sites are occupied (35 kDa band), monoglycosylated forms occur when either one of the two sites are occupied (28 kDa band) and unglycosylated forms occur when neither site is occupied (22 kDa band) (Collinge *et al*., 1996). Western blotting has the advantage of recognising different forms of PrP^{Sc} through the analysis of molecular mass in addition to relative abundance of di- mono- and unglycosylated bands. This characterisation produces a "PrP signature" which varies among different forms of TSEs (Collinge *et al*., 1996).

Western blot sensitivity can be increased by concentration of PrP^{Sc} in tissue samples by precipitation with sodium phosphotungstic acid (NaPTA). Traditionally used in histology with haematoxylin for staining tissue elements such as striated muscle, smooth muscle and neuroglia fibres (Mallory, 1900), NaPTA ($Na_2H[PW_{12}O_{40}]$) features the nearly spherical trianion $[PW_{12}O_{40}]$ which belongs to a broad class of polynuclear transition metal-oxo complexes known as polyoxometalates (POM). POMs have been shown to non-specifically precipitate proteins (Yeang *et* al., 1995; Scott *et al*., 1971) and amino acids (Thimann, 1930). Although the precise molecular mechanism of PrP^{Sc} interaction with NaPTA is largely unknown there is evidence to suggest that this is specific for both protease resistant and sensitive forms of PrP^{Sc} (Safar *et al*., 1998; Tremblay *et al*., 2004). By incorporating a NaPTA concentration

step, Western blot sensitivity has been increased up to three-fold, allowing for the identification of extraneural abnormal PrP in spleen and muscle from sporadic CJD patients (Glatzel *et al*., 2003).

This chapter describes the development and optimisation of a Western blot immunoassay for conventional PK resistant PrP^{Sc}, using scrapie-infected sheep brain homogenates. The aim of these experiments was to maximise the sensitivity of the assay using NaPTA concentration and exploiting a panel of novel PrP specific monoclonal antibodies, before applying it to detection of PrP^{Sc} in scrapie-infected sheep blood.

3.1.1. Antibodies

The panel of novel PrP specific monoclonal antibodies described in this thesis were generously donated by Dr S. McCutcheon (Roslin Institute). The antibodies were raised by immunising PrP null mice with truncated recombinant ovine PrP using conventional techniques, the details of which will soon be published.

At the beginning of this project, many of the commercial antibodies available were specific for epitopes present in the N- terminal octapeptide repeat region of PrP. For detection of PrP^{Sc}, this is not ideal as following PK digestion, the N terminal region of the protein is cleaved. Therefore, the vaccination strategy to immunise PrP null mice with truncated recombinant ovine PrP was used in order to generate an immune response directed against epitopes in the C- terminal region of the PrP protein.

Some of the novel PrP specific monoclonal antibodies (BC6, BH1) do share a similar binding region (C-terminal globular domain) to the commercially available 6H4 antibody, but due to reasons of cost, studies to compare the characteristics of the novel antibodies with other commercial products were not carried out.

The reason the panel of antibodies were described as novel was simply a reference to the fact that they were a “new” panel of antibodies available for use. The detailed

biological characterisation and functionally of these antibodies will be described in detail by Dr S. McCutcheon (Roslin Institute) who is currently writing up this work for publication.

Following epitope mapping studies carried out by Dr S. McCutcheon (Roslin Institute) the location of four antibody core binding regions were identified and are summarised below.

Anti-PrP Antibody	**Amino Acids**	**Core Binding Region**
FH10	198-207	TETDIKMMER
BC6	141-152	GSDYEDRYYREN
BH1	136-152	PHILFGSDYEDRYYREN
JB10	216-225	QRESQAYYQR

Table 3.1: Epitope locations of anti-PrP antibodies. Courtesy of Dr S. McCutcheon (Roslin Institute).

3.2. Materials and methods

3.2.1. Sensitive Western blot method

The methods developed from Safar *et al*., 1998 and Wadsworth *et al*., 2001 are described in detail in chapter 2, section 2.3.4.5. Figure 3.1 shows a simplified diagram of the sensitive Western blot method.

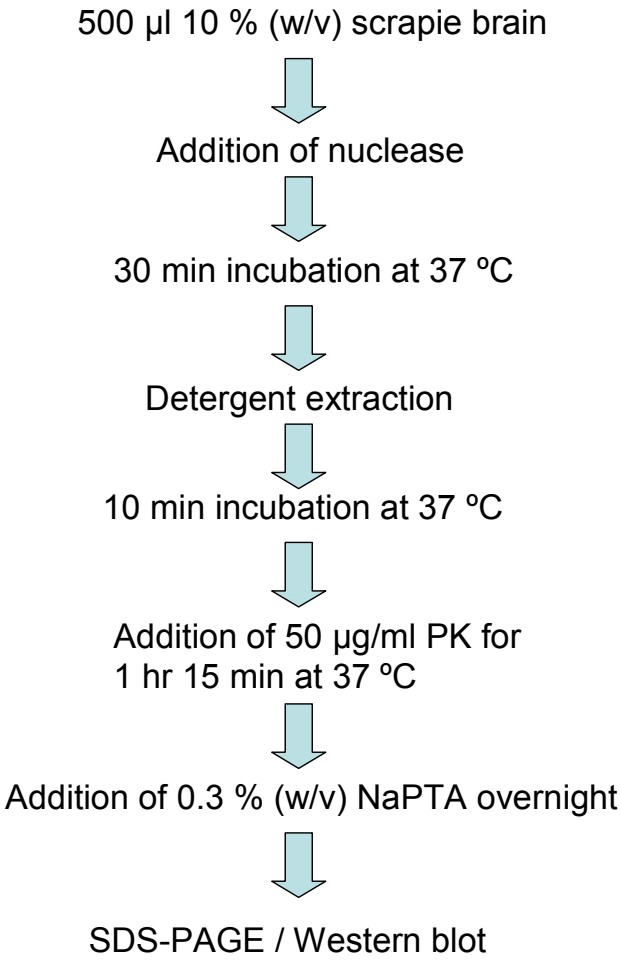


Figure 3.1: Simplified flow diagram of the sensitive Western blot method

3.3. Results

3.3.1. Standard Western blotting technique

Western blotting (see chapter 2, section 2.3.5.5) is a method for detection of conventional PrP^{Sc} that relies on PK digestion to distinguish the abnormal protein from PrP^{C}. Figure 3.2 shows the detection of PrP from normal and scrapie brain homogenates using a standard Western blot method. Without the PK step both forms of the protein give the same distinct pattern on Western blots, where three distinct bands (that represent differential glycosylation as described in the introduction) can be identified and resolve between 22-35 kDa (figure 3.2, lanes 1 and 3). After PK digestion, PrP^{C} is fully degraded (figure 3.2, lane 2). PrP^{Sc} is partially resistant to treatment with PK where the N terminal region of the protein is cleaved, resulting in a PK resistant core called p27-30 (figure 3.2, lane 4).

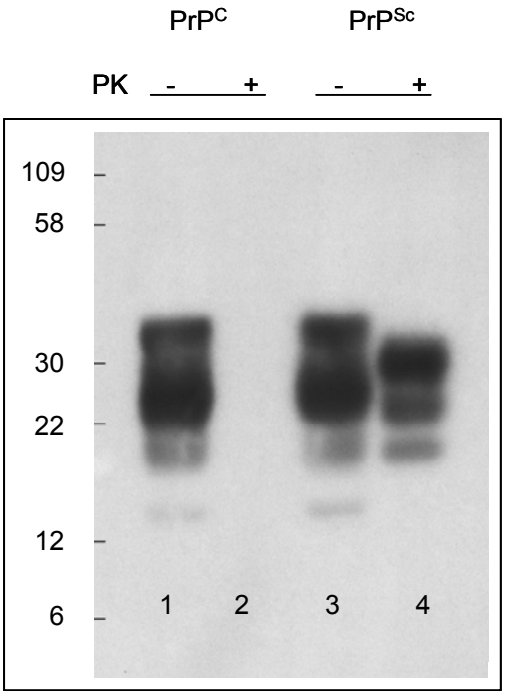


Figure 3.2: Western blotting for PrP^{Sc} detection. Key: Lane 1 & 2, uninfected sheep brain homogenate; lane 3 & 4 scrapie-infected sheep brain homogenate. As indicated lane 1 & 3 was not PK digested, lane 2 & 4 was digested with PK. 150 µg wet tissue equivalent was loaded in each well. The blot was probed with BC6 at 1 µg/ml. The blot was developed using standard ECL methods; the exposure time was 2 min. Molecular masses are shown on the left (kDa).

3.3.2. Screening a panel of PrP-specific monoclonal antibodies on sheep and mouse brain homogenates.

A panel of new monoclonal antibodies to sheep PrP (see table 3.1) were screened for specific PrP^{Sc} binding on Western blots of sheep scrapie brain homogenate samples. Figure 3.3 is a representative blot of the results. All antibodies were used at the same concentration (1 µg/ml) and some gave a more intense signal on blots than others. For instance, BH1 (figure 3.2, lanes 11 and 12), BC6 (lanes 9 and 10) and IH9 (lanes 7 and 8) gave the most intense signal and therefore these antibodies may have a high affinity for ovine PrP. Antibodies such as AE11 (lanes 3 and 4) and JB10 (lanes 1 and 2) also gave a positive, yet weaker signal. FH6 (lanes 17 and 18) was weaker still, whilst DC12 (data not shown) was unable to detect PK -resistant PrP.

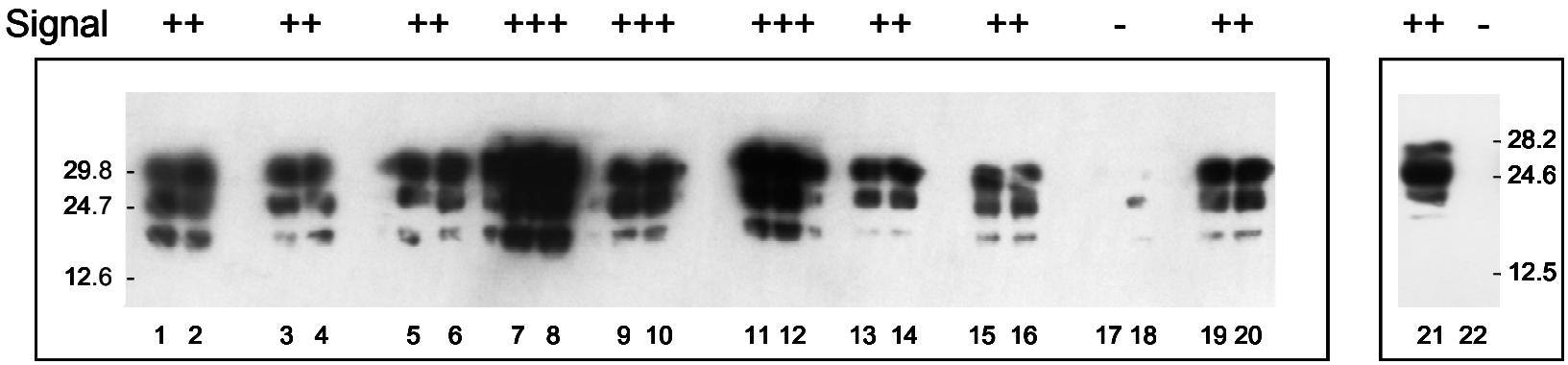


Figure 3.3: Screening of monoclonal antibodies on Western blots of PK-resistant PrP^{Sc} from scrapie- infected sheep brains. Wells were loaded with 2.1 mg wet tissue equivalent of PK digested scrapie sheep brain homogenate, and the membrane cut so it could be probed using different monoclonal antibodies at 2 µg/ml. Samples were loaded in duplicate. In some cases sample spilled over into a fresh lane (between lanes 6 and 7, 8 and 9, and 12 and 13). Key: lanes 1 & 2, JB10; lanes 3 & 4, AE11; lanes 5 & 6, EA6; lanes 7 & 8, IH9; lanes 9 & 10, BC6; lanes 11 & 12, BH1; lanes 13 & 14, BF5; lanes 15 & 16, IH11; lanes 17 & 18, FH6 and lanes 19 & 20, FD12. In lane 21 & 22 uninfected sheep brain homogenate (300 µg) was probed with BC6 (1 µg/ml). Lane 22, the sample was PK digested. The blot was developed using standard ECL methods; the exposure time was 1 min. Molecular masses are shown on the left and far right (kDa).

Since the objective was to apply the optimized Western blot to cellular components of blood, a source of scrapie-infected cells was required as a positive control as an alternative to scrapie-infected brain homogenates. As there are no readily available scrapie-infected sheep cell lines, the SMB mouse cell line (Clarke and Haig, 1970) was chosen for this purpose. Therefore it was important to select monoclonal antibodies from the panel of 24 which allow sensitive detection of both mouse and sheep PrP. To achieve this, the same panel of antibodies were also screened against PK treated brain homogenates from mice infected with the scrapie strain 139A (figure 3.4). A group of 10 antibodies bind to mouse PrP^{Sc}, with varying signal intensities. As for sheep, BH1 (lane 8), BC6 (lane 9) and IH9 (lane 6) produced the most intense signal, whereas CF5 (lane 5) was weaker. In contrast to the blots of sheep PrP^{Sc}, the monoglycosylated band in the 139 A mouse scrapie strain is more pronounced, producing a different glycosylation pattern.

After comparison with the sheep blots a group of 6 antibodies were selected for further study (IH9, AE11, BH1, BC6, EG6, and FD12). Based on the high signal intensity obtained with these antibodies on Western blots, it is likely that in both species they bind PrP^{Sc} with high affinity (table 3.2). The three bands representing diglycosylated, monoglycosylated and unglycosylated PrP^{Sc} were clearly visible. Subsequent Western blots of brain (for assay optimisation) were carried out using scrapie sheep brain homogenate only.

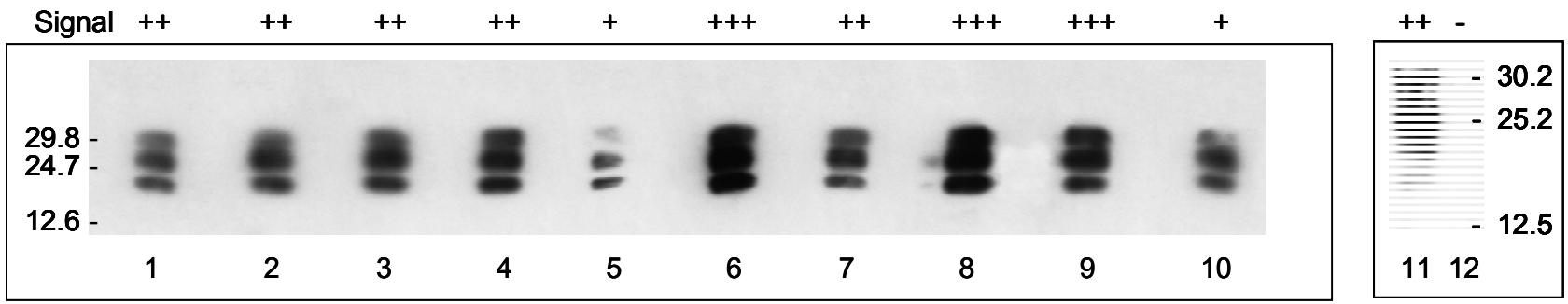


Figure 3.4: Screening of monoclonal antibodies on Western blots of PK-resistant PrP^{Sc} from scrapie-infected mouse brains. Wells were loaded with 2.1 mg wet tissue equivalent of PK digested scrapie-infected (139A) mouse brain homogenate, and the membrane cut so it could be probed using various monoclonal antibodies at 1 µg/ml. In lane 1, HC2; lane 2, AE11; lane 3, EG6; lane 4, FD12; lane 5, CF5; lane 6, IH9; lane 7, BF5; lane 8, BH1; lane 9, BC6; lane 10; JC4. Lane 11, 300 µg uninfected mouse brain homogenate and lane 12, 300 µg PK digested uninfected mouse brain homogenate. The blot was developed using standard ECL methods; the exposure time was 5 sec. Molecular masses are shown on the left and far right (kDa).

Monoclonal antibody	Species cross-reactivity	Isotypes	Signal intensity
AE11	Sheep, mouse	IgG1	++
EA6	Sheep	IgG1	++
EG6	Sheep, mouse	IgG1	+++
FD12	Sheep, mouse	IgG1	++
CF5	Sheep, mouse	IgG2a	+
EC9	Sheep	IgG2a	+
IH9	Sheep, mouse	IgG2b	+++
IH11	Sheep	IgG2a	++
FD1	Sheep	IgG1	+
FH10	Sheep	IgG2a	++
HB4	Sheep	IgG1	++
IF1	Sheep	IgG1	+
DE3	Sheep	IgG1	+
BF5	Sheep, mouse	IgG1	++
JB10	Sheep	IgG1	++
BD12	Sheep	IgG1	+
DC12	Sheep	IgG1	-
DB12	Sheep	IgG1	+
FH6	Sheep	IgG1	-
HC2	Sheep, mouse	IgG1	++
BH1	Sheep, mouse	IgG1	+++
BC6	Sheep, mouse	IgG1	+++
HC7	Sheep	IgG1	+
JC4	Sheep, mouse	IgG1	++

Table 3.2 Cross-reactivity of PrP specific monoclonal antibodies used in SDS-PAGE and Western blotting to detect low PrP^{Sc} amounts. Antibodies were used at 2 µg/ml and 2.1 mg wet tissue was loaded. Key: +++ = very intense signal , ++ = intense signal , + = good signal , - = no signal

3.3.3. Concentration of PrP^{Sc} by NaPTA precipitation

The sensitivity of Western blots can be improved by concentrating PrP^{Sc} using NaPTA precipitation (Wadsworth *et al*., 2001; Glatzel *et al*., 2003; Safar *et al*., 1998; McCutcheon *et al*., 2005) and therefore this concentration step was included in the method. Figure 3.5 shows experiments where scrapie sheep brain homogenates were PK treated and either NaPTA precipitated or not. Samples were serially diluted (from 2500 µg down to 5 µg wet tissue equivalent) then resolved and Western blotted. After NaPTA precipitation, PrP^{Sc} could be detected in the equivalent of 9 µg of wet tissue (figure 3.5, lane 8, with NaPTA). Without this step the detection limit was 78 µg of wet tissue (figure 3.5, lane 6, without NaPTA).

This result confirms that NaPTA precipitation increases sensitivity of PrP^{Sc} detection when compared to control samples in which PBS has been substituted in place of NaPTA, and this is in agreement with the findings of other investigators (Wadsworth *et al*., 2001; McCutcheon *et al*., 2005). The 6 selected antibodies (IH9, AE11, BH1, BC6, EG6, and FD12) were then tested incorporating this concentration step to establish which gave the most sensitive detection of PrP^{Sc}.

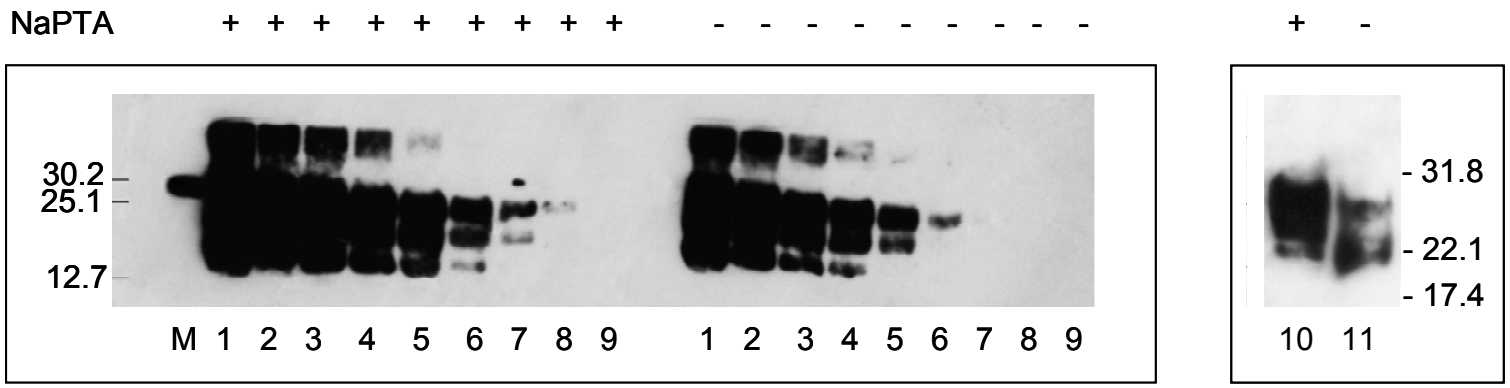


Figure 3.5: NaPTA precipitation before Western blot analysis of PK-resistant PrP^{Sc} increases sensitivity of detection. Samples were treated with or without NaPTA following PK digestion as shown. Wells were loaded with serial dilutions of the PK digestion products from scrapie infected sheep brains. In lane 1 the equivalent of 2500 µg wet tissue was loaded, in lane 2, 1250 µg, in lane 3, 625 µg, lane 4, 313 µg, lane 5, 156 µg, lane 6, 78 µg, lane 7, 19 µg, lane 8, 9 µg, and lane 9, 5 µg. Lane 10, 500 µg NaPTA precipitated uninfected sheep brain homogenate; lane 11, 500 µg uninfected sheep brain homogenate without NaPTA precipitation. M = molecular weight marker. The blot was probed with the mAb IH9 (1 µg/ml), and developed using standard ECL methods; with an overnight exposure. Molecular masses are shown on the left and far right (kDa).

3.3.4. Comparing the end-points of detection for different primary antibodies

The selected antibodies (IH9, AE11, EG6, FD12, BH1 and BC6) were initially tested at a concentration of 1 µg/ml on titrated samples of scrapie brain homogenate from prepared bulk samples (described in chapter 2, section 2.3.1.2). The scrapie brain homogenate pellet was serially diluted to produce a range of dilutions; 313 µg, 156 µg, 78 µg, 39 µg, 19 µg and 9 µg wet tissue equivalent, respectively. When used at 1 µg/ml there were clear differences between antibodies. The limit of detection for EG6 and FD12 was 39 µg and 19 µg respectively (figure 3.6, EG6 and FD12, lanes 3 and 4). The limit of detection for AE11 was also 19 µg (figure 3.6, lane 2) however unlike FD12 the 3 glycoform bands were visible in all of the higher brain concentrations (figure 3.6, AE11, lanes 1-4). The remaining 3 antibodies (IH9, BH1 and BC6) could detect down to 9 µg wet tissue equivalent (figure 3.6). Therefore these antibodies and AE11 were selected for further testing.

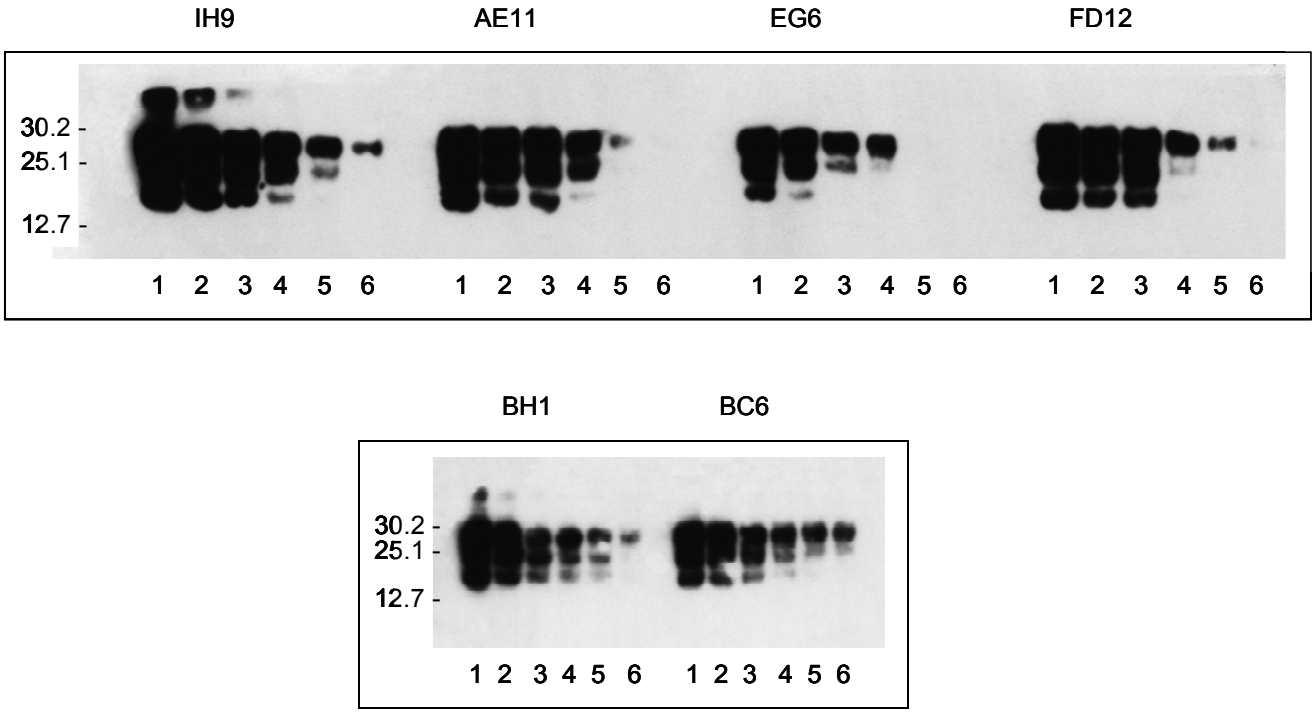


Figure 3.6: Comparing the end-points of detection for different antibodies on Western blots of NaPTA precipitated PK-resistant PrP^{Sc} from scrapie-infected sheep brains. Wells were loaded with serial dilutions of the PK digestion products from natural scrapie infected sheep brains. In lane 1 the equivalent of 313 µg wet tissue was loaded, in lane 2, 156 µg, in lane 3, 78 µg, lane 4, 39 µg, lane 5, 19 µg, and lane 6, 9 µg. The blot was probed with various mAb, IH9, AE11, EG6, FD12, BH1 and BC6. The antibody used is labelled above each blot and all were used at a concentration of 1 µg/ml. The blot was developed using standard ECL methods; the exposure time was 30 min. Molecular masses are shown on the left (kDa).

To determine whether a higher concentration of the primary antibody would further increase the sensitivity of PrP^{Sc} detection, the 4 selected antibodies were tested at concentrations of 2 and 5 µg/ml. Because they were being used at higher concentrations than before (1 µg/ml), a lower range of dilutions were used. A range from 78 µg wet tissue down to 2 µg was used for testing at 2 µg/ml, whereas for the higher antibody concentration (5 µg/ml) a dilution range between 39 µg wet tissue to 1 µg was used. At a concentration of 2 µg/ml, IH9 detected the diglycosylated PrP^{Sc} band in the equivalent of 5 µg wet tissue, while BC6 and BH1 detected PrP^{Sc} in as little as 2 µg wet tissue equivalent (figure 3.7). This antibody concentration also allowed for the detection (in some cases) of all three bands, where 2 bands had previously been visible when probed with antibody at 1 µg/ml (e.g. IH9, 2 bands in 19 µg wet tissue at 1 µg/ml, but all three bands in the same wet tissue equivalent at 2 µg/ml). No difference in sensitivity was observed with AE11 (figure 3.7).

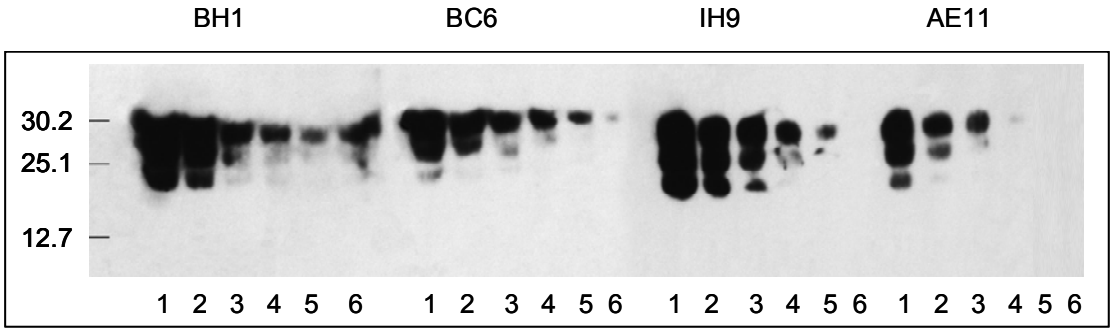


Figure 3.7: The effect of primary antibody concentration on the end-points of detection of PrP^{Sc} by Western blot. Wells were loaded with serial dilutions of the PK digestion products from natural scrapie infected sheep brains. In lane 1 the equivalent of 78 µg wet tissue was loaded, in lane 2, 39 µg, in lane 3, 19 µg, lane 4, 9 µg, lane 5, 5 µg, and lane 6, 2 µg. The blot was probed with various mAb, BH1, BC6, IH9 and AE11 all at a concentration of 2 µg/ml. The antibody used is labelled above each blot. The blot was developed using standard ECL methods; the exposure time was 3 h. Molecular masses are shown on the left (kDa).

No further increase in sensitivity was observed when antibody concentrations were increased to 5 µg/ml, in fact in some cases binding was reduced compared to blots incubated with 2 µg/ml primary antibody (figure 3.8). It is possible if concentrations

are too high, and too much unbound primary antibody remains after washing, some of the secondary antibody epitopes will be blocked, allowing less secondary antibody to bind. This would decrease the sensitivity of detection. The results of the titration of primary antibodies indicate that the highest sensitivity was achieved using BH1 and BC6 at an optimal concentration of 2 µg/ml (table 3.3).

mAb	**Ab concentration**	**313 µg**	**156 µg**	**78 µg**	**39 µg**	**19 µg**	**9 µg**	**4.5 µg**	**2 µg**	**1 µg**
IH9	1 µg/ml	+++	+++	+++	+++	++	+			
	2 µg/ml	n/a	n/a	+++	+++	+++	++	+		
	5 µg/ml	n/a	n/a	n/a	+++	++	++	+		
AE11	1 µg/ml	+++	+++	+++	++	+	+			
	2 µg/ml	n/a	n/a	+++	++	++	+			
	5 µg/ml	n/a	n/a	n/a	+	+				
EG6	1 µg/ml	+++	+++	++	+					
FD12	1 µg/ml	+++	+++	+++	++	+				
BH1	1 µg/ml	+++	+++	+++	+++	+++	+			
	2 µg/ml	n/a	n/a	+++	+++	+++	+++	+	+	
	5 µg/ml	n/a	n/a	n/a	+++	+++	++	++	+	
BC6	1 µg/ml	+++	+++	+++	+++	+++	++			
BC6	2 µg/ml	n/a	n/a	+++	+++	+++	+	+	+	
BC6	5 µg/ml	n/a	n/a	n/a	+++	+++	++	+		
					++	+	+			

Table 3.3: Summary table showing primary antibody concentrations and sensitivity of detection. Each experiment was carried out at least three times.
Key : +++ 3 bands, ++ 2 bands, + 1 band.

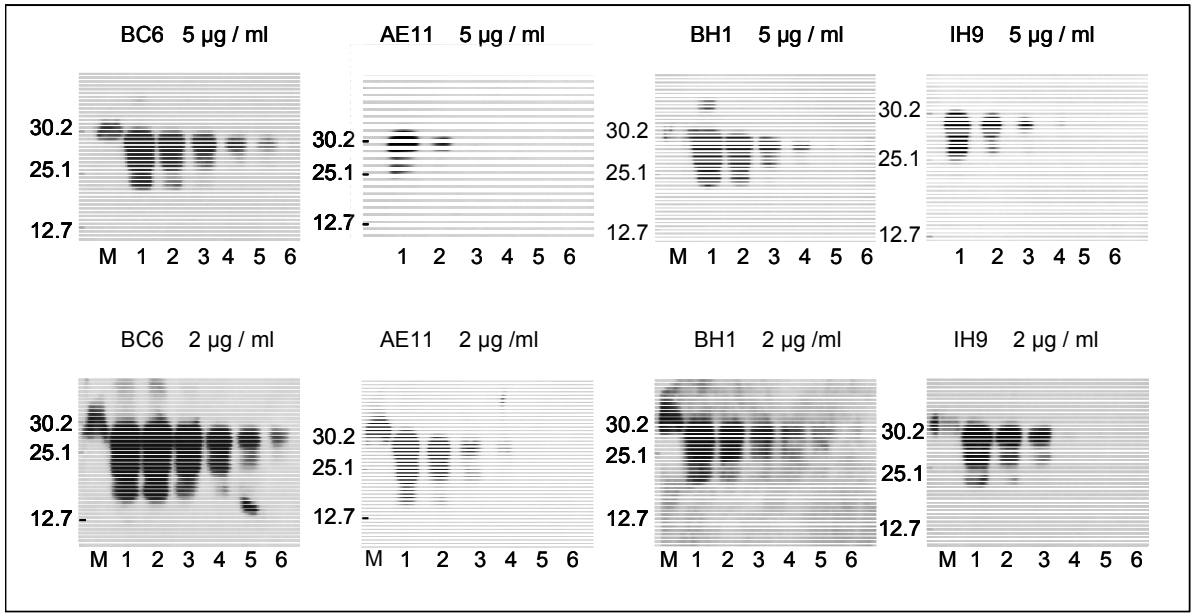


Figure 3.8: Increasing primary antibody concentrations does not increase sensitivity. Wells were loaded with serial dilutions of the PK digestion products from natural scrapie infected sheep brains. The blot was probed with various mAb, BH1, BC6, IH9 and AE11 at a concentration of 2 µg/ml or 5 µg/ml. In 2 µg/ml concentrations in lane 1 the equivalent of 78 µg wet tissue was loaded, in lane 2, 39 µg, in lane 3, 19 µg, lane 4, 9 µg, lane 5, 5 µg, and lane 6, 2 µg. In 5 µg/ml concentrations in lane 1 the equivalent of 39 µg wet tissue was loaded, in lane 2, 19 µg, in lane 3, 9 µg, lane 4, 5 µg, lane 5, 2 µg, and lane 6, 1 µg. M = molecular weight markers. The blot was developed using standard ECL methods; the exposure time was 3 h. Molecular masses are shown on the left (kDa).

3.3.5. Secondary antibody titration

Using BH1 and BC6 as primary antibodies, the secondary antibody (goat anti-mouse HRP- Appendix 1) was titrated. Previously used at a 1:7000 dilution, concentrations of 1:10 000, 1:5000 and 1:2.500 were also tested. Results previously for BH1 and BC6 indicate a maximum detection limit of 2 µg wet tissue equivalent seen with a secondary antibody concentration of 1:7000. By using the secondary antibody at the 1:5000 and 1:2500 concentrations stated above, it was difficult to determine the limit of detection due to high background (particularly with BH1) suggesting an overload of HRP in the system (figure 3.9 shows 1:5000 results as an example). The maximum detection limit observed using a secondary antibody concentration of 1:10 000 was 20 µg equivalent wet tissue (Figure 3.9). Using the secondary antibody at a concentration of 1:7000 is optimal and when used in conjunction with primary antibodies at 2 µg/ml there is no background staining and the maximal detection limit is 2 µg wet tissue equivalent (figure 3.7).

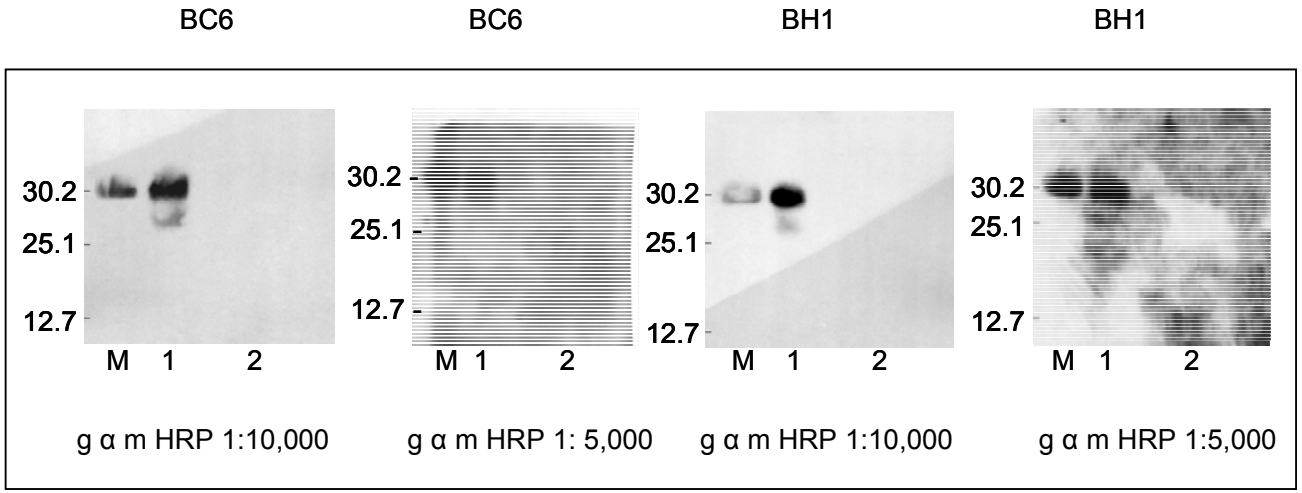


Figure 3.9: Secondary antibody titration does not improve sensitivity of detection. Wells were loaded with serial dilutions of the PK digestion products from natural scrapie infected sheep brains. The blot was probed with BH1or BC6 at a concentration of 2 µg/ml. The secondary antibody was used at a concentration of 1:10,000 or 1:5000 as indicated. M = molecular weight markers. In lane 1 the equivalent of 20 µg wet tissue was loaded, in lane 2, 2 µg. The blot was developed using standard ECL methods; the exposure time was 5 min. Molecular masses are shown on the left (kDa).

3.3.6. Comparison of an alternative sensitive detection system

Western blot sensitivity has been increased by using different detection reagents (Wadsworth *et al*., 2001). Therefore the enhanced sensitivity substrate, West Femto reagent (Pierce) was tested. Because the substrate is extremely sensitive, less primary and secondary antibody is required to obtain a signal. Therefore, the primary (BH1 and BC6) and secondary antibodies were re-titrated using this system. Primary antibodies were used at concentrations of 0.2 µg/ml, 0.5 µg/ml and 1 µg/ml. Secondary antibodies were tested at 1:100,000, 1:200,000 and 1:500,000 dilutions. Figure 3.10 is a representative blot using BC6 and shows that the signal produced was similar using the 3 different primary antibody concentrations. The optimal secondary antibody concentration appeared to be 1:200,000. A dilution of 1:500,000 diminished the signal and concentrations higher than 1:200,000 produced an increased background. PrP^{Sc} present in 20 µg wet tissue equivalent was clearly apparent (figure 3.10). The diglycosylated band of disease-associated PrP in the equivalent of 2 µg wet tissue was also faintly visible, although the result was not conclusive because the background signal was high in all cases beyond a 30 second blot exposure.

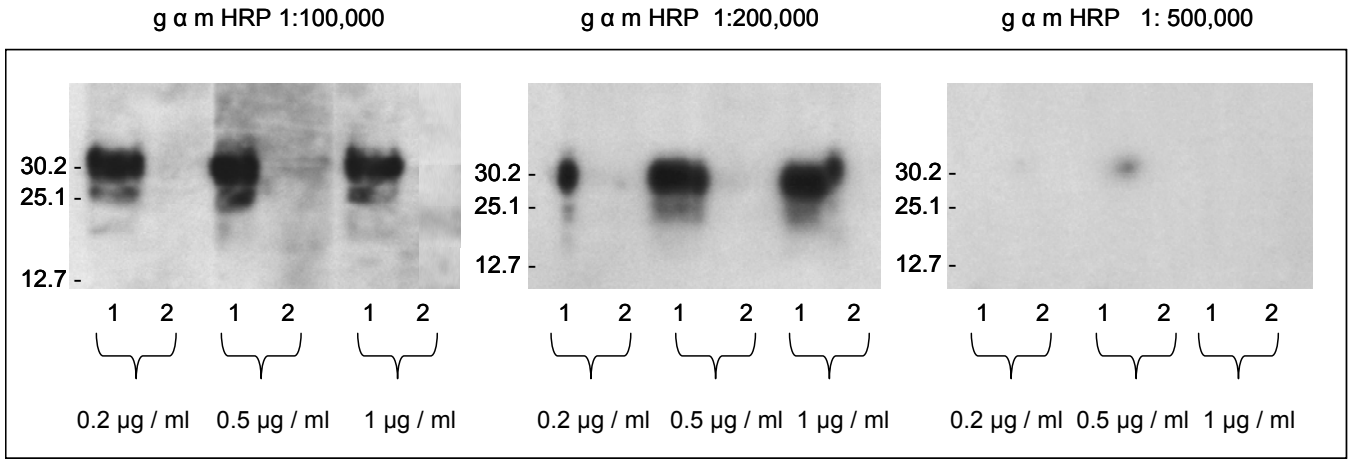


Figure 3.10: An enhanced sensitivity substrate does not increase sensitivity of detection. Wells were loaded with serial dilutions of the PK digestion products from natural scrapie infected sheep brains. The blot was probed with BC6 at a concentration of 0.2 µg/ml, 0.5 µg/ml and 1 µg/ml as shown. The equivalent of 20 µg wet tissue (lane 1) and 2 µg wet tissue (lane 2) was loaded. The secondary antibody was used at 3 different concentrations, 1:100,000, 1:200,000, and 1:500,000 as indicated. The blot was developed using the enhanced sensitivity West Femto substrate according to the manufacturer's instructions. The exposure time was 30 sec. Molecular masses are shown on the left (kDa).

To reduce background, different blocking buffers such as 3% casein in 0.05% Tween 20, and 5% milk powder were tried (figure 3.11), but none successfully reduced the background signal on the blots. Increasing the concentration of Tween detergent in wash buffers (0.05%, 0.1%, and 0.5%) was also unsuccessful at reducing background (data not shown). Although West Femto is an extremely sensitive enhanced chemiluminescent substrate there was no enhanced detection of PrP^{Sc} when either BH1 or BC6 were used. Background signals were high even after titration of primary and secondary antibodies. Testing different blocking and wash buffers did not reduce the level of background signal. The results obtained suggest that the limit of PrP^{Sc} detection by this Western blot assay has been reached and that the sensitivity could not be increased further by using this alternative substrate.

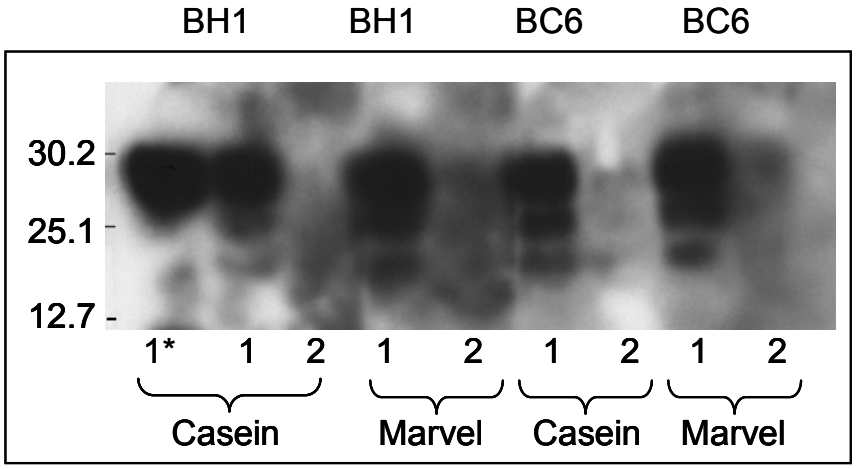


Figure 3.11: The use of different blocking buffers does not reduce background when using West Femto. Wells were loaded with serial dilutions of the PK digestion products from natural scrapie infected sheep brains. The blot was probed with BC6 or BH1 at a concentration of 0.2 µg/ml. The equivalent of 20 µg wet tissue (1) and 2 µg wet tissue (2) was loaded. Lane 1* indicates where sample spilled over from lane 1. The secondary antibody was used at a concentration of 1:200,000. The membrane was cut and blocked in either casein or marvel milk powder blocking buffer as indicated. The blot was developed using the enhanced sensitivity West Femto substrate according to the manufacturer's instructions. The exposure time was 30 sec. Molecular masses are shown on the left (kDa).

3.3.7. Confirmation of the sensitivity of the optimised Western blot using scrapie brain spikes

Spiking infected brain into uninfected brain confirms that the sensitivity of infection is maintained when the spike is recovered against a high background of normal PrP^{C} and other proteins. The maximum sensitivity achieved with the optimized Western immunoassay was detection of PrP^{Sc} in the equivalent of 2 µg scrapie-infected brain (figure 3.8). To confirm that a similar level of sensitivity could be achieved using brain spikes, decreasing amounts of scrapie sheep brain homogenate were diluted into uninfected sheep brain homogenate (chapter 2, section 2.3.1.2). Detection of PrP^{Sc} was tested using the monoclonal antibodies BH1 and BC6 as previously described. Both antibodies consistently detected PrP^{Sc} in samples spiked with 20 mg, 2 mg, and 200 µg wet tissue equivalent (figure 3.12, BH1 and BC6, lanes 1, 2 and 3).

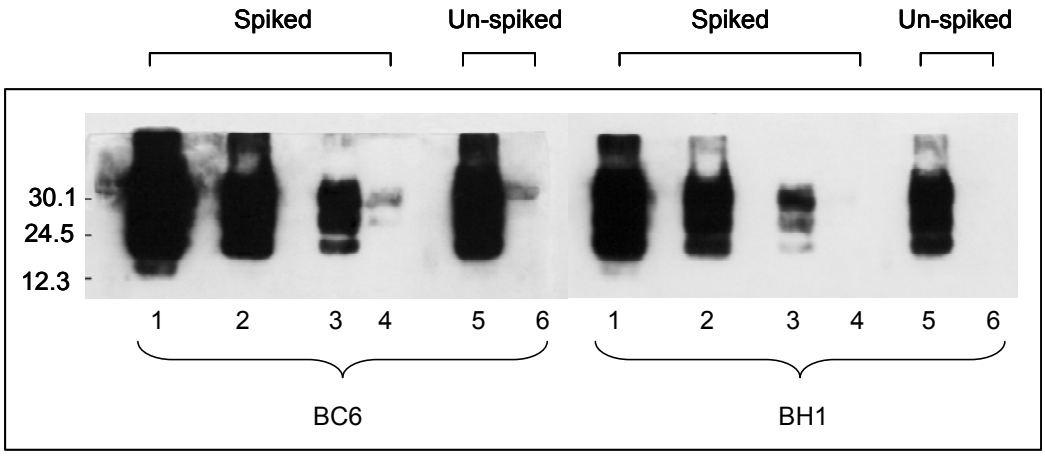


Figure 3.12: Western blot analysis of PrP^{Sc} recovered from spikes of scrapie-infected brain homogenate in a background of uninfected brain homogenate. Wells were loaded with the PK digestion products from scrapie infected sheep brains spiked into an uninfected sheep brain homogenate to give a combined wet tissue equivalent of approx 500µg per well. Key: lanes 1-4, brain spikes of 20 mg, 2 mg, 200 µg & 20 µg wet tissue equivalent, respectively; lane 5, 500µg wet tissue equivalent of scrapie-infected brain homogenate (PK digested). Lane 6, 500 µg wet tissue equivalent of uninfected brain homogenate (PK digested). The blot was probed with BC6 and BH1 mAb at a concentration of 2 µg/ml. The blot was developed using standard ECL methods; the exposure time was 1 min. Molecular masses are shown on the left (kDa).

The diglycosylated band in the 20 µg brain spike was consistently visible using BC6 although the intensity of the signal varied (data not shown). Detection of the same band was not obvious with BH1 which was surprising as both antibodies had previously been shown to detect PrP^{Sc} in the same amount of scrapie infected brain (figures 3.7, 3.8 and table 3.2). As brain spiked samples were prepared separately for each antibody it is possible that there is slight sample variation which may account for this result. Previously using these antibodies the di-, mono- and unglycosylated band were detected in the equivalent of 20 µg wet tissue scrapie brain homogenate and the diglycosylated band was detected in as little as 2 µg wet tissue (section 3.3.4 and table 3.2). It is possible in the spiking experiments that the PrP present in the normal brain caused the ratio of PK to total protein concentration to be altered and more undigested PrP^{C} was present. This high background of PrP^{C} may have contributed to the reduction in the signal on the immunoblot as less PrP^{Sc} could be detected against the background of undigested PrP^{C}. However, the levels of undigested PrP^{C} would have to be lower than are detectable by sensitive Western blotting as a signal was not observed in uninfected brain homogenate following digestion with the same concentration of PK as used in the spiked samples (figure 3.12, lane 6). In addition to 50 µg/ml, higher concentrations of 75 µg/ml and 100 µg/ml of PK were also tested. Detection was not improved although further repeat experiments are required to confirm the effect of higher PK concentrations (data not shown). Brain contains material such as deoxyribonucleic acid (DNA) and connective tissues that are not easily disrupted by sonication. The presence of such material may be affecting the recovery of spikes from uninfected brain. Therefore to try to overcome these matrix effects and to maximise solubility of the infected and normal brain material, samples were solubilised using different sonicators and in different buffers (6M guanidinium hydrochloride and 0.1 % sarcosyl). However again, the smallest recoverable spike was 20 µg wet tissue equivalent (data not shown).

To overcome potential variability in results (see above), spiked scrapie brain samples were prepared in bulk and directly compared to non-spiked brain samples (figure 3.13, panel 1 and 2).

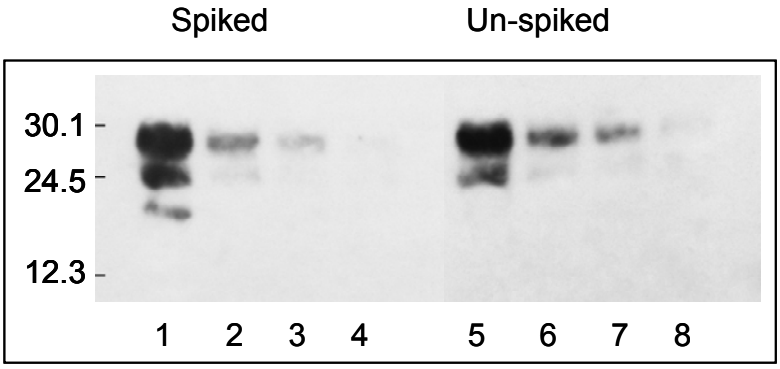


Figure 3.13: The sensitivity of Western blot detection of PrP^{Sc} is similar for spiked and unspiked samples. Wells were loaded with the PK digestion products from scrapie-infected sheep brains spiked into an uninfected sheep brain homogenate to give a combined wet tissue equivalent of approx 500 µg per well. The equivalent unspiked samples are also shown. Key: lanes 1 & 5, 200 µg; lanes 2 & 6,100 µg; lanes 3 & 7, 50 µg and lanes 4 & 8, 25 µg wet tissue equivalent, respectively. The blot was probed with BC6 at a concentration of 2 µg/ml. The blot was developed using standard ECL methods; the exposure time was 30 min. Molecular masses are shown on the left (kDa).

These experiments consistently demonstrated a reproducible detection limit that was similar for spiked scrapie brain samples when compared to scrapie brain that was not spiked into normal brain homogenate (figure 3.13). Again there were differences between the overall recovery of detection in spiked samples (faint diglycosylated band in 25 µg wet tissue in figure 3.13 yet a more intense signal seen with 20 µg wet tissue in figure 3.12). Also, based on previous results it would be expected that all PrP glycoforms would be visible in 200, 100, 50 and 25 µg wet scrapie brain tissue although this is not apparent (figure 3.13). These differences may be due to slight variations of the amount of PrP^{Sc} in individual scrapie brain aliquots or loss of PrP during long term sample storage. Additionally, although great care was taken to ensure that samples were processed in an identical manner and that equal amounts were loaded onto the gels, it is possible that variability in protein loading could also account for

differences in immunoblot signals. However, because recovery of brain spikes is similar to non-spiked samples this demonstrates that the sensitivity of the Western immunoassay is not substantially altered when recovering small quantities of PrP^{Sc} against a high background of other proteins. This confirms that the assay is suitable for PrP^{Sc} detection in tissue samples where the protein in present at low concentrations.

3.4. Discussion

The aim of the research presented in this chapter was to develop a sensitive Western blot immunoassay that could be applied to conventional PK-resistant PrP^{Sc} detection in blood. Overall sensitivity was increased by incorporating a NaPTA precipitation step to concentrate PrP^{Sc}, and screening a panel of novel monoclonal antibodies raised against recombinant sheep PrP. Using the optimised method, conventional PrP^{Sc} was detected in as little as 2 μg wet brain equivalent, although in brain spiking experiments to mimic *in vivo* conditions, the sensitivity was slightly reduced, with consistent recovery of 20 μg brain spikes.

Sensitive methods are required for TSE diagnosis, especially when applied to peripheral tissues during pre-clinical infection. Even in clinical cases of some TSE diseases (e.g. vCJD, scrapie), PrP^{Sc} positive peripheral tissues contain PrP^{Sc} levels in the range of 0.1-15 % of those found in brain (Wadsworth *et al*., 2001; Andreoletti *et al*., 2002). Therefore, a variety of procedures to concentrate PrP^{Sc} have been employed to improve assay sensitivity. NaPTA precipitation has previously been shown to concentrate PrP^{Sc} (Safar *et* al., 1998; Wadsworth *et* al., 2001), allowing for detection of PrP^{Sc} in tissues previously thought to be negative (Glatzel *et* al., 2003). In agreement with other investigators, when a NaPTA concentration step was included in the protocol for Western blotting of sheep PrP^{Sc}, the sensitivity of detection was increased approximately four-fold. The exact molecular mechanism of PrP^{Sc} interaction with NaPTA is unknown but may be specific for PK resistant and sensitive forms of PrP^{Sc} (Safar *et al*., 1998; Tremblay *et al*., 2004). The extent of PrP^{Sc} concentration may also be dependent on the size, shape and charge of the NaPTA molecules (Lee *et al*., 2005) and it is possible that this could be experimentally manipulated to further maximise PrP^{Sc} concentration. In addition to NaPTA precipitation, other approaches have been used to concentrate PrP^{Sc}. Examples include commercially developed ligands for specific PrP^{Sc} capture (Nazor *et al*., 2005), use of fluorescence labelled palindromic PrP peptides to detect misfolded PrP (Pan *et al*., 2007) and ELISA based assays using the same monoclonal antibody for both capture and detection (Pan *et al*., 2005). However, these methods use commercially developed reagents that are not available for general

use, which is why these approaches were not employed to develop or combine with the sensitive Western blot.

The European Commission validated three diagnostic tests in 1999 for cattle PrP^{Sc}. The Prionics test is based on a Western blot using the monoclonal antibody 6H4 (epitope in the C-terminus, sequence DYEDRYYRE). The Enfer test (produced by Enfer Technology Ltd) is a high throughput chemiluminescent ELISA that uses a polyclonal anti-PrP antibody for detection. The TeSeE test (Bio-Rad) is also based on an ELISA where PrP^{Sc} is treated with PK, solubilised and denatured before detection by a sandwich ELISA using two monoclonal antibodies. These validated tests are for cattle and BSE and have not been found to directly cross over to small ruminants. To address this, Bio-Rad have developed a separate sheep and goat TeSeE rapid assay kit as the original cattle TsSeE kit did not contain monoclonal antibodies with high affinities to sheep and goat PrP^{Sc}. PrP^{Sc} detection limits in cattle brain can be estimated using reports in previous publications where the Prionics test has a detection limit of 5-20 pmol, the TeSeE test, 0.5-2 pmol and the Enfer test, 1-10 pmol (Brown *et al*., 2001; Ingrosso *et al*., 2002; Soto *et al* 2004). However, it is very difficult to compare the sensitivities of commercial TSE tests with the sensitive Western blot described in this thesis. This is because commercial reagents are often tested using dilutions of brain from species other than sheep. The concentration of PrP in brain material will vary depending on the species, the TSE agent and the area of brain sampled. In order to perform a true comparison, commercial antibodies and reagents would have to be tested alongside the panel of novel monoclonal antibodies described in this thesis using standardized brain samples. Due to time considerations, these experiments were not carried out.

Screening of the panel of novel anti-PrP monoclonal antibodies against PK digested sheep and mouse scrapie brain samples identified antibodies that appeared to bind PrP^{Sc} with high affinity on Western blots, as reflected by differences in the signal intensities. The epitopes of these antibodies are located towards the C terminus of ovine PrP (between amino acids 90-233), but the conformation of PrP^{Sc} might influence how accessible certain epitopes are to antibody binding, and this could also explain some of

the observed difference in signal intensity with different antibodies. Titrations of primary and secondary antibodies were performed to optimise the concentrations required to achieve maximum assay sensitivity, while minimising the effects of non-specific binding (at higher antibody concentrations), resulting in high background staining. Attempts to increase sensitivity by using an alternative chemiluminescence substrate resulted in unacceptably high levels of background staining, and did not improve assay sensitivity, although it did have the advantage that less primary and secondary antibody was required to obtain a signal.

The level of sensitivity achieved with the optimized Western blot for sheep PrP^{Sc} compares well with that reported by other authors using similar methods. Wadsworth *et al.* (2001) were able to detect PrP^{Sc} in 5 nl of 10 % vCJD brain homogenates (equivalent to 0.5 µg wet tissue weight) by using NaPTA precipitation in conjunction with high sensitivity detection systems, which is only slightly more sensitive than the optimised Western blot assay developed here. However, using the hamster 263K scrapie model, Lee *et al*. (2000) demonstrated PrP^{Sc} detection in 10-20 ng brain tissue equivalents, which is approximately 100 fold more sensitive than the detection limit achieved using the method described here. A likely explanation for the observed differences is that the PrP^{Sc} concentration in the starting samples is not the same, because of differences in the species and/or TSE agent. In particular, hamsters infected with the 263K scrapie strain are known to have unusually high concentrations of PrP^{Sc} in brain and other tissues, whereas in sheep with natural scrapie there is much variability in the amount of PrP^{Sc} deposited in brain. Evidence suggests that this could be influenced by sheep PrP genotype with the highest levels of PrP^{Sc} deposition found in VRQ/VRQ sheep, the genotype of the sheep samples used in these experiments (McCutcheon *et al.*, 2005). The different detection limits reported for different immunoassays may also reflect apparent variations in the relative amounts of PK resistant and PK sensitive PrP^{Sc} shown in different TSE strains (Safar *et al.*, 1998) or species. If samples contain high levels of PK sensitive PrP^{Sc}, overall assay sensitivity may be reduced.

In conclusion, a highly sensitive, rapid, Western blot assay for detection of conventional sheep PK-resistant PrP^{Sc} has been developed. The sensitivity of the method was increased by incorporation of a NaPTA precipitation step and use of new monoclonal antibodies raised against sheep PrP, to give a reliable detection limit of approximately 20µg scrapie brain tissue equivalents, similar to that achieved in other published Western blot methods. The next aim was to apply the optimised assay to detection of conventional PK resistant PrP^{Sc} in blood samples from scrapie-infected sheep.

Chapter 4

Can PrP^{Sc} Be Detected In Blood From Scrapie-Infected Sheep Using A Sensitive Western Blot Immunoassay?

4.1. Introduction

Despite a wealth of research in recent years, several fundamental issues concerning the transmissibility of TSE disease through blood transfusion remain unsolved, such as the nature of the infectious agent and the distribution of infectivity in blood components. The latter is of particular importance for the development of safer methods of blood transfusion in humans, where commonly used blood components include plasma, platelets and red cells. Since October 1999 leukocytes have been depleted from all blood used for transfusion in the United Kingdom, but the effectiveness of this measure in preventing or reducing vCJD transmission is unknown. Leucoreduction appears to remove only about 42 % of TSE infectivity (Gregori *et al*., 2004), although leucoreduced blood that is passed through prion removal filters containing resin appears to remove all the residual infectivity (Gregori *et al* 2006). However, leucoreduction does not remove all TSE infectivity and defining exactly which blood components are infectious remains crucial.

In some TSE diseases such as scrapie, vCJD and some experimental rodent TSE models, the agent is replicated in lymphoreticular tissues before infection of the CNS (Eklund *et al*., 1967; Hadlow *et al*., 1982; Hill *et al*., 1999). Lymphocytes recirculate between lymphoid tissues and blood and in TSE infected animals it is likely that infectivity is spread in this manner, although it is not believed significant for neuroinvasion (Blattler *et al*., 1997). Experimental rodent TSE models have demonstrated low levels of infectivity in blood during preclinical and clinical stages of disease (Brown *et al*., 1998, 1999; Cervenakova *et al*., 2003). Despite these experiments it has proved difficult until recently to demonstrate that blood from natural TSE cases contains infectivity (Hadlow *et al*., 1980; Hadlow *et al*., 1982; Fraser *et al*., 1994). This is because in rodents only small volumes of blood or blood components can be assayed by i/c inoculation. Also transmission of infection may be limited by species barrier effects. However, transfusion of large volumes (up to 500ml) of whole blood or buffy coat from sheep with natural scrapie or experimentally infected with BSE resulted in efficient transmission of disease to recipient sheep in the absence of a species barrier (Hunter *et al*., 2002; Houston *et al*., 2000; Houston *et al*., 2008). Transmission can occur with blood collected early enough in the incubation period that

the donor sheep had no accumulation of abnormal PrP elsewhere (palatine tonsils, mesenteric lymph nodes and spleen) (Siso *et al*., 2006; Houston *et al*., 2008). Transmission of BSE from the blood of an experimentally infected primate (*Microcebus murinus*) has also been reported (Bons *et al*., 2002) and more recently CWD was transmitted *via* blood in white tailed deer fawns (Mathiason *et al*., 2006). In addition, four cases of vCJD infection in recipients of non-leucodepleted red blood cells from known vCJD cases have occurred in humans (Llewelyn *et al*., 2004; Peden *et al*., 2004; Wroe *et al*., 2006; Health Protection Agency, 2007). One of the individuals was also heterozygous for codon 129 of the prion protein gene (Peden *et al*., 2004).

In humans and sheep, the distribution of infectivity between different blood components is unknown, but evidence from rodent TSE models suggests that the highest levels of infectivity are found in the buffy coat fraction of blood. In mice infected with vCJD or a Gerstmann-Straussler-Scheinker (GSS) disease isolate (Fukuoka-1), there were similar infectivity levels of approximately 18-30 infectious units (IU) per ml in buffy coat, but no detectable infectivity in red cells (Cervenakova *et al*., 2003). Brown *et al* (1998) carried out experiments where scrapie-infected hamster brain homogenate was "spiked" into normal human blood and found that the highest levels were also in the buffy coat fraction with slightly lower levels in plasma. In follow up studies using the GSS mouse model described above, they found that preclinical blood contained 6-12 IU per ml in buffy coat, but infectivity was absent or present only in trace amounts in plasma. Infectivity in the blood of clinical mice rose to 100 IU per ml in buffy coat, and 20 IU per ml in plasma (Brown *et al*., 1999). In hamster scrapie, very little infectivity was found in association with purified platelets, although 22.6 IU per ml was associated with the buffy coat subset of mononuclear leukocytes (Holada *et al*., 2002). In sheep, TSE infectivity was found in the buffy coat fraction of blood (Hunter *et al*., 2002; Houston *et al*., 2000) and more recent results revealed that infectivity appears mainly linked to the monocyte ($CD14^{+}$) subset of leukocytes in sheep (Andreoletti *et al*., 2007). In addition to cell-associated PrP^{Sc} it is possible that PrP^{Sc} may be associated with blood cell membrane fragments. Evidence exists which demonstrates an association between PrP^{Sc} released into the extracellular

environment and exosomes, suggesting their role in cell to cell agent transmission (Fevrier *et al*., 2004).

The detection of PK resistant PrP^{Sc} is used as a biochemical marker for the presence of infectivity. So far, most attempts to detect PrP^{Sc} in blood have been unsuccessful. Methods used to date include immunocapillary electrophoresis (Schmerr *et al*., 1999), immunocytochemistry (Hermann *et al*., 2002), and Western blotting (Wadsworth *et al*., 2001), but the results so far have been negative or inconclusive. Levels of PrP^{Sc} in blood may be low, which presents challenges for assay sensitivity. Although little is known regarding the distribution of TSE infectivity in blood, studies on the distribution of host PrP^{C} in blood have been carried out. In healthy sheep there are significant levels of cell-surface PrP^{C} on peripherial blood mononuclear cells (PBMCs) with platelets containing high levels of intracellular PrP^{C} (Barclay *et al*., 2002; Halliday *et al*., 2005). Therefore, PrP^{Sc} has to be identified against a high background of PrP^{C}. There is evidence for the existence of PK sensitive forms of PrP^{Sc} (Tremblay *et al*., 2004) and very little is known about the biochemical properties of blood-associated PrP^{Sc}. For instance, because PrP^{Sc} is present in very low amounts, in an environment with constant biochemical changes, it is unclear if PrP^{Sc} in blood can form aggregates resistant to PK digestion.

Despite these challenges PrP^{Sc} has been amplified to detectable levels in TSE infected blood using a relatively new technique termed protein misfolding cyclic amplification (PMCA) therefore showing that if the assay has the required sensitivity then PrP^{Sc} detection in blood is possible (Castilla *et al*., 2005 ; Saa *et al*., 2006). Other recent detection techniques include aggregate specific ELISA where PrP^{Sc} aggregates were detected in plasma from mice or deer infected with scrapie or CWD (Chang *et al*., 2007) or detection of misfolded PrP in TSE-infected plasma from mice using fluorescence labelled pallindromic PrP peptides (Pan *et al*., 2007). These are very recent methods however, and use reagents that are not yet commercially available, hence they were not used/combined with the sensitive Western blot.

Chapter 3 describes a sensitive Western blot immunoassay for PK resistant PrP^{Sc} using scrapie infected sheep brain homogenates. The next step was to apply this new method to blood. PrP^{Sc} is recognised conventionally as PK resistant, having a distinctive triple band pattern (between 27-30 kDa) on Western blots. One aim of this thesis was to use the sensitive Western blot method to address the question of whether PrP^{Sc} is present in sufficient quantities to explain infectivity levels detected by bioassay and to be able to state clearly whether or not TSE infectivity in blood is associated with conventional PrP^{Sc}. Attempts to detect PrP^{Sc} in blood using Western blotting have so far been unsuccessful. When the method developed by Wadsworth *et al.* (2001) was applied to a buffy coat fraction from a single vCJD case PrP^{Sc} was not detected. However only one 15 ml sample was tested and it is possible that assaying larger volumes of blood from multiple patients may allow detection PrP^{Sc} in at least some individuals. The optimised Western blot assay for sheep PrP^{Sc} clearly does not have the analytical sensitivity of the PMCA assay which can detect as few as 8,000 equivalent molecules of PrP^{Sc} in the hamster scrapie model (Saa *et al*., 2006). However, the advantage of applying the new method to blood samples from sheep experimentally infected with scrapie is that larger volumes of blood from multiple animals can be tested, which may overcome the need for amplification of PrP^{Sc}. In Chapter 4, the detection limits of the new assay were established for PK resistant PrP^{Sc} in cellular components using SMB cells. This allowed the development of modifications for use with cell extracts (rather than brain homogenate) before applying to blood.

4.2. Results

4.2.1. Establishing SMBs15 and SMB-PS cell lines

The SMBs15 (Scrapie-infected mouse brain) cell line was established originally in culture from a brain taken from a mouse affected by the Chandler scrapie isolate (Clarke and Haig, 1970) and shown to be of mesodermal origin (Haig and Clarke, 1971). Cells continuously produce low levels of PrP^{Sc}, but can be cured of infection with pentosan sulphate. Such uninfected cells are referred to as SMB-PS cells (Birkett *et al*., 2001). SMBs15 and SMB-PS cells obtained from the TSE Resource Centre at IAH Compton were grown as described in chapter 2, section 2.3.2. Figure 4.1 illustrates the phenotypic characteristics of these cells.

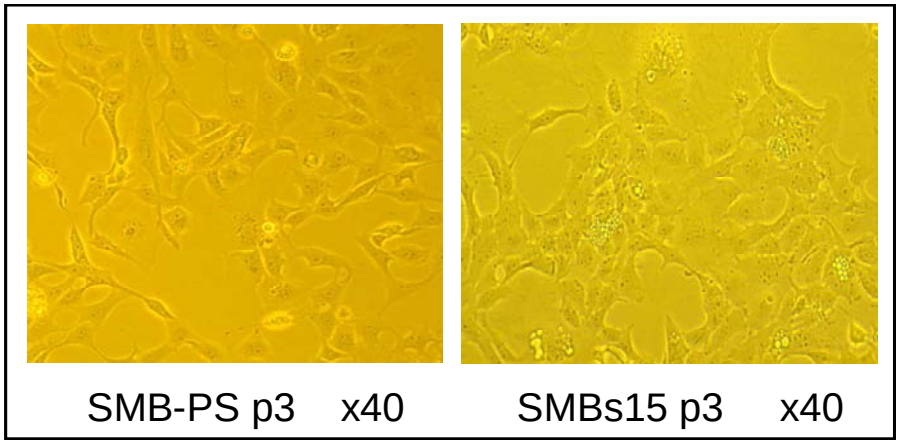


Figure 4.1: Phenotypic characteristics of SMB-PS and SMBs15 cells. Cells were passaged three times (p3) and photographed at forty times magnification (x 40).

4.2.2. Application of the sensitive Western blot method developed in brain to SMB cells

Using Western blot analysis it was confirmed that infected SMBs15 cells showed evidence of conventional PK resistant PrP^{Sc} (figure 4.2, lanes 6-8) unlike uninfected SMB-PS cells (figure 4.2, lanes 2-4). It was surprising that a signal on the Western blot was not seen in SMB-PS cells without PK digestion (figure 4.2, lane 1) as these cells have been shown to contain PrP^{C} (Birkett *et al*., 2001). A signal may have been apparent if more sample was used, although this was not pursued as all subsequent experiments were carried out on infected SMBs15 cells.

Following treatment with varying concentrations of PK, a similar signal was observed in SMBs15 cells under all PK conditions (figure 4.2, lanes 6-8). As an intense PrP^{Sc} signal was seen using 75 µg/ml PK and PK concentrations of 75-100 µg/ml are routinely used in SMB blotting methods (Birkett *et al*., 2001; Rudyk *et al*., 2000) a final PK concentration of 75 µg/ml was used in all subsequent Western blot experiments with SMB cells.

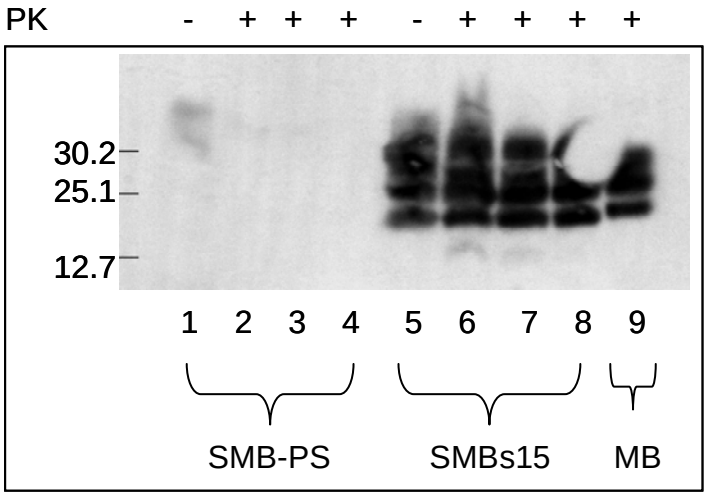


Figure 4.2: Testing optimal PK digestion on Western blots of PrP^{Sc} and PrP^{c} from SMBs15 and SMB-PS cell lysates (approx 1 x 10^6 cells). Key: lanes 1-4, SMB-PS lysates: lanes 5-8, SMBs15 lysates. Lane 1 & 5, cells were not PK digested: lane 2 & 6, 20 µg/ml PK: lanes 3 & 7, 50 µg/ml PK: lanes 4 & 8, 75 µg/ml PK. Lane 9, 500 µg wet tissue equivalent of PK digested (50 µg/ml) 139 A scrapie infected murine brain homogenate (MB). The blot was probed with the mAb BH1, and developed using standard ECL methods; the exposure time was 30 sec. Molecular masses are shown on the left (kDa).

The effect of NaPTA precipitation was then examined and resulted in an enhancement in the signal for PrP^{Sc} (figure 4.3, lane 1 compared to lane 2) and was therefore used in all subsequent experiments. The antibodies BH1 and BC6 were tested as these gave the highest sensitivity of detection on blots using scrapie brain as described in chapter 3. Similarly in SMBs15 cells a signal was observed when both antibodies were used (figure 4.3). The primary and secondary antibody concentrations (2 µg/ml and 1:7000) that were used in the optimised Western for brain homogenates were also used in SMBs15 cell experiments.

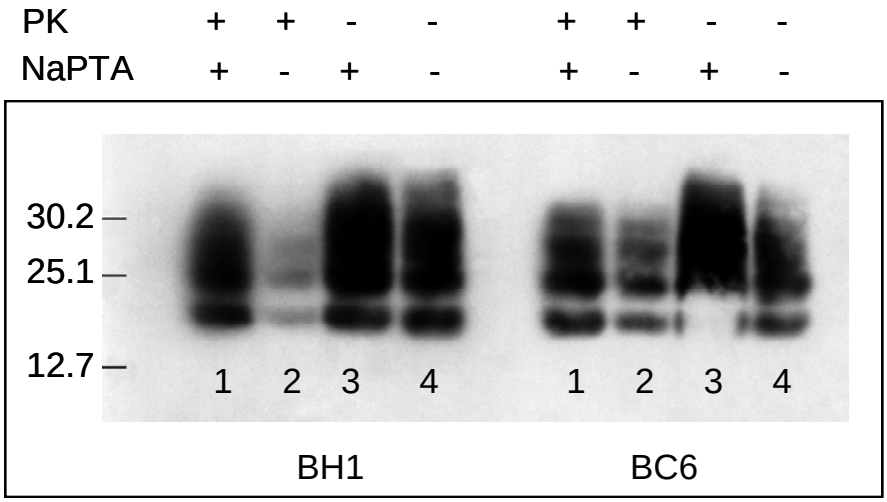


Figure 4.3: Testing the effect of NaPTA and different mAbs on Western blots of PrP^{Sc} and PrP^{c} from SMBs15 cell lysates. Samples were NaPTA precipitated and/or PK digested and probed with antibodies as indicated at a concentration of 2 μg/ml. In each lane approx 1 x 10^6 cells were lysed. The blot was developed using standard ECL methods; the exposure time 30 sec. Molecular masses are shown on the left (kDa).

4.2.3. Estimation of the sensitivity of Western blotting for detection of PrP^{Sc} positive cells in blood using SMB cell spikes

A titration series of SMBs15 cells were analysed by Western blot to indicate the limits of detection of PrP^{Sc} in this cell line, and to establish the approximate number of cells needed to represent a PrP^{Sc} signal (well resolved bands). A PrP^{Sc} signal showing the three glycosyl bands without over-saturation of the signal was obtained with 1.25 x 10^5 and 6 x 10^4 cells (figure 4.4, lanes 4 and 5, red stars). The detection limit was reached at approximately 6 x 10^4 SMBs15 cells (figure 4.4, lane 5).

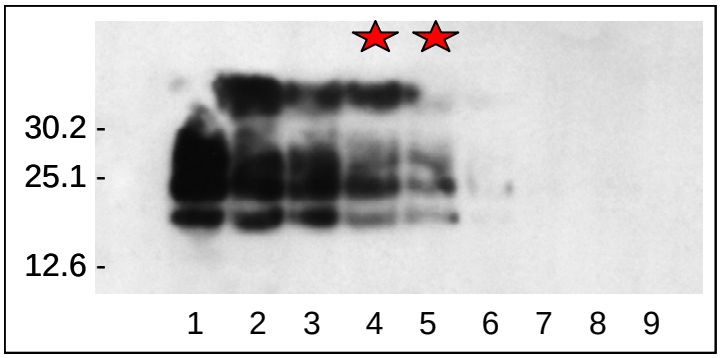


Figure 4.4: Establishing limits of detection of PrP^{Sc} from SMBs15 cells. Cells were serially diluted in PBS from approx 1 x 10^6 cells in lane 1, 5 x 10^5 cells in lane 2, 2.5 x 10^5 in lane 3, 1.25 x 10^5 in lane 4, 6 x 10^4 in lane 5, 3 x 10^4 in lane 6, 12 x 10^3 in lane 7, 6 x 10^3 in lane 8 and 3 x 10^3 cells in lane 9. The blot was probed with the mAb BH1 (2 µg/ml) and developed using standard ECL methods; the exposure time was 1 min. Molecular masses are shown on the left (kDa).

The sensitivity of the Western immunoassay was also assessed following spiking of SMBs15 cells into uninfected peripherial blood mononuclear cells (PBMC). Uninfected PBMCs were used as diluent because ultimately the Western assay would be applied to buffy coat samples (which include PBMCs) from clinical scrapie cases. Decreasing numbers of SMBs15 cells were added to increasing numbers of uninfected PBMCs, and these results were compared with a titration of SMBs15 cells only (figure 4.5). The results were very similar, confirming that the limit of PrP^{Sc} detection was reached at 6 x 10^4 SMBs15 cells, in both spiked and unspiked samples. Thus the sensitivity of the assay is maintained even when SMBs15 cells are diluted into much larger numbers of uninfected cells.

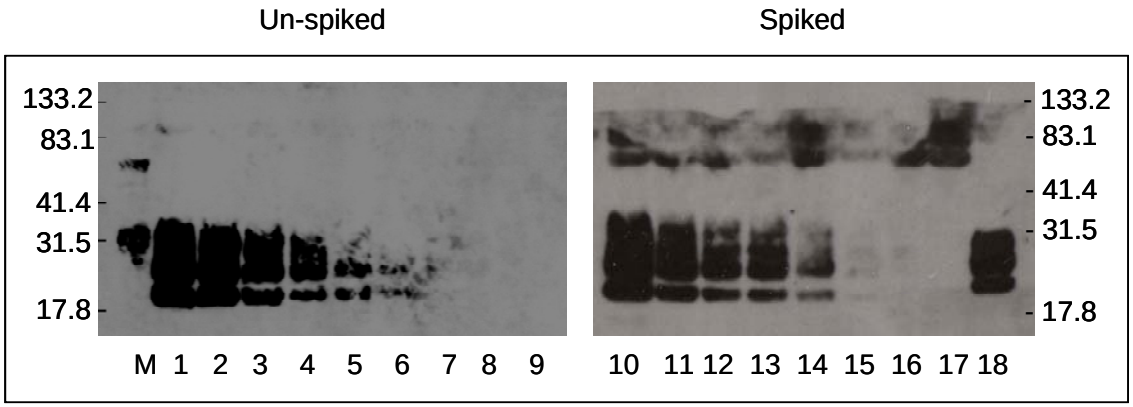


Figure 4.5: The detection limit of PrP^{Sc} from SMBs15 cells and SMBs15 cells spiked into uninfected PBMCs on Western blots is the same. Numbers of SMBs15 cells per well: lanes 1 & 10, 2 x 10^6 : lanes 2 & 11, 1 x 10^6 : lanes 3 & 12, 5 x 10^5 : lanes 4 & 13, 2.5 x 10^5 : lanes 5 & 14, 12 x 10^4 : lanes 6 & 15, 6 x 10^4 : lanes 7, 8, 9 & 16, 1.5 x 10^4. SMBs15 cells were mixed with differing numbers of uninfected PBMCs to give a total number of 4 x 10^6 cells per well (shown as spiked above the blot). Lane 17, 4 x 10^6 PBMC cells only. Lane 18, 500 µg wet tissue equivalent of 139 A scrapie infected murine brain homogenate (PK digested). Lane M indicates the presence of markers. The blot was probed with the mAb BC6 at a concentration of 2 µg/ml and developed using standard ECL methods; the exposure time was 1 min. Molecular masses (kDa) are shown on the left on the un-spiked blot and the right on the spiked blot.

To test this further and to establish how many cells could physically be loaded and well resolved on a gel, SMBs15 cell spikes (12 x 10^4 and 6 x 10^4) were mixed with greater numbers of PBMCs (1 x 10^7, 5 x 10^7 and 1 x 10^8). The results obtained give an indication of numbers of PBMCs that can be pooled into one sample to be resolved on a gel. The signal from 12 x 10^4 and 6 x 10^4 SMBs15 cells could still be recovered even after dilution into 1 x 10^8 PBMCs, with a similar signal intensity to that obtained from unspiked SMBs15 cells (figure 4.6, lanes 5 and 6 compared to lanes 7 and 8). When SMBs15 cells were spiked into uninfected PBMCs, in addition to the conventional PrP^{Sc} signal from the cell line, high molecular weight bands were also visible (40-133 kDa) which are discussed in more detail in section 4.3.

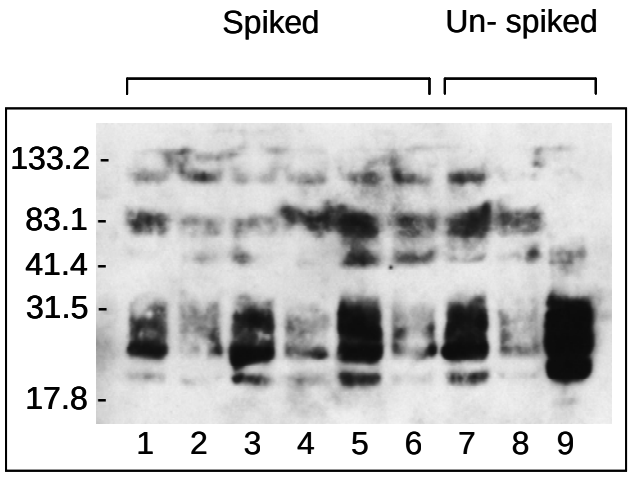


Figure 4.6: Cell loading limits and recovery of PrP^{Sc} from SMBs15 cells spiked into increasing numbers of uninfected PBMCs. Numbers of SMBs15 cells per well: lanes 1, 3, 5 & 7, 12 x 10^4 : lanes 2, 4, 6 & 8, 6 x 10^4. Numbers of PBMCs per well: lanes 1 & 2, 1 x 10^7: lanes 3 & 4, 5 x 10^7: lanes 5 & 6, 1 x 10^8. Lane 9, 500 µg wet tissue equivalent of 139 A scrapie infected murine brain homogenate (PK digested). The blot was probed with the mAb BC6 at a concentration of 2 µg/ml and developed using standard ECL methods; the exposure time was 1 min. Molecular masses are shown on the left (kDa).

4.2.4. Application of the sensitive Western blot method to frozen buffy coat preparations from uninfected sheep blood

Given the success of results when the sensitive Western blot method was applied to SMBs15 cells, this assay was tested on uninfected blood in order to establish the biochemical conditions for these cell types. There is strong evidence from rodent (Brown *et al*., 1998, 1999), sheep (Hunter *et al*., 2002; Houston *et al*., 2008) and primate models (Bons *et al*., 2002) to suggest that infectivity is mainly associated with leukocytes and this was the reason for initially studying buffy coat (which largely consists of leukocytes, including PBMC and granulocytes) and PBMC subsets.

Experiments (figures 4.7, 4.8, 4.9, 4.12 and 4.14) were carried out using a starting volume of 50 ml of blood from four uninfected sheep. The number of buffy coat or PBMC cells obtained from 50 ml of blood ranged from between 1 x 10^6 to 1 x 10^8 cells.

The cell numbers analysed in each blot are detailed in each figure legend. Each experiment was repeated three times and representative blots are shown.

One main difference between the sample preparation when using blood compared to either brain or SMBs15 cells was that buffy coat samples were extremely viscous, which not only proved difficult to load but also resulted in 'smearing' of the samples during SDS-PAGE. After Western blotting, samples did produce a signal which was in the region of 83-41 kDa (figure 4.7 lanes 1 and 2). Conventional PrP^C has a molecular weight of around 25-35 kDa so the signal produced in the region of 83-41 kDa was unexpected. It is possible that this represents non-specific binding. Alternatively NaPTA could be concentrating PrP associated with other proteins or possibly causing the artificial aggregation of PrP in the buffy coat.

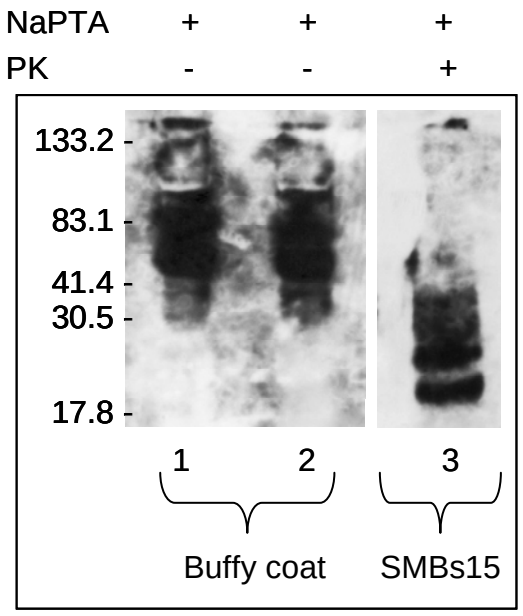


Figure 4.7: Western blot analysis of PrP^C from uninfected buffy coat samples. Key: Lanes 1 & 2, approx 1 x 10^7 buffy coat cells from two different uninfected sheep. In lanes 1 & 2 samples were lysed, before NaPTA precipitation. Samples were not PK digested. Lane 3, 1 x 10^6 PK digested SMBs15 cell lysate. The blot was probed with the mAb BC6 at a concentration of 2 µg/ml and developed using standard ECL methods; the exposure time was 4 hr. Molecular masses are shown on the left (kDa).

To reduce the viscosity of the samples and to improve loading, uninfected buffy coat samples were treated with four buffers (summarised in table 4.1) each containing varying concentrations/combinations of nucleases in PBS. It was found that viscosity was reduced significantly when a combination of 2.5 µg DNase and 50 U/ml Benzonase was added (buffer 1, table 4.1, figure 4.8, lane 5). To check how efficiently the samples had resolved using the varying nuclease conditions the gel was also stained with Ponceau S to show total protein on the membrane (figure 4.8). It was found that the buffy coat proteins had resolved well. After blotting with an anti-PrP antibody bands as observed previously in the range of 83-40 kDa and also between 133 and 83 kDa (in figure 4.7) were again apparent (figure 4.8, all lanes).

Buffer	Amount of Benzonase	Amount of DNase 1
1	50 units / ml	2.5 µg
2	50 units / ml	5 µg
3	100 units / ml	2.5 µg
4	100 units / ml	5 µg

Table 4.1: Buffers containing varying concentrations/combinations of nucleases used to improve sample preparation of buffy coat samples.

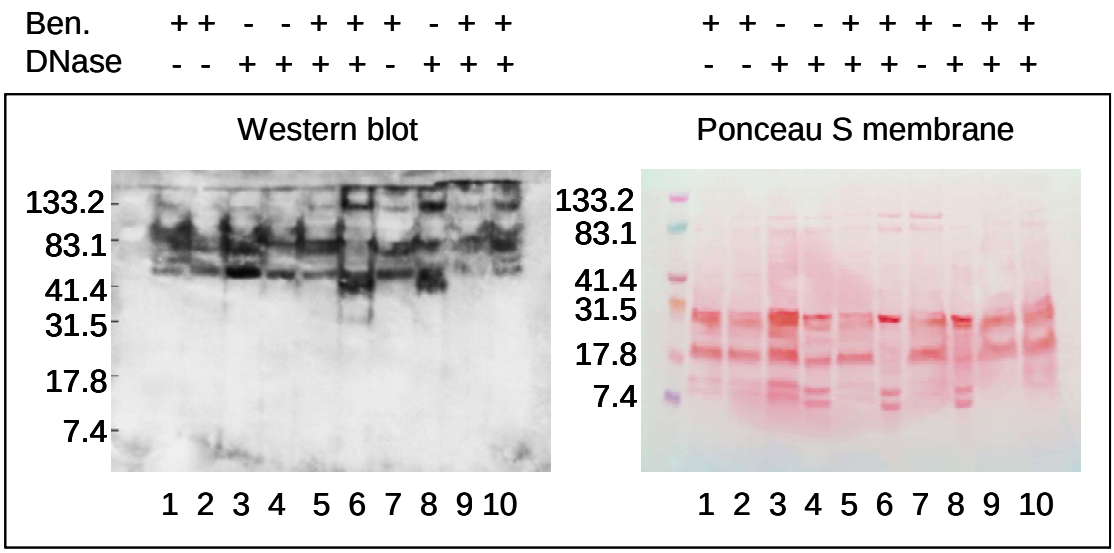


Figure 4.8: Screening of nuclease conditions on Western blots of NaPTA precipitated PrP^C from uninfected buffy coat samples. A Western blot and Ponceau S stained membrane are shown. Buffy coat samples tested were obtained from a single uninfected sheep. A combination of Benzonase (Ben.) and DNase 1 was used as shown. Key: Lanes 1, 5, 6, 9 & 10, 50 U/ml Benzonase.: lanes 2 & 7, 100 U/ml Benzonase : lanes 3, 5 & 9, 2.5 µg DNase 1: lanes 4, 6, 8 & 10, 5 µg DNase 1 Samples in lane 7-10 were pre-washed in PBS before nuclease treatment. The blot was probed with the mAb BC6 at a concentration of 2 µg/ml and developed using standard ECL methods; the exposure time was 4 hr. Molecular masses are shown on the left (kDa).

Because sheep granulocytes do not express PrP^C (Halliday *et al.*, 2005) and the contents of lysed granulocytes could be compromising the assay and possibly contributing to the production of bands in the 40-133 kDa region, it was decided to remove the granulocytes from the buffy coat samples. Platelets which are also present in buffy coat fractions have been shown, when activated, to produce high molecular weight complexes on Western blots (Jones *et al.*, 2005), although minimal numbers of platelets within the buffy coats tested here are likely to be present. However, to increase purity, buffy coat fractions were subjected to density gradient centrifugation to isolate PBMCs (chapter 2, section 2.3.3.2). The 40-133 kDa bands were again evident in uninfected PBMCs, although there was also a band seen around 35-40 kDa which could correspond to the diglycosylated form of the PrP protein. Bands corresponding to the mono and unglycosylated forms of PrP may also have been present in non-

NaPTA treated uninfected PBMCs (figure 4.9, lanes 7-10, indicated by the arrows). However, to conclusively show conventional PrP^C in blood by Western blotting, further optimisation of the method (carried out in section 4.5.) was required.

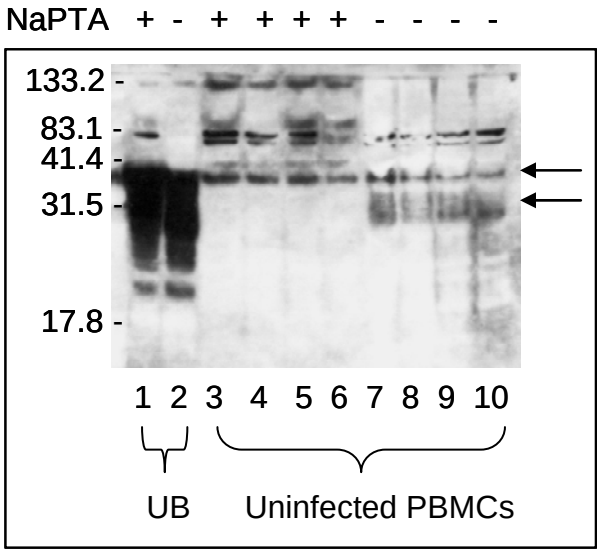


Figure 4.9: Establishing the effect of NaPTA precipitation on titrations of uninfected PBMCs. Number of PBMCs per well: lane 3 & 7, 5 x 10^7: lane 4 & 8, 1 x 10^7: lane 5 & 9, 5 x 10^6: lane 6 & 10,1 x 10^6 . PBMC samples tested were obtained from a single uninfected sheep. Lanes 3-6, samples have been NaPTA precipitated, lanes 7-10 samples have not been NaPTA precipitated. Lane 1, 500 µg wet tissue equivalent of NaPTA precipitated uninfected sheep brain homogenate: lane 2, 500 µg wet tissue equivalent of uninfected sheep brain homogenate (UB). Signals in lane 1 &2 are almost identical due to blot exposure. The blot was probed with the mAb BC6 at a concentration of 2 µg/ml and developed using standard ECL methods; the exposure time was 2 hr. Molecular masses are shown on the left (kDa).

4.2.5. Major modification to the sensitive Western blot method to allow for the detection of conventional PrP^C in blood components from uninfected and scrapie-infected sheep

Many months were spent changing and developing new biochemical extraction procedures which would allow for detection of 'conventional' PrP^C in blood. In fact each step of the original method (which although worked well for both brain and SMB cells) was re-optimised. The changes are schematically illustrated in figure 4.10 and the output results, from initial tests on uninfected and scrapie brain, shown in figure 4.11.

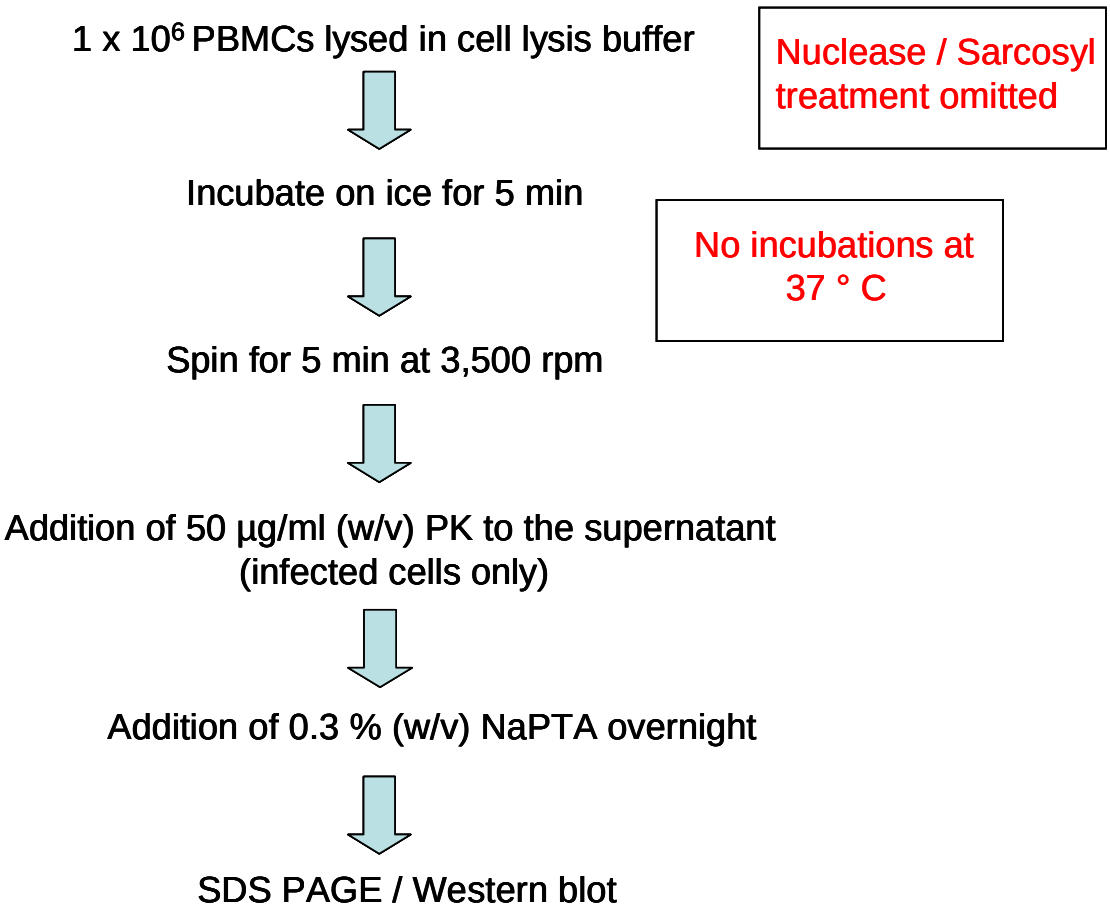


Figure 4.10: Major modifications to the sensitive Western blot method allow for detection of conventional PrP^C. Each step of the original method was re-optimised. As indicated by the red text in the boxes, the final method did not include treatment with nucleases or sarcosyl or incubation at 37 ° C. Following optimisation, the flow diagram represents the final Western blot method used in blood.

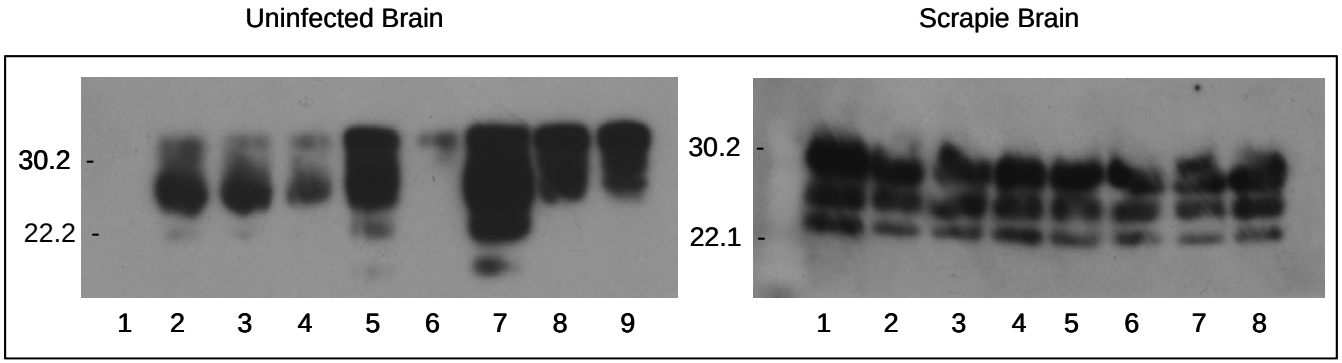


Figure 4.11: Harsh detergents and long incubation steps used in the Western blot protocol result in degradation of PrP^C. Wells were loaded with 300 µg wet tissue equivalent of uninfected sheep brain homogenate or the PK digestion products from 300 µg wet tissue equivalent of scrapie infected sheep brain homogenate as indicated. The sensitive Western blot method (chapter 3) was applied to samples in lane 1. In lane 2, the nuclease step and incubation for 30 min at 37 º C was omitted. In lane 3, the detergent extraction and incubation step for 10 min at 37 ºC were omitted. In lane 4 & 5, samples were lysed in cell lysis buffer, the pellet and supernatant loaded after incubation for 10 min at 37 ºC and NaPTA precipitation respectively. This was repeated but without the incubation step for 10 min at 37 º C, the supernatant loaded in lane 7, the pellet in lane 8, repeated in lane 9. In lane 6, cell lysis buffer instead of 4 % sarcosyl was used in the detergent extraction step. The blot was probed with the mAb BC6 at a concentration of 2 µg/ml and developed using standard ECL methods; the exposure time was 10 min. Molecular masses are shown on the left (kDa).

It was discovered that when the original sensitive Western blot was applied to uninfected brain, there was no signal for conventional PrP^C (figure 4.11, lane 1). This was unusual, but highlighted the need for these further optimisation experiments. When sample incubation steps at 37 º C were omitted from the methodology, the amount of PrP^C recovered in uninfected sheep brain was increased (figure 4.11, lanes 2 and 3 compared to lane 1). Additionally the use of sarcosyl appeared to result in PrP^C degradation (figure 4.11, lane 6) whereas cell lysis buffer (chapter 2, section 2.2.4) which contains 0.5 % (v/v) igepal and 0.5 % (v/v) sodium deoxycholate results in a greater PrP^C recovery (figure 4.11, lanes 7 and 8). Interestingly no difference in the Western blot signal was observed in PK digested scrapie brain homogenates. It may be that the conformation of PrP^{Sc} is more robust and less prone to degradation in certain detergents and at 37 º C (figure 4.11).

Optimum recovery of PrP^C was achieved using cell lysis buffer with incubation on ice (figure 4.11, lane 7) and this method was then applied to uninfected PBMCs purified from whole blood taken from healthy sheep as shown in figure 4.12. All subsequent experiments were carried out using the modified method which will be referred to as the blood Western blot method.

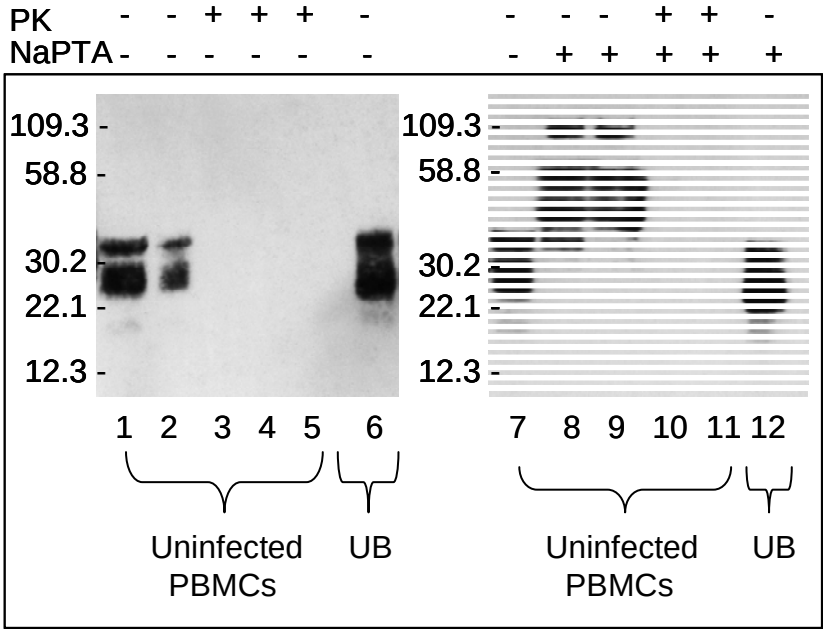


Figure 4.12: PrP^C detection in uninfected PBMCs and the appearance of high molecular weight bands after NaPTA precipitation. Key: Lanes 1 – 5 and 7 – 11 approx 1 x 10^6 uninfected PBMCs. PBMC samples tested were obtained from a single uninfected sheep. Lane 6 & 12 wells were loaded with 300 μg wet tissue equivalent of uninfected brain homogenate (UB). Samples were PK digested and NaPTA precipitated as indicated. The blot was probed with the mAb BC6 at a concentration of 2 μg/ml and developed using standard ECL methods; the exposure time was 10 min. Molecular masses are shown on the left (kDa).

Conventional PrP^C was detected in uninfected frozen PBMCs (figure 4.12, lanes 1, 2) and the glycoform profile was similar to that seen in uninfected brain (figure 4.12, lane 6). As expected PK digestion resulted in degradation of PrP^C (figure 4.12, lanes 3, 4 and 5). Interestingly, bands of higher molecular weight than PrP^C (approximately 40 – 60 kDa and 109 kDa) were seen after NaPTA precipitation of PBMC samples (figure 4.12, lanes 8 and 9) but were absent in PK treated samples (figure 4.12, lanes 10 and 11). This effect was not observed in uninfected brain (figure 4.12, lane 12). High molecular weight bands were still present even when low concentrations of NaPTA (0.0075 %) were used (data not shown given that the outcome was the same as seen in figure 12). Uninfected animals were confirmed as TSE free by immunohistochemical analysis of medulla brain sections (data not shown).

After testing uninfected PBMCs, the blood Western blot method was applied to frozen PBMCs from sheep clinically affected with scrapie. Blood (50 ml) was obtained from ten scrapie-infected sheep. The number of buffy coat or PBMC cells obtained from 50 ml of blood ranged from between 1 x 10^6 to 1 x 10^8 cells. The cell numbers analysed in each blot are detailed in each figure legend. Each experiment was repeated three times. Figure 4.13 shows a representative blot from two scrapie-infected sheep. Figure 4.14 is a representative blot from a third scrapie-infected animal. Representative blots where blood from the remaining seven sheep clinically affected with scrapie was tested are shown in Appendix 5.

As observed for the uninfected PBMC samples, without PK digestion or NaPTA precipitation the glycoform profile for PrP in PBMCs (figure 4.13, lane 1) from scrapie- infected animals and brain were similar (figure 4.13, lanes 5 and 6). When PBMC samples were PK digested no signal was observed (figure 4.13, lane 3 and 4). This indicated that the conventionally defined PrP^{Sc} is undetectable in the sample using this very sensitive method.

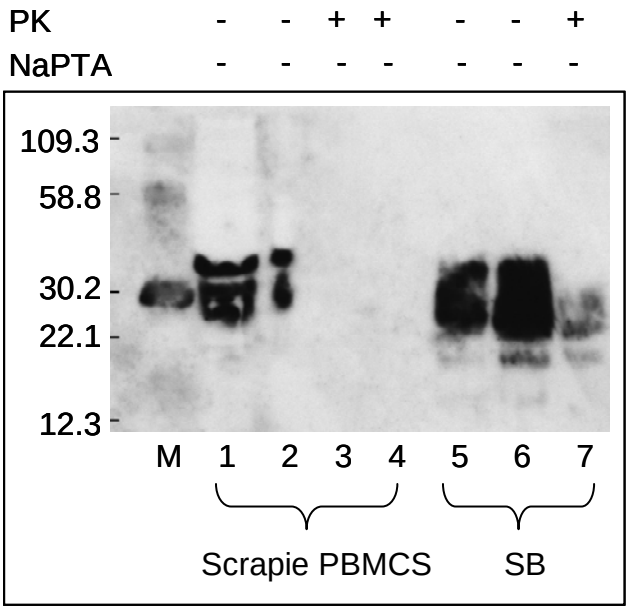


Figure 4.13: Western blot analysis of PBMCs from scrapie-infected sheep without NaPTA precipitation. Key: Lanes 1 & 3 and 2 & 4, approx 1 x 10^6 PBMC from two different scrapie infected sheep. Lanes 5 – 7, wells were loaded with 300 µg wet tissue equivalent of scrapie brain homogenate (SB). M indicates markers. Samples were PK digested without subsequent NaPTA precipitation as shown. The blot was probed with the mAb BC6 at a concentration of 2 µg/ml and developed using standard ECL methods; the exposure time was 5 min. Molecular masses are shown on the left (kDa).

NaPTA precipitation of samples resulted in high molecular weight bands of a similar molecular weight (approximately 40-60 kDa) as observed in uninfected PBMC samples. Unlike uninfected PBMC samples (figure 4.14, lanes 1 and 2) the high molecular weight bands present in NaPTA treated samples appeared to be PK resistant (figure 4.14, lanes 3 and 4). Conventional PrP bands were also observed in NaPTA treated PBMCs from uninfected (figure 4.14, lane 1) and scrapie-infected sheep and these were sensitive to PK (figure 4.14, lane 3). Also, as previously observed, no high molecular weight bands were seen in scrapie infected (figure 4.14, lane 7 and 8) or uninfected brain homogenates treated the same way (figure 4.14 lanes 5 and 6).

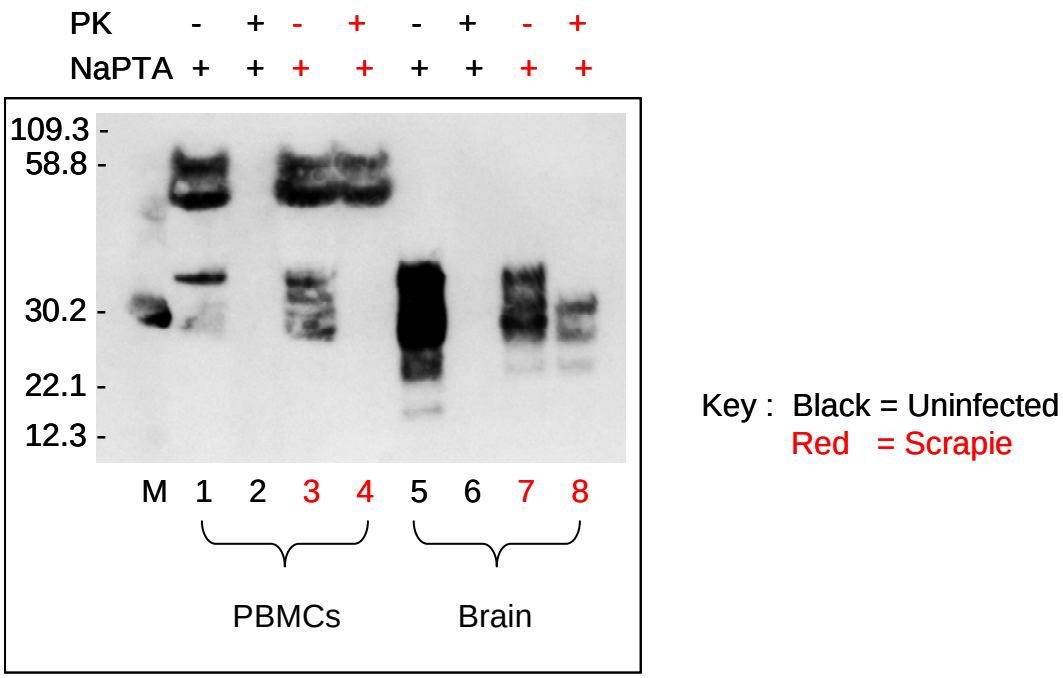


Figure 4.14: High molecular weight bands are PK resistant in PBMCs from scrapie-infected sheep. Key: In lanes 1 & 2 approx 1 x 10^6 uninfected PBMCs from one uninfected sheep. Lanes 3 & 4, approx 1 x 10^6 PBMCs from one scrapie-infected sheep. In lanes 5 & 6 wells were loaded with 300 µg wet tissue equivalent of uninfected sheep brain homogenate. In lanes 7 & 8 wells were loaded with 300 µg wet tissue equivalent of sheep scrapie brain homogenate. Samples were PK digested and NaPTA precipitated as indicated. M indicates markers. The blot was probed with the mAb BC6 at a concentration of 2 µg/ml and developed using standard ECL methods; the exposure time was 10 min. Molecular masses are shown on the left (kDa).

4.3. Discussion

TSE agents can be transmitted via blood using sheep models (Houston *et al*., 2000; Hunter *et al*., 2002; Houston *et al*., 2008) and in humans vCJD cases have been reported that were probably the result of infected blood transfusions (Llewelyn *et* al., 2004; Peden *et* al., 2004; Health Protection Agency, 2007). However, it is not known which blood components carry infection and in previous studies using various approaches, PrP^{Sc} had not been detected in TSE-infected blood (Wadsworth *et al*., 2001; Schmerr *et al*., 1999). More recently a new technique (where PrP^{Sc} is amplified to detectable levels) termed PMCA has been applied to TSE infected buffy coat samples successfully (Castilla *et al*., 2005; Saa *et al*., 2006). However, the levels of PrP^{Sc} in blood prior to PMCA amplification are very low. Using a sensitive Western blot assay (described in chapter 3) the aim of this chapter was to establish if conventional PrP^{Sc} is present in blood. The sheep model has the advantage of having large blood volumes and combined with this new sensitive Western blot analysis, it is clear that the patterns of protein detected with an anti-PrP antibody on Western blots are very different from the conventional triple banded pattern of PrP^{Sc}. These observations are intriguing and could form the basis of a TSE diagnostic blood test if confirmed.

Initially the detection limit of the assay for cellular components was tested using SMB cells that express low levels of conventional PrP^{Sc}. The minimum number of SMB cells in which a PrP^{Sc} signal could be detected using the optimized Western blot was 6×10^4 cells. In spiking experiments where 6×10^4 SMB cells were spiked into increasing numbers of uninfected PBMCs, the same signal could be recovered even after dilution into 1×10^8 PBMCs. This means that the ratio of infected cells in the total population was 0.01 %. Therefore it can loosely be assumed in a buffy coat where conventional PrP^{Sc} was not detected that the number of infected blood cells is less than 0.01 %. This indicates that a high level of sensitivity has been achieved, although it is based on the assumption that infected sheep blood buffy coat cells contain similar levels of PrP^{Sc} to SMB cells. Also SMB cells are murine brain cells and therefore physiologically different to sheep blood cells and it is possible that not all of the SMB cells within the

population were infected. However, although not ideal carrying out spiking experiments using a cellular system gives more of an indication of the level of sensitivity required for conventional PrP^{Sc} detection in blood than preparing brain homogenate spikes only.

In order to apply the sensitive Western blot to buffy coat samples from sheep clinically affected with scrapie, the method had to be modified to incorporate additional nuclease treatments to reduce viscosity and improve sample loading. Experiments to optimise conditions for electrophoresis and blotting of buffy coat samples were carried out using samples from uninfected sheep. However, bands corresponding to the molecular weights expected for PrP^{C} (between 20 and 40 kDa) were not observed on the blots, although higher molecular weight signals were consistently seen (40-133 kDa range). It is possible that the high molecular weight bands represent non-specific binding or that NaPTA precipitation is concentrating PrP associated with other proteins, or causing artifical aggregation of PrP. This result was unexpected, since PrP^{C} has previously been detected in uninfected sheep PBMC by Western blotting (Halliday *et al*., 2005). There were, however, differences in the methods used, for instance Halliday and colleagues did not use NaPTA precipitation. The buffy coat fractions tested in the present study contain granulocytes and platelets as well as PBMC, so it is possible that components of these cells may have interfered with the detection of PrP^{C}. However, when PBMC were isolated from the sheep buffy coat samples, and analysed by Western blot, bands corresponding to the molecular weight of diglycosylated PrP^{C} were still inconclusive.

Because of the problems detecting conventional PrP^{C}, the original method was significantly altered for PrP^{C} detection using uninfected and scrapie infected brain homogenate. Although PrP^{C} and PrP^{Sc} have the same primary sequence and are covalently indistinguishable, they can be discriminated in several ways. PrP^{C} exists as a monomer and has 43 % α-helix and 3 % β-sheet, whereas PrP^{Sc} tends to form aggregates and has 34 % α-helix and 43 % β-sheet (Pan *et al*., 1993). Due to these structural differences, degradation of PrP^{C} in brain and blood samples may be more likely under conditions that include treatment with harsh detergents or incubation at

certain temperatures. It was found that some steps in the methodology (i.e sample treatment with sarcosyl and long incubations at 37 º C) resulted in degradation of PrP^{C} from uninfected brain. Structural analysis of PrP^{C} and PrP^{Sc} has proved difficult but refolded recombinant PrP (rPrP), which is soluble, monomeric and α-helical in structure, is thought to be representative of PrP^{C} and has been used for NMR analysis of the tertiary structure (Zahn *et al*., 2000; James *et al*., 1997; Lopez Garcia *et al*., 2000; Riek *et al*., 1997) and x-ray crystal structures have been published (Knaus *et al*., 2001; Eghiaian *et al*., 2004; Haire *et al*., 2004). Also models of PrP^{Sc} have been described (Wille *et al*., 2002). Whether these models are accurate in terms of blood associated PrP^{C} and PrP^{Sc} remains to be seen but the different structural qualities of both forms of the prion protein may account for the differences seen when steps in the method were altered and tested on uninfected and scrapie infected brain homogenates.

The re-optimised method (referred to as the blood Western blot method) was re-applied to PBMC samples from six uninfected and seven scrapie infected sheep. Bands corresponding to the molecular weights expected for PrP^{C} (between 25 and 35 kDa) were observed on the blots after the modifications, but after the addition of NaPTA to this protocol, higher molecular weight signals were still consistently seen (40-133 kDa range). High molecular weight bands have been reported in blood previously. In activated human platelets, Jones and colleagues (2005) described a band of approximately 250 kDa on Western blots which they suggested was cellular PrP complexed with an unidentified protein. One vCJD blood sample has also been tested using NaPTA precipitation (Wadsworth *et al*., 2001). However bands between 40 – 133 kDa in buffy coats or PBMCs have not been reported. These bands may represent unspecific binding or it is possible they may be PrP that has been complexed with other blood proteins as a result of NaPTA precipitation. These results are intriguing though as the bands appeared to be PK resistant in scrapie infected but PK sensitive in uninfected PBMCs.

Using the blood Western blot it was not possible to detect conventional PrP^{Sc} in blood components from TSE infected animals although bands of other molecular weights (approximately 40-60 kDa, 80-83 kDa and 109 kDa) were detected and could represent a blood specific form of $PrP^{Sc.}$ If TSE infectivity in sheep blood is related to a non-

conventional form of PrP^{Sc}, this could explain the difficulty found in many labs, including this one, in detection of the conventional form of PrP^{Sc} in blood.

After NaPTA precipitation high molecular weight complexes that cross-react with an anti-PrP antibody are present in blood. In uninfected PBMCs these bands appear to be sensitive to PK degradation. However in PBMCs from scrapie-infected animals these bands appear resistant to PK. These results are interesting as it is possible that the bands may represent a blood specific form of PrP^{Sc}, different from the conventionally defined PrP^{Sc} found in brain and lymphoid tissues. It may be that PrP is being complexed with other proteins due to an effect of NaPTA. PrP in blood may have a different conformation to PrP in brain and there may be PK sensitive forms of PrP in blood. These results warrant further investigation which is discussed in chapter 5.

Chapter 5

Investigating High Molecular Weight Bands In Scrapie-Infected And Uninfected Sheep Blood

5.1. Introduction

The previous chapter described attempts to detect the conventional form of PrP^{Sc} in scrapie-infected sheep blood, using a sensitive immunoassay incorporating a NaPTA concentration step. Although it was not possible to detect conventional PrP^{Sc}, results showed that after the addition of NaPTA, high molecular weight bands that cross react with a PrP antibody were present in blood. In uninfected frozen PBMC samples these bands appeared to be sensitive to PK digestion. In frozen PBMCs from scrapie-infected sheep the bands were resistant to PK. These initial results indicated that NaPTA precipitation of PBMC preparations enables discrimination between scrapie-infected and uninfected sheep which may be extremely important in the development of a diagnostic TSE blood test. To characterise the high molecular weight bands in more detail and to investigate if they could be used as a marker for TSE disease in blood the following questions were addressed:

Are the high molecular weight bands present in different TSE agents?

Are the high molecular weight bands disease-specific?

Are the high molecular weight bands present in PBMC subsets?

Are the high molecular weight bands artefacts?

Are the high molecular weight bands sensitive to high concentrations of PK?

Are the high molecular weight bands PrP?

5.2. Results

5.2.1. Testing purified BSE-infected cell subsets for PrP^{Sc}

To investigate if conventional PrP^{Sc} and high molecular weight bands could be detected in blood infected with a different TSE agent, the blood Western blot method (chapter 4, section 4.4) was applied to frozen PBMCs (chapter 2, section 2.3.3.2) $CD21^+$ B cells (chapter 2, section 2.3.3.4) and unbound cells (the $CD21^+$ B cell negative population) from one pre-clinical BSE-infected sheep. Figure 5.1 represents these results

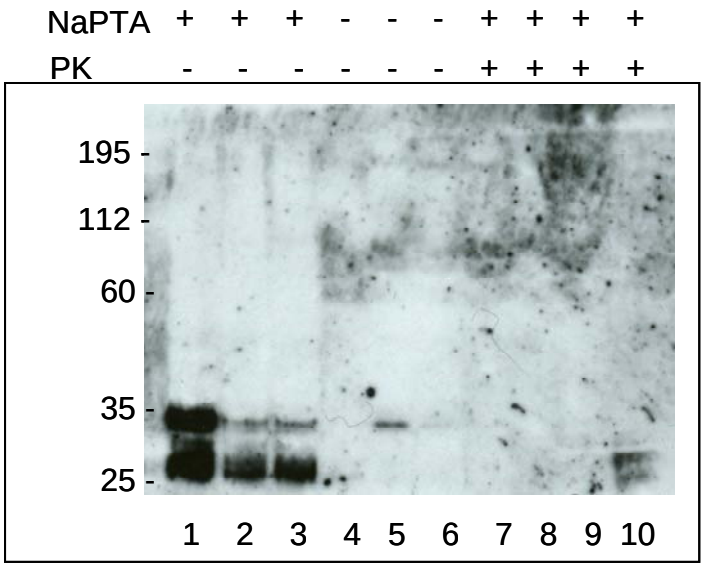


Figure 5.1: Conventional PrP^{Sc} and high molecular weight bands were not detected in PBMCs, unlabelled cells or $CD21^+$ B cells from one pre-clinical BSE-infected sheep. Key; lanes 1, 4 and 7, 1 x 10^6 PBMCs, lanes 2, 5, and 8, 1 x 10^6 unlabelled cells, lanes 3, 6, and 9, 1 x 10^6 $CD21^+$ B cells. Lane 10, 300 µg scrapie-infected brain homogenate. Samples were PK digested and NaPTA precipitated as shown. The blot was probed with BC6 at a concentration of 2 µg/ml. The blot was developed using standard ECL methods; the exposure time was 20 min. Molecular masses are shown on the left (kDa).

As expected, NaPTA precipitation increases the overall sensitivity of detection (figure 5.1, lanes 1-3 compared to lanes 4-6). When samples were PK digested, no conventional PrP^{Sc} signal was observed (figure 5.1, lanes 7-9). This indicates that conventional PrP^{Sc} was undetectable in PBMCs, unlabelled cells and $CD21^{+}$ B cell subsets from a pre-clinical BSE infected animal (figure 5.1, lanes 7-9). Detection may have been possible if clinical blood from different animals was tested, or experiments applied to a larger volume of blood although this was not tested due to unavailability of samples.

There was a possible signal present at 60-112 kDa (with and without NaPTA) in PBMCs and subsets from pre-clinical BSE-infected animals (figure 5.1, lanes 1-3 and 7-9) which is similar to the size of high molecular weight bands observed in uninfected or clinical scrapie blood (figure 5.1 and chapter 4, figures 4.12, 4.13 and 4.14, 40-109 kDa). However, the signal was weak, had previously only been observed in NaPTA treated samples and may not represent high molecular weight bands. However, if high molecular weight bands with lower signal intensities were confirmed, they may not be as prominent in the BSE-infected blood cell fractions as these are pre-clinical unlike clinical scrapie-infected samples. Alternatively, the conformation of PrP^{Sc} in BSE compared to scrapie may be different enough to change the effect of NaPTA in forming possible complexes.

5.2.2. High molecular weight bands are present in uninfected and scrapie-infected sheep blood

Varying numbers of buffy coat or PBMC cells (in a starting volume of 50 ml of blood) from ten scrapie infected sheep and four uninfected sheep (chapter 4 and Appendix 5) were analysed using the blood Western blot method. Although conventional PrP^{Sc} was undetectable, high molecular weight bands were observed (chapter 4). To further characterise the high molecular weight bands, fresh uninfected PBMCs from an additional six disease free sheep were analysed. Frozen PBMCs from an additional four clinical scrapie-infected sheep were also tested. PBMC samples (cell numbers tested shown in the figure legends) were resolved on 4-12 % Bis-Tris gels (chapter 2, section

2.3.5) which separate with accuracy high molecular weight proteins. Figure 5.2 shows a representative blot of these experiments.

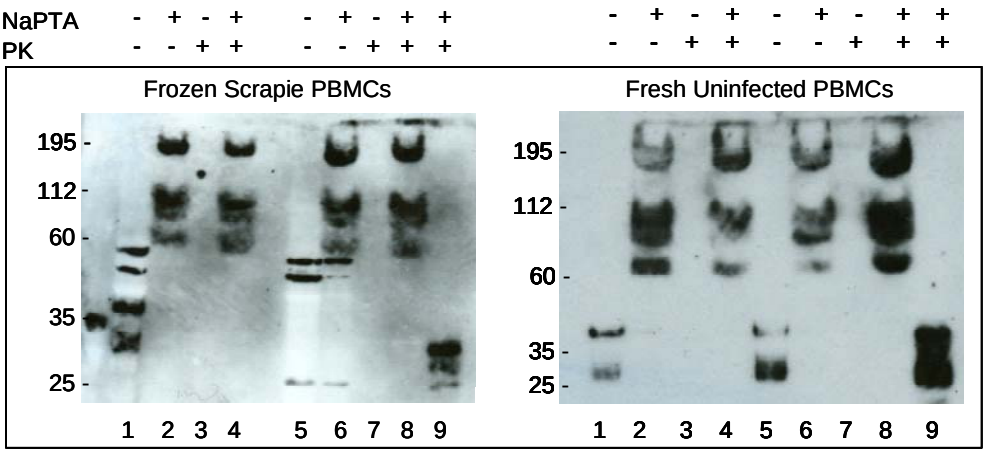


Figure 5.2: High molecular weight bands are present in PBMCs from fresh uninfected and frozen scrapie-infected sheep blood following NaPTA precipitation. Wells were loaded with 1 x 10^6 PBMCs in lanes 1-8. Lane 9 was loaded with 300 µg wet tissue equivalent of PK digested scrapie sheep brain homogenate. Samples were treated with PK and NaPTA as indicated. Lanes 1-4 represent PBMCs from one animal and lanes 5-8 from a second animal. The blot was probed with BC6 at a concentration of 2 µg/ml. The blot was developed using standard ECL methods; the exposure time was 5 min. Molecular masses are shown on the left (kDa).

High molecular weight bands were observed in NaPTA precipitated frozen clinical scrapie-infected (figure 5.2, lanes 2 and 4) and fresh uninfected PBMCs (figure 5.2, lanes 6 and 8) with or without PK digestion. This suggests that the high molecular weight bands are not disease-specific. As previously shown in chapter 4 (figures 4.12, 4.13 and 4.14) these high molecular weight bands were not seen in scrapie or uninfected brain samples processed under the same conditions. The molecular weights of the bands were approximately 195 kDa, 60 kDa, and 112 kDa where a doublet band was seen. In initial experiments (chapter 4 figure 4.14) the high molecular weight bands appeared PK resistant in frozen PBMCs from scrapie-infected animals and PK sensitive in frozen uninfected PBMCs. However, in fresh uninfected PBMCs, high

molecular weight bands were present in both PK treated and non-PK treated samples (figure 5.2, lanes 4 and 8). The uninfected PBMCs tested in chapter 4 had been stored at – 80 º C for up to 12 months and when these samples were lysed there were no protease inhibitors in the cell lysis buffer (chapter 2, section 2.2.4). It is possible that some sample degradation occurred as a result of long-term storage combined with lack of protease inhibitors, resulting in no signal on a Western blot following PK digestion and NaPTA precipitation. Uninfected PBMCs described in this chapter were fresh and lysed in cell lysis buffer containing protease inhibitors. These factors may have contributed to the intense signals observed following NaPTA percipitation with and without prior PK digestion (figure 5.2, panel 2). Uninfected animals were confirmed to be TSE free by immunohistochemical analysis (data not shown).

Although the high molecular weight bands are not disease-specific, it was still important to characterise them further. Bands with molecular weights of 60 kDa, 112 kDa and 195 kDa have not been described in blood before using PrP specific antibodies, and subtle differences may be revealed between uninfected and scrapie-infected sheep blood on more detailed examination. If levels of TSE infectivity in sheep blood are related to a non-conventional form of PrP^{Sc} it is possible these high molecular weight bands may represent where the infectivity resides.

5.2.3. High molecular weight bands are present in CD21 $^{+}$ B cell subsets

To characterise the expression of high molecular weight bands in sheep blood components in greater detail, CD21 $^{+}$ B cell PBMC subsets from uninfected and scrapie-infected sheep were isolated (chapter 2, section 2.3.3.4) and analysed. PrP expression appears to be up-regulated on CD21 $^{+}$ B cells in some scrapie-infected sheep so these cells were of particular interest (Halliday *et al*., 2005). Acquired CD21 $^{+}$ B cell populations were assessed for purity by flow cytometry (chapter 2, section 2.3.3.5) which is shown in figures 5.3a and 5.3b.

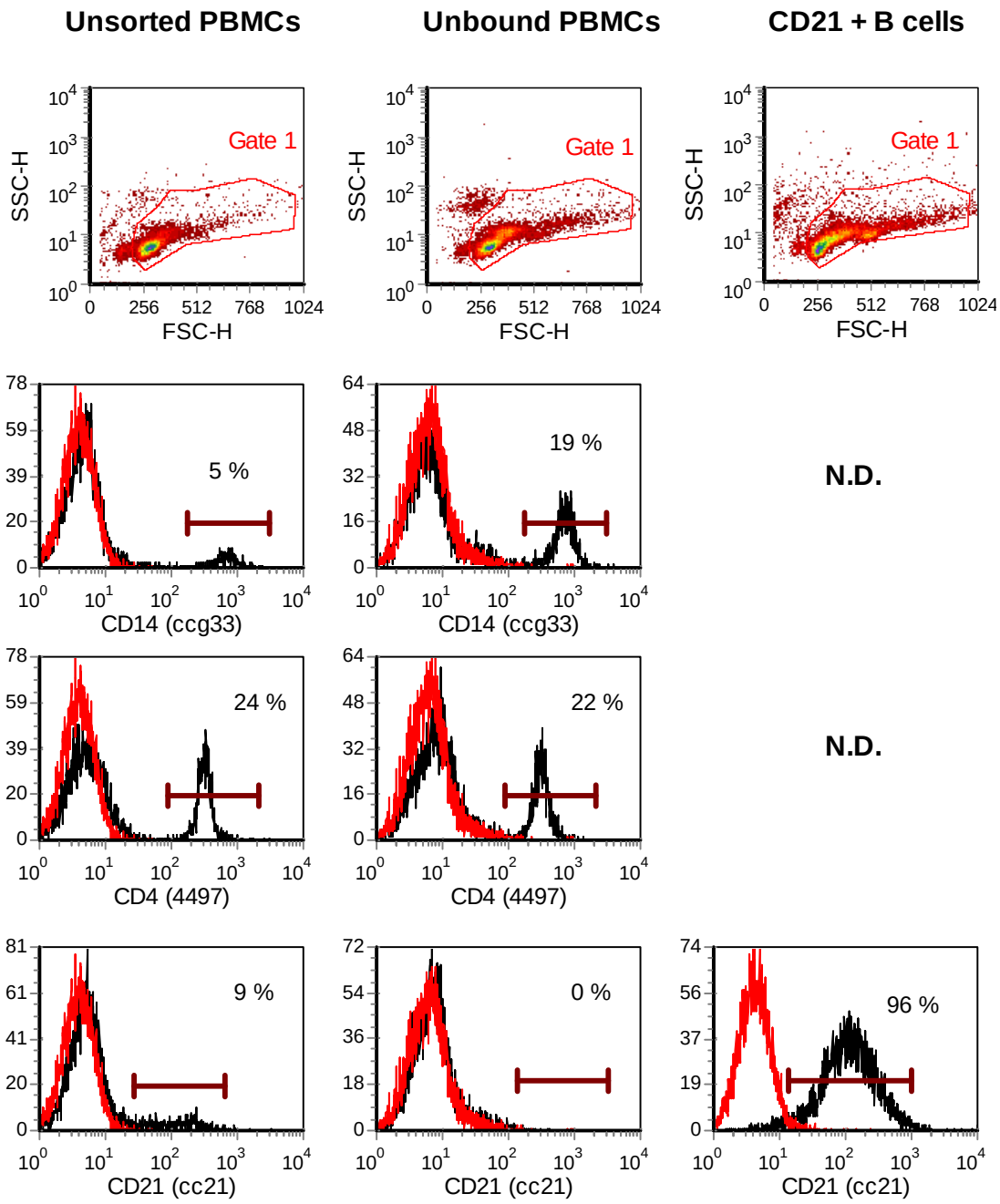


Figure 5.3 A: Flow cytometry analysis of PBMCs from uninfected sheep blood prior to CD21 $^{+}$ B cell sorting, unbound cells following sorting and the final CD21 $^{+}$ B cell sort. Cells were gated according to their light scattering characteristics. Histograms depicting surface expression levels of various cell markers (as shown) on PBMCs, unbound cells and CD21 $^{+}$ B cells. Red lines, isotype matched negative controls (TrT1: IgG1 isotype matched control for CD14, CD4 and CD21). Analysis was carried out using FACS express version 3. Key: N.D. represents no data.

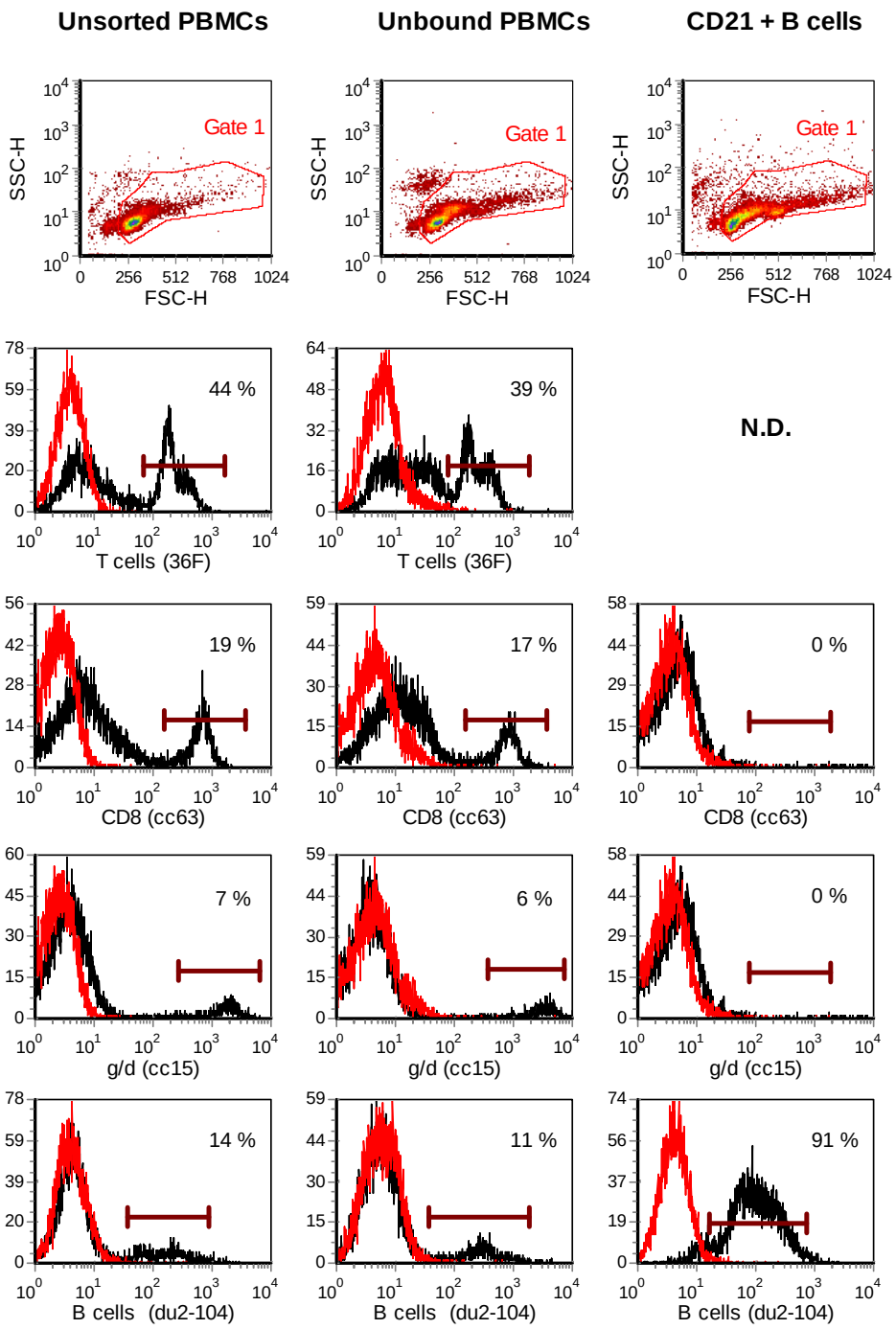


Figure 5.3 B: Flow cytometry analysis of PBMCs from uninfected sheep blood prior to CD21 $^{+}$ B cell sorting, unbound cells following sorting and the final CD21 $^{+}$ B cell sort. Cells were gated according to their light scattering characteristics. Histograms depicting surface expression levels of various cell markers (as shown) on PBMCs, unbound cells and CD21 $^{+}$ B cells. Red lines, isotype matched negative controls (TrT1: IgG1 isotype matched control for T cells. TrT2: IgG2a isotype matched control for CD8 and g/d. IgM-FITC: secondary only control for B cells). Analysis was carried out using FACS express version 3. Key: N.D. represents no data.

Sheep PBMCs appeared to consist of approximately 5% monocytes (CD14), 44% T cells expressing CD2 alpha/beta T cells, 24% $CD4^+$ T cells , 19% $CD8^+$ T cells, 7% γ/δ T cells, and 14 % B cells (figures 5.3, A and 5.3, B). B cells were labelled with a pan B cell antibody (Du2-104) that is thought to bind to CD72 (Young *et al*., 1997). CD21 is expressed on a subset of B cells and was found on approximately 9% of ovine PBMCs. Other investigators report higher percentages of $CD21^+$ B cells in sheep blood (17%, personal communication with Dr E. Lefreve (IAH, Compton) and 20%, personal communication with Dr F. Houston (Glasgow University) although the age, sex and disease status of the animal may account for such differences (Chevallier *et al*., 1998).

The efficiency of the separation of $CD21^+$ B cells was confirmed by antibody staining of the unlabelled cells and purified cell fraction, following cell sorting (figures 5.3, A and 5.3, B). No $CD21^+$ B cells were present in the unlabelled fraction and the total number of B cells was reduced from 14% (unsorted PBMCs) to 11% (figure 5.3, B). CD21 was expressed on 91% of the cells isolated by magnetic cell sorting (figure 5.3 B). There was no contamination from other cell types, indicating that the majority of the purified cells were $CD21^+$ B cells (figure 5.3, B).

The blood Western blot was applied to PBMCs, unlabelled cells (following $CD21^+$ B cell sorting) and purified $CD21^+$ B cell fractions from uninfected and scrapie-infected sheep. Figure 5.4 however shows a representation of PBMCs and $CD21^+$ B cells from uninfected sheep, and unlabelled cells from scrapie-infected sheep. Examples of all cell fractions in uninfected and scrapie-infected animals are not shown as the high molecular weight bands have similar characteristics in both. Alongside blots, samples were also resolved on gels which were stained using coomassie techniques (chapter 2, section 2.3.5.2). If high molecular weight bands observed on blots could be matched with gels, this would aid in excising the correct bands for ultimate identification using mass spectrometry.

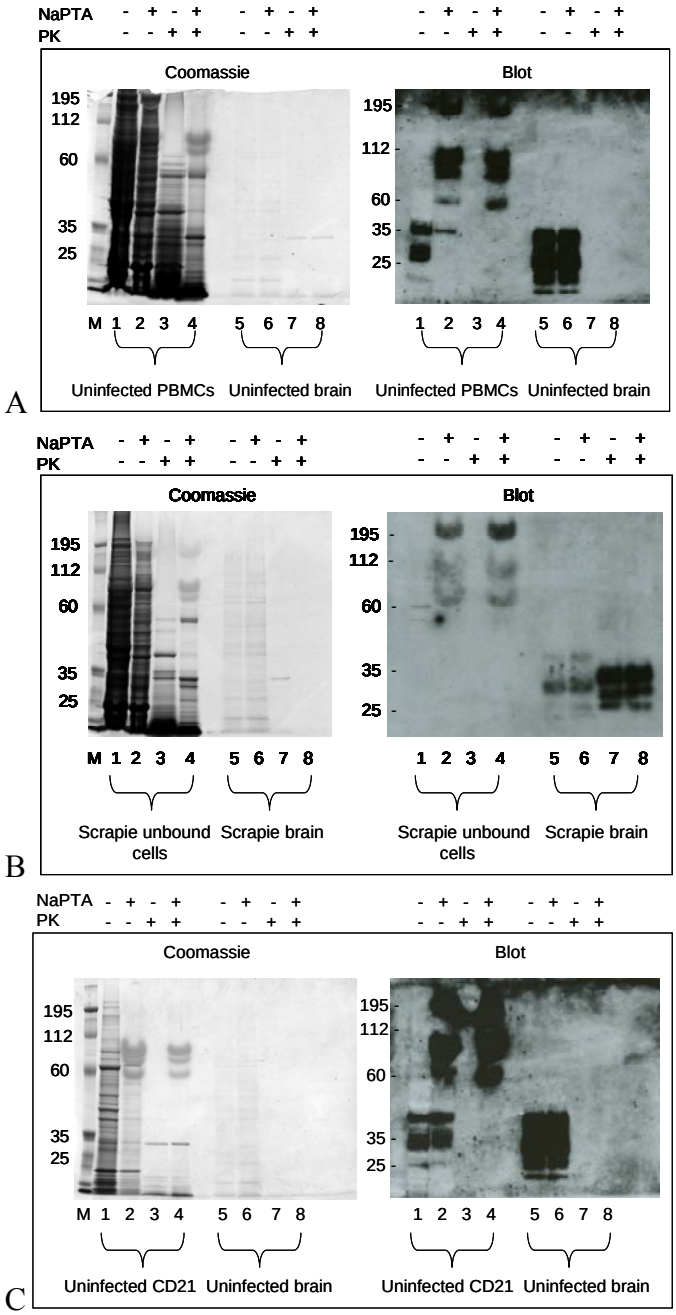


Figure 5.4: High molecular weight bands are present in PBMCs, unlabelled cells and CD21 $^{+}$ B cells from uninfected and scrapie-infected sheep. Key; A = uninfected PBMCs. B= unlabelled cells from scrapie-infected sheep, C = uninfected CD21 $^{+}$ B cells . In all cases, wells were loaded with 1 x 10^{6} cells. In A and C, lanes 5 - 6, wells were loaded with 300 µg wet tissue equivalent of uninfected sheep brain homogenate. In B, wells were loaded with 300 µg wet tissue equivalent of scrapie sheep brain homogenate. The blots were probed with BC6 at a concentration of 2 µg/ml or gels stained with coomassie as shown. The blot was developed using standard ECL methods; the exposure time was 5 min. Molecular masses are shown on the left, and indicated by M in the coomassie gel (kDa).

High molecular weight bands were observed in NaPTA treated uninfected PBMCs (figure 5.4, A) and CD21 $^{+}$ B cells (figure 5.4, C) in addition to unlabelled cells from scrapie-infected sheep (figure 5.4, B). However, high molecular weight bands were present in all three cell fractions from uninfected and scrapie-infected sheep (data not shown). The high molecular weight bands on the blot appeared to have a similar molecular weight to diffuse bands present on the gels stained with coomassie blue. The PBMC fraction of blood (lymphocytes and monocytes) appears to contain more cellular proteins than the same number of purified CD21 $^{+}$ B cells only. This was reflected in the stained gels, resulting in diffuse bands possibly corresponding to the observed high molecular weight bands being more apparent in NaPTA precipitated CD21 + B cells compared to PBMCs (figure 5.4, A compared to C).

5.2.4. High molecular weight bands are recognised using several PrP antibodies and are not caused by non-specific cross-reactivity

To determine whether the high molecular weight bands are caused by non-specific cross-reactivity of the particular monoclonal antibody used (BC6) experiments testing other anti-PrP antibodies with different specificities were carried out. These experiments were carried out on PBMCs from uninfected sheep only (as further characterisation of uninfected and scrapie-infected bands was not the objective). Figure 5.5 represents experiments where non-PK treated uninfected PBMCs and brain homogenates were probed with four different anti-PrP antibodies.

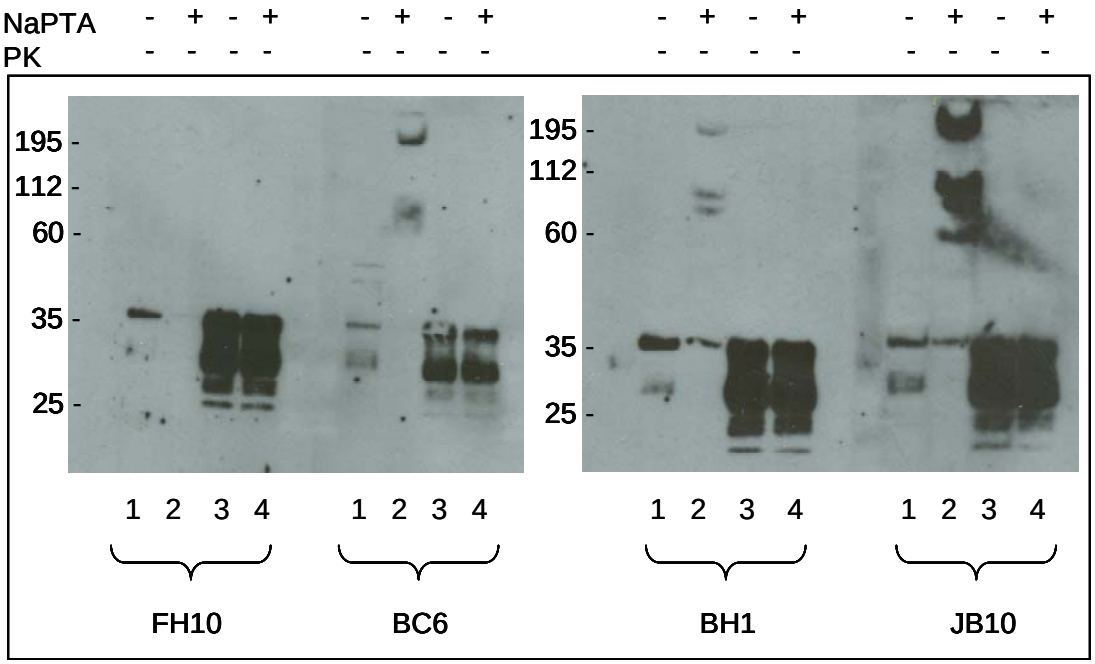


Figure 5.5: High molecular weight bands are recognised by several PrP antibodies. In lanes 1 and 2, wells were loaded with 1 x 10^6 uninfected PBMCs. In lanes 3 and 4, wells were loaded with 300 µg wet tissue equivalent of uninfected sheep brain homogenate. Samples were treated with or without NaPTA as indicated. The blots were probed with different primary antibodies as indicated at a concentration of 2 µg/ml. The blot was developed using standard ECL methods; the exposure time was 10 min. Molecular masses are shown on the left (kDa).

The weak signal observed in uninfected PBMCs with and without NaPTA precipitation using BC6 was surprising (figure 5.5, lanes 1 and 2). Previously this antibody produced intense signals on Western blots (figure 5.2 and 5.4). Following several repeats of this experiment, intense signals on blots were always seen when JB10 was the primary antibody, weak to intense signals using BC6 and BH1, and weak to no signals using FH10. This may reflect conformational changes in the prion protein due to NaPTA where the JB10 epitope (at the extreme C terminal end of the molecule) is exposed, and other epitopes buried.

To determine if the high molecular weight bands observed in blood were due to non-specific antibody binding, experiments were repeated and blots probed with the secondary antibody, a monoclonal antibody with an irrelevant specificity, or BC6 and JB10 as positive controls.

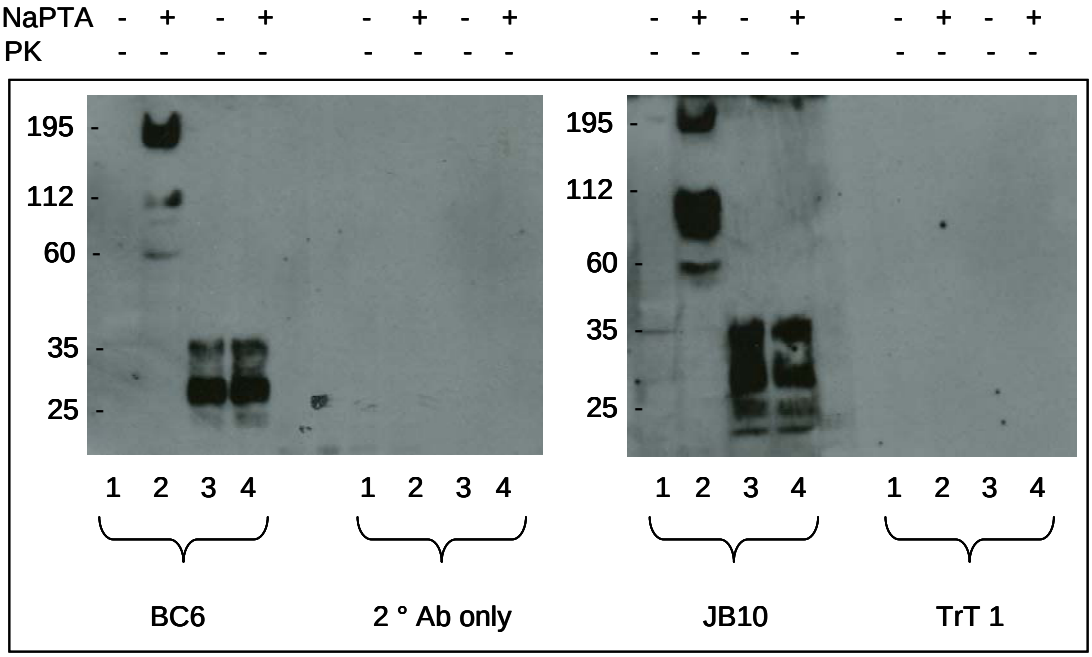


Figure 5.6: High molecular weight bands are not due to non-specific antibody binding. In lanes 1 and 2, wells were loaded with 1 x 10^6 uninfected PBMCs. In lanes 3 and 4, wells were loaded with 300 µg wet tissue equivalent of uninfected sheep brain homogenate. Samples were treated with or without NaPTA as indicated. The blots were probed with BC6, turkey rhinotracheitis (TrT 1) or JB10 at a concentration of 2 µg/ml. One blot was probed with the secondary antibody only (1:7000 dilution). The blot was developed using standard ECL methods; the exposure time was 30 min. Molecular masses are shown on the left (kDa).

Figure 5.6 shows that as expected, high molecular weight bands were recognised by the anti-PrP antibodies BC6 and JB10 in NaPTA precipitated uninfected PBMCs (lane 2). When the membrane was probed with secondary antibody only or an antibody raised against turkey rhinotracheitis virus (TrT 1) there was no signal. This supports the view that the high molecular weight bands observed in lane 2 result from specific binding of anti-PrP primary antibodies.

Further evidence indicating that the high molecular weight bands may include PrP was obtained following monoclonal antibody blocking studies. Incubation with full length recombinant ovine PrP sufficiently blocked the binding sites of the anti-PrP

monoclonal BC6 antibody, as no signal was observed on Western blots after antibody blocking compared to BC6 alone (figure 5.7).

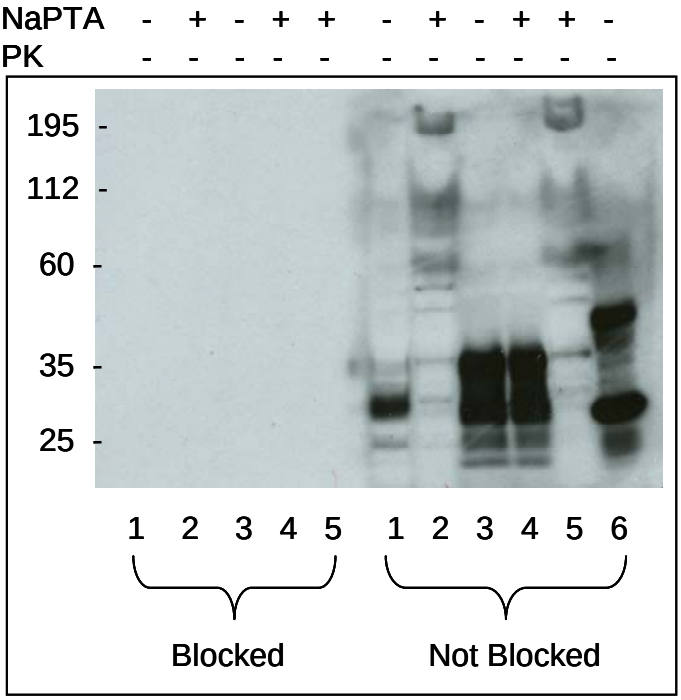


Figure 5.7: Monoclonal antibody blocking studies revealed that the high molecular weight bands may be PrP. In lanes 1, 2, and 5 wells were loaded with 1 x 10^6 uninfected PBMCs. In lanes 3 and 4, wells were loaded with 300 µg wet tissue equivalent of uninfected sheep brain homogenate. Lane 6 was loaded with 50 ng of full length ARR alpha recombinant ovine PrP. Samples were treated with or without NaPTA as indicated. The blots were probed with BC6 at a concentration of 1 µg/ml, or BC6 blocked with full length ARR genotype, α-conformation recombinant ovine PrP as indicated. The secondary antibody was used at a 1:7000 dilution. The blot was developed using standard ECL methods; the exposure time was 10 min. Molecular masses are shown on the left (kDa).

5.2.5. High molecular weight bands may not be composed of protein

Strong evidence has been described to suggest that high molecular weight bands observed in clinical scrapie-infected and uninfected blood may be composed of PrP. However conclusive evidence is required, particularly as the high molecular weight bands remain in uninfected PBMCs, unlabelled cells and CD21 $^{+}$ B cell subsets following PK digestion and NaPTA precipitation. Therefore experiments investigating if high molecular weight bands are sensitive to higher PK amounts were carried out.

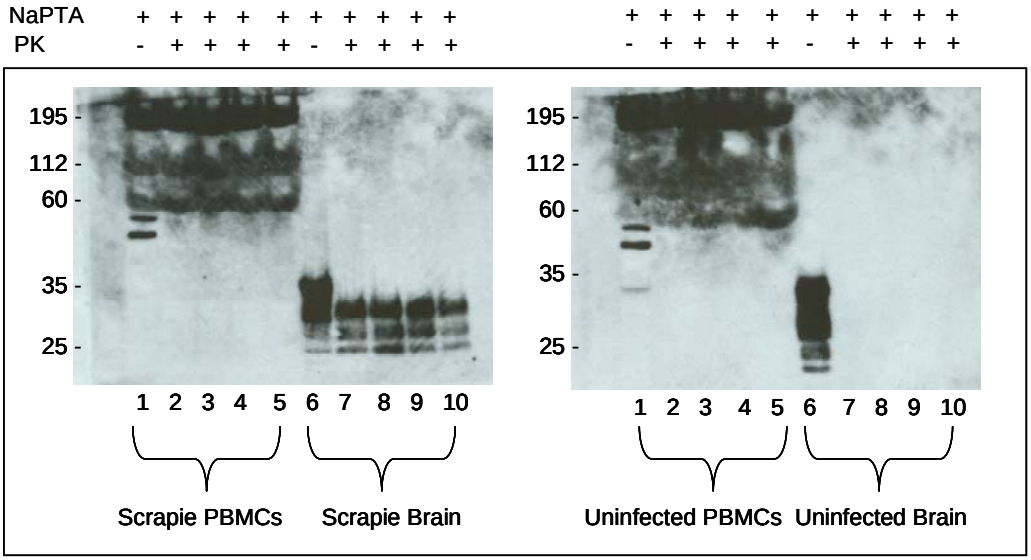


Figure 5.8: High molecular weight bands are not sensitive to PK digestion. In lanes 1 - 5, wells were loaded with 1 x 10^6 uninfected or scrapie-infected PBMCs as shown. In lanes 6 - 10, wells were loaded with 300 µg wet tissue equivalent of uninfected or scrapie-infected sheep brain homogenate. Samples were treated with or without PK as indicated. All samples were NaPTA precipitated. In lanes 1 and 6, samples were not PK digested, lanes 2 and 7, 50 µg/ml PK was used, lanes 3 and 8, 100 µg/ml PK, lanes 4 and 9, 500 µg/ml PK and lanes 5 and 10, 5000 µg/ml PK. Blots were probed with BC6 at a concentration of 2 µg/ml and developed using standard ECL methods; the exposure time was 20 min. Molecular masses are shown on the left (kDa).

High molecular weight bands were still apparent in scrapie-infected and uninfected PBMCs, even following digestion with 5000 μg PK (figure 5.8, lane 5). The signal was diminished in the equivalent brain control samples suggesting that PK digestion was occurring (figure 5.8, lane 10). It was surprising to find no difference between scrapie and uninfected PBMCs as digestion with such high concentrations of enzyme should have degraded the PrP^C and most other proteins in the uninfected blood. This should have left no PrP^C to concentrate, yet following precipitation with NaPTA high molecular weight bands were still present. This strongly suggests that the high molecular weight bands do not contain protein. Alternatively, the protein is in a form that is protected from PK digestion.

To further investigate if the high molecular weight bands do or do not contain protein, methods that visualise proteins were used.

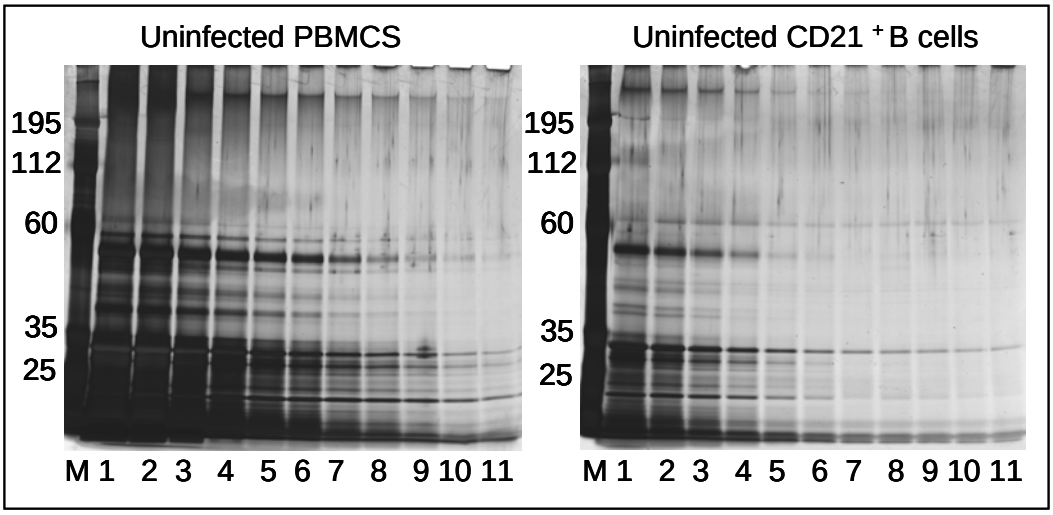


Figure 5.9: High molecular weight bands were not seen in uninfected PBMCs or CD21 $^+$ B cells following silver staining. Cells were titrated, PK digested and NaPTA precipitated. Gel electrophoresis was performed and proteins visualised using standard silver staining techniques. Lane 1, 1 x 10^6 cells, lane 2, 5 x 10^5, lane 3, 2.5 x 10^5, lane 4, 12 x 10^4, lane 5, 6 x 10^4, lane 6, 1.5 x 10^4, lane 7, 7 x 10^3, lane 8, 3.5 x 10^3, lane 9, 18 x 10^2, lane 10, 9 x 10^2 and lane 11, 4.5 x 10^2 . The exposure time was 6 min. M indicates molecular masses (kDa).

Figure 5.9 shows that the high molecular weight bands were not visible following staining with silver. It is possible that the high molecular weight bands may be glycosylated as glycoproteins do not stain well using silver (Goldberg *et al*., 1997).

SYPRO® orange dyes interact with the SDS coat around proteins in gels and give more consistent staining between different types of proteins compared to gels stained with Coomassie blue. Unlike silver, SYPRO® orange dyes also stain glycoproteins well and because of this characteristic this dye was used to stain the high molecular weight proteins.

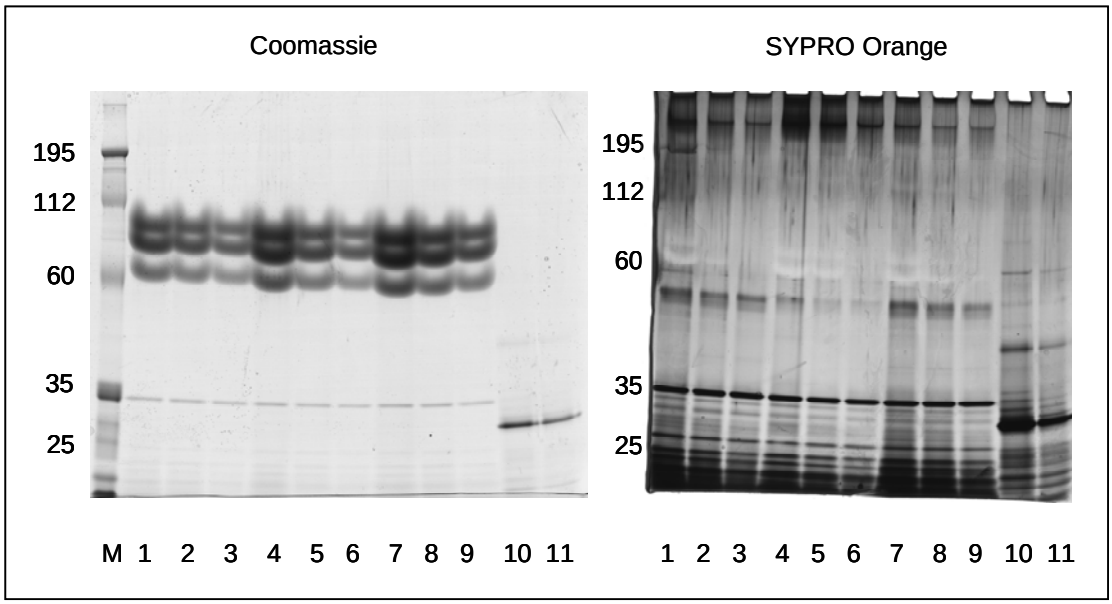


Figure 5.10: High molecular weight bands were not seen in uninfected PBMCs, CD21 $^{+}$ B cells or unbound cells following SYPRO® orange staining. Cells were titrated, PK digested and NaPTA precipitated. Gel electrophoresis was performed and proteins visualised using standard coomassie blue or SYPRO® orange techniques. Lanes 1, 4, and 7, 3.5 x 10^3 PBMCs, CD21 $^{+}$ B cells or unbound cells respectively , lanes 2, 5 and 8, 18 x 10^2 PBMCs, CD21 $^{+}$ B cells or unbound cells respectively, lanes 3, 6, and 9, 9 x 10^2 PBMCs, CD21 $^{+}$ B cells or unbound cells respectively. In lanes 10 and 11, wells were loaded with 10 µg and 5 µg wet tissue equivalent of PK digested, NaPTA precipitated uninfected sheep brain homogenate respectively. The SYPRO® orange gel was scanned using a Typhoon Trio imager laser scanner (GE Healthcare, UK). M indicates molecular masses (kDa) on the coomassie gel, but these were not included for SYPRO® orange to prevent fluorescence quenching of samples.

However, high molecular weight bands were also not visible using SYPRO® orange techniques (figure 5.10). Although high molecular weight bands were evident on Coomassie Blue stained gels, they were not seen with silver (figure 5.9) and SYPRO® orange staining (figure 5.10) which supports the idea that they are not proteinaceous.

Experiments have revealed evidence suggesting NaPTA precipitated high molecular weight bands in blood may be protein, possibly PrP. However this could not be confirmed as the high molecular weight bands were not sensitive to high PK amounts, and could not be visualised on gels using several methods. Therefore to conclusively demonstrate the identity of these high molecular weight bands, bands were excised (figure 5.11) and analysed using mass spectrometry.

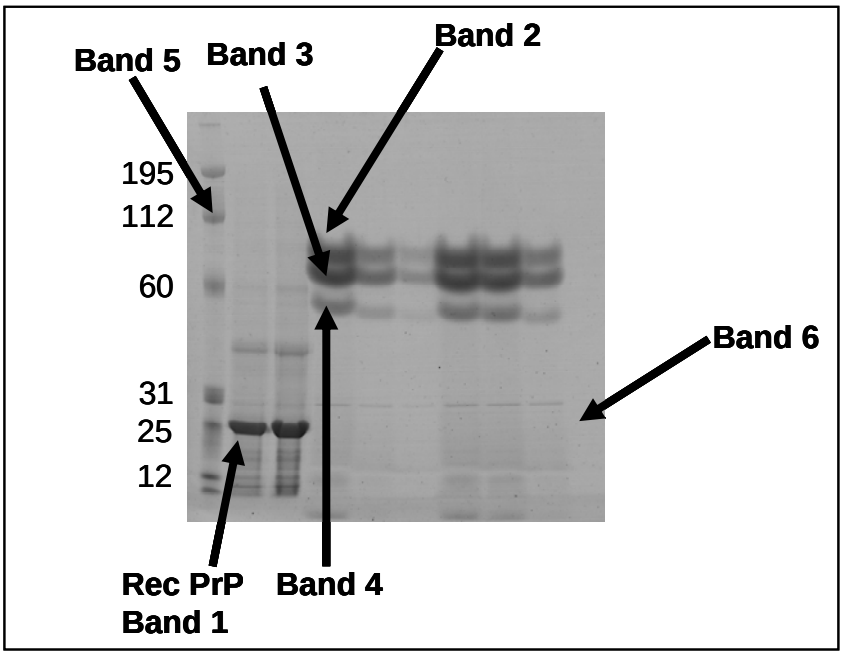


Figure 5.11: High molecular weight bands in PK digested, NaPTA precipitated CD21 $^{+}$ B cells were analysed using mass spectrometry. The two lanes nearest to the markers were loaded with 8 µg of recombinant full length ARR alpha recombinant ovine PrP. The following six lanes were loaded with 1 x 10^6, 5 x 10^5 and 2.5 x 10^5 CD21 $^{+}$ B cells from two uninfected sheep respectively. Bands were excised from the gel as indicated. Band 6 represents a slice of blank gel that was excised as an additional control.

Mass spectrometry data was obtained from Dr. P. Skipp (Proteomics Manager, Southampton University). Results were entered into MASCOT where peptide sequences were searched against sequence databases to identify possible proteins. Results initially looked promising as the high molecular weight bands in CD21 $^{+}$ B cells were identified as PrP. However, band 1 (recombinant PrP control) contained high amounts of protein which was analysed first. Therefore all bands were re-analysed in a different order to validate the initial result and to check for possible contamination from the recombinant PrP positive control. Unfortunately, following further analysis, contamination was confirmed. Mass spectrometry for the high molecular weight bands gave a negative result for protein. The control bands were identified correctly (Band 1, PrP, band 5, Beta-Galactosidase). Table 5.1 summarises mass spectrometry data.

Table 5.1: Summary of mass spectrometry data revealed the high molecular weight bands may not be composed of protein. Mass spectrometry data was obtained from Dr. P. Skipp (Proteomics Manager, Southampton University).

Band	Mascot search ID	NCBI accession number	Matching peptides	Homology threshold	% sequence coverage
1	Prion protein (Ovis aries)	gi/ 38145699	R.ESQAYYQR.G	41	47 %
			R.YPGQGSPGGNR.Y	44	
			R.VVEQMCITQYQR.E	33	
			R.YPNQVYYRPVDR.Y	35	
			R.VVEQMCITQYQR.E + Oxidation (M)	35	
			K.GENFTETDIKIMER.V	38	
			K.GENFTETDIKIMER.V + Oxidation (M)	20	
			R.YSNQNNFVHDCVNITVK.Q	32	
			K.QHTVTTTTKGENFTETDIK.I	21	
			K.HVAGAAAAGAVVGGLGGYMLGSAMSRPLIHFGNDYEDR.Y	34	
2	No protein hits to report	None	None	None	None
3	No protein hits to report	None	None	None	None
4	No protein hits to report	None	None	None	None

Table 5.2: Summary of mass spectrometry data revealed the high molecular weight bands may not be composed of protein. Mass spectrometry data was obtained from Dr. P. Skipp (Proteomics Manager, Southampton University).

Band	Mascot search ID	NCBI accession number	Matching peptides	Homology threshold	% sequence coverage
5	Beta-Galactosidase (E.Coli)	gi/50513329	R.GDFQFNISR.Y	32	19 %
			R.IDPNAWVER.W	33	
			R.LAAHPPFASWR.N	36	
			K.HQQQFFQFR.L	38	
			R.VDEDQPFPAVPK.W	39	
			K.LWSAEIPNLYR.A	42	
			R.APLDNDIAVSEATR.I	37	
			R.LSGQTIEVTSEYLFR.H	40	
			R.IENGLLLLNGKPLLIR.G	25	
			R.DVSLLHKPTTQISDFHVATR.F	31	
			R.VVQPNATAWSEAGHISAWQQWR.L	25	
			R.VTVSLWQGETQVASGTAPFGGEIIDER.G	35	
6	No protein hits to report	None	None	None	None

Evidence presented in this chapter indicates that high molecular weight bands are present in scrapie-infected and uninfected blood following NaPTA precipitation. Work was carried out to identify these high molecular weight bands which is summarised in table 5.2. Experiments did not conclusively show that the high molecular weight bands were PrP although there was some evidence to suggest this. Additionally there was also evidence to indicate use of NaPTA may result in possible artefacts and that the high molecular weight bands may not be protein based. Although this work was not continued due to time constraints it raises thought-provoking questions regarding the use of NaPTA in a diagnostic capacity.

Evidence high molecular weight bands are PrP	**Evidence high molecular weight bands are not protein**
High molecular weight bands were recognised by several PrP antibodies in PBMCs and $CD21^{+}$ B cell subsets	High molecular weight bands were still present in uninfected blood after PK digestion prior to NaPTA precipitation
Secondary and non-PrP antibody blots were negative	High molecular weight bands were not sensitive to high amounts of PK
High molecular weight bands were observed using coomassie protein staining techniques	High molecular weight bands were not observed using silver or SYPRO orange staining techniques
	Mass spectrometry results suggested there was no protein in the high molecular weight bands

Table 5.2: Summary table of the results presented in this chapter to identify the high molecular weight bands observed in scrapie-infected and uninfected blood.

5.3. Discussion

The strategy of the research presented in this chapter involved characterisation of potentially novel high molecular weight bands as markers for TSE disease in blood. The starting point for this strategy was to compare high molecular weight band expression in blood cell subsets from clinical scrapie-infected, pre-clinical BSE-infected and uninfected sheep. Identifying the high molecular weight bands was also important and to establish if they represented aggregates of PrP, several PrP antibodies (recognising different epitopes on the PrP molecule) were tested with appropriate controls. Although initially promising, further evidence revealed high molecular weight bands were not sensitive to enzymatic digestion with PK, prior to NaPTA precipitation in uninfected and scrapie-infected samples. Additionally high molecular weight bands were not always visualised using various protein staining techniques and mass spectrometry data indicated the high molecular weight bands were not proteinaceous. Therefore, as the high molecular weight bands were not disease-specific and had similar characteristics in both scrapie and uninfected blood they could not be used as markers for TSE disease. However bands with molecular weights of 60 kDa, 112 kDa and 195 kDa have not been described before, and if infectivity is associated with a non-conventional form of PrP^{Sc} it is possible these high molecular weight bands may represent where infectivity resides. Additionally, subtle differences in infected blood may be revealed following more detailed examination. Unfortunately it was not possible to conclusively identify the high molecular weight bands although some intriguing observations were made, particularly concerning the use of NaPTA in PrP^{Sc} blood detection methods.

NaPTA was used to concentrate PrP in blood and increase the sensitivity of the Western blot assay. NaPTA ($Na_2H[PW_{12}O_{40}]$) features the nearly spherical (termed Keggin-type) trianion $[PW_{12}O_{40}]$ which belongs to a broad class of polynuclear transition metal-oxo molecules known as polyoxometalates (POM). POM anions, including those with Keggin structures are known to undergo degradation due to changes in pH and/or buffer characteristics (Hill *et al.*, 1990). In many published methods that incorporate NaPTA, samples are pre-incubated in buffers containing

sarcosyl (Safar *et al*., 1998; Wadworth *et al*., 2001). Initial method development experiments within this thesis (chapter 3) also employ sarcosyl. However in further experiments (chapter 4) sarcosyl was omitted and samples (brain and blood) were suspended in cell lysis buffer prior to NaPTA precipitation (chapter 2, section 2.2.4). It is possible that a change in buffer may result in a partial breakdown of POM anion structure, altering POM size, shape and charge. Breakdown in POM structure may result in size-specific electrostatic interactions between Keggin-type anions and sample proteins, causing the presence of high molecular weight bands in blood. However, this requires that the interacting proteins (whether PrP or not) survive digestion after digestion with very high levels of PK. The same effect may not have been observed in brain due to the presence of different proteins, differing charges and therefore modified electrostatic interactions. It would be interesting to carry out spectroscopic studies to establish the POM structure in solution, such as the cell lysis buffer containing the blood and brain proteins.

Evidence suggests that NaPTA and PrP^{Sc} can form large high molecular weight bands which can be identified by electron microscopy, due to the tungsten content (Safar *et al*., 1998). Additionally the association (and dissociation) of NaPTA with PrP^{Sc} does appear to be dependent on POM size, shape and charge (Lee *et al*., 2005). POMs can also influence polymerisation of amyloid fibrils in a charge-dependent manner, with Keggin-type species, differing in their charge, shifting the equilibrium of PrP 27-30 polymers between amyloid fibrils and POM crystals, resulting in the growth of lattices (personal communication Dr J Safar – manuscript in preparation). This suggests that NaPTA could contribute to the formation of PrP^{Sc} aggregates in blood although it is difficult to conclusively state that the aggregates are abnormal prion protein as they are present in uninfected blood samples following PK digestion. However, these aggregates require further investigation. Conventional PrP^{Sc} is not detectable by anything other than amplification methods in blood, so infectivity may be associated with a form of PrP^{Sc} such as these aggregates which have not been described before.

Conformation–dependent immunoassays (CDI) have been developed that use denaturation with guanidinium hydrochloride, rather than PK-resistance to distinguish

between cellular and disease associated PrP (Safar *et al*., 1998). The basis of the CDI is that the detector antibody recognises an epitope that is always exposed in PrP^{c}, but only becomes exposed in PrP^{Sc} following denaturation. As a result of this PrP^{Sc} can be identified and measured by comparing antibody binding to denatured and native forms of the prion protein. Recently a CDI-formatted sandwich immunoassay was described for the measurement of PrP^{Sc} in sheep brain (McCutcheon *et al*., 2005). Scrapie-infected samples showed significant increases in PrP measured in the denatured fraction (which represents PrP^{Sc}) compared to uninfected sheep. When a NaPTA concentration step was incorporated into this method, interestingly results suggested that a higher concentration of PrP was also present in the denatured than in the native fractions of brain samples from uninfected sheep. This provides further evidence that NaPTA may be able to induce a change in the conformation of PrP (McCutcheon *et al*., 2005). It would be intriguing to test this CDI-based assay on NaPTA precipitated ovine blood from scrapie-infected and uninfected animals.

There was evidence suggesting the novel high molecular weight bands observed in NaPTA precipitated blood included PrP. Several PrP monoclonal antibodies were tested, all of which cross-reacted with the high molecular weight bands. When the membrane was probed with the secondary antibody only or a monoclonal antibody with an irrelevant specificity, there was no signal, indicating that the high molecular weight band result from specific binding of anti–PrP primary antibodies. Interestingly the intensity of the Western blot signal varied depending on the PrP antibody used. All the antibodies recognised epitopes within the C terminus of the PrP molecule (residues 90-233) although the epitope for JB10 was located at the extreme C terminal end. When JB10 was used signal intensity was increased. All the epitopes are accessible but it may be that if NaPTA is inducing a change in conformation, the JB10 epitope becomes more accessible in re-folded PrP.

A PrP band of approximately 250 kDa has been described in activated platelets (Jones *et al*., 2005) and carbohydrate-dependent aggregates of PrP-like proteins have been described in plasma (Tsukui *et al*., 2007) although NaPTA precipitation was not used. Apart from these reports there is no other published evidence (at the time of writing)

suggesting that PrP aggregates are present in blood or that NaPTA precipitation may be inducing conformational change in blood-associated PrP. This made the initial results presented in this chapter potentially very interesting. Studies using brain material have shown that TSE infectivity is associated with a wide range of PrP^{Sc} aggregate states (Caughey *et al*., 1997). In addition to large amyloid fibrils there are smaller subfibrillar oligomers. Evidence suggests these non-fibrillar particles are more infectious than larger aggregates, and are more efficient initiators of TSE disease (Silveira *et al*., 2005). It is conceivable that such aggregates, induced by NaPTA precipitation may be present in blood. High molecular weight bands were not observed in NaPTA precipitated brain which suggests that if PrP aggregates were being formed in blood then possibly one or more biochemical factors present only in blood were enhancing aggregate formation. High molecular weight bands were observed in $CD21^{+}$ B cell subsets in addition to PBMC fractions, also indicating there is a common factor present on different blood cells that is involved in aggregate formation. Due to time constraints it was not possible to test plasma or other leukocyte cell subsets such as monocytes, which have recently been implicated in the pathogenesis of scrapie infectivity in sheep (Andreoletti *et al*., 2007). It would have also been interesting to test the effect of other concentration methods such as classical salt precipitation or cyclic amplification techniques (Soto *et al*., 2002) to establish if high molecular bands are only apparent using NaPTA.

Despite initial findings it was not possible to conclusively show that the high molecular weight bands were composed of PrP. Some evidence suggested the bands were not composed of protein. The characteristics of the high molecular weight bands were similar in scrapie-infected and uninfected sheep blood, and even following PK digestion (prior to NaPTA treatment) high molecular weight bands remained in uninfected samples. This result was unusual as proteolytic digestion with PK should degrade all the PrP^{C}, leaving none left for the NaPTA to concentrate. It was possible that PK digestion was compromised due to the presence of protease inhibitors in the cell lysis buffer (chapter 2, section 2.2.4). However high molecular weight bands were still present (in uninfected and scrapie-infected) even after high concentrations of PK were tested. One interpretation is that the high molecular weight bands are artefacts,

and that the presence of NaPTA is causing a cross-reaction that is recognised by the PrP antibodies. However, it is also possible that the NaPTA is causing a complex to form, including PrP, but which is physically protected from degradation by the action of NaPTA or some other factor.

Various staining techniques for the detection of protein exist such as silver staining (Switzer *et al*., 1979) coomassie blue (Schaffner and Weissmann, 1973) and fluorescent dyes (Steinberg *et al*., 1996; Patton, 2002). Coomassie blue binds non-specifically to virtually all proteins stoichiometerically, and the high molecular weight bands observed on blots were seen using this staining method. Surprisingly when more sensitive methods such as silver staining were used, the high molecular bands were no longer visible. Protein detection using silver depends on the binding of silver ions to the amino acid side chains, primarily the sulfhydril and carboxyl groups of proteins (Switzer *et al*., 1979; Oakley *et al*., 1980; Merril *et al*., 1981; Merril *et al*., 1990) followed by reduction to free metallic silver (Rabilloud, 1990 and 1999). Lack of detection using silver does not necessarily mean that the high molecular weight bands are not protein as not all proteins are stained equally using this technique. For instance, several classes of highly negatively charged proteins including proteoglycans, glycoproteins and mucins, which contain high levels of sulphated sugar residues, are detected poorly by silver staining (Goldberg *et al*., 1997).

Fluorescent SYPRO® orange dyes interact with the SDS coat around proteins in the gel and give more consistent staining between different types of protein compared to coomassie blue. Unlike silver, these dyes also stain glycoproteins well (Steinberg *et al*., 1996). It is conceivable that the high molecular weight bands contain large amounts of carbohydrates which can not be visualised using silver staining. However, when SYPRO® orange staining techniques were applied, the high molecular weight bands were again not visible. The exact mechanism of the interaction between proteins and SYPRO® orange dyes has not been fully characterised but the interaction does seem dependent upon an initial binding of SDS to the proteins. Evidence suggests that SDS binding is occurring as the high molecular weight bands are being resolved by electrophoresis. It is possible that NaPTA precipitation of samples is effecting

SYPRO® orange stain efficiency, by causing any protein present to become so surrounded by glycans that the dye is unable to detect it. Alternately the high molecular weight bands are not composed of protein, which is why no detection was observed with SYPRO® orange of silver staining techniques.

Mass spectrometry was applied to try to identify the high molecular weight bands. Results were intriguing as they indicated that the high molecular weight bands did not contain any peptides. Positive control samples (one of the protein molecular weight markers and recombinant PrP) were analysed and correctly identified, indicating that the mass spectrometer was working optimally. This data suggests that the high molecular weight bands are not protein, or that NaPTA is causing protein to complex into a state not compatible with mass spectrometry. Due to time constraints it was not possible to test the effect of NaPTA precipitation on the positive controls although it would be interesting to see if NaPTA prevents identification of these samples. It may be that NaPTA is causing resistance to enzymatic digestion prior to mass spectrometry and again this could be tested.

High molecular weight bands in scrapie-infected and uninfected blood could not be identified. Initial results indicated that the high molecular weight bands may include PrP but additional evidence showed that the bands could be non-protein artefacts present due to NaPTA precipitation. NaPTA may be promoting the association of carbohydrate moieties (glycans) with proteins. Various methods can be used to detect glycoproteins on blots although these were not employed due to time constraints. Lectins are carbohydrate binding proteins which can be used to discriminate and analyse the glycan structure of glycoproteins, and there are various commercial lectin stains available. Additionally proteoglycans can be detected with cationic dyes such as alcian blue. Other glycan detecting methods include periodic acid-Schiff staining, digoxigenin or Emerald 300. Testing such methods may have revealed the presence of glycans within the high molecular weight bands.

Novel high molecular weight bands have been characterised in blood. Although evidence suggested the bands were not protein, they were reactive to several PrP

antibodies. It is possible that the high molecular weight bands contain carbohydrate. Recently a potential blood test for TSE disease has been described by detecting carbohydrate-dependent aggregates of PrP^{Sc} – like proteins in scrapie-infected hamster plasma (Tsukui *et al*., 2007). In this study the authors detected PK-resistant 3F4-reactive protein in scrapie plasma following protein concentration using SDS and acetic acid (termed acidic SDS precipitation). The 3F4-reactive proteins in scrapie-infected animals resulted in multiple high molecular weight bands (37, 40 and 50 kDa) on gels. Interestingly, pre-treatment with PNGase F (to remove carbohydrate side chains) prior to acidic SDS percipitation resulted in the disappearance of these high molecular weight bands. This data indicates that PrP^{Sc} may aggregate with blood proteins (in this case plasma) through carbohydrate side chains.

There were several differences between the study described by Tsukui and colleagues and the data presented in this chapter. Different concentration methods were tested (acidic SDS precipitation compared to NaPTA) in addition to different blood components from different animal species, infected with different scrapie strains. The size of the high molecular weight bands observed also differed (37, 40 and 50 kDa compared to 60, 112 and 195 kDa). Additionally in this chapter high molecular weight bands were not only observed in scrapie-infected blood but also uninfected samples. It is possible that NaPTA causes more aggregation than acidic SDS precipitation, resulting in high molecular weight bands in uninfected blood. It is also conceivable that plasma proteins do not promote aggregation in the same way as PBMC subset proteins.

The observations from the experiments described in this chapter show that NaPTA precipitation of scrapie and uninfected PBMC and $CD21^{+}$ B cell subsets results in the presence of high molecular weight bands. These are only present in blood and were not observed in brain. It was not possible to discriminate between scrapie-infected and uninfected sheep blood, which may be an extremely important finding for the development of diagnostic TSE blood tests that incorporate PrP concentration using NaPTA. NaPTA precipitation clearly increases the sensitivity of detection of

immunoassay methods for PrP^{Sc} detection in blood, but these results highlight that NaPTA precipitation may produce artefacts that could interfere with such tests.

Chapter 6

The Effect Of Scrapie Infection On Protein Expression In Sheep Leukocytes

6.1. Introduction

PrP^{Sc} is used as a biochemical marker for the presence of TSE infectivity. However, rodent models exist where TSE disease develops but abnormal PrP is not detectable indicating that PrP does not always correlate with infectivity (Manson *et al*., 1999). Detection of conventional PK resistant PrP^{Sc} in blood was not possible using the sensitive Western blot assay described in Chapter 3. It may be that levels of PrP^{Sc} are too low in blood and/or the assay did not have the required sensitivity for detection. PrP^{Sc} has not yet conclusively been detected in TSE-infected blood (Wadsworth *et al*., 2001; Schmerr *et al*., 1999) although more recently a new technique termed protein misfolding cyclic amplification (PMCA) has successfully been applied to TSE infected buffy coat samples, where PrP^{Sc} is amplified to detectable levels (Castilla *et al*., 2005; Saa *et al*., 2006). Although there is still considerable research into PrP^{Sc} based blood diagnostic tests, these difficulties have led researchers to look for alternative TSE biomarkers which may be indicators of the onset of TSE disease.

It has been shown that TSE infection can effect the expression of other molecules. Non prion protein biomarkers such as 14-3-3 proteins (Green *et al*., 2002) and the protease inhibitor Cystatin C (Sanchez *et al*., 2004) have been suggested as markers of TSE disease, but these are not specific and altered concentrations of both markers are observed in other neurological disorders such as Alzheimers disease. The acidic calcium binding protein S100B which is produced by astrocytes, microglial cells and oligodendroglial cells is also elevated in TSE as well as Alzheimers disease (Beaudry *et al*., 1999; Cepek *et al*., 2005). However, in hamster scrapie models serum levels of S100B are elevated only towards the end of scrapie progression (Otto *et al*., 1998; Beekes *et al*., 1999), suggesting that S100B may not be suitable for use as a preclinical TSE diagnostic marker.

In blood, TSE associated biochemical changes in metabolites and hormones have been described. TSE infected hamsters show elevated levels of plasma noradrenalin after comparison with control animals (Pollera *et al*., 2007). Moorby and co-workers (2000) sampled blood from 47 healthy and BSE-infected dairy cows over a 28 week period, and observed increased levels of plasma β-hydroxybutyrate in diseased animals. In a

follow up study, increased concentrations of lactic acid and amino acids were observed in pre-clinical and clinical BSE-infected animals (Moorby *et al*., 2002). However, changes in metabolite concentrations may be attributed to other conditions such as ketosis (Lindner, 1959). Other novel molecular biomarkers in blood have also been described. Miele *et al*., (2001) found that transcription of the gene encoding erythroid differentiation related factor (EDRF), now known as erythroid-associated factor (ERAF) was down-regulated in TSE infected mice. However, ERAF transcription in healthy individuals was found to occur with a high degree of variation which questions the suitability of using EDRF as a marker for TSE disease.

It is clear that a wide range of approaches have identified potential markers of TSE disease other than the prion protein, but it is evident that further work is required before routine testing for specific TSE biomarkers in blood can be implemented. The primary objective of this chapter therefore was to investigate changes in protein expression in buffy coat samples from sheep clinically affected with scrapie compared with uninfected animals. After observing differences in protein expression using relatively insensitive methods such as Coomassie Blue staining of 1D PAGE separated samples, clinical scrapie-infected and uninfected buffy coats were analysed further by two-dimensional gel electrophoresis (2-D PAGE). The buffy coat proteomes were screened using SameSpots PG240 Software to identify any differences between the two samples. Any changes of significance were taken forward for identification by mass spectrometry. The ultimate aim was to identify biochemical markers other than PrP^{Sc} that are identifiable during a TSE infection and may therefore have future diagnostic value.

This chapter contains preliminary work performed with assistance from Dr S. Banner (Roslin Institute). Preliminary isoelectric focussing (IEF) runs and 2D gels shown in sections 6.2.2 and 6.2.3 as well as initial small format 2D Western blots shown in Figure 6.8 were performed by Dr S. Banner. These experiments were carried out by Dr S. Banner during the process of demonstrating the 2D PAGE technique which was then subsequently used for the remainder of the work presented in this chapter.

6.2. Results

6.2.1. Coomassie Blue staining reveals differences in protein expression between scrapie-infected and uninfected buffy coats

Conventional PrP^{Sc} was not detected in buffy coat samples from sheep clinically affected with scrapie (chapter 4, section 4.3) when tested by Western blot. However, some unusual PrP-related bands were detected but these proved difficult to identify, therefore other protein markers of TSE disease were also sought. Initially, buffy coat samples from two scrapie cases and two uninfected sheep were resolved on a gel and stained by Coomassie Blue (chapter 2, section 2.3.5.2) to compare the protein profiles of the two samples (figure 6.1).

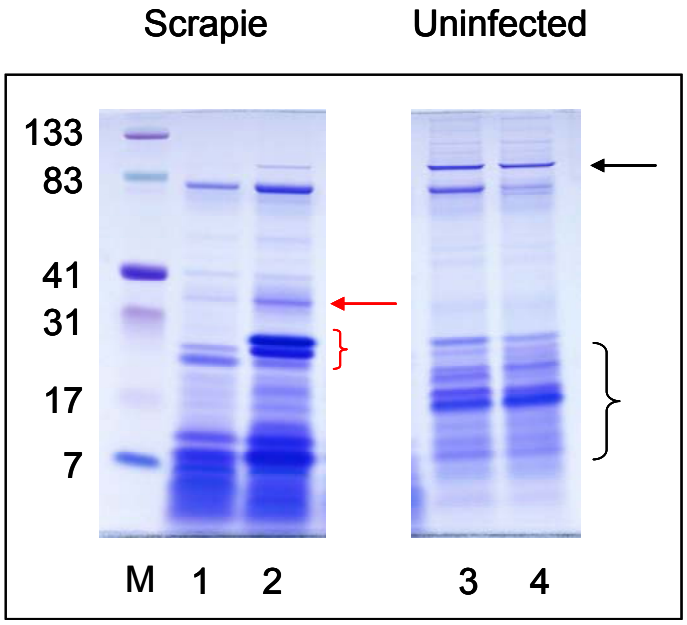


Figure 6.1: Coomassie staining reveals differences in the protein profiles of scrapie-infected and uninfected buffy coats (leukocytes and platelets). Scrapie-infected and uninfected buffy coat samples were PK digested and NaPTA precipitated. The number of buffy coat cells loaded per well was 12 x 10^6 cells. Lanes 1 and 2 represent two different scrapie animals, lanes 3 and 4, two different uninfected animals. Differences in protein expression are highlighted by the red (scrapie) and black (uninfected) arrows and brackets. M indicates molecular weight markers. The gel was stained using standard coomassie staining methods. Molecular masses are shown on the left (kDa).

Coomassie staining revealed some differences between the protein profiles of scrapie-infected and uninfected buffy coat samples (figure 6.1). A protein of approximately 35 kDa appeared more highly expressed in scrapie-infected buffy coats (red arrow). Additionally, a protein with an approximate molecular mass of 84 kDa was up-regulated in uninfected buffy coats (black arrow). The expression of lower molecular weight proteins also differed between diseased and healthy animals (black bracket). Buffy coat protein profiles from the two uninfected sheep were similar, but differences in protein expression were observed among the two scrapie-infected buffy coats (red bracket). This may have been due to natural variation between animals, and it is possible that the buffy coat of the second animal may have contained more platelet or plasma proteins than the sample from the first animal. Silver staining confirmed these differences in protein expression (data not shown). These preliminary results warranted more detailed investigation to identify the proteins that appear to be up or down-regulated in disease.

Due to the number and complexity of proteins present in the buffy coat fraction of blood it was considered not sensible to excise single protein bands from one dimensional gels for analysis by mass spectrometry. Mixed protein samples would invariably become an issue and proteins would not be easily identifiable. Therefore, the method of 2-D polyacrylamide electrophoresis (2D PAGE) first described by O'Farrell (1975) was used as this allows greater separation of proteins, initially by isoelectric point (pI) using isoelectric focussing (IEF) and then by mass using standard sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteomes can then be compared and differences identified more easily. Protein spots in these gels are more likely to consist of a single protein and therefore identification by mass spectrometry will be more reliable.

6.2.2. Buffy coat protein precipitation with NaPTA inhibits isoelectric focussing

Iso-electric focussing is an electrophoretic method that separates proteins according to their isoelectric points (pI). The IEF technique relies on use of a stable pH gradient under the influence of an electric field. Proteins migrate towards the cathode if positively charged or the anode if negatively charged until they reach their pI. At this point they have a net charge of zero. If the protein diffuses away from its pI, its charge changes because it enters a different pH and therefore migrates back to its pI. This is termed the focusing effect of IEF.

In previous experiments (described in more detail in Chapter 3) buffy coat proteins were concentrated using NaPTA. For continuity NaPTA precipitation was also included in buffy coat sample preparation for 2-D PAGE. Initially, uninfected buffy coat pellets containing 1 x 10^7 cells were incubated in cell lysis buffer before NaPTA precipitation (described in Chapter 2, section 2.2.4). Following protein concentration with NaPTA, samples were combined with DeStreak rehydration buffer containing 0.5 % pH 3-11 immobilised pH gradient (IPG) buffers. The IPG buffers, developed by Gorg and colleagues (1998; 2000) help to define the range and shape of the pH gradient produced for IEF as well as retaining protein solubility. Following sample loading into the IPG strips, using passive in-gel rehydration (Chapter 2, section 2.3.6.2) buffy coat proteins were subjected to IEF. The progress of an IEF run with NaPTA precipitated samples is shown in Figure 6.2.

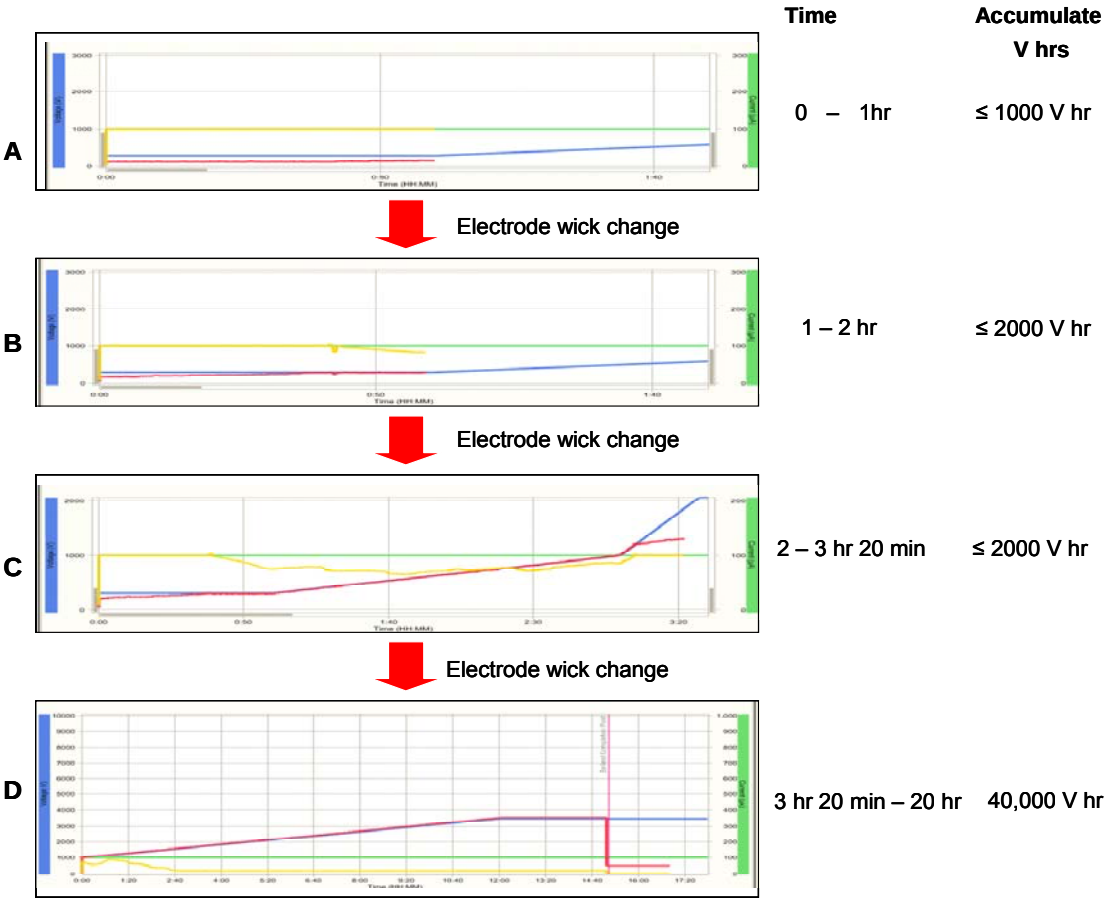


Figure 6.2: NaPTA precipitation adversely affects isoelectric focussing. Uninfected buffy coats (1 x 10^7 cells) were NaPTA precipitated before IEF. During the first 2 hours of IEF the current stays high and prevents volts reaching the set point. IEF was stopped periodically to replace wicks so that the current could reduce (D). Panel C shows after several electrode wick changes the current begins to drops after approximately 2 hr 40 min. The lines corresponding to the current and voltage are described in the key above. A description of an expected IEF profile is described in the text below and shown in Figure 6.4 later.

NaPTA precipitation of buffy coat proteins appears incompatible with separation of proteins by IEF. IPG strips are subjected to IEF for a total of 40 kV hr, which ordinarily takes approximately nine hours. However, when samples were precipitated using NaPTA it took 23 hours to accumulate the required kV hr. In a standard IEF run the initial high current should fall and remain low until focussing completes. If the samples contain high levels of salts or other interfering components, this will not occur. Changing the electrode wicks can be used as a method of "desalting" a sample, and in this case replacing electrode wicks on an hourly basis did help to reduce the current, but only after approximately 2 hours 40 min (figure 6.2, C). The binding of NaPTA directly to the proteins appears to adversely affect IEF and it was therefore deemed incompatible with 2D gel electrophoresis.

6.2.3. NaPTA precipitated buffy coats do not produce satisfactory 2D proteomes

Despite the problems associated with IEF in the presence of NaPTA, the samples did reach 40 kV hrs and so were separated by 2D PAGE. This was carried out as described in chapter 2, section 2.3.6 and the resultant silver stained gel shown in figure 6.3.

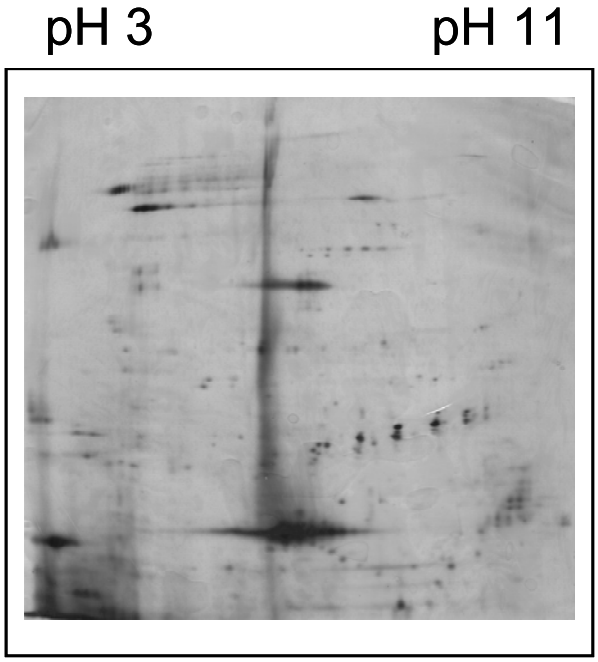


Figure 6.3: NaPTA precipitation of uninfected buffy coats results in poor protein resolution by 2D PAGE. NaPTA precipitated protein samples (50 µg) were electrophoresed on 12 % homogenous SDS-PAGE gels. Samples were visualised by silver staining according to the Plus One method (GE Healthcare).

NaPTA precipitation of uninfected buffy coats resulted in the apparent uniform loss of the majority of the protein spots (figure 6.3). This was not due to insufficient sample as 50 µg of protein was loaded. NaPTA may adversely affect the protein charge such that many proteins may take on a very high positive or negative charge. Proteins could then migrate beyond pH3 or pH10 and are excluded from the IPG strips and hence are not observed in the 2D gel.

Protein concentration steps are often included during sample preparation prior to 2D when the starting tissue proves problematic, for instance there is a low protein concentration. Buffy coat samples are protein rich (approximately 300 µg of protein per 1 x 10^7 cells) and so the lack of starting material is not anticipated to be a problem here. NaPTA precipitation was used in buffy coat sample preparation because this step had been used in previous experiments outlined in chapter 3. However, the use of NaPTA does not appear compatible with 2D PAGE (figures 6.2 and 6.3) and although

other methods could be employed to potentially improve compatibility with NaPTA, ultimately the aim of this work was to identify any changes in protein expression between scrapie and uninfected buffy coats. In order to avoid protein losses it is important to minimise manipulation of the starting tissue and because of this as well as the problems observed with IEF, NaPTA precipitation for 2D-PAGE sample preparation was not pursued.

6.2.4. Buffy coat IEF is optimal without a NaPTA concentration step

Buffy coats containing 1 x 10^7 cells from three clinical scrapie–infected and three uninfected sheep were prepared without NaPTA. They were assayed for total protein using a non-interfering protein assay (2D quant kit, described in chapter 2, section 2.3.6.1), and 50 µg of protein was added to each IPG strip by passive in-gel rehydration (Chapter 2, section 2.3.6.2). Following sample rehydration the buffy coat proteins were separated by IEF.

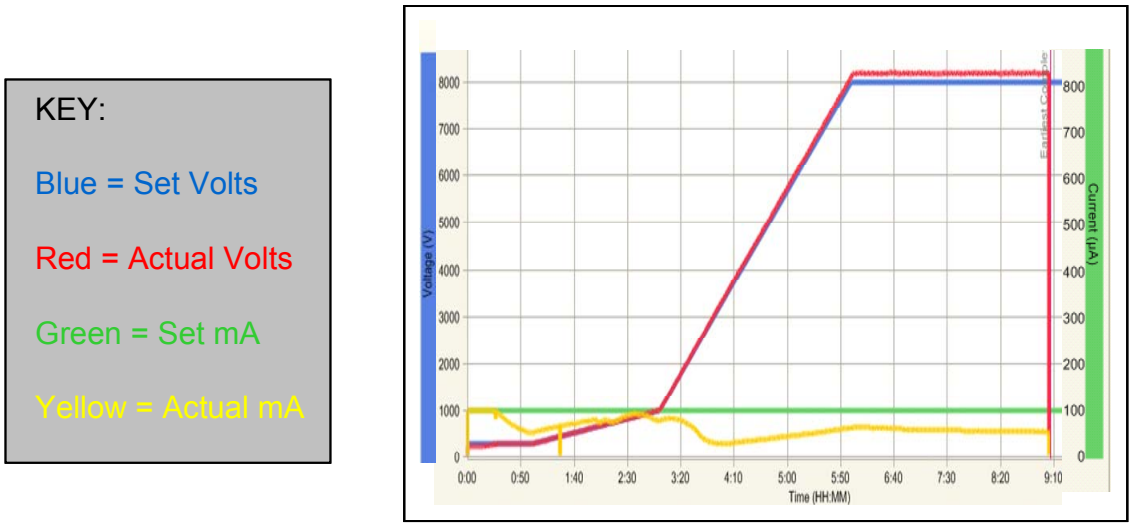


Figure 6.4: Standard IEF profile of ovine buffy coats. The traces corresponding to the current and voltage are described in the key above. IPG strips containing scrapie–infected and uninfected buffy coat proteins were run together and subjected to IEF for 40 kV hr.

Figure 6.4 shows a typical profile observed after scrapie and uninfected buffy coat IEF. The required 40 kV hr were accumulated over 9 hours, the initial high current dropped after approximately 30 min and then remained below the set limit throughout the focussing. No manual intervention (electrode wick exchange) was required indicating samples were appropriately prepared, and contained no interfering components.

6.2.5. Buffy coat proteome analysis from scrapie-infected and uninfected sheep

Buffy coat proteins from the three infected and uninfected animals were then separated by 2D PAGE and representative 2D gels from one scrapie and one uninfected animal are shown in figure 6.5

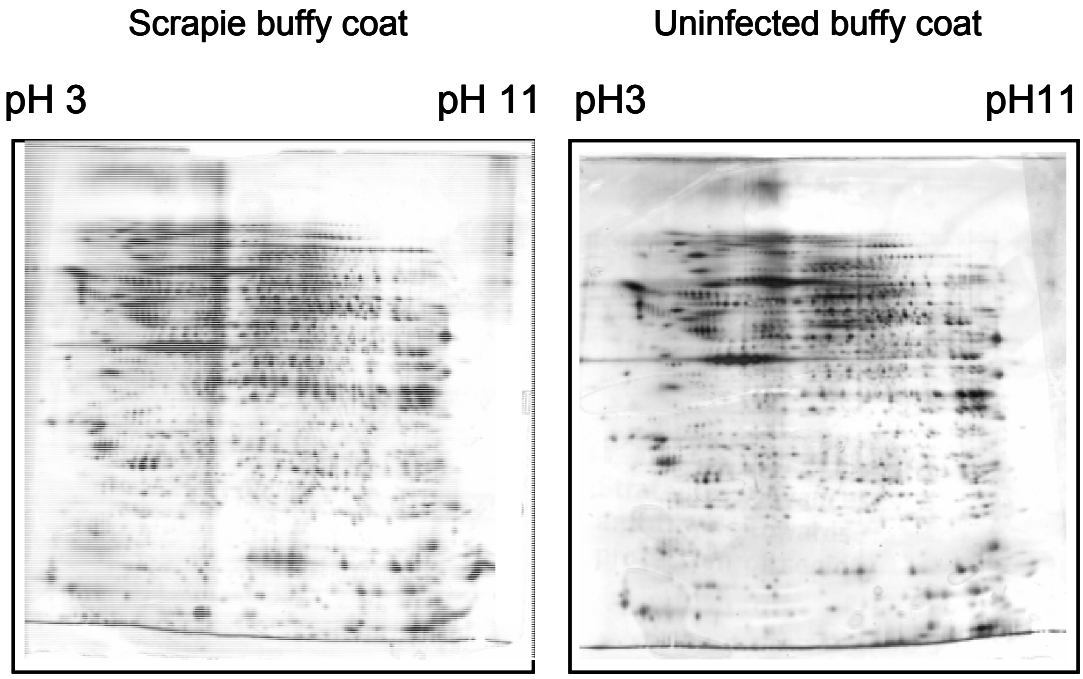


Figure 6.5: Buffy coat proteome analysis from scrapie-infected and uninfected sheep show reproducibly similar proteomes. 12 % homogenous SDS PAGE gels were loaded with 50 µg of protein from clinical scrapie infected or uninfected buffy coats. Gel electrophoresis was performed at 12 W overnight at 30ºC. Proteins were visualised using Plus One silver staining methods (GE Healthcare).

A total of 50 μg of protein appeared optimal as the 2D gels were not overcrowded with protein spots, allowing visibility of less abundant proteins and an accurate representation of buffy coat proteins. On visual inspection, the proteomes looked generally similar in diseased and uninfected animals (figure 6.5). This is not surprising as disease may only cause subtle differences in protein expression. However, the slight overall variation of staining intensity between these two gels is most likely caused by small variations in protein loading and/or silver staining procedures. These issues are counteracted by the software (SameSpots PG240, see section 6.2.6) used for subsequent analysis of these gels as it allows for minor variations in staining intensities using normalisation algorithms. Therefore any differences observed are anticipated to be real and not due to silver staining variations.

In order to identify any differences in protein expression between healthy and clinical scrapie animals, buffy coat samples were taken from three clinical scrapie and three uninfected sheep. After IEF and 2–D PAGE specific protein changes between diseased and uninfected blood were analysed using SameSpots PG240 analysis software (Nonlinear Dynamics).

6.2.6. SameSpots PG240 software analysis

SameSpots PG240 is a specific 2D analysis tool that has significantly streamlined the analysis of sample proteomes. Analytical gels are aligned perfectly using an automated image alignment programme. Gel images are then analysed rapidly in a single step that includes detection of protein spots, background subtraction, normalisation and protein spot matching. Maximum fold change based on the spots normalised volume and one way ANOVA *p*-values are calculated. Protein spots are then ranked according to their *p*-values and fold change, so that the most significantly different spots can be identified quickly. Protein spots can then be accepted or rejected for further study.

In total, eighteen buffy coat proteomes (three clinical scrapie and three uninfected, with three repeats of each) were analysed using SameSpots software. Collectively six protein spots appeared to be significantly up-regulated in scrapie buffy coats and one in

uninfected buffy coats. Figure 6.6 shows an example of a protein spot (spot 33) that was significantly up-regulated in scrapie buffy coats.

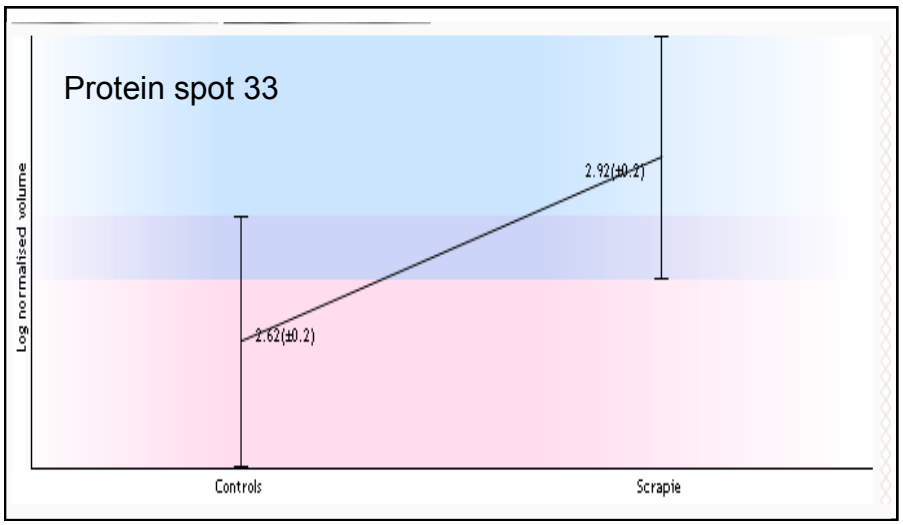


Figure 6.6: SameSpots analysis revealed subtle differences in protein expression between scrapie-infected and uninfected buffy coats. The maximum fold change based on the spots normalised volume is displayed. Pink shading represents control buffy coat, blue shading represents scrapie-infected buffy coat. Colours have been assigned arbitrarily by SameSpots software.

After analysis protein spots of interest were displayed statistically as shown in table 6.1

Protein spot	Fold-difference	ANOVA *p*-value	Scrapie regulation
20	2.218598	0.008731	Up-regulated
32	2.020842	0.015532	Up-regulated
33	2.000824	0.015562	Up-regulated
82	1.9161	0.024771	Down-regulated
52	1.80308	0.039457	Up-regulated
50	1.776065	0.046021	Up-regulated
73	1.74703	0.046543	Up-regulated

Table 6.1: Summary of the protein spots that were up and down–regulated in clinically infected scrapie buffy coats

Table 6.1 shows the fold difference between scrapie-infected and control animals, in addition to the calculated analysis of variance (ANOVA) for the selected proteins. The column of interest is the ANOVA which demonstrates that all the proteins have a *p*-value less than the critical *p*-value of 0.05, which is classed as significant. SameSpots analysis revealed seven buffy coat protein spots that were significantly up or down-regulated following scrapie infection. The next step was to try and identify these proteins.

6.2.7. Mass spectrometry identified lactate dehydrogenase, annexin 1 and elongation factor 1 as proteins of interest

Preparative gels were prepared so that proteins of interest could be identified by mass spectrometry. A total of 400 μg of buffy coat protein from either a scrapie–infected or an uninfected sheep were separated by IEF and then 2D PAGE as normal. These gels were stained using a modified silver staining protocol as described in chapter 2, section 2.3.6.5 (Yan *et al*., 2000). It was important to resolve more sample than before to ensure there was sufficient protein available for mass spectrometry analysis and also to allow for the reduction in sensitivity when using a modified silver staining protocol. The resultant gels were compared and matched to the analytical gels. However, due to the greater protein load, silver impurities and gel to gel variation it was not possible to excise with confidence all seven protein spots. This could be achieved by repeating these experiments with lower protein amounts or using narrower pH ranges and different gels to separate proteins over a greater distance. However, it was possible to excise five protein spots for identification (protein spots 32, 82, 52, 73, and 33).

The excised proteins were digested with trypsin by Dr A. Gill (Roslin Institute) and the resulting digest mixture analysed by mass spectrometry. The experimental mass values of the resultant peptides were entered into MASCOT where peptide sequences were searched against sequence databases to identify possible proteins. This work was carried out by Dr D. Kurian (IAH, Compton). In total, three protein spots were identified, spot 82 was recognised as elongation factor 1 (EF-1), spot 52, lactate dehydrogenase (LDH) and spot 32, annexin 1 (Table 6.2).

Table 6.2: Summary of mass spectrometry results. Three potential biomarkers (lactate dehydrogenase B, elongation factor 1 and annexin 1) were identified.

Spot	Mascot search ID	NCBI accession number	Matching peptides	Homology threshold	% sequence coverage
52	Lactate dehydrogenase B	gi/27806561	K.FIIPQIVK.Y	33	18 %
			K.SADTLWGIQK.D	36	
			K.MVVESAYEVIK.L	33	
			K.SLTDELALVDVLEDK.L	45	
			K.SLTDELALVDVLEDKLK.G	27	
			K.GEMMDLQHGSLFLQTPK.I	17	
			K.GEMMDLQHGSLFLQTPK.I	26	
			K.LKGEMMDLQHGSLFLQTPK.I	22	
82	Elongation factor 1	gi/86827651	R.LPLQDVYK.I	22	9 %
			K.IGGIGTVPVGR.V	38	
			R.EHALLAYTLGVK.Q	47	
			K.YYVTIIDAPGHR.D	50	
32	Annexin 1	Gi/74	K.GVDEATIIEILTK.R	33	19 %
			K.GTDVNVFTTILTTR.S	33	
			K.GLGTDEDTLNEILASR.T	49	
			K.GGPGSAVSPYPTFNPSSDVEALHK.A	19	

Mass spectrometry and subsequent analysis produced a short list of three candidate biomarkers, LDH, and annexin 1 which appeared up-regulated in sheep clinically affected with scrapie, and EF-1 which appeared down-regulated. These proteins require validation of their changing expression levels in other formats such as Western blotting. However, due to time constraints and antibody availability, it was not possible to investigate all three proteins. Due to the availability of an anti-LDH antibody (details described in Appendix 1) and evidence suggesting a possible role for LDH elevation in plasma induced by scrapie infection (Adams and Field, 1967) this protein was initially selected for further study.

6.2.8. Localisation and elevation of lactate dehydrogenase in scrapie–infected buffy coats

Experiments to localise LDH on small format 2D PAGE were carried out. Following electrophoresis, gels containing scrapie–infected and uninfected buffy coat proteins were Western blotted and probed for LDH (as described in chapter 2, section 2.3.5.5). Figure 6.7 represents these preliminary results.

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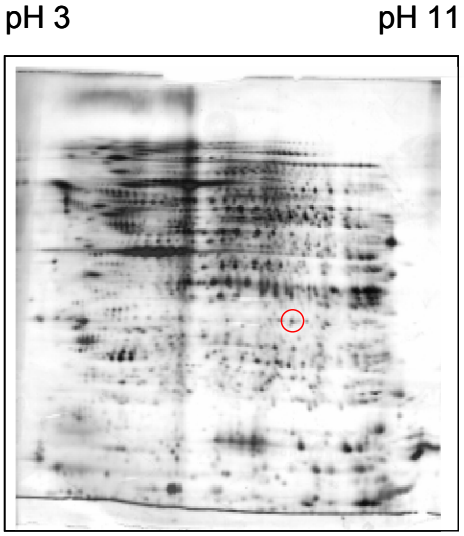
A)



B)

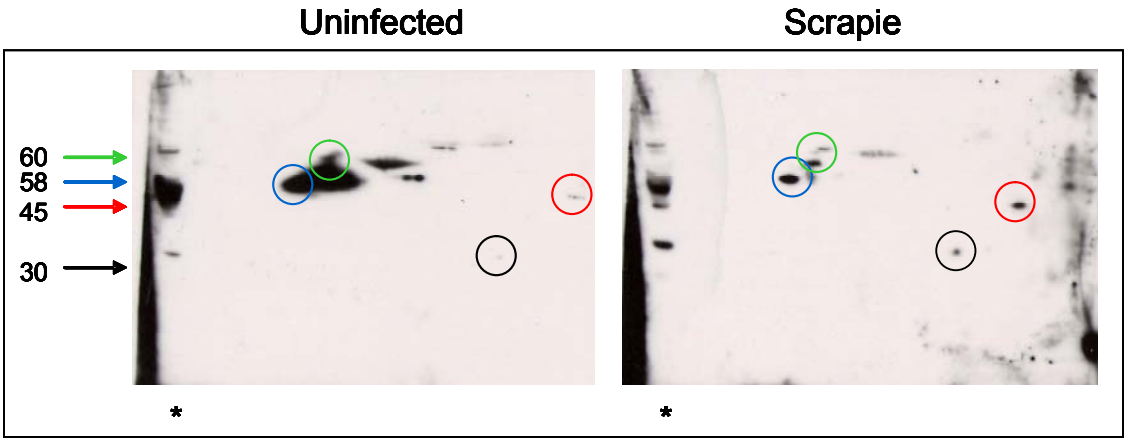


Figure 6.7: Localisation of LDH in scrapie–infected buffy coats. In A), the location of spot 52, corresponding to LDH is highlighted on a silver stained gel (red circle). In B) 2D Western blots are shown. Each 2D Western blot contains 50 µg of buffy coat protein from uninfected or scrapie–infected sheep. Samples were separated by IEF before second dimension separation. 1D wells were loaded with 10 µg of buffy coat protein from uninfected or scrapie–infected sheep as indicated by *. The blots were probed with anti-LDH at 1:1000 dilution. The secondary antibody, rabbit α-goat HRP was used at a dilution of 1:1000. The circles indicate immunoreactivity to LDH. Key: The arrows show approximate molecular weights (kDa) for each spot. The blots were developed using standard ECL methods; the exposure time was 2 min.

LDH has a molecular weight of approximately 37 kDa and the protein spot selected from the 2D gel for mass spectrometry (figure 6.7, A) was approximately 38 kDa and up-regulated in scrapie-infected buffy coats. The 2D blots (figure 6.7, B) demonstrate immunoreactivity to LDH, which appears elevated in scrapie–infected buffy coat (as indicated by the black and red circles), but also down-regulated in scrapie-infected buffy coat (as indicated by the green and blue circles). There are two isoenzymes of LDH in mammals, LDH–M and LDH–H (Sass *et al*., 1989). Each molecule consists of four subunits, where each subunit is either H (heart-specific) or M (muscle-specific) so the protein spots observed on the 2D blot may represent the H or M tetrameric isoenzymes. The pI of the LDH protein spots are approximately pH 4.5 (blue circle) pH 5 (green circle) pH8 (black circle) and pH 9.5 (red circle). LDH has an expected pI of 7. However, the shift may depend on the isoenzyme of LDH being detected here. The protein spots on the 2D blot may represent different LDH subunit aggregation states.

Although this blot did not contain standard molecular weight markers (a 1D buffy coat sample was resolved instead), after comparison with previous 2D blots generated by Dr S. Banner (Roslin Institute) it appears that the lowest immunoreactive band present in the 1D samples does migrate at approximately 37 kDa (molecular weight of LDH) corresponding to the lowest protein spot in the 2D blot (black circle). Interestingly, this band appears more abundant in the scrapie–infected buffy coat when compared to the uninfected sample. Similarly, the immunoreactive bands in the 1D samples that migrate at approximately 45 kDa, 58 kDa and 60 kDa all correspond to 2D protein spots (red circle, blue circle and green circle respectively) and show up or down-regulation in the same way. This result is promising as it begins to validate the 2D methodology and SameSpots analysis.

To further characterise the LDH antibody and also quantify the level of change in LDH expression in scrapie–infected buffy coat, 1D blots were carried out as shown in figure 6.8. These blots are quicker and generally more reproducible than 2D blotting and will allow better quantification of LDH levels in scrapie and uninfected buffy coats.

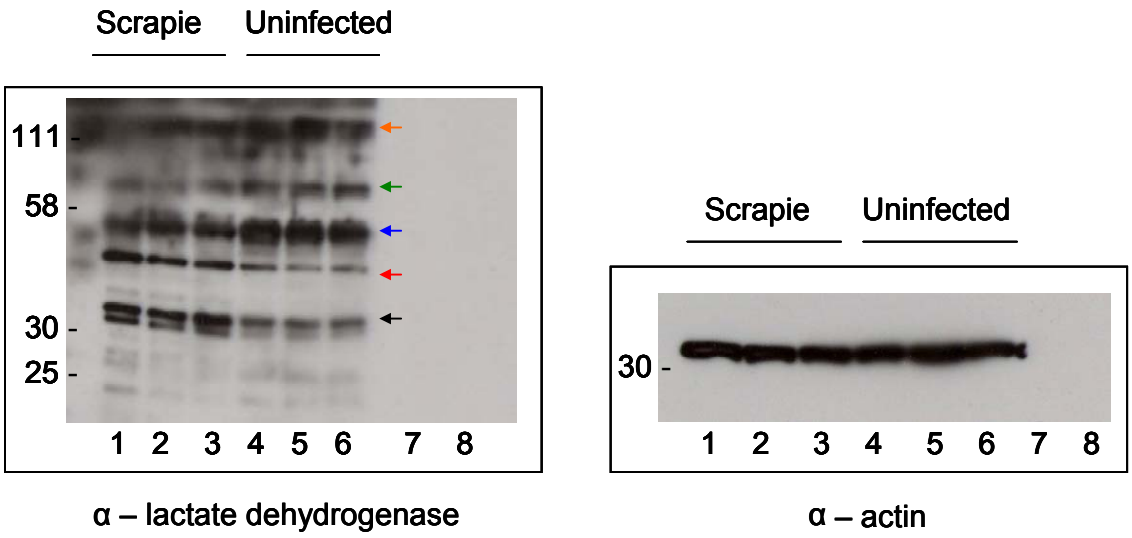


Figure 6.8: LDH and actin expression in 1D blots of scrapie–infected and uninfected buffy coats. Wells were loaded with 10 µg of buffy coat protein from three scrapie–infected sheep (lanes 1, 2, 3 and 7), or 10 µg of buffy coat protein from three uninfected sheep (lanes 4, 5, 6 and 8). Blots were probed with anti–lactate dehydrogenase at a 1:1000 dilution or anti–actin at a 1:5000 dilution. Secondary antibodies used were goat α mouse HRP, 1:2000 (actin) and rabbit anti-goat HRP, 1:1000 (lactate dehdrogenase). Lanes 7 and 8 in each gel were probed with the appropriate secondary antibody only. The blots were developed using standard ECL methods; the exposure time was 2 min for anti–lactate dehydrogenase and flash for anti–actin. Differences in LDH expression between scrapie and uninfected animals are indicated using arrows. Molecular weight masses are shown on the left (kDa).

As shown in the 2D blots (figure 6.7), LDH appeared elevated in the scrapie–infected buffy coat samples in the 1D blots (figure 6.8). In particular, a stronger signal was present in the lower protein bands (just above 30 kDa and 45 kDa, black and red arrows) when compared to the uninfected controls. Interestingly, protein bands with a higher molecular weight (around 58 kDa, blue and green arrows and 111 kDa, orange arrow) were elevated in the uninfected buffy coats. When the membrane was probed with secondary antibody only there was no signal, indicating that the immunoreactivity observed in lanes 1–6 was due to the anti–lactate dehydrogenase primary antibody. Actin was used as a protein loading control.

The 1D blots (figure 6.8) appear to validate the 2D (figure 6.7) blots. Therefore, to help illustrate this, figure 6.9 shows a comparison of the two, highlighting that changes in LDH protein expression between scrapie and uninfected buffy coats in 1D blots are the same as shown in 2D blots.

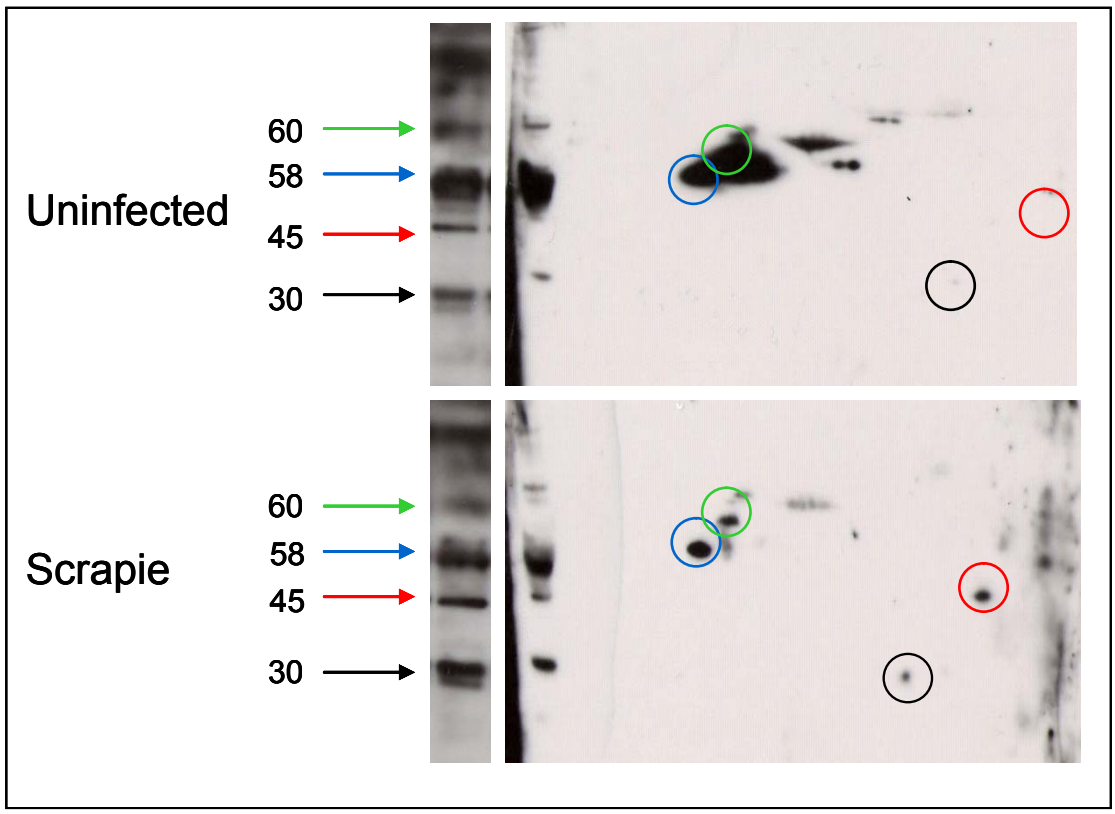


Figure 6.9: LDH protein expression in uninfected and scrapie-infected buffy coats appears the same on 1D and 2D blots. Differences in LDH expression between scrapie and uninfected animals are indicated using arrows and circles. Key: The arrows show approximate molecular weights (kDa) for each spot.

Although preliminary, these results are promising as they identify that certain LDH isoforms are possibly elevated in the buffy coat fraction of blood after scrapie infection. LDH was initially localised in 2D gels because of its relative increase compared to uninfected. Expression levels analysed by 1D PAGE do seem to increase in scrapie-infected buffy coats. Further work is required to confirm this result and may reveal LDH as a potential biomarker for scrapie infection.

6.3. Discussion

The diagnosis of TSE disease remains a fundamental problem for animal health and welfare. Existing methods rely on post-mortem immunohistochemistry or immuno-detection of PK resistant PrP^{Sc} in brain and tonsil tissue by Western blotting, but obtaining this material involves invasive procedures. Therefore there is a need for validated, non-invasive diagnostic tests and the use of blood as a test material would be ideal. It has been shown that TSE disease can be transmitted between animals via the blood system (Houston *et al*., 2000), but previous studies to detect PrP^{Sc} in blood samples have met with limited success. Only PMCA technology appears sensitive enough for PrP^{Sc} detection in clinical and pre-clinical TSE infected blood (Castilla *et al*., 2005; Saa *et al*., 2006). However, this technique requires highly specialised equipment, making automated high throughput diagnosis difficult. Consequently, there is a need for the identification of biomarkers other than PrP^{Sc} in blood that indicate the presence of a TSE infection, and can be used in the development of diagnostic blood-based tests.

2D gel analysis of buffy coats from clinical scrapie-infected and uninfected sheep blood revealed a panel of seven proteins that were significantly up or down-regulated after scrapie infection. Using Q-TOF-MS analysis, three candidate protein biomarkers were identified, lactate dehydrogenase (LDH), elongation factor 1 (EF-1) and annexin 1 (A1). LDH and A1 were elevated in clinical scrapie-infected sheep while EF-1 was down-regulated. Even though preliminary, these results may have potential diagnostic value which could be explored in a follow-up study.

Appropriate sample preparation is essential for good 2D electrophoresis results. Before preparation for 2D PAGE, buffy coat proteins from infected and uninfected animals were concentrated using NaPTA precipitation. However, problems during IEF were encountered when this was performed. IPG strips were subjected to IEF for a total of 40 k V hr which takes approximately nine hours during a standard IEF run. However, NaPTA precipitated samples required 23 hours to accumulate 40 k V hr. It is possible that in addition to protein, NaPTA was concentrating non-protein components such as

salts. This could leave the sample in a highly conductive state. Focussing of proteins at their pI would not occur until the salt ions had moved out of the strip, prolonging the time for complete IEF. The presence of salts can also result in non-focussing of regions at either end of the IPG strip. This effects second dimension separation of proteins and promotes horizontal streaking on the final 2D gel, which was observed after 2D electrophoresis. Salts can be removed using gel filtration or precipitation and resuspension methods but this was not carried out due to possible protein loss. Additionally, dialysis would be time consuming to perform and so was not pursued.

NaPTA-based protein precipitation is not a standard procedure commonly used in 2D electrophoresis. There is evidence to suggest that NaPTA precipitation is specific for both protease resistant and sensitive forms of PrP^{Sc} (Safar *et al*., 1998; Tremblay *et al*., 2004), although the precise molecular mechanism of PrP^{Sc} interaction with NaPTA is largely unknown. Therefore it is conceivable that poor 2D separations were due to NaPTA only precipitated PrP or proteins associated with PrP.

Protein precipitation is an optional step in sample preparation for 2D electrophoresis which is often employed to concentrate protein from a dilute source. Because the buffy coat samples were rich in protein and precipitation with NaPTA caused the problems outlined above it was not pursued. It was important to obtain a complete and accurate representation of all buffy coat proteins in the scrapie-infected and uninfected samples. Therefore for effective proteome analysis, sample preparation was kept as simple as possible to avoid protein losses (chapter 2, section 2.3.6.1).

Lactate dehydrogenase (LDH) is an enzyme responsible for converting muscle lactic acid into pyruvic acid which is an essential step in producing cellular energy. LDH consists of five tetrameric isoenzymes composed of the random association of two subunits (H and M) that are encoded by different genes (Cahn *et al*., 1962). LDH was found to be up-regulated in the buffy coat blood fraction from sheep clinically infected with scrapie. These results are in agreement with Schmidt and colleagues (2004) who saw elevated LDH levels in CJD patients when compared to individuals suffering from other dementias (Schmidt *et al*., 2004). This study was conducted using cerebrospinal

fluid (CSF) samples, but does provide further evidence of elevation of LDH in TSE disease. Additionally, a possible role for LDH elevation induced by scrapie infection in mouse plasma has also been described, although this was reported to have been due to contamination of the mouse scrapie strain with a LDH elevating virus (Adams and Field, 1967). LDH enzymes are widely distributed throughout the body and studies in humans show high levels in the heart, kidney, liver, muscle, brain, red blood cells and lungs (Abeloff *et al*., 2004). Following cellular damage LDH is released into the blood stream. Therefore it is conceivable that in TSE disease LDH may be elevated in blood as a result of tissue damage from PrP^{Sc} accumulation in the brain and other organs. If tissue damage is required before detection, pre-clinical diagnosis using LDH may not be possible, which would rule out this protein as a true diagnostic biomarker However, this will be the subject of further studies and ongoing experiments in our laboratory.

It was observed that protein bands in 1D Western blots were more intense around 30 and 45 kDa in scrapie infected buffy coats, whereas in uninfected samples, bands were more intense around 58 kDa. This may represent LDH H and M tetrameric isoenzymes. Because many disease processes cause elevations in total LDH levels (i.e. cancer, heart and liver disease) a breakdown of the five different isoenzymes that comprise LDH may be helpful for diagnosis. For example, in humans after myocardial infarction LDH-1 rises over LDH-2 which is normally the dominant isoenzyme present in blood (Lott and Stang, 1980). If the levels of individual isoenzymes were measured in the blood of sheep this may help to differentiate a diagnosis of scrapie, providing the normal limits in healthy animals were also determined.

The annexins are a family of closely related calcium and membrane-binding proteins expressed in most eukaryotic cell types. There are over one hundred and sixty unique annexin proteins (Morgan *et* al., 1997). Although structurally and biochemically similar annexins have diverse functions that include vesicle trafficking, cell division, apoptosis, calcium signalling and growth regulation. Annexin 1 in particular also has an anti-inflammatory role (Ferlazzo *et al*., 2003). Annexin 1 was found to be up-

regulated in clinical scrapie-infected buffy coats, although this requires further experimental confirmation.

To date there appears to be no evidence suggesting a link between annexin 1 expression and TSEs. However, annexin 5 has been shown to be up-regulated in cerebellar brain tissue from transgenic mouse models of inherited prion disease (Biasini *et al*., 2006). There is also good evidence to suggest that in other clinical conditions (i.e cancer and diabetes) changes in annexin expression levels may contribute to pathological disease (Xin *et al*., 2003; Ghitescu *et al*., 2001). Annexins 1, 2, 4 and 6 have been isolated from mammalian brain and two patterns of annexin localization are characteristic of Alzheimer's disease. Annexin 6 appears associated with granulovacuolar bodies in degenerating pyramidal neurons, while annexin 2 is expressed in astrocytes associated with some β-amyloid plaques (Eberhard *et al*., 1994). Annexin 1 is also increased in injured brain tissue (Johnson *et al*., 1989) and it may be that in scrapie-infected sheep this protein is released from brain or inflammatory cells.

Elongation-factor-1 (EF-1) is responsible for aminoacyl-tRNA transfer on the ribosome in the process of protein synthesis, and is comprised of two components, a G-protein named eEF-1A and a nucleotide exchange factor, eEF-1B. In several cancers EF-1 is up-regulated which is thought to be detrimental to cell cycle checkpoint mechanisms (Ogawa *et al*., 2004). There appears to be no reported evidence suggesting a role for EF-1 in TSE disease, but results in this study indicate a possible down-regulation in scrapie-infected buffy coats. The precise biological involvement of EF-1 in scrapie infection remains to be determined.

Although three potential TSE biomarkers have been described, it is important to note that caution is required when comparing protein expression in blood from clinically sick animals with healthy animals. Sheep that are suffering from any type of disease may exhibit abnormal behaviours (for instance, a reduction in appetite or an increase in stress hormone levels) causing changes in blood protein levels that are not specific to actual TSE infection. Therefore, when proteomic studies are carried out, it is important that appropriate controls are considered.

Sheep are susceptible to a wide range of disorders of which bacterial infections are common. For example, animals can become infected with Johne's disease, a contagious fatal bacterial infection of the intestinal tract. Also called Paratuberculosis, this disorder is caused by the *Mycobacterium avium* bacteria (Greig. 2000). Sheep are also prone to viral infections such as Maedi Visna which is a common disease caused by an ovine letivirus. This disorder affects the lungs, causing these organs to fill with fluid and imflammatory cells (Stroub. 2004). However, bacterial and viral infections such as these will cause increased numbers of leucocytes in the blood and cause an immune response. Therefore, these can not really be used as appropriate controls for brain degenerative diseases like scrapie which has no classical immune response.

Neurological disorders that are not related to TSE disease would represent suitable controls. Polioencephalomalacia (caused by a deficiency in thiamin) occurs sporadically in sheep at pasture. This disease causes acute cerebral oedema with cerebral necrosis. There are also other CNS disorders such as plant associated toxicoses where animals suffer paralysis and gait disturbances after accidentally ingesting toxins from plants (Chattopadhyay *et al*., 1985). These would be more appropriate control sheep diseases although blood samples from such animals may be difficult to acquire. Centres specialising in such disorders may have to be approached. However, comparison of blood from animals suffering from non-TSE related neurological disorders is required to identify biomarkers specific for TSE disease.

Disease-specific PrP^{Sc} accumulation represents the only available molecular marker of TSE disease. However, PrP^{Sc} is not detectable in all TSE-affected animals (Manson *et al*., 1999) and though the detection of PrP^{Sc} in blood may prove useful, current techniques such as PMCA will be technically demanding to perform on a high throughput scale (Castilla *et al*., 2005; Saa *et al*., 2006). Although there are other potential biomarkers for TSE disease, many have been identified in body fluids such as CSF which are difficult to obtain. Currently no reliable biomarkers have been identified in sheep blood. In this study three potential biomarkers were identified. LDH and annexin 1 appeared up-regulated in buffy coat following scrapie infection, while EF-1

appeared down-regulated. Further work is required to determine how valuable these markers may be for diagnostic testing of scrapie, but these preliminary findings may be of interest in further studies investigating possible surrogate markers for TSE disease.

Chapter 7
Final Discussion

7.1. TSEs and blood

TSE animal models have provided important information on the presence and distribution of infectivity in peripheral blood during the pre-clinical and clinical stages of TSE disease. Preliminary studies in sheep show that TSEs can be transmitted to animals following blood transfusion from donor sheep with natural scrapie or experimental BSE (Houston *et al*., 2000; Hunter *et al*., 2002). Recently, the final results of this long-term study have shown that whole blood and buffy coat obtained during the pre-clinical and clinical phase of disease infection transmitted BSE infection to 36 % of transfusion recipients and scrapie to 43 % of recipients (Houston *et al*., 2008). These experiments provide evidence that TSE infectivity is present in the peripheral blood of TSE-infected animals. However, it is unclear exactly where in the blood such infectivity resides.

Although it has been shown that TSE disease can spread *via* blood transfusion it is possible that different TSE strains may act in different ways. There are two groups of CJD, classical (which include sporadic CJD (sCJD), inherited, and iatrogenic CJD) and variant CJD (vCJD). In humans there have been four blood transfusion-related vCJD infections in individuals who received non-leucodepleted red cells (Peden *et al*., 2004; Llewelyn *et al*., 2004; Health Protection Agency, 2007). However, there is no evidence to suggest that sCJD can be transmitted via blood. This may be because like bacterial or viral infections, different TSE strains may target different tissues within the body. For example, hepatitis B primarily invades the liver (Wu *et al*., 1991) and HIV attacks the immune system (Zheng *et al*., 2005). In sCJD, the infectivity may target the brain and central nervous system (CNS) and may not accumulate in other tissues or within the blood system.

In individuals infected with vCJD, however, the infectious agent and/or PrP^{Sc} is known to replicate in the lymphoreticular tissues such as the spleen, tonsil and lymph nodes before neuroinvasion (Hill *et al*., 1999). Involvement of the lymphoid tissues in sCJD cannot completely be ruled out as recently PrP^{Sc} has been demonstrated in the spleen in some cases of sCJD, however the role of the lymphoreticular system is thought to be

minimal (Glatzel *et al*., 2003). It is known that lymphocytes continually recirculate between blood and lymphoreticular tissues, which is why the blood of vCJD patients may be more efficient in spreading infection unlike sCJD.

The sheep TSE model may help in the study of the pathogenesis of human TSE disease as the distribution of infectivity and/or PrP^{Sc} is similar in TSE-infected sheep and humans infected with vCJD (Hadlow *et al*., 1982; Van Keulen *et al*., 2000; Jeffrey *et al*., 2001). Therefore results presented in this thesis are may reflect the human situation.

One characteristic of TSE disease is the accumulation of an abnormal form of the prion protein, PrP^{Sc}, which can form amyloid plaques (Prusiner, 1998). Detection of conventional PK-resistant PrP^{Sc} is used as a biochemical marker for TSE infection, and it is possible that PrP^{Sc} may exclusively be the causative agent of TSE disease. This is termed the "protein-only" hypothesis and is supported by experiments which show that scrapie infectivity is reduced by procedures that hydrolyze or modify proteins (Diener *et al*., 1982). Additionally, PrP knockout mice ($Prnp^{o/o}$) appear completely protected against scrapie disease and fail to propagate infectious agent (Bueler *et al*., 1993). If murine PrP transgenes are re-introduced into these mice susceptibility is restored (Fischer *et al*., 1996).

Conventional PK-resistant PrP^{Sc} gives a distinct pattern on Western blots where three distinct bands can be identified and resolve between 27-30 kDa (Collinge *et al*., 1996). The original objective of the research presented in this thesis was to use conventional PrP^{Sc} as a marker for TSE disease in blood to investigate which blood components carry the abnormal PrP isoform and therefore likely infectivity, which would aid in our understanding of disease pathogenesis. So far, most attempts to detect conventional PrP^{Sc} in blood have been unsuccessful. This protein may be difficult to detect in blood as it may only be present at very low levels and as a consequence detection methods require excellent analytical sensitivity. One major aim of this project was give a definitive answer to the question of whether conventional PrP^{Sc} is present in TSE-infected sheep blood in sufficient quantities to explain the infectivity levels shown by

bioassay. It was first therefore necessary to develop and optimise a sensitive Western blot immunoassay for conventional PK resistant PrP^{Sc} using scrapie-infected sheep brain homogenates.

In some rodent models TSE disease develops but abnormal PrP is not detectable indicating that PrP may not always correlate with infectivity (Manson *et al*., 1999). Therefore, another aim of the project was to use a proteomics approach to identify biochemical markers other than conventional PrP^{Sc} to demonstrate TSE infection in blood.

7.2. Conventional PrP^{Sc} may not correlate with the level of TSE infectivity found in sheep blood by bioassay

There are relatively few published records of attempts to detect PrP^{Sc} in blood samples using Western blotting. Wadsworth and colleagues (2001) increased the sensitivity of a Western blot by incorporating a NaPTA concentration step, but when applied to blood, they were unable to detect PrP^{Sc} in the buffy coat fraction of blood from a single vCJD case. However, the starting sample contained only 15 ml of whole blood, and the advantage of using scrapie-infected sheep is that the methodology could be applied to much larger volumes of blood.

Using scrapie-infected sheep brain homogenates, the overall sensitivity of a Western blot immunoassay was increased by incorporating a NaPTA precipitation step to concentrate PrP^{Sc}, and screening a panel of novel monoclonal antibodies raised to recombinant sheep PrP (this thesis). Using the optimised method, PrP^{Sc} was detected in as little as 2 µg wet brain equivalent, although in brain spiking experiments to mimic *in vivo* conditions, the sensitivity was slightly reduced, with consistent recovery of 20 µg brain spikes. This level of sensitivity compared well with the assay developed by Wadsworth and co-workers, and was applied to 50 ml of blood and blood cell subsets (PBMCs and $CD21^{+}$ B cells) from several clinical scrapie-infected sheep. However conventional PrP^{Sc} was still undetectable.

High levels of infectivity are found in the brain of TSE-infected individuals. In sheep and goats clinically affected with natural scrapie, infectivity levels are greater than or equal to 10^4 infectious units (IU)/g of brain homogenate (Hadlow *et al*., 1979, 1980, 1982; Pattison *et al*., 1964, 1972; Groschup *et al*., 1996). Titres of the CJD infectious agent are higher than that of natural scrapie, with levels of 10^8 to $10^{8.3}$ IU/g of brain homogenate (Sklaviadis *et al*., 1989). Rodents infected with the 263 K scrapie strain contain very high levels of infectivity in brain ($10^{9.6}$ IU/g of brain homogenate) (Kimberlin *et al*., 1986). The levels of infectivity clearly differ depending on the TSE agent, the bioassay host and the strain of agent. Levels of infectivity may also vary considerably between different individuals of the same species. Unless the particular isolate being used has been titrated and characterised it is difficult to equate infectivity per gram in this way. However, based on titres of natural scrapie infectious agent, it is possible to estimate that 2 µg of scrapie-infected brain homogenate contains approximately 0.02 infectious units, whereas 20 µg wet brain equivalent contains approximately 0.2 infectious units. It is difficult to estimate the amount of PrP^{Sc} molecules present in these brain amounts as PrP^{Sc} alone may not cause infection. An infectious unit may comprise PrP^{Sc} alone, but could also contain a subspecies of PrP^{Sc} or other molecules yet to be identified.

Attempts have been made to physically separate PrP^{Sc} and TSE infectivity in order to establish the nature of the infectious agent and estimate how many PrP^{Sc} molecules are required to cause infection (Sklaviadis *et al*., 1989; Somerville *et al*., 1999). Recent experiments to disaggregate purified PrP^{Sc} from 263 K scrapie-infected hamster brain using detergents and sonication have produced a wide range of different sized particles. These particles (from very small units up to large amyloid fibrils) can be separated using fractionation techniques. Subsequent bioassay studies indicate that the most infectious units of PrP are 17-27 nm particles of 300-600 kDa, and if composed completely of PrP, would give an oligomer of 14-28 PrP molecules. Clusters of less than six molecules have virtually no infectivity (Silveira *et al*., 2005). This data suggests that between 14-28 molecules of protein monomers are required in a single particle of infectious PrP^{Sc}. This may differ depending on the TSE strain and although

possible, it is currently unknown if infectious PrP in blood could be in the form of these small oligomers.

Because the number of infectious PrP^{Sc} particles within an infectious unit in each TSE strain may differ, it is difficult to estimate exactly how many PrP^{Sc} molecules are present in the scrapie-infected brain homogenate and also in the SMB cells used in this study. Quantification of PrP^{Sc} in brain to give an idea in absolute terms of how much abnormal prion protein in actually detected in wet tissue could be carried out using ELISA or Western blotting techniques and then comparing signals with known recombinant PrP concentrations. SMB cells expressing PrP^{Sc} could be quantified using a colony-lift assay, where PrP^{Sc}-expressing cell colonies can be identified.

Evidence from rodent models show that TSE infected blood contains much lower levels of infectivity than brain. The highest levels of infectivity have been found in the buffy coat fraction of blood. In mice infected with vCJD or a Gerstmann-Straussler-Scheinker (GSS) disease isolate (Fukuoka-1), there were similar infectivity levels of approximately 18-30 IU/ml in buffy coat, but no detectable infectivity in red cells (Cervenakova *et al*., 2003). Infectivity in the blood of clinical mice can rise to 100 IU/ml in buffy coat, and 20 IU/ml in plasma (Brown *et al*., 1999). Whole blood and buffy coat obtained from pre-clinical and clinical sheep infected with BSE or scrapie has also been transfused (intravenously) into healthy animals (Houston *et al*., 2000; Hunter *et al*., 2002; Houston *et al*., 2008). If titres of infectivity in sheep blood are similar to those in rodents (for example, 10 IU/ml) then administration of up to 500 ml of blood would deliver a significant dose (approximately 5000 IU). This suggests there could still be a good chance of transmission, even if the titre of infectivity was relatively low.

It appears that 500 ml of scrapie infected blood is sufficient to transmit infection via the transfusion route in sheep ((Houston *et al*., 2000; Hunter *et al*., 2002; Houston *et al*., 2008). However, the number of infectious units present in 500 ml of blood is unknown. In theory, titres of infectivity in sheep blood may be similar to those in rodents (10 IU/ml) but it is also possible that there is less infectivity (for example 1

IU/ml). Infectivity levels of 1 IU/ml would mean that administration of 500 ml of blood would deliver approximately 500 IU. It is difficult to determine the levels of infectivity present within the starting volume of 50 ml of blood used in this study and the number of cells tested (1 x 10^6 to 1 x 10^8 cells). In addition, regardless of the infectivity levels it does not necessarily mean that a large amount of PrP^{Sc} is present.

In order to establish how the sensitivity limit of the developed Western blot assay (optimised using brain) relates to blood volume, a titration of blood in sheep would have to be carried out in parallel with titrations of the brain material used in this study. This would show if large blood volumes (500 ml) or the 50 ml of blood tested contains infectivity and detectable PrP^{Sc}. Such experiments are required as blood associated PrP^{Sc} is not well defined and there may be differences in sensitivity limits of detection compared to PrP^{Sc} found in brain. If PrP^{Sc} is detected (by Western blot in brain) and correlates with infectivity in smaller blood volumes, it may then be possible to estimate what the concentration of infectivity may be. Presently, this is very difficult to define.

The levels of PrP^{Sc} present in TSE-infected blood remain unknown. Soto and colleagues (2005) reported that using PMCA technology, PrP^{Sc} could be detected in clinical scrapie hamster buffy coat blood samples. They found that 7 rounds of 144 PMCA cycles were optimal for detection of as little as 20 fg/ml or as few as 4 x 10^5 equivalent molecules of PrP^{Sc} /ml. Based on the total buffy coat volume of 20 µl used in their experiments it was estimated (by Western blotting and ELISA, comparing signals with known recombinant PrP concentrations) that approximately 8,000 equivalent molecules of PrP^{Sc} could be detected (Castilla *et al.*, 2005). The PMCA method is extremely sensitive to be able to detect such low PrP^{Sc} levels. Therefore, it is not surprising that when the optimised Western blot was applied to clinical scrapie-infected blood and blood cell subsets, conventional PrP^{Sc} was not detected (this thesis). The 263 K scrapie model contains very high levels of infectivity in brain tissue ($10^{9.6}$ IU/g of brain homogenate) and although 8,000 equivalent molecules of PrP^{Sc} were detected in hamster blood by PMCA, it is conceivable that infection with different TSE agents (such as natural scrapie with a lower titre of infectivity) may even contain lower blood-associated levels of PrP^{Sc}. Low levels of PrP^{Sc} may contribute to transmission of

TSE infection *via* blood with success rates of 36 % (BSE) and 43 % (natural scrapie) in sheep when large volumes of blood are used, resulting in a relatively large dose (Houston *et al*, 2008). However, lower volumes of blood may contain extremely low levels of PrP^{Sc} which may not be present in sufficient quantities to contribute to the spread of infectivity.

7.3. Comparison with virus infectivity in blood.

Viruses such as HIV and hepatitis also infect the blood system and studying mechanisms of virus infection may provide clues as to how TSE infectivity may spread within the body. The HIV virus for example infects the blood by attaching to the $CD4^+$ receptor and co-receptor on the host $CD4^+$ T helper cell (Berger *et al*., 1999), using the enzyme reverse transcriptase to convert viral RNA to DNA, which is then incorporated in to the host cell genes (Zheng *et al*., 2005). The cell then begins to make the proteins of the HIV virus, using the HIV mRNA as a template. Viral envelope proteins are also produced which come together within the host cell membrane to package the viral RNA, enzymes and core proteins. The virus then pinches off the cell, buds and is released into the extracellular environment (Zheng *et al*., 2005). This mechanism is used by many viruses to infect host cells (i.e. HIV, hepatitis B and C). Interestingly, evidence suggests that during the replication process of HIV, defective virus particles are also produced (Bernier and Tremblay, 1995). These defective particles possess a genome which contains deletions of the infectious virus genome, and they can only infect and replicate in a host cell if fully infectious virus is present. This is also true for hepatitis viruses (Terre *et al*., 1991).

Following infection, the TSE agent may replicate in the blood using a similar mechanism. Levels of infectivity increase as infection is released from one blood cell allowing further blood cells to become infected. As replication of the TSE agent occurs, small numbers of defective particles (as described in HIV and hepatitis infection) are also produced. These particles are not required for infection but instead are a by product of the infection process. Defective particles also become apparent in the brain and nervous system as infection spreads and reaches these tissues. It is

possible that the defective particles could be conventional PrP^{Sc}, and that the molecules really responsible for infection consist of a different form of PrP or they may be molecules that as yet have not been identified.

Some viruses (for example, Epstein-Barr virus) have the ability to mimic host proteins which help protect the infectious viral proteins (preventing detection by the host immune system) as they continue to infect the host (Schaadt *et al*., 2005). These protective "mimicked" proteins are not infectious. Following TSE infection, it is possible that the infectious agent may try to mimic host PrP^{C}. However, this process may be inefficient and the mimic of PrP^{C} that is produced may not have all the characteristics of the host PrP^{C}. Instead the infectious agent could produce a protein with a different conformation, which represents conventional PrP^{Sc}. The real molecules causing infection may be buried deep within the protective PrP^{Sc} coat, which is required to enable efficient spread of the infectious agent.

Some viruses have the ability to produce viral proteins which interfere in the normal processing of host proteins on the surface of the cell. Herpes simplex virus for example produces viral proteins able to make subtle changes to host major histocompatibility complex 1 (MHC) molecules. MHC class 1 molecules exist on all cells where they hold and present foreign antigens to CD8 cytotoxic T lymphocytes if the cell has been infected by a virus or other microbe. Following the alteration of the MHC molecules by the virus, the host cell recognises the MHC as defective and ejects it from the cell. This process disrupts the normal host immune response allowing the herpes infection to spread (Fruh *et al*., 1995). Like the herpes virus, the TSE infectious agent may be able to make subtle changes to PrP^{C} processing on the cell membrane, which produces conventional PrP^{Sc} that is not recognised as PrP^{C} and is therefore ejected by the host cell. The conventional PrP^{Sc} is not infectious but as a result of it being produced, the function of PrP^{C} is disrupted enough to allow the real infectious agent (which could be an unrelated molecule) to spread more efficiently. This is theoretical as the exact function of PrP^{C} is unknown and there is no evidence to suggest the proposed biological roles of PrP^{C} could be disrupted following TSE infection. However if PrP^{C} for example played a role in efficient immune system function (and there is evidence

suggesting PrP^C may help in activation and proliferation of T cells (Bainbridge and Walker, 2005), disrupting the host immune system would help promote TSE agent infection. PrP^C may also have several different roles in the central nervous system, and PrP knockout mice show defects in synaptic function (Manson *et al*., 1995), increased vulnerability of neurones to oxidative stress (Brown *et al*., 1999), reduced Cu^{2+}/Zn^{2+} dependent superoxide dismutase (SOD) activity (Brown *et al*., 1999), and changes in membrane localisation of nitric oxide synthase (Ovadia *et al*., 1996). Therefore if the infectious agent is able to disrupt this normal function of PrP^C (by producing PrP^{Sc}) the loss of PrP^C may help contribute to neurodegeneration which is observed in TSE-infected individuals.

7.4. Isoforms of PrP, other than PrP^{Sc}, may be associated with infectivity

The possibility that TSE infectivity is not directly related to PrP^{Sc} may explain why some experimental mouse models (for instance, transgenic mice with a P102L mutation) develop TSE disease but have no detectable conventional PrP^{Sc} (Manson *et al*., 1999; Barron *et al*., 2001). Pathology studies of scrapie-infected mouse brain have shown damaged brain areas without accumulation of PrP^{Sc} (Jeffrey *et al*., 1994; Jeffrey *et al*., 1996) which also indicates that PrP^{Sc} itself my not constitute the infectious agent. TSE infectivity appears to be associated with a wide range of PrP^{Sc} aggregate states (Caughey *et al*., 1997; Silveira *et al*., 2005) and it is possible that smaller particles are more infectious than larger aggregates or amyloid fibrils. It is possible that smaller particles and not aggregated PrP^{Sc} may be responsible for spread of TSE infection, and that these particles may be present in blood.

Attempts have been made to assess if different forms of PrP are associated with infectivity. Recently Piccardo and colleagues (2007) performed bioassay studies using human brain from two cases of Gerstmann- Straussler-Scheinker (GSS) with the P102L prion protein gene mutation. The first case showed accumulation of PrP amyloid in the brain, whereas the second case showed PrP amyloid accumulation and spongiform encephalopathy. TSE disease was transmitted efficiently to transgenic mice (PrP101LL) when brain tissue from the second case was inoculated into the animals.

The recipient mice showed PrP amyloid accumulation in the brain and spongiform degeneration. However, there was almost a complete absence of disease transmission in transgenic mice inoculated with brain material from the first GSS case. PrP amyloid was still present in the brain but there were no clinical signs of disease (Piccardo *et al*., 2007). This data may suggest that infectivity is not associated with PrP amyloid. Alternatively, it is possible that large aggregates of amyloid forming PrP have a protective role and sequester smaller infectious particles into inert non-infectious aggregates.

These explanations may explain why high titres of TSE infectivity are observed in blood, yet conventional PrP^{Sc} is undetectable unless using ultra-sensitive methods of detection (PMCA). However, it should also be noted that the nature of blood-associated PrP^{Sc} is unknown, including sensitivity to digestion with proteinase K. Evidence suggests that PK sensitive forms of PrP^{Sc} exist (Tremblay *et al*., 2004) and it is conceivable that this form of PrP^{Sc} may be present in blood. Therefore this would not be detected by current methods such as Western blotting which rely on treatment with PK to distinguish PrP^{C} from PrP^{Sc}, and could explain why high levels of TSE infectivity may not correlate with conventional PrP^{Sc}. Western blots for blood-associated PrP were carried out with and without PK. A signal for conventional PrP was observed in scrapie-infected blood without prior PK treatment between 30-35 kDa, although it is unclear if this represents PrP^{Sc}. However, following PK digestion no signal was observed indicating that any PrP^{Sc} present in the scrapie-infected blood sample was undetectable at the level achieved by the immunoassay. The PrP^{Sc} may also have been PK sensitive and therefore degraded. PrP^{Sc} can exist in a number of different conformations (Safar *et al*., 1998) and sensitivity to PK digestion may depend on how tightly the PrP^{Sc} molecule is folded. Tightly folded aggregated PrP^{Sc} molecules may be less prone to PK degradation, whereas protein monomers and smaller oligomers may be more easily degraded. If blood-associated PrP^{Sc} in blood is in the form of smaller oligomers, high concentrations of PK would degrade the protein very quickly. However, it is possible that very low PK concentrations would have less of an effect and may produce a signal on a Western blot, although it would still be difficult to

conclude if this represents conventional abnormal prion protein as cellular PrP^{C} may still be present.

7.5. Factors other than conventional PrP^{Sc} in TSE infected sheep blood.

Although conventional PrP^{Sc} was not detected in TSE-infected blood, interestingly bands with higher molecular weights were detected with anti-PrP antibodies in blood samples with prior NaPTA treatment before Western blotting. Initially these bands appeared to be present only in scrapie-infected blood. However, further investigation revealed these bands were also present in uninfected blood. After sample resolution on gels that separate high molecular weight proteins the exact molecular weights were determined. Bands of 60, 112 (a doublet) and 195 kDa were observed. High molecular weight bands in blood have been described previously (Jones *et al*., 2005; Tsukui *et al*., 2007) although the bands described in this thesis are of different molecular weights and have not previously been described in blood using NaPTA based Western blot methods.

Although high molecular weight bands were detected in both scrapie-infected and uninfected blood, it is still possible that they may have importance in TSE disease. The Western blot method does not have the capabilities to show subtle differences/characteristics that may exist in high molecular weight bands from TSE-infected animals compared to controls. The biological role of the high molecular bands is unclear. It is possible that the bands may contain carbohydrate. Following NaPTA precipitation, carbohydrates could cause aggregation with proteins or other molecules, creating high molecular weight complexes in blood. The level of carbohydrate or other components required for aggregation may be different in TSE-infected blood. The infectivity of the high bands has also yet to be tested and it may be that bands from uninfected blood are not infectious but bands from scrapie-infected blood do contain levels of TSE infectivity. PK-resistant PrP molecules have recently been reported in uninfected mouse, hamster and human brain (Yuan *et al*., 2006). These molecules (called “silent prions”) may also exist in blood and could aggregate with themselves, with other proteins or non proteinaceous components. This could explain why high molecular weight bands are also observed in uninfected blood and highlights the need

for further study to try to discriminate the bands present in blood of diseased and healthy animals. "Silent prions" could be present in uninfected and TSE-infected blood, but only contribute to the disease process by interaction with the infectious agent in TSE affected animals.

In addition to high molecular weight bands, a preliminary proteomics study to assess changes in protein expression in buffy coat samples from sheep clinically affected with scrapie against uninfected animals was also carried out. 2D gel and Q-TOF-MS analysis identified three candidate protein biomarkers, lactate dehydrogenase (LDH), elongation factor 1 (EF-1) and annexin 1 (A1). LDH and A1 were elevated in clinical scrapie-infected sheep while EF-1 was down-regulated.

LDH is an enzyme responsible for converting muscle lactic acid into pyruvic acid which is an essential step in producing cellular energy. LDH consists of five tetrameric isoenzymes composed of the random association of two subunits (H and M) that are encoded by different genes (Cahn *et al*., 1962). LDH was found to be up-regulated in the buffy coat blood fraction from sheep clinically infected with scrapie. These results are in agreement with Schmidt and colleagues (2004) who saw elevated LDH levels in CJD patients when compared to individuals suffering from other dementias (Schmidt *et al*., 2004). Further evidence supporting a role for LDH elevation in TSE-infected plasma is also described (Adams and Field, 1967). LDH enzymes are widely distributed throughout the body and following cellular damage LDH is released into the blood stream. Therefore it is conceivable that in TSE disease LDH may be elevated in blood as a result of tissue damage from PrP^{Sc} accumulation in the brain and other organs.

The annexins are a family of closely related calcium and membrane-binding proteins expressed in most eukaryotic cell types (Morgan *et* al., 1997). Although structurally and biochemically similar annexins have diverse functions that include vesicle trafficking, cell division, apoptosis, calcium signalling and growth regulation. Annexin 1 (A1) in particular also has an anti-inflammatory role (Ferlazzo *et al*., 2003). A1 was found to be up-regulated in clinical scrapie-infected buffy coats. To date there appears

to be no evidence suggesting a link between annexin 1 expression and TSEs. However, A1 appears to increase in injured brain tissue (Johnson *et al*., 1989) and it may be that in scrapie-infected sheep this protein is released from brain or inflammatory cells.

Elongation-factor-1 (EF-1) is responsible for aminoacyl-tRNA transfer on the ribosome in the process of protein synthesis, and is comprised of two components, a G-protein named eEF-1A and a nucleotide exchange factor, eEF-1B. Results indicate a possible down-regulation in scrapie-infected buffy coats. The precise biological involvement of EF-1 in TSE infection remains to be determined.

The detection of markers for TSE infection (such as LDH, A1, EF-1 or the high molecular weight bands for example) allows interesting pathogenesis studies to be carried out. In the case of the high molecular weight bands, initially, it would need to be shown that these are significantly different in uninfected and scrapie-infected blood, and then they would have to be identified. If the high molecular weight bands or LDH, A1 or EF-1 genuinely proved to be blood biomarkers of TSE infectivity, it would be interesting to challenge transgenic mice deficient in the biomarker with infectious TSE agent. This may result in a longer incubation period before onset of disease or alternatively, animals may not succumb to disease. This would highlight that the biomarker is essential for pathogenesis. It should be noted though that not all biomarkers may have the same effect on disease. Although PrP^{Sc} has a direct effect on disease (PrP null mice are resistant to scrapie) other proteins or molecules may just be up-regulated and play less of a role in the disease progress (for instance 14-3-3 proteins are up-regulated in neurological disorders but do not have a direct effect on TSE infection (Green *et al*., 2002).

To assess which blood cells might carry the biomarker, blood could be separated into components and blood cell subsets and a proteomics study (similar to the one described in this thesis) could be carried out and validated by Western blotting. This would show which blood cells are important in spreading the infection. Subsequent bioassay studies may establish whether TSE infectivity correlates with accumulation of the marker.

7.6. Summary

This study has demonstrated that conventional PrP^{Sc} may not present in TSE-infected sheep blood in sufficient quantities to explain the infectivity levels observed in TSE-infected sheep blood. An optimised sensitive Western blot, incorporating a NaPTA concentration step was tested on large sheep blood volumes. The pattern of protein detected with novel anti-PrP monoclonal antibodies on Western blots, was very different from the conventional triple banded pattern of PrP^{Sc}. High molecular weight bands of 60, 112 (doublet) and 195 kDa reactive with a number of PrP-specific monoclonal antibodies were apparent in Western blots of NaPTA precipitated scrapie-infected and uninfected blood samples, both before and after PK digestion. These bands were not present in equivalent brain homogenate samples. Such bands have not been reported before using NaPTA based Western blot methods, and may represent a novel form of blood-specific PrP. It is possible that TSE infectivity may be associated with a non-conventional form of PrP although further analysis and identification of the high molecular weight bands is required to conclusively show this. Proteins other than PrP^{Sc} could also be used as markers of TSE infection, and using a proteomics approach three candidate biomarkers, lactate dehydrogenase (LDH), elongation factor 1 (EF-1) and annexin 1 (A1) were identified.

The results presented in this thesis may explain the difficulties found in many labs in developing sensitive detection methods for conventional PrP^{Sc} in blood. TSE infectivity in sheep blood is considerable (Houston *et al*., 2008) but using a very sensitive Western blot immunoassay conventional PrP^{Sc} was not detected. The nature of PrP^{Sc} in blood is unknown, and identification of a non-conventional form would enable some advances within the TSE field. For instance levels of various forms of PrP could be established on various blood cells to gain insights into which cells contribute to TSE pathogenesis. Additionally, such findings would aid in the development of ante

mortem blood diagnostic tests. The effectiveness of control measures currently used by the National Blood Service to safeguard the UK blood supply could also be assessed.

7.7. Future plans

From this study it has become apparent that sheep blood may contain a non-conventional form of PrP. Sensitive western blot analysis using anti-PrP antibodies revealed the presence of high molecular weight bands in scrapie-infected and uninfected sheep blood. The conventional triple protein pattern of PrP^{C} and/or PrP^{Sc} was not observed. However, further investigations are required to conclusively show that the nature of PrP^{Sc} in blood is different to the form associated with brain and lymphoid tissue. Therefore aims for future research include the following:

1. Studies to conclusively identify the PrP-related high molecular weight bands. Initially, mass spectrometry experiments would be carried out on known proteins with or without NaPTA precipitation to establish if NaPTA is causing protein to complex into a state that is not compatible with mass spectrometry. If positive control samples are not identified with NaPTA, it may be that the concentration step is causing possible resistance to enzymatic digestion prior to mass spectrometry so this would also be tested.

2. If non conventional PrP is identified by mass spectrometry techniques, plasma and leukocyte subsets will be examined for the presence of non-conventional forms of PrP. Such subsets would include monocytes as these may be associated with TSE infectivity (Andreoletti *et al*., 2007).

3. Perform a titration of scrapie infected blood in a sheep model testing a range of blood volumes alongside a parallel study of titrated scrapie brain using the blood Western blot method developed in this thesis. Parallel titrations are required because the nature of PrP^{Sc} in blood is not well defined and there may be differences in the sensitivity of detection compared to brain associated PrP^{Sc}.

4. Carry out bioassay studies on blood cell subsets simultaneously with amplification of conventional PrP^{Sc} by PMCA and subsequent Western blotting. This would also help to establish if conventional PrP^{Sc} correlates with TSE infectivity. Additionally, the infectivity of the high molecular weight bands could be tested, and PMCA carried out to establish if this material can be amplified alongside bioassay experiments.

5. If PrP^{Sc} is not identified in the high molecular weight bands by mass spectrometry, test for the presence of non-protein components such as carbohydrates. Experiments could include lectin staining, alcin blue staining, periodic acid-Schiff staining, digoxigenin or Emerald 300. Such experiments may reveal the presence of glycans within the high molecular weight bands. By investigating the characteristics of the high molecular weight bands, disease-related differences may be identified.

6. Establish other protein markers for TSE disease. Preliminary proteomic studies indicate three potential TSE candidate biomarkers; LDH and A1 appeared up-regulated in buffy coat following scrapie infection, while EF-1 appeared down-regulated. It is important to repeat these preliminary studies, establish normal levels of these proteins in a range of healthy animals. If levels appear similar in uninfected sheep, it would then be interesting to see the effect of scrapie incubation periods on knock out mice. Additionally, it would be intriguing to establish if LDH and A1 are up-regulated on leukocyte subsets during scrapie infection and if EF-1 is down-regulated. The TSE infection process may be responsible for up or down-regulation of these proteins, as apposed to these proteins influencing infection. It is possible though that these candidate biomarkers may correlate with TSE infectivity. By measuring the levels of blood-associated LDH, A1 and EF-1 it may be possible to establish where the infection travels in the body and which tissues/blood cells become infected.

Chapter 8

Appendices

Appendix 1: Antibodies

Antibodies used in this study are tabulated below

Name	Subclass	Antigen	Supplier	Reference	Use
CC15	IgG2a	γ/δ T cells	IAH, Compton	Clevers *et al*., 1990	Flow Cytometry 1:400
CC21	IgG1	CD21 (B cells)	IAH, Compton	Sopp (1996)	Flow Cytometry 1:10 MACS 1:10
CC63	IgG2a	CD8 (T cell subset)	IAH, Compton	MacHugh *et al*., 1991	Flow Cytometry 1:10 MACS 1:10
CC-G33	IgG1	CD14 (monocytes)	IAH, Compton	Sopp *et al*., 1996	Flow Cytometry 1:10
DU2-104	IgM	CD72 (B cells)	Dr W. Hein, Basel Institute for Immunology, Switzerland	Young *et al*., 1997	Flow Cytometry 1:10
36F	IgG1	CD2 (α/β T cell receptor)	Dr W. Hein, Basel Institute for Immunology, Switzerland	Mackay *et al*., 1988	Flow Cytometry 1:10

Appendix 1: Antibodies

Name	Subclass	Antigen	Supplier	Reference	Use
44-97	IgG1	CD4 (T cell subset)	Dr E. Meeusen, University of Melbourne, Australia	Maddox *et al*., 1985	Flow Cytometry 1:10
TRT 1	IgG1	Turkey rhinotracheitis	IAH, Compton	Cook *et al*., 1993	Flow Cytometry 1:10 Western Blotting 1:200
TRT 3	IgG2a	Turkey rhinotracheitis	IAH, Compton	Cook *et al*., 1993	Flow Cytometry 1:10
Monoclonal antibody AE11	IgG1	Recombinant sheep PrP (residues 90-233)	Dr S McCutcheon, IAH, Compton	Dr S McCutcheon (manuscript in preparation)	Western Blotting 1:200 1:500 1:1000

Appendix 1: Antibodies

Name	Subclass	Antigen	Supplier	Reference	Use
Monoclonal antibody BC6	IgG1	Recombinant sheep PrP (residues 90-233)	Dr S McCutcheon IAH, Compton	Dr S McCutcheon (manuscript in preparation)	Western Blotting 1:200 1:500 1:1000 1:2000 1:5000
Monoclonal antibody BD12	IgG1	Recombinant sheep PrP (residues 90-233)	Dr S McCutcheon IAH, Compton	Dr S McCutcheon (manuscript in preparation)	Western Blotting 1:1000
Monoclonal antibody BF5	IgG1	Recombinant sheep PrP (residues 90-233)	Dr S McCutcheon IAH, Compton	Dr S McCutcheon (manuscript in preparation)	Western Blotting 1:1000

Appendix 1: Antibodies

Name	Subclass	Antigen	Supplier	Reference	Use
Monoclonal antibody BH1	IgG1	Recombinant sheep PrP (residues 90-233)	Dr S McCutcheon IAH, Compton	Dr S McCutcheon (manuscript in preparation)	Western Blotting 1:200 1:500 1:1000 1:2000 1:5000
Monoclonal antibody CF5	IgG2a	Recombinant sheep PrP (residues 90-233)	Dr S McCutcheon IAH, Compton	Dr S McCutcheon (manuscript in preparation)	Western Blotting 1:1000
Monoclonal antibody DB12	IgG1	Recombinant sheep PrP (residues 90-233)	Dr S McCutcheon IAH, Compton	Dr S McCutcheon (manuscript in preparation)	Western Blotting 1:1000
Monoclonal antibody DC12	IgG1	Recombinant sheep PrP (residues 90-233)	Dr S McCutcheon IAH, Compton	Dr S McCutcheon (manuscript in preparation)	Western Blotting 1:1000

Appendix 1: Antibodies

Name	Subclass	Antigen	Supplier	Reference	Use
Monoclonal antibody DE3	IgG1	Recombinant sheep PrP (residues 90-233)	Dr S McCutcheon IAH, Compton	Dr S McCutcheon (manuscript in preparation)	Western Blotting 1:1000
Monoclonal antibody EA6	IgG1	Recombinant sheep PrP (residues 90-233)	Dr S McCutcheon IAH, Compton	Dr S McCutcheon (manuscript in preparation)	Western Blotting 1:1000
Monoclonal antibody EC9	IgG2a	Recombinant sheep PrP (residues 90-233)	Dr S McCutcheon IAH, Compton	Dr S McCutcheon (manuscript in preparation)	Western Blotting 1:1000
Monoclonal antibody EG6	IgG1	Recombinant sheep PrP (residues 90-233)	Dr S McCutcheon IAH, Compton	Dr S McCutcheon (manuscript in preparation)	Western Blotting 1:1000
Monoclonal antibody FD1	IgG1	Recombinant sheep PrP (residues 90-233)	Dr S McCutcheon IAH, Compton	Dr S McCutcheon (manuscript in preparation)	Western Blotting 1:1000
Monoclonal antibody FD12	IgG1	Recombinant sheep PrP (residues 90-233)	Dr S McCutcheon IAH, Compton	Dr S McCutcheon (manuscript in preparation)	Western Blotting 1:1000

Appendix 1: Antibodies

Name	Subclass	Antigen	Supplier	Reference	Use
Monoclonal antibody FH6	IgG1	Recombinant sheep PrP (residues 90-233)	Dr S McCutcheon IAH, Compton	Dr S McCutcheon (manuscript in preparation)	Western Blotting 1:1000
Monoclonal antibody FH10	IgG2a	Recombinant sheep PrP (residues 90-233)	Dr S McCutcheon IAH, Compton	Dr S McCutcheon (manuscript in preparation)	Western Blotting 1:500 1:1000
Monoclonal antibody HB4	IgG1	Recombinant sheep PrP (residues 90-233)	Dr S McCutcheon IAH, Compton	Dr S McCutcheon (manuscript in preparation)	Western Blotting 1:1000
Monoclonal antibody HC2	IgG1	Recombinant sheep PrP (residues 90-233)	Dr S McCutcheon IAH, Compton	Dr S McCutcheon (manuscript in preparation)	Western Blotting 1:1000
Monoclonal antibody HC7	IgG1	Recombinant sheep PrP (residues 90-233)	Dr S McCutcheon IAH, Compton	Dr S McCutcheon (manuscript in preparation)	Western Blotting 1:500 1:1000
Monoclonal antibody IF1	IgG1	Recombinant sheep PrP (residues 90-233)	Dr S McCutcheon IAH, Compton	Dr S McCutcheon (manuscript in preparation)	Western Blotting 1:1000

Appendix 1: Antibodies

Name	Subclass	Antigen	Supplier	Reference	Use
Monoclonal antibody IH9	IgG2b	Recombinant sheep PrP (residues 90-233)	Dr S McCutcheon IAH, Compton	Dr S McCutcheon (manuscript in preparation)	Western Blotting 1:200 1:500 1:1000
Monoclonal antibody IH11	IgG2a	Recombinant sheep PrP (residues 90-233)	Dr S McCutcheon IAH, Compton	Dr S McCutcheon (manuscript in preparation)	Western Blotting 1:1000
Monoclonal antibody JB10	IgG1	Recombinant sheep PrP (residues 90-233)	Dr S McCutcheon IAH, Compton	Dr S McCutcheon (manuscript in preparation)	Western Blotting 1:500 1:1000
Monoclonal antibody JC4	IgG1	Recombinant sheep PrP (residues 90-233)	Dr S McCutcheon IAH, Compton	Dr S McCutcheon (manuscript in preparation)	Western Blotting 1:1000
Polyclonal antibody Anti-Lactate Dehydrogenase	IgG	Lactate Dehydrogenase	Rockland Immunochemicals, USA	Sass *et al*., 1989	Western Blotting 1:1000

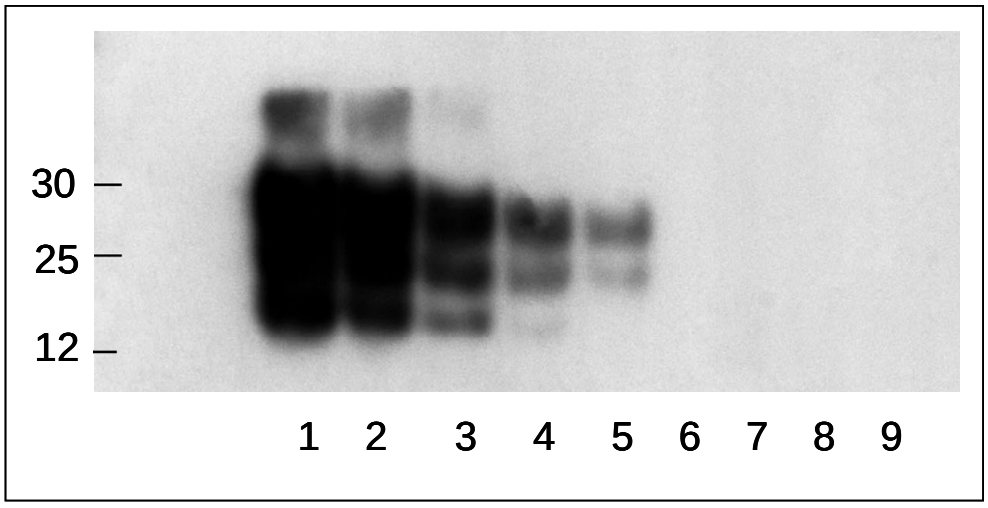
Appendix 1: Antibodies

Name	Subclass	Antigen	Supplier	Reference	Use
Monoclonal antibody Anti β-actin clone AC15	IgG1	Actin	Sigma Aldrich, UK	North *et al*., 1994	Western Blotting 1:5000
Goat anti-mouse HRP	IgG	Mouse IgG F(ab')$_2$	Sigma Aldrich ,UK	Sigma Aldrich, UK	Western Blotting 1:2000 1:2500 1:5000 1:7000 1:10,000 1:100,000 1:200,000 1:500,000
Rabbit anti-goat HRP	IgG	Goat IgG F (ab')$_2$	Sigma Aldrich, UK	Sigma Aldrich, UK	Western Blotting

Appendix 1: Antibodies

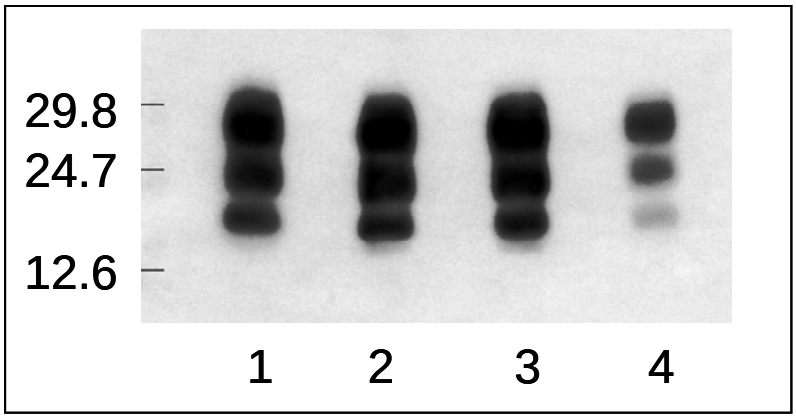
Name	Subclass	Antigen	Supplier	Reference	Use
Goat anti-mouse FITC	IgG1 IgM	Mouse IgG1 Mouse IgM	Southern Biotechnology, USA	The *et al*., 1970	Flow Cytometry 1:200
Goat anti-mouse PE	IgG	Mouse IgG	Southern Biotechnology, USA	Southern Biotech	Flow Cytometry 1:400

Appendix 2: Serial dilutions of PK-resistant PrP^{Sc} from scrapie-infected sheep brains



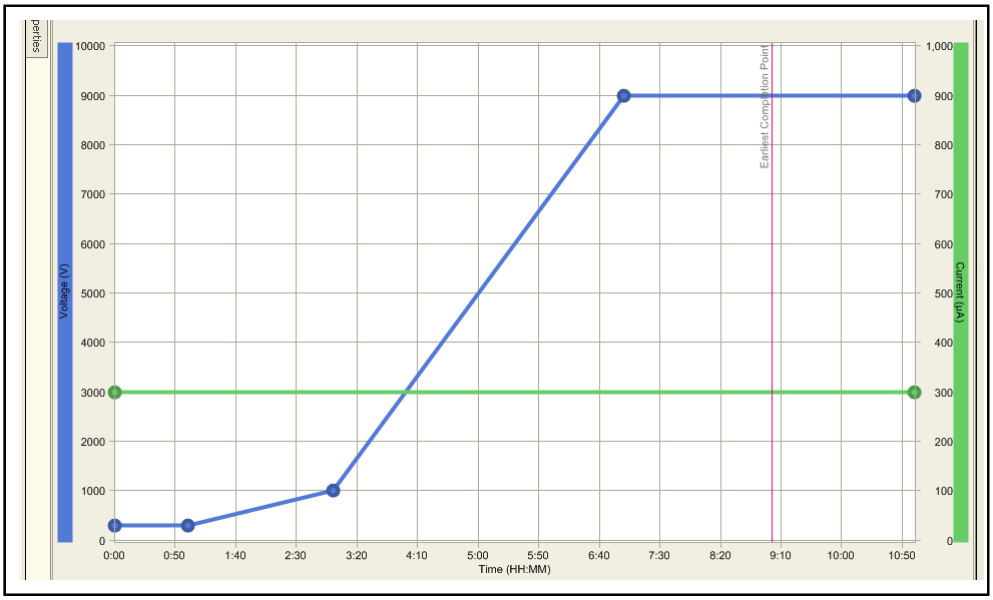
Appendix 2: Western blot analysis of serial dilutions of NaPTA precipitated PK-resistant PrP^{Sc} from scrapie-infected sheep brains. Wells were loaded with serial dilutions of the PK digestion products from natural scrapie infected sheep brains. In lane 1 the equivalent of 2500 µg wet tissue was loaded, in lane 2, 1250 µg, in lane 3, 625 µg, lane 4, 313 µg, lane 5, 156 µg, lane 6, 78 µg, lane 7, 19 µg, lane 8, 9 µg, and lane 9, 5 µg. The blot was probed with the mAb IH9, and was developed using standard ECL methods; the exposure time was 10 sec. Molecular masses are shown on the left (kDa).

Appendix 3. Detection of PK-resistant PrP^{Sc} is not affected by sample storage Conditions



Appendix 3: Detection of NaPTA precipitated PK-resistant PrP^{Sc} is not affected by sample storage conditions (lanes 1-3). Wells were loaded with the PK digestion products from scrapie infected sheep brains. 2.1 mg wet tissue equivalent was loaded in each well. Fresh samples were loaded into lane 1, samples stored at -20 ºC in lane 2, and samples stored at -80 ºC in lane 3. The blot was probed with the mAb IH9 at a concentration of 1 µg/ml. In lane 4 the sample was not NaPTA precipitated. The blot was developed using standard ECL methods; the exposure time 2 sec. Molecular masses are shown on the left (kDa).

Appendix 4. Buffy coat IEF template



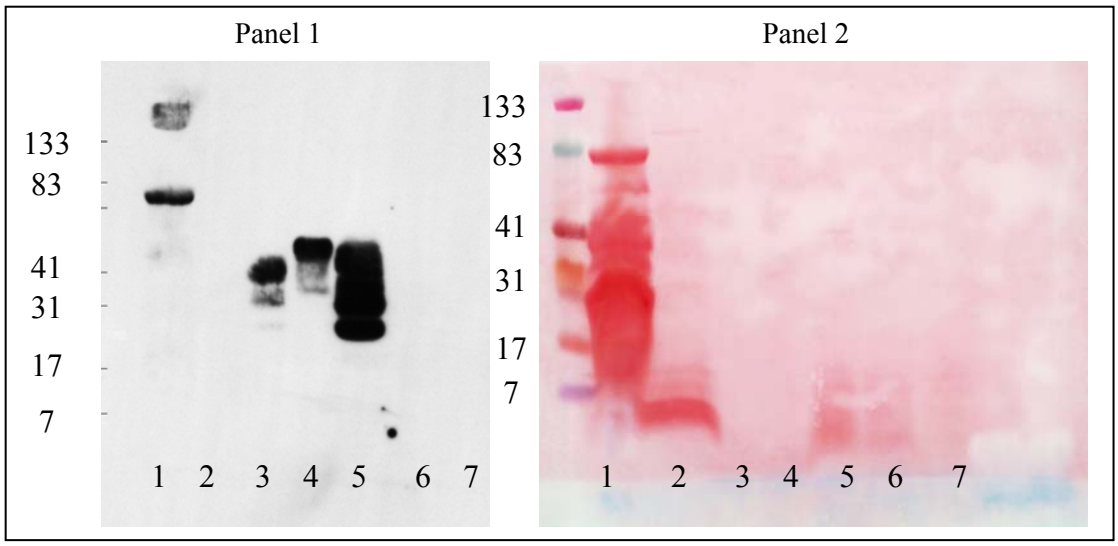
Key: Blue = set volts, Green = set mA

Appendix 4: Buffy coat IEF template. The traces corresponding to the current and voltage are described in the key above. IPG strips containing scrapie-infected and uninfected buffy coat proteins are subjected to IEF for 40 k V hr using this template.

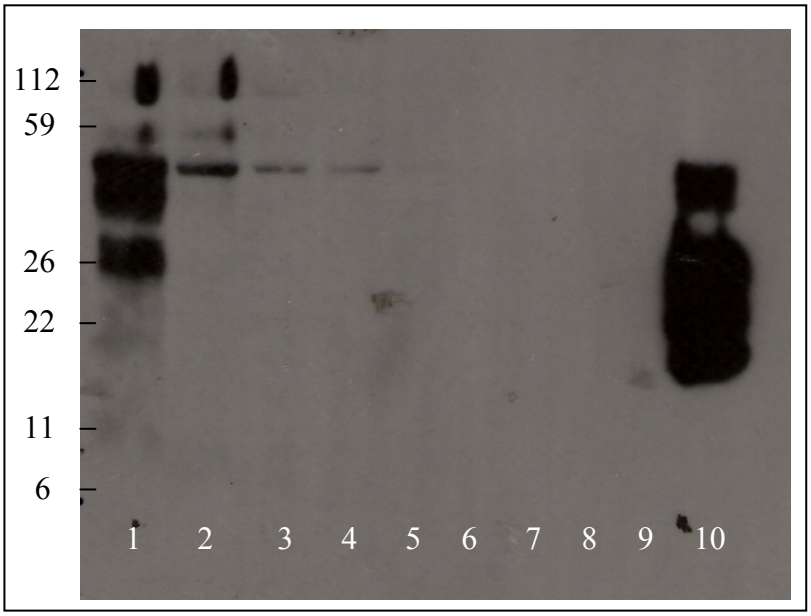
Appendix 5. Application of the blood Western blot to buffy coat and PBMC samples from seven scrapie infected sheep.

The blood Western blot method was applied to frozen buffy coat and PBMCs from a starting volume of 50 ml obtained from ten sheep clinically affected with scrapie. The number of buffy coat or PBMC cells obtained from 50 ml of blood ranged from between 1 x 10^6 to 1 x 10^8 cells. The cell numbers analysed in each blot are detailed in each figure legend. Each experiment was repeated three times.

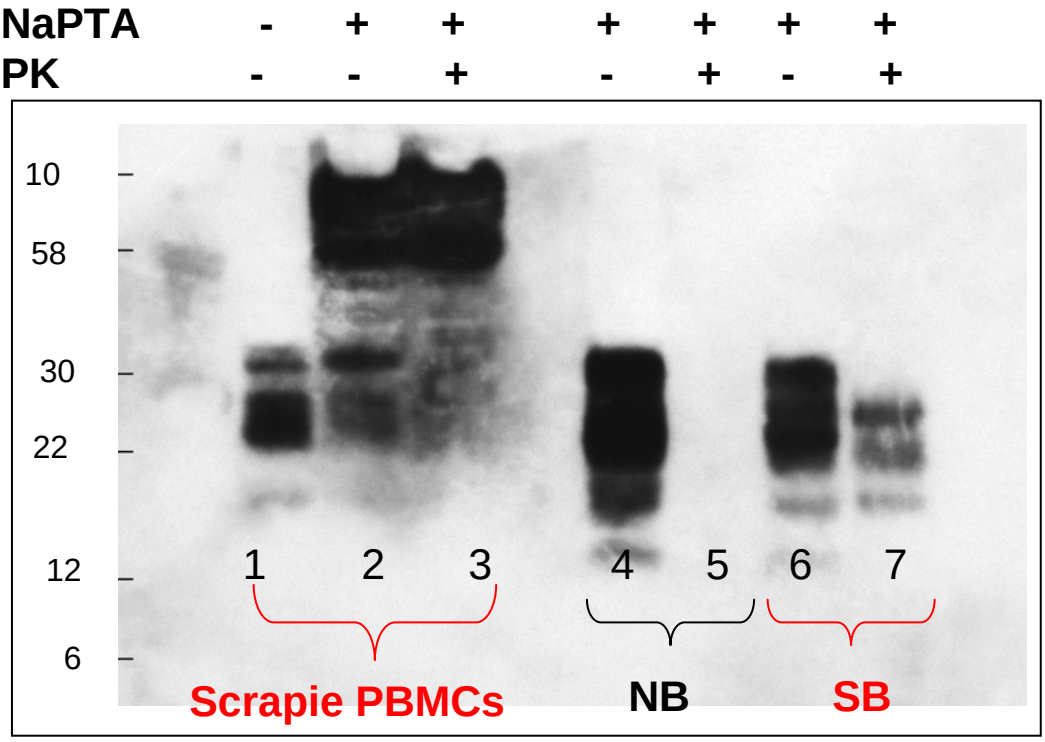
In chapter 4, representative blots from three animals clinically affected with scrapie are shown. Representative blots where blood from the remaining seven sheep clinically affected with scrapie was tested are shown below.



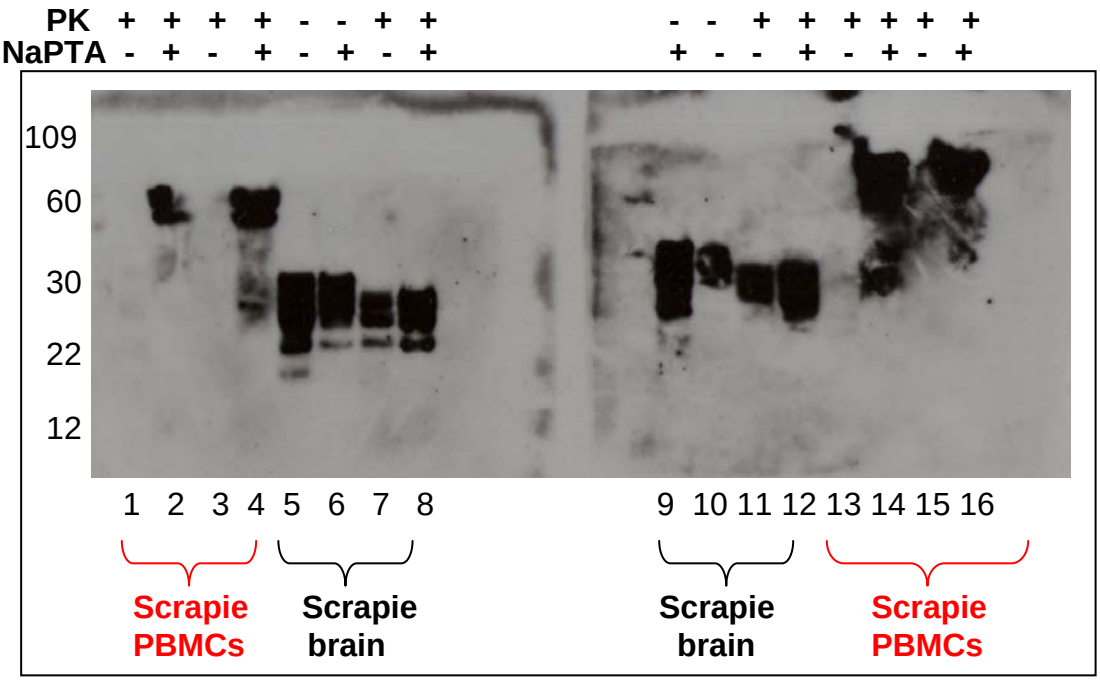
Appendix 5.1: Western blot analysis of a PK digested, NaPTA precipitated buffy coat sample from one clinical scrapie infected sheep. Key: Panel 1, Western blot: panel 2, Ponceau S stained membrane . Lane 1, 1 x 10^8 buffy coat cells: lane 2, freezing media: lane 3, 5 x 10^5 SMB cells: lane 4, 500 µg wet tissue equivalent of normal brain homogenate: lane 5, 500 µg wet tissue equivalent of scrapie infected brain homogenate: lane 6 & 7, 300µl of wash buffer (PBS). The blot was probed with the mAb BC6 at a concentration of 2 µg/ml and developed using standard ECL methods; the exposure time was 1 hr. Molecular masses are shown on the left (kDa).



Appendix 5.2: Western blot analysis of PK digested, NaPTA precipitated titrated buffy coat cells from one clinical scrapie infected sheep. Key : lane 1, 5 x 10^7 buffy coat cells: lane 2, 1 x 10^7 , lane 3, 5 x 10^6 : lane 4, 1 x 10^6 : lane 5, 5 x 10^5: lane 6, 1 x 10^5: in lane 7, 5 x 10^4 : lane 8, 1 x 10^4 : lane 9, 200 µg wet tissue equivalent of scrapie infected brain homogenate. The blot was probed with the mAb BC6 at a concentration of 2 µg/ml and developed using standard ECL methods; the exposure time was 30 min. Molecular masses are shown on the left (kDa).



Appendix 5.3: High molecular weight bands are PK resistant in PBMCs from scrapie-infected sheep. Key: In lanes 1, 2 and 3, approx 1 x 10^6 scrapie infected PBMCs from one scrapie infected sheep. In lanes 4 & 5 wells were loaded with 300 µg wet tissue equivalent of uninfected sheep brain homogenate. In lanes 6 & 7 wells were loaded with 300 µg wet tissue equivalent of sheep scrapie brain homogenate. Samples were PK digested and NaPTA precipitated as indicated. The blot was probed with the mAb BC6 at a concentration of 2 µg/ml and developed using standard ECL methods; the exposure time was 5 min. Molecular masses are shown on the left (kDa).



Appendix 5.4: High molecular weight bands are PK resistant in PBMCs from scrapie-infected sheep. Key: In lanes 1 & 2, approx 1 x 10^6 scrapie infected PBMCs from one scrapie infected sheep. In lanes 3 & 4, approx 1 x 10^6 scrapie infected PBMCs from a second scrapie infected animal. Lanes 13 & 14 and 15 & 16 represent approx 1 x 10^6 scrapie infected PBMCs from a third and fourth scrapie infected sheep respectively. In lanes 5 – 12, wells were loaded with 300 µg wet tissue equivalent of sheep scrapie brain homogenate. Samples were PK digested and NaPTA precipitated as indicated. The blot was probed with the mAb BC6 at a concentration of 2 µg/ml and developed using standard ECL methods; the exposure time was 5 min. Molecular masses are shown on the left (kDa).

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