

**An Investigation of Scrapie Pathogenesis in Neonatal  
Mice with Special Reference to Germinal Centre  
Maturation**

**Michelle Ierna**

**A thesis submitted in partial fulfillment of the requirements of the  
University of Edinburgh for the degree of Doctor of Philosophy.**

The programme of research was carried out at the Institute for Animal Health,  
Neuropathogenesis Unit, Edinburgh.

August 2001



## **Declaration**

I declare that the work presented in this thesis is my own, except where otherwise stated. All experiments were designed by myself, in collaboration with my supervisors Dr Moira Bruce and Dr John Fazakerley unless otherwise stated. No part of this work has been, or will be, submitted for any other degree, or professional qualification.

Michelle Ierna

August 2001

## Acknowledgements

I would like to express my gratitude to my supervisors **Moira Bruce** and **John Fazakerley** for giving me the opportunity to undertake this project. The development of the thesis would not have been possible without their constant support, mentoring, insightful discussions and careful scrutiny of the manuscript. Thankyou both for your encouragement over the past 4 years.

I would like to acknowledge the work carried out by **George Outram, Hugh Fraser and Alan Dickinson**, at the Animal Breeding Research Organisation. Their experiments laid the foundations for this thesis and made the project possible in the first place.

Respect and many thanks also to **Christine Farquhar, Neil Mabbott, Karen Brown and Patricia McBride** (The Peripheral Pathogenesis group, NPU) who were never too busy to share their time and for many hours of discussion, mentorship and training. Again, their work has been crucial in the development of the thesis and I will be forever indebted to them.

I would also like to express my gratitude to the following people for their excellent technical support during the course of the project:

To **Irene McConnell, Jenny Beaton and Nicola McAllister** (NPU animal facility), for assistance provided with animal husbandry over the past four years.

To **Aileen Boyle** and **Wing Gee Liu**, for scoring of brains and generally keeping me amused in the microscope room.

To **Anne Suttie** (NPU) for initiating me into the world of histology and who was never too busy to prepare sections for me in moments of desperation.

To **Diane Ritchie**, for assisting with intravenous injections.

To **Neil McLennan and Kathleen Rennison** (National CJD Surveillance Unit, Edinburgh), who at short notice generously assisted with *in situ* hybridisations.

To **Jill Sales** (Napier University) for her advice and assistance with the statistical analysis of the incubation period data.

To **Robert Burns** who kindly helped with some last minute ELISAs whilst I was writing up.

I would like to take this opportunity to say a **BIG** thankyou to **Adrian, Mum and Dad** and to the rest of my family and friends for all their support and patience over the past few years. Hopefully I will now regain some sense of normality (and a social life!) in the coming months.

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## Abbreviations

ABC	Avidin Biotin Complex method used in immunocytochemistry
APAAP	Alkaline phosphatase anti- alkaline phosphatase immune complex
BCIP	5-bromo-4-chloro-indolyl-phosphate
BLB	Brain Lysis Buffer
BLC	B lymphocyte chemoattractant
BSE	Bovine spongiform encephalopathy
CJD	Creutzfeldt-Jakob Disease
CoVF	Cobra venom factor
CR1	Complement receptor 1
CR2	Complement receptor 2
cRNA	complementary ribonucleic acid
d.o	days old
d.p.i	days post inoculation
DAB	Diaminobenzidine
DIG	Digoxigenin
DNA	deoxyribonucleic acid
Dpl	Doppel
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
FDC	Follicular dendritic cell
FFI	Fatal Familial Insomnia
FITC	Fluorescein Isothiocyanate
FMS	Fresh mouse serum
FRC	Follicular Reticular Cell
FSE	Feline Spongiform Encephalopathy
HRP	Horse Radish Peroxidase
i.c	Intracerebral inoculation
i.p	Intraperitoneal inoculation

i.v	Intravenous inoculation
ICC	Immunocytochemistry
Ig	Immunoglobulin
IHSB	Iodine High Salt Buffer
IL-6	Interleukin 6
ISH	<i>In situ</i> hybridisation
kDa	KiloDaltons
Lt $\beta$	Lymphotoxin $\beta$
Lt $\beta$ R-Ig	Lymphotoxin $\beta$ receptor –human immunoglobulin fusion protein
MBM	Meat and bone meal
mRNA	messenger RNA
NBT	Nitroblue Tetrazolium Salt
NEM	N- ethylmaleimide
NPU	Neuropathogenesis Unit
PAP	Peroxidase anti peroxidase immune complex
PK	Proteinase K
PLP	periodate lysine paraformaldehyde
PMSF	Phenylmethylsulphonyl Fluoride
PNA	Peanut agglutinin
PNS	Peripheral nervous system
<i>Prn-p</i>	Mouse prion protein gene
PrP <sup>c</sup>	Cellular prion protein
PrP <sup>res</sup>	Protease resistant PrP
PrP <sup>sc</sup>	Scrapie specific PrP
PrP <sup>sen</sup>	Protease sensitive PrP
RML	Rocky Mountain Laboratory
RT-PCR	Reverse transcriptase polymerase chain reaction
s.c	Subcutaneous inoculation
SAF	Scrapie associated fibrils
SCID	Severe Combined Immunodeficient mice
SDS	Sodium dodecyl sulphate

SFV	Semliki Forest Virus
<i>Sinc</i>	Scrapie incubation period gene
SSC	Saline sodium citrate
TCR- $\alpha$	T cell receptor $\alpha$
TE	Tris/EDTA
TME	Transmissible Mink Encephalopathy
TNF- $\alpha$	Tumour necrosis factor $\alpha$
TNFR1	Tumour necrosis factor receptor 1
tRNA	Transfer RNA
TSE	Transmissible Spongiform Encephalopathy
vCJD	Variant Creutzfeldt Jakob Disease

## Abstract

Studies carried out during the 1970s-1980s, demonstrated that neonatal mice are less susceptible than adult mice to intraperitoneal (i.p) but not intracerebral (i.c) challenge with the ME7 strain of scrapie. Using doses that are 100% lethal to adult mice, some peripherally challenged neonatal mice resist scrapie infection and survive or have significantly longer incubation periods than mice challenged as adults. Susceptibility to scrapie increases between 7-10 days after birth and no survivors are observed.

These experiments were repeated in order to determine whether neonatal mice could sustain scrapie replication within the spleen. The presence of scrapie associated protease resistant prion protein ( $\text{PrP}^{\text{Sc}}$ ) was used as a marker for infectivity.  $\text{PrP}^{\text{Sc}}$  could not be detected by Western blotting, 70 days after infection in the spleens of neonatally challenged mice, a time when infectivity has plateaued within spleens of mice inoculated as adults. This suggests that neonatal mice have an impaired ability to replicate scrapie and therefore resist scrapie infection.

In adult mice, cellular prion protein ( $\text{PrP}^{\text{C}}$ ) expressing follicular dendritic cells (FDCs) in the spleen are required for replication of scrapie after i.p challenge. It is therefore hypothesised that neonatal mice resist scrapie infection due to lack of mature  $\text{PrP}^{\text{C}}$  expressing FDCs within spleen. The presence of  $\text{PrP}^{\text{C}}$  and the maturation of FDCs were studied in the spleens of developing mice using immunocytochemistry and confocal microscopy. It was found that FDC-M1+ cells can be detected from birth within the immature spleen white pulp. However detectable levels of the FDC functional marker, complement receptor 1 were not observed until 14 days after birth, inferring that the FDCs are functionally immature prior to this timepoint.  $\text{PrP}^{\text{C}}$  was detected from 10 days old immunocytochemically, and was found to co-localise with FDC-M1+ cells with an immature dendritic morphology compared to 30 day old mice. It can be concluded that  $\text{PrP}^{\text{C}}$  is expressed on immature FDCs in the 10 day old mouse. The detection of  $\text{PrP}^{\text{C}}$  on FDC-M1+ cells between 10 and 14 days coincides with a dramatic increase in susceptibility to scrapie. This is consistent with

the hypothesis that neonatal mice have an impaired ability to replicate scrapie due to insufficient PrP<sup>c</sup> expressing FDCs in the spleen.

Paradoxically, previous experiments have shown a proportion of peripherally inoculated neonatal mice to develop scrapie after very short incubation periods ranging between 150-230 days. During the first two weeks of life, the number of cases with short incubation periods decreases. It is hypothesised that due to the immaturity of peripheral nerves at birth, infectivity can be taken up more efficiently by neonatal peripheral nerve endings and reach the central nervous system (CNS) without prior replication in the spleen. Adult severe combined immunodeficient mice (SCID) are resistant to scrapie due to a lack of mature FDCs. Neonatal SCID mice offer a model with which to test this hypothesis without the complicating factor of a developing lymphoreticular system. After subcutaneous challenge, a large proportion of neonatally inoculated SCID mice developed scrapie after a very short incubation period compared to SCIDs challenged as adults. This suggests that infectivity can access the CNS with greater ease in neonates compared to adults and since SCID mice do not possess functional FDCs, demonstrates that the mechanism is not dependent on prior FDC replication.

In conclusion, the results presented in this thesis demonstrate that FDC dependent and non-FDC dependent mechanisms exist in the neonatal mouse for peripheral scrapie pathogenesis.

# Chapter 1

## Introduction

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# 1.0 Introduction

Scrapie is a naturally occurring, invariably fatal neurodegenerative disease affecting the brains of sheep (Dickinson, 1976; Hunter, 1972), which was first documented in England over three hundred years ago by Comber in 1772 (quoted by Stamp, 1964). The earliest signs of scrapie include pruritus (itching of the skin resulting in sheep rubbing themselves against fixed objects and causing wool loss and skin inflammation), hyperexcitability and trembling (Stamp, 1964). These signs are followed by motor incoordination, abnormal posture, paralysis and death (Stamp, 1964). There is no specific immune response associated with scrapie (Clarke and Haig, 1966) and as a consequence there is still no serological test to identify exposed animals at any stage of scrapie infection.

Scrapie belongs to a family of Transmissible Spongiform Encephalopathies (TSEs) that also include Bovine Spongiform Encephalopathy (BSE) in cattle (Wells et al., 1987) and various forms of Creutzfeldt-Jacob disease (CJD) in humans (Will et al., 1996). These diseases occur as familial, sporadic or acquired infections as detailed in Table 1.1.

Public interest in the TSEs has been fuelled by the recent emergence of BSE in cattle, and the subsequent appearance of variant CJD (vCJD) in humans (Will et al., 1996). The origins of BSE are still widely debated. However, a close correlation has been found between feeding meat and bone meal (MBM- an animal derived protein source) to cattle during the 1970s-1980s and the appearance of the BSE. (Horn et al., 2001). During this period in the United Kingdom, (UK), sheep outnumbered cattle by 2:1 (Horn et al., 2001). It is likely that a higher percentage of sheep entered MBM processing, compared to cattle. Although difficult to ascertain, it is therefore possible that scrapie infected sheep entering the MBM manufacturing process could have been responsible for the emergence of BSE in cattle (Horn et al., 2001).

<b>TSE</b>	<b>Species</b>	<b>Comments</b>	<b>Ref</b>
Scrapie	Sheep	Prototype TSE, first documented 300 years ago by Comber, 1772. Transmitted experimentally to goats and rodent species	Chandler, 1961.
Bovine Spongiform Encephalopathy (BSE)	Cattle	May have arisen sporadically within the cattle population and passaged through ingestion of BSE contaminated MBM. Alternatively may have arisen through ingestion of scrapie contaminated MBM.	Wells et al, 1987.
Feline Spongiform Encephalopathy (FSE)	Domestic cats	Caused by same agent as BSE. Probably acquired through ingestion of BSE contaminated food	Pearson et al, 1991.
Novel TSEs	Exotic ungulates and large cats in zoos	Caused by same agent as BSE.	Bruce et al, 1997.
Transmissible Mink Encephalopathy (TME)	Mink	Observed in farmed mink possibly due to ingestion of scrapie contaminated offal	Marsh and Kimberlin, 1975.
Chronic Wasting Disease	Mule Deer and elk	Observed in wild and farmed deer	Williams and Young, 1980.
Sporadic Creutzfeldt-Jakob Disease (spCJD)	Human	A rare disease, not linked to PRN-P mutations. It has a worldwide distribution. No environmental risk factors identified	Creutzfeldt, 1920. Jakob, 1921.
Iatrogenic CJD (iCJD)	Human	Transmission through neurosurgery and human pituitary derived growth hormone treatment	Collinge et al, 1991.
Familial CJD (fCJD)	Human	Associated with PRN-P mutations	Goldfarb et al, 1996.
Variant CJD (vCJD)	Human	Acquired disease affecting mainly young people. vCJD and BSE caused by same TSE strain. Probably transmitted from cattle to humans through eating BSE contaminated beef products.	Will et al, 1996. Bruce et al, 1997.
Gerstmann Straussler Scheinker Syndrome (GSS)	Human	CJD-like illness associated with PRN-P mutations	Gerstmann et al, 1936.
Fatal Familial Insomnia (FFI)	Human	CJD-like illness associated with PRN-P mutations	Gambetti et al 1992.
Kuru	Human	Acquired disease which was probably passaged in humans through ritual cannibalism of dead relatives.	Gajdusek and Zigas, 1957.

**Table 1.1: Natural TSE diseases**

It is of interest that during the 1970s, the use of MBM to supplement feed for newborn calves of 1-12 weeks of age was common practise in the U.K (Horn et al., 2001). This practise was not adopted in continental Europe or the United States (Horn et al., 2001). Prior to 1970 in the U.K, cattle would not have received MBM until 2 years old. This suggests that young calves may be more susceptible than older cattle to BSE. In sheep, certain studies have demonstrated that lambs can acquire scrapie via maternal transmission either *in utero* or shortly after birth, and this is discussed in more detail in Chapter 7.

Recently vCJD affecting mainly young adults has been shown to be caused by the same TSE strain as BSE (Bruce et al., 1997). The most likely mode of BSE transmission into the human population is by ingestion of BSE contaminated beef products. However, the reasons why relatively young people succumb to vCJD are still unclear.

The current interest in the possible age-related susceptibility of humans, cattle and sheep to natural TSEs has prompted a re-examination of experimental data demonstrating an age-related susceptibility of mice to scrapie. These studies were carried out in the Neuropathogenesis Unit, Edinburgh during the 1970s-1980s. It was found that a proportion of neonatal mice were resistant to scrapie after intraperitoneal (i.p) challenge with doses that were 100% lethal to adult mice (Outram et al., 1973). Paradoxically, it was found that a proportion of peripherally challenged neonatal mice succumbed to scrapie after significantly shorter incubation periods than would be expected for adult mice.

Two hypotheses (outlined in section 1.3) were developed to predict the possible biological mechanisms responsible for the age-related differences in susceptibility of neonatal mice to scrapie peripheral challenge. These hypotheses have been tested during the course of the project with a view to gaining insights into the possible reasons for the apparent age-related susceptibility of humans, cattle and sheep to natural TSEs.

## 1.1 The nature of the scrapie agent

Scrapie is a disease with unusual characteristics, some of which were originally discovered by misfortune. The scrapie agent is extraordinarily resistant to formalin inactivation, a property that resulted in the accidental inoculation of sheep in Scotland in the 1940s (Gordon, 1946). 18,000 animals were vaccinated against louping ill virus with a formalin-treated suspension of ovine brain and spleen unknowingly contaminated with scrapie (Gordon, 1946). As a result, 1500 sheep developed the disease after two years, leading to the conclusion that the disease was the result of a slow viral infection.

Multiple distinct TSE strains have been identified by passaging natural and experimental sheep scrapie through mice. The strains differ in their absolute incubation period in genetically uniform mice, in the relative incubation periods between mice of different genotypes (Dickinson and Outram, 1964) and in quantitative neuropathology (Fraser and Dickinson, 1968).

Experiments attempting to inactivate the scrapie agent using ultra-violet radiation demonstrated that the scrapie agent was radiation resistant. Consequentially, if the scrapie agent contained nucleic acid then the radiation target size was very small (Alper et al., 1966; Alper, 1978). This led to the proposal that the scrapie agent was devoid of nucleic acid.

Other experiments were devised in order to test the possibility that other macromolecules could be responsible for agent replication. Polysaccharides are extremely complicated structures, which offer a plethora of permutations that could potentially carry information like DNA. However early attempts to extract intact polysaccharides also resulted in the inactivation of the agent (Hunter, 1972).

The unusual properties of scrapie, led to the development of various hypotheses that predict the composition of the scrapie agent and its mechanism of replication.

### 1.1.2 The Virus Hypothesis

The virus hypothesis predicts that the scrapie agent is composed of a foreign nucleic acid coding for a coat protein. The hypothesis is supported indirectly by evidence that the scrapie agent is filterable and transmissible to other species (Stamp, 1962). Certain studies have reported the presence of viral particles in infectious fractions of terminal scrapie brain homogenates (Cho, 1976; Cho and Greig, 1975; Cho et al., 1977). However further investigation as suggested that the putative virus structures were artefacts of the preparation process required of electron microscopy.

Although the strongest evidence against the virus hypothesis comes from the fact that nucleic acid was not detected during the ultraviolet irradiation experiments (Alper et al., 1966; Alper, 1978), and the resistance of scrapie to formalin inactivation (Gordon, 1946). The existence of multiple strains on the other hand still argues strongly for scrapie being of viral origin. The existence of strains suggests that an informational molecule such as DNA or RNA must be involved despite the fact that no nucleic acid containing molecule has been found.

Another feature of viruses is their ability to mutate. Detailed analysis of certain strains such as 87A has revealed that mutations occur during serial passage resulting in the production of ME7. It is thought that the mutation results in the *de novo* production of ME7 and is not due to the co-existence of ME7 and 87A in the original isolate (Bruce and Dickinson, 1987).

It should also be borne in mind that twin-stranded nucleic acid molecules can react very slowly with formalin and that the polyoma virus is relatively resistant to inactivation by this chemical (Weil, 1963). In addition, the pathogenic properties of scrapie have similarities with known viruses, a feature which also lends support to the agent being of viral origin. Infection mechanisms of scrapie are akin to known gastrointestinal viruses such as Herpes Simplex Virus Type I (HSV-1) (Gesser and Koo, 1996). Following oral inoculation, HSV-1 gains access to the enteric nervous system via intramucosal nerve fibres within the

gastrointestinal lumen (Gesser and Koo, 1996). A recent study using hamsters orally inoculated with a hamster adapted scrapie strain 263K found evidence that scrapie infects enteric and autonomic ganglia of the splanchnic and vagus nerve circuits (McBride et al., 2001).

### **1.1.2 The Virino Hypothesis**

In an attempt to define the scrapie agent, the term virino was used to denote the basic infectious unit of scrapie (Dickinson and Outram, 1979; Dickinson and Outram, 1988). Since the scrapie agent had peculiarly high physicochemical stability and no scrapie associated nucleic acid had been identified, it was thought inappropriate to define the scrapie agent as a virus in the classical sense. Nevertheless, the agent had certain similarities to a virus, for example its ability to mutate (Dickinson, 1976) and the fact that strains could be changed selectively (Dickinson and Meikle, 1971).

The virino hypothesis has its origins in the replication site hypothesis which Dickinson and Outram put forward to explain the biological properties of scrapie infectivity and its interaction with the host. The virino hypothesis postulates that the scrapie agent is composed of an agent specific informational molecule bound tightly by a host protein (Dickinson and Outram, 1979; Dickinson and Outram, 1988). The informational molecule may or may not be a nucleic acid but would be protected from degradation by a host protein.

The primary host gene controlling the scrapie incubation period is known as *Sinc*, an acronym for scrapie *inc*ubation (Dickinson and Outram, 1964). It was first recognised approximately 30 years ago in a random-bred mouse colony at the Moredun Institute in Edinburgh (Dickinson and Outram, 1964) and has two alleles, either *s7* (short incubation period with the ME7 scrapie strain) or *p7* (prolonged incubation period with ME7). Details of the passage history of the ME7 scrapie strain and its importance in the experiments carried out in this project are given in section 2.2. A similar gene is found in sheep called *Sip* (an

acronym for Scrapie Incubation Period) which also controls the scrapie incubation period.

Dickinson, Fraser and colleagues demonstrated that the mouse *Sinc* gene was the major host locus controlling the survival time and neuropathology of mice infected with various strain isolates of scrapie (Dickinson and Meikle, 1971). Influenced by the work on allosteric interactions of enzymes by Jacob and Monod, they proposed that protein products encoded by the *Sinc* locus formed an oligomeric complex which acted as a binding and replication site for the infectious agent genome (Dickinson and Outram, 1979; Dickinson and Outram, 1988). It is predicted that the informational molecule acts by disrupting or blocking important cell metabolic processes.

### **1.1.3 The Protein Only Hypothesis**

The failure to detect a scrapie specific nucleic acid led to the development of a hypothesis that predicted that the scrapie agent was composed solely of protein and that a protein alone was capable of self-replication. The basis for the protein only hypothesis, was first proposed by a mathematician in the late sixties (Griffith, 1967). He suggested that it would be thermodynamically feasible for an aberrant protein to be formed by changing the conformation of a normal protein. The normal protein would be modelled on the conformation of the aberrant form.

Stanley Prusiner later provided experimental evidence to support this hypothesis (Prusiner, 1982). When attempting to purify the scrapie infectious agent from the brains of TSE affected animals, he discovered that a protein critical for infectivity had been denatured during the cell fractionation process. The experiments which led to the discovery of this scrapie-associated protein are described below.

### **1.1.4 The scrapie specific prion protein (PrP<sup>sc</sup>)**

During electronmicroscopical studies of scrapie and CJD infected brains, abnormal scrapie associated fibrils (SAF) were found (Merz et al., 1981). These fibrils are composed of paired helical filaments of 100-1000nm in length and are

uniquely associated with TSE disease.

Biochemical analysis of these fibrils in scrapie affected hamster brains by Stanley Prusiner, led to the discovery that they contained an abnormal protease resistant protein of 27-30 kilodaltons (kDa) (Prusiner, 1982). Since this protein was critical for infectivity he coined the name “prion” derived from the term *proteinaceous infectious agent* (Prusiner, 1982) to describe the novel protein he considered to be the scrapie agent.

The purification of the prion protein allowed the N terminus to be sequenced and subsequent isolation of cDNA clones from libraries derived from scrapie-affected rodents was carried out. Surprisingly, it was found that the abnormal protein was not virally encoded but was the product of a host encoded gene known as the cellular prion protein or PrP<sup>c</sup> (Oesch et al., 1985). PrP<sup>c</sup> is described in section 1.1.5.

The abnormal prion protein is known as PrP<sup>Sc</sup> (scrapie) since it is associated with scrapie infectivity. Due to its partial resistance to protease treatment, PrP<sup>Sc</sup> is sometimes referred to as PrP<sup>res</sup>. PrP<sup>Sc</sup> exists as a full length protein of 33-35 kDa *in vivo*. Following protease digestion, PrP<sup>Sc</sup> is shortened to 27-30 kDa and is therefore distinguishable from full length PrP<sup>33-35</sup> on Western blots due to the molecular weight difference (Oesch et al., 1985).

PrP<sup>Sc</sup> is thought to be the only protein component in the scrapie agent, as infectious fractions of the purified agent retain PrP<sup>Sc</sup> after digestion with amino-acid specific proteinases such as trypsin and proteinase K (McKinley et al., 1983). However it is not known how many PrP<sup>Sc</sup> molecules are required to make one infectious unit of scrapie. It is generally accepted that the full length PrP<sup>Sc</sup> protein forms aggregates which result in scrapie associated fibril (SAF) formation (Merz et al., 1981). These fibrils accumulate extracellularly to form amyloid plaques often seen in TSE affected brains.

PrP<sup>sc</sup> is undisputedly an important disease marker during infection since it deposited in peripheral lymphoid tissue in experimental and natural TSE infection, often at a very early stage during infection. Scrapie associated PrP<sup>sc</sup> can be detected by Western blotting (Farquhar et al., 1994; Mabbott et al., 2000a) by immunocytochemistry (McBride et al., 1992) and electronmicroscopy (Jeffrey et al., 2000) in the spleens of experimentally infected mice. It can also be detected immunocytochemically in the spleens of mice experimentally infected with mouse passaged CJD (Kitamoto et al., 1991). In natural TSEs, PrP<sup>res</sup> has been detected in lymphoid tissue of scrapie affected sheep, in the appendix and tonsil of humans infected with vCJD (Hill, 1999) and in alimentary tract associated lymphoid tissue of mule deer fawns infected with Chronic Wasting Disease (Sigurdson et al., 1999). However it cannot be assumed that PrP<sup>sc</sup> is solely responsible for TSE disease. A study has shown that 55% of BSE infected mice did not test positive for PrP<sup>sc</sup> despite developing clinical symptoms and their brains containing infectivity (Lasmezaz et al., 1997).

### **1.1.5 The Cellular Prion protein (PrP<sup>c</sup>)**

PrP<sup>c</sup> is a 33-35 kDa encoded by the Prn-p gene (Oesch et al., 1985) found on the cell surface as a glycoposphatidyl inositol (GPI) anchored glycoprotein. It is also known as PrP<sup>sen</sup> due to its sensitivity to protease digestion where it is completely degraded (Oesch et al., 1985). Protease treatment of samples derived from TSE affected animals is therefore a useful distinguishing factor between host encoded PrP<sup>c</sup> and TSE associated PrP<sup>sc</sup>. The conformational properties of PrP<sup>c</sup> are also different from PrP<sup>sc</sup>, whereby PrP<sup>c</sup> has a high  $\alpha$  helix content and PrP<sup>sc</sup> has a high  $\beta$  sheet composition (Nguyen et al., 1995; Pan et al., 1993).

Mice carrying a null mutation in the Prn-p gene do not express PrP<sup>c</sup> and are resistant to TSE infection (Bueler et al., 1993). PrP<sup>c</sup> is therefore required for onset and development of TSEs. Further experiments were carried out to determine the effect of Prn-p gene dosage on TSE pathogenesis. Using Prn-p heterozygous mice (Prn-p<sup>+/-</sup>) infected with various TSE strains, it was found that the overall intensity of TSE associated lesions and the severity of the clinical

signs were not affected in the terminal stages of the disease (Manson et al., 1994). However, the overall incubation period was prolonged as expected and there was a delay in the onset of clinical symptoms (Manson et al., 1994). It was found that the onset of PrP<sup>Sc</sup> deposition in the brains of Prn-P+/- was the same for wildtype Prn-P+/+ mice. However, the build up of PrP<sup>Sc</sup> in the brains of heterozygotes occurred more slowly compared to wildtype mice. In the terminal stages there was no difference in the intensity of PrP<sup>Sc</sup> deposition (Manson, 1996; Manson et al., 1994). Therefore, the accumulation of PrP<sup>Sc</sup> is not limited by the levels of PrP<sup>C</sup> in the brain.

Of particular importance is the finding that the *Sinc* and Prn-p genes are congruent and therefore one and the same. As discussed previously *Sinc* has two alleles s7 and p7 corresponding to short and long incubation periods respectively (Dickinson et al., 1968). Restriction Fragment Length Polymorphism (RFLP) analysis of a mouse Prn-p restriction fragment, demonstrated a segregation of Prn-p with incubation period length (Carlson et al., 1986; Hunter et al., 1987). The two alleles were named Prn-p<sup>a</sup> (short incubation period and producing the PrP A allotype) and Prn-p<sup>b</sup> (long incubation period and producing the PrP B allotype). These Prn-p alleles are analogous to the *Sinc*<sup>s7</sup> and *Sinc*<sup>p7</sup> alleles described by Dickinson 30 years previously. The same situation is found in sheep, where scrapie incubation periods are controlled by two alleles named Sip sA (short incubation period) and Sip pA (prolonged incubation period (Hunter et al., 1989).

PrP mRNA is expressed at high levels in the brain (Oesch et al., 1985) and to varying degrees in non- neural tissues such heart lung kidney, spleen and liver (Caughey et al., 1988; Oesch et al., 1985). PrP mRNA has been found in a variety of mouse and human derived cell lines of B cell, T cell and fibroblast origin (Caughey et al., 1988). Using immunocytochemistry, PrP<sup>C</sup> can be found at high levels in spleen and is associated with follicular dendritic cells (Brown et al., 1999 ; McBride et al., 1992; Ritchie et al., 1999). Using flow cytometry, PrP<sup>C</sup> has also been detected at low levels on T cells (Mabbott et al., 1997).

#### 1.1.5.1 The function of PrP<sup>c</sup>

Despite its established role in TSE pathogenesis, little is known about the function of PrP<sup>c</sup>. The primary sequence of PrP<sup>c</sup> has been highly conserved among many species suggesting a fundamental role. However, Prn-p<sup>-/-</sup> mice develop normally (Bueler et al., 1993; Manson et al., 1994). Nevertheless several theories regarding its function have arisen. For example, a role in tissue development during mouse embryogenesis has been proposed (Manson et al., 1992). PrP mRNA has been found at high levels in the developing brain and spinal cord pre-natally (McKinley et al., 1988). Transcripts have also been detected in non-neural tissue such as the kidney during development.

Recent studies have shown that the earliest age at which PrP mRNA can be detected using whole mount *in situ* hybridisation is between embryonic day 8.5 and embryonic day 9 (Miele, 1999) within the mouse CNS, coinciding with a switch from anaerobic metabolism to aerobic metabolism. This is discussed in more detail in Chapter 5. However, the upregulation of the Prn-p gene at such a critical stage in development suggests that PrP<sup>c</sup> may be involved in respiration pathways. Further support for this hypothesis comes from differential analysis of gene expression between organs in Prn-p<sup>+/+</sup> and Prn-p<sup>-/-</sup> mice. Using the differential display reverse transcriptase- polymerase chain reaction technique (DDRT-PCR), it was demonstrated that NADH B14.5b is downregulated in the Prn-p<sup>-/-</sup> liver (Miele, 1999). This molecule is involved in the synthesis of the mitochondrial respiratory chain complexes. In addition, electronmicroscopic studies of Prn-p<sup>-/-</sup> brain have shown a 40% reduction in the number of mitochondria. 28% of these mitochondria had abnormal pathology. This evidence supports the hypothesis that PrP<sup>c</sup> may be developmentally regulated to cope with the new burden of oxidative stress as the organism switches from anaerobic to aerobic respiration. There is evidence to suggest that PrP<sup>c</sup> can bind copper (Brown et al., 1997). If this is the case, PrP<sup>c</sup> may be involved in a relay system, trafficking copper to copper dependent super oxide dismutases that metabolise reactive oxygen species.

There is some evidence to suggest that PrP<sup>c</sup> may be a receptor or adhesion molecule since studies involving T lymphocytes have demonstrated that Prn-p/- T cells have a reduced ability to respond to the T cell mitogen Concanavilin A (Mabbott et al., 1997). However, GPI anchored proteins do not contain a transmembrane region and therefore are not involved directly in intracellular signalling, so the exact role of PrP<sup>c</sup> in T cell activation is still not certain.

Finally, it has been found that adult Prn-p/- mice appear to develop cerebellar ataxia in later life depending on the knockout line used. This had previously been attributed to a loss of normal synaptic function resulting in epileptiform activity reminiscent of CJD and terminal scrapie (Collinge et al., 1994). However, recent studies of Prn-p/- mice have revealed an alternative explanation for the observed ataxia. Certain Prn-p/- mouse lines upregulate a PrP-like protein called Doppel (Dpl) (Moore et al., 1999). Similar to PrP<sup>c</sup>, the function of Dpl is unknown and is expressed minimally in the CNS in wildtype mice. However, the Dpl gene (Prnd) is under the control of the Prn-p promoter and hence becomes upregulated in the Prn-p deficient mouse lines since the promoter is intact.

#### 1.1.5.2 The role of PrP<sup>c</sup> in scrapie

The absolute requirement of PrP<sup>c</sup> in TSE pathogenesis makes this protein central to predictions regarding the composition of the scrapie agent and the mechanisms of scrapie agent replication.

On the one hand, Prusiner extended the protein only hypothesis (Griffith, 1967) proposed by Griffith to include the properties of PrP<sup>sc</sup> and PrP<sup>c</sup>. This became known as the prion hypothesis. It predicted that the scrapie agent was composed of PrP<sup>sc</sup> alone and that donor PrP<sup>sc</sup> could convert host PrP<sup>c</sup> to the pathogenic form by conformational modification i.e. from predominantly  $\alpha$ -helix to  $\beta$ -sheet (Pan et al., 1993) either at the cell surface or after internalisation of PrP<sup>sc</sup>.

The most compelling evidence to date in support of the prion hypothesis comes from *in vitro* studies demonstrating that the protease resistant form of scrapie can

be produced *de novo* when incubated with radioactively labelled PrP<sup>C</sup> molecules (Kocisko et al., 1994). The implications of this experiment are twofold. Firstly, no nucleic acid is required since the reaction was carried out without cell involvement. Secondly, it suggests the conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup> may be a viable pathogenic mechanism. However, it must be noted that in this experiment infectivity analysis of the new PrP<sup>Sc</sup> protein was not carried out. Even if they had been, it would have been impossible to detect the *de novo* production of PrP<sup>Sc</sup> due to excess of PrP<sup>Sc</sup> used to initiate the reaction.

The difficulties in separating newly formed PrP<sup>Sc</sup> molecules from PrP<sup>Sc</sup> used to initiate the reaction, has been a major barrier to testing the prion hypothesis. A newly developed *in vitro* technique called the cyclic amplification of protein misfolding (Saborio et al., 2001) offers a way of amplifying PrP<sup>Sc</sup> to high titre with only a small amount of PrP<sup>Sc</sup> needed to start the reaction. Since the majority of the PrP<sup>Sc</sup> produced will be newly formed abnormal protein, then it will be of interest if this can induce TSE disease in mice after injection.

Although it is clear that PrP<sup>Sc</sup> deposition occurs during TSE pathogenesis, it is disputed whether PrP<sup>Sc</sup> can carry the information resulting in the observed strain diversity of scrapie. The virino hypothesis on the other hand predicted that a *Sinc* encoded protein formed a multimeric replication site for the scrapie associated informational molecule to replicate (Dickinson and Outram, 1979). Since *Sinc* and Prn-p genes are congruent (Hunter et al., 1992), the virino hypothesis can be extended to include PrP<sup>C</sup> as the putative multimeric replication site for the scrapie associated informational molecule. Interestingly, a recent study has demonstrated that PrP<sup>C</sup> can bind Human Immunodeficiency Virus (HIV) RNA, in a similar manner to the HIV-1 encoded nucleic acid binding protein (NC protein) (Gabus et al., 2001). Binding of NC protein to HIV RNA controls proviral DNA synthesis and viral assembly. These findings suggest that under normal circumstances, PrP<sup>C</sup> may participate in nucleic acid metabolism. In terms of the nature of the scrapie agent, the results provide support for the hypothesis that PrP<sup>C</sup> can form a close, stabilising interaction with a scrapie specific nucleic acid.

Despite the hypotheses that have arisen attempting to describe the scrapie agent its identity remains an enigma. PrP<sup>c</sup> is undoubtedly involved in TSE propagation and pathogenesis in general, but the extent to which PrP<sup>sc</sup> is involved may vary between TSE models. The virino hypothesis unifies both the prion and viral hypotheses by acknowledging the role of host encoded PrP<sup>c</sup> in pathogenesis and the strain diversity associated with scrapie.

## 1.2 Peripheral scrapie pathogenesis

Lack of knowledge of the precise nature of the scrapie agent has not hampered studies of scrapie pathogenesis attempting to define the mechanisms leading to TSE disease onset. Natural scrapie infection, like other TSEs such as BSE and vCJD, is probably acquired via the peripheral route. In cattle, eating TSE contaminated meat and bone meal has been linked to the emergence of BSE (Horn et al., 2001). In humans, eating BSE contaminated foodstuffs is believed to be responsible for the subsequent appearance of vCJD (Will et al., 1996). In sheep, scratching of the skin (Taylor et al., 1996) ewe to lamb transmission during the birthing process (Foster et al., 1992) and horizontal transmission by eating contaminated foetal materials, present after parturition (Pattison et al., 1972) have been proposed as possible mechanisms for scrapie spread.

Early scrapie pathogenesis experiments studied the course of events only after direct inoculation of scrapie into the brain. At the time it was realised that clinically relevant models for scrapie were required and so many scrapie pathogenesis studies were set up to look at the organs involved in scrapie replication after peripheral challenge (Eklund et al., 1967) as this is the most likely route of exposure to scrapie. The main aims of scrapie peripheral pathogenesis studies are to determine the cell targets of scrapie infectivity in the periphery and elucidate the mechanisms leading to subsequent neuroinvasion with a view to developing preventative strategies for natural TSEs.

The most sensitive technique available for measuring infectivity levels in TSE affected tissue is the bioassay. Pathogenesis studies often make use of the bioassay to determine the order in which tissues become infected with scrapie and to quantify the level of infectivity in each tissue. Bioassaying for scrapie infectivity involves removing tissue of interest from mice previously inoculated with scrapie and inoculating the homogenised tissue intracerebrally into a panel of indicator mice. A shortening of the incubation period in the indicator mice correlates with an increase in infectivity titre within the tissue of interest. In order to determine the temporal distribution of scrapie infectivity after peripheral challenge, tissue would be removed and bioassayed at various time points after scrapie challenge. This is a time consuming procedure especially if low levels of infectivity are present in tissue as this will lead to prolonged incubation periods in the indicator mice. Bioassays also involve a considerable number of mice.

In order to avoid these problems, PrP<sup>Sc</sup> detection using Western blotting can be used to obtain the same information in a shorter amount of time (Race et al., 1992). Following experimental peripheral inoculation with certain strains of scrapie, PrP<sup>Sc</sup> accumulates rapidly in lymphoid tissue and is associated with increase in infectivity levels during scrapie replication (Farquhar et al., 1994). The correlation between increasing levels of infectivity and PrP<sup>Sc</sup> detection is particularly high after peripheral challenge with the ME7 strain of scrapie (see section 2.3.2 for passage history of this strain). The ME7 strain has been well characterised at NPU and detailed studies of the correlation of infectivity with PrP<sup>Sc</sup> in peripheral lymphoid tissue have been carried out. For these reasons PrP<sup>Sc</sup> has been used as a disease specific marker for scrapie pathogenesis studies carried out in this project.

### **1.2.1 Organs involved in scrapie pathogenesis**

Many of the early peripheral scrapie pathogenesis studies were carried out using the “Chandler” scrapie isolate (Eklund et al., 1967). The Chandler isolate was derived from a scrapie affected goat and passaged intracerebrally into Sinc<sup>s7</sup> mice at NPU. Following at least 21 i.c passages, the 139A scrapie strain was derived.

The Chandler isolate was also passaged into Sinc<sup>s7</sup> mice at the Rocky Mountain Laboratory in the USA, from which the Rocky Mountain Laboratory (RML) scrapie strain was obtained. Recent studies carried out in Zurich have used the RML scrapie strain (Klein et al., 1997; Klein et al., 1998).

Most of the scrapie peripheral pathogenesis studies at the NPU have been carried out using the ME7 scrapie strain (Brown et al., 1999; Fraser et al., 1996), derived from a different natural scrapie isolate (see section 2.3.2). It should be noted that the peripheral pathogenesis of the Chandler isolate and the 139A, RML and ME7 mouse adapted scrapie strains are very similar. However, subtle differences between RML and ME7 regarding the cell targets of these strains have been revealed in recent published reports (Blattler et al., 1997; Brown et al., 1999) and the implications of these findings will be discussed in more detail in section 1.2.3.

The original studies on the temporal kinetics of scrapie replication were carried out using the Chandler isolate. By bioassaying a variety of tissue at various sequential time points after subcutaneous challenge of mice with Chandler, it was found that infectivity could be detected in the spleen after 1 week post-inoculation. However, by 2 weeks after scrapie challenge, infectivity in the spleen was undetectable and did not reappear until 3-4 weeks after inoculation (Eklund et al., 1967). Simultaneously, replication could be detected in peripheral lymph nodes. Scrapie replication begins in the spinal cord at 12 weeks post-inoculation, followed by the brain between 12-16 weeks (Eklund et al., 1967). These observations on scrapie replication kinetics are summarised in Fig 1.1.

The high levels of infectivity detected 1 week post inoculation have been attributed to the presence of infectivity in the inoculum (Eklund et al., 1967). The subsequent disappearance of infectivity suggests that infectivity is cleared from the system. The mechanism by which this occurs is as yet unknown, although it is possible that macrophages sequester infectivity (Beringue et al., 2000). It should be noted that the undetectable levels of infectivity after 1 week does not exclude

the possibility that scrapie replication is occurring and could be due to the limits of sensitivity of the bioassay. Indeed it is possible that replication of scrapie occurs immediately after inoculation but is masked by the concentration of infectivity present in the inoculum.

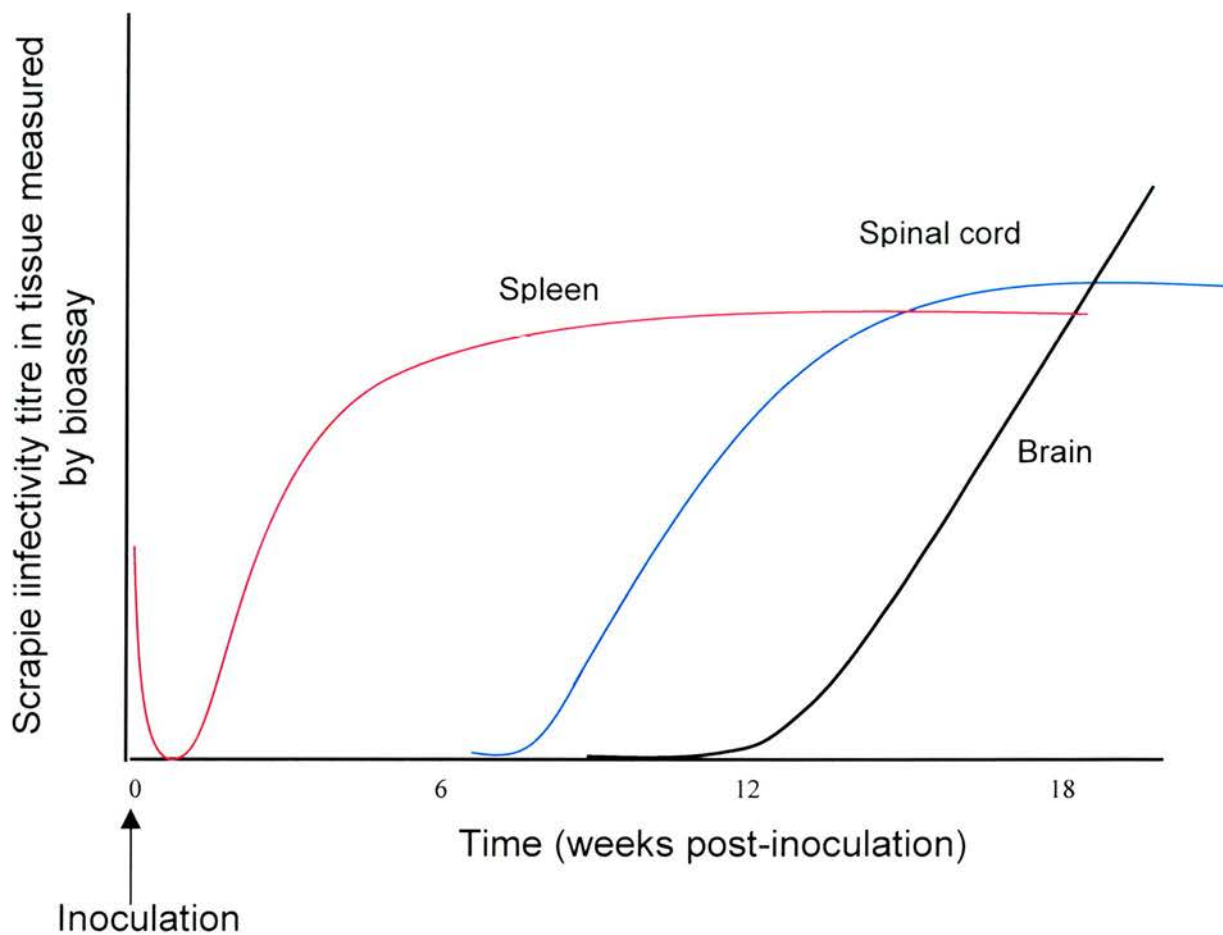
Consistent with the findings by Eklund et al, later studies using the 139A scrapie strain demonstrated that high levels of scrapie infectivity can be observed in the spleen, 4 weeks after subcutaneous challenge with scrapie. However, infectivity was not assayed prior to 4 weeks hence the precise time when replication commenced is unknown. Replication commenced in the spinal cord 9 weeks after inoculation and in the brain at 13 weeks as expected (Kimberlin and Walker, 1979).

The role of the spleen in peripheral scrapie pathogenesis was investigated further by removal of the spleen (splenectomy). Using the ME7 strain of scrapie it was found that splenectomy shortly before or after intraperitoneal scrapie challenge prolongs the incubation period by at least 7 weeks (Fraser and Dickinson, 1970). Similar results have been found using the Chandler strain, whereby the incubation period is prolonged by 25-30 weeks when the spleen is removed before or up to 2 weeks after intraperitoneal challenge (Clarke and Haig, 1971). The influence of the spleen on scrapie peripheral pathogenesis is not absolute since splenectomy does not lead to survival. However it suggests that other scrapie replication sites are available elsewhere in the body such as peripheral lymph nodes.

Analysis of possible cell targets for scrapie ruled out the possibility of T cell involvement, since neither adult nor neonatal thymectomy has any effect on incubation period prolongation after intraperitoneal challenge (Fraser and Dickinson, 1978; McFarlin et al., 1971). This was true for both the Chandler strain (McFarlin et al., 1971) and the ME7 strain of scrapie (Fraser and Dickinson, 1978). More recent studies have shown that T cell receptor  $\alpha$  (TCR- $\alpha$ ) knockout mice deficient in T cells peripherally challenged with RML are ~~not~~

susceptible to scrapie, consistent with the original thymectomy data (Klein et al., 1998).

Mice subjected to non-lethal doses of  $\gamma$ -radiation prior to peripheral scrapie challenge with ME7, maintain normal incubation periods when compared to non-irradiated controls (Fraser and Farquhar, 1987). Since mitotically active T and B cells are sensitive to radiation, this suggests that B cells may not be involved in ME7 pathogenesis. However, follicular dendritic cells are radiation resistant (Jaroslav and Nossal, 1968), and were thus proposed as the candidate cells for peripheral scrapie replication. However, these radiation studies cannot rule out the possibility of post-mitotic differentiated B cells being involved in peripheral pathogenesis. The involvement of FDCs in peripheral scrapie pathogenesis was also supported indirectly with experiments carried out in Severe Combined Immunodeficient mice (SCID) mice (explained in section 2.3.1). These mice lack T and B cells and possess immature non-functional FDCs since the maturation of FDCs is B cell dependent maturation (Cerny et al., 1988). Severe Combined Immunodeficient Mice (SCID) resist intraperitoneal scrapie challenge using doses that are 100% lethal to immunocompetent control mice (Fraser et al., 1996). Replication of the scrapie agent could only be restored after reconstitution with bone marrow (Fraser et al., 1996), which induces FDC maturation (Cerny et al., 1988; Yoshida et al., 1995). The conclusion that the lack of FDCs was responsible for the resistance of SCID mice to scrapie was reached on the basis of the previous irradiation experiments (Fraser and Farquhar, 1987). Further experimentation using immunodeficient knockout mice have been consistent with FDC-dependent replication of infectivity (discussed in section 1.2.3).



**Fig 1.1 Temporal kinetics of scrapie replication after peripheral challenge.** Infectivity levels are detected after one week in spleen but rapidly decline to undetectable. This probably represents adherence of inoculum to spleen. Infectivity levels in spleen reach plateau levels in spleen 2-4 weeks after challenge long before accumulation is detected in the CNS. Replication commence in spinal cord between 9-12 weeks after challenge and commences finally in brain 12-16 weeks after challenge. This graph was compiled using data from Eklund et al, 1967; Kimberlin and Walker, 1979.

### **1.2.2 Follicular Dendritic Cells**

FDCs are unique non-lymphoid cells found only in follicles and germinal centres (Szakal and Hanna, 1968). They are characterised by long dendritic processes that form networks of membrane, which are intimately associated with B cells. The main function of FDCs is to retain antigen in a native state within immune complexes (van Rooijen, 1975). Immune complexes are composed of antigen bound by antibody and complement components that have become deposited on the immune complex following complement cascade activation. Indeed it is possible for certain antigens to activate the complement cascade without the need for antibody forming part of the immune complex. An overview of this is given in Chapter 4.

Immune complex trapping is mediated through the presence of Fc receptors that trap the Fc portion of antibodies within the immune complexes. In addition C3b and C3d complement component receptors on the surface of FDCs, trap breakdown products of the complement cascade (C3b and C3d) that have become deposited on the immune complex (Papamichail et al., 1975). In the non-immunised mouse, trapping of immune complexes occurs predominantly via complement component receptors such as CR1 (binds C3b and C4b) and CR2 (binds C3d) (Yoshida et al., 1993). However, in immunised mice both Fc and complement receptors are involved in antigen retention (Papamichail et al., 1975; Yoshida et al., 1993). This is discussed in more detail in Chapter 4.

The retention of antigen on FDCs in this manner is associated with the maintenance of memory B cells (Gray et al., 1990). After stimulation with immunogen germinal centres are formed within 2-3 weeks. Two days after stimulation with an immunogen, B cells are activated in T cell areas and form extrafollicular foci adjacent to the red pulp outside the primary follicle. This migratory event may be influenced by the presence of FDCs, which trap the B cells using their cell adhesion molecules (Kosco et al, 1992). After a further two days, secondary responsive foci are found in the primary B cell follicle. The B cells here are distinguished by their ability to bind the plant lectin peanut

(PNA) (Coico et al., 1983; Rose et al., 1980). During the next 14 days, these B cells give rise to the germinal centres, and the germinal centre develops two distinct regions: the dark and light zones (Kosco-Vilbois, 1997). Germinal centre B cells populate the dark zone, whereas FDCs are found in the light zone.

#### 1.2.2.1 The origins of FDCs

Firstly, certain experiments suggest a mesenchymal/fibroblastic origin (Kapasi et al., 1998). In this model it is thought that FDCs are formed from reticular cells characterised by BP-3 antigen (McNagny et al., 1991; McNagny et al., 1988) immunolabelling on the cell surface (Yoshida et al., 1995). These reticular cells develop into CR1+, BP-3+ pre-FDCs due to B cell and antigen stimulation and subsequently into mature FDCs which can trap immune complexes (Berg and Dijkistra, 1995). The first trapping of immune complexes has been observed in the rat between 2-4 weeks after birth (Dijkistra et al., 1982). A summary of FDC maturation is illustrated in Fig 1.2

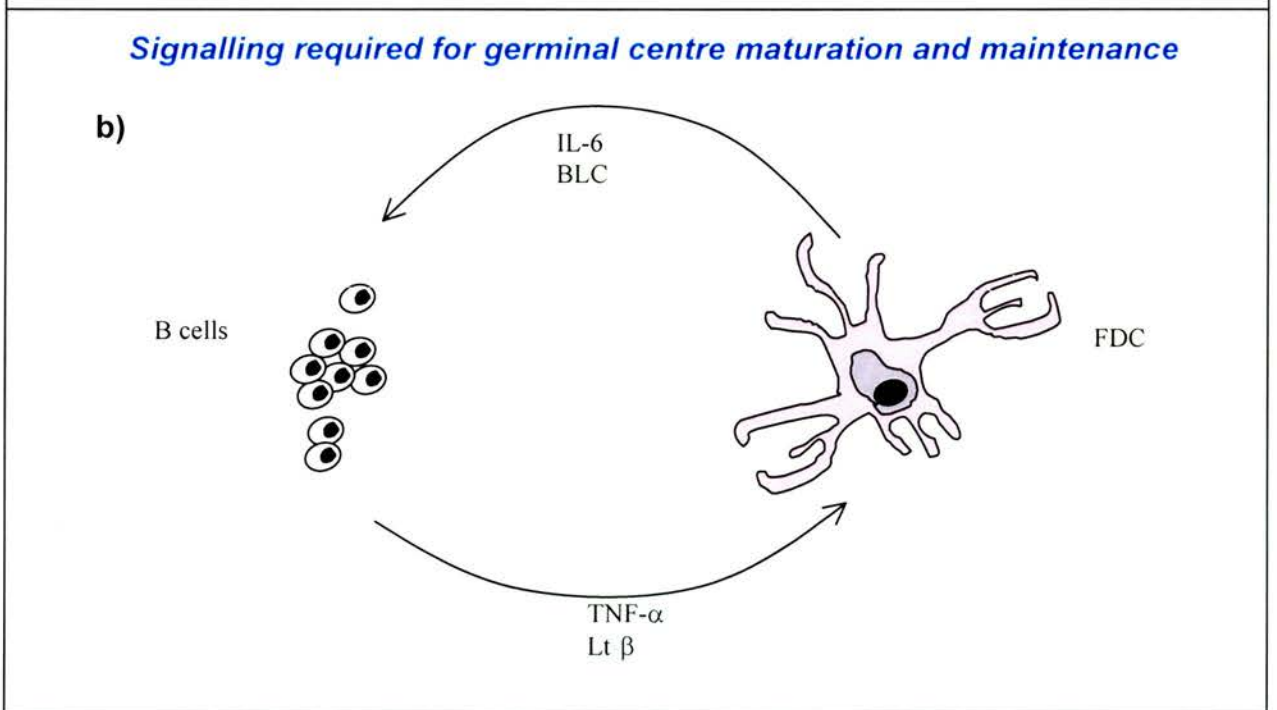
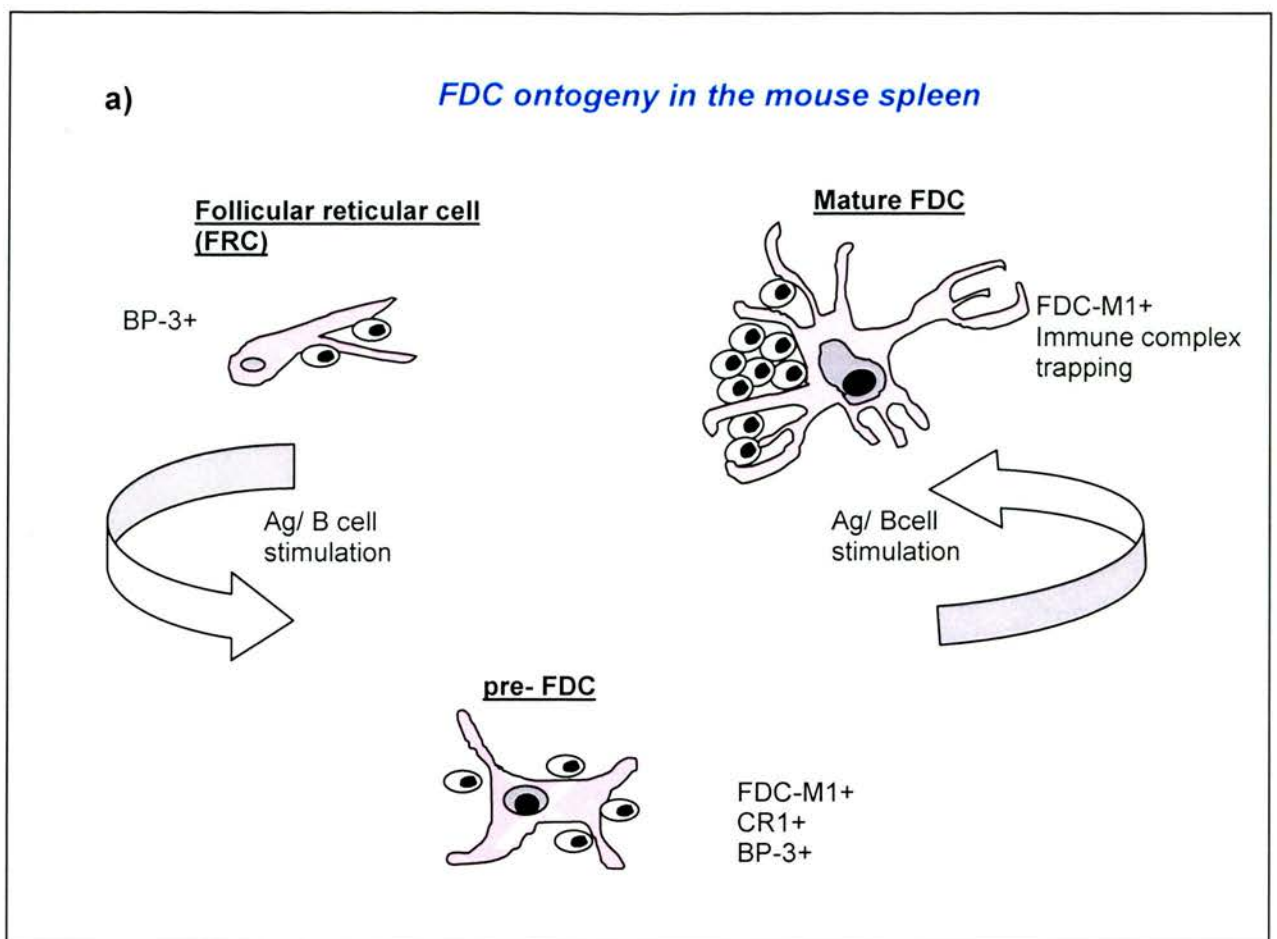
Other experiments support a bone marrow origin for FDCs (Szakal et al., 1995). In adult SCID mice, FDCs of donor and host origin can be found after reconstitution with adult bone marrow (Kapasi et al., 1998). However, there appeared to be a predominance of host derived FDCs within spleen sections. Since quantitation of donor versus host FDCs was not carried out, conclusions about the true proportion of donor derived FDCs cannot be made. Reconstitution of neonatal SCID mice with adult bone marrow or foetal liver cells again resulted in a mixed population of FDCs from both host and donor origin (Kapasi et al., 1998). On a subjective level, there appeared to be more donor derived FDCs in neonatally reconstituted mice compared to adult SCIDs (Kapasi et al., 1998). Thus neonatal mice may be more receptive to bone marrow derived FDCs during early postnatal life compared to adults. Although it is evident that some resident cells within lymphoid tissue mature into FDCs during postnatal life. In adults, either bone marrow derived FDC replacement slows down or secondary lymphoid tissue becomes less receptive to bone marrow FDC. In this case, it is possible that resident reticular cell derived FDCs will predominate.

Recent studies at the Neuropathogenesis Unit aimed at elucidating the cell target for scrapie pathogenesis has also been useful in determining the cell origin of the FDC (see Table 1.2 for summary of data). These studies have revealed evidence for a reticular origin for FDCs in adult mice.

The study exploited the lack of mature FDCs in the lymphoid tissue of SCID mice. PrP<sup>c</sup> is present at high levels on FDCs in immunocompetent mice and can be readily detected using immunocytochemistry (McBride et al., 1992; Ritchie et al., 1999) However, SCID mice lack mature FDCs and do not possess detectable PrP<sup>c</sup> within their spleens (Fraser et al., 1996).

Two chimaeric mouse lines were produced by crossing the *scid* mutation into either Prn-p<sup>-/-</sup> or Prn-p<sup>+/+</sup> mouse lines. These were called SCID/PrP<sup>-/-</sup> and SCID/PrP<sup>+/+</sup> mice. Using PrP<sup>c</sup> as an FDC marker, it was predicted that if FDC precursors are present in the bone marrow, then reconstitution of SCID/PrP<sup>-/-</sup> mice with PrP<sup>+/+</sup> bone marrow should result in the detection of PrP<sup>c</sup> immunolabelling associated with FDC markers. However if the reticular origin hypothesis was correct then reconstitution of SCID/PrP<sup>-/-</sup> mice with PrP<sup>+/+</sup> bone marrow, should not result in PrP<sup>c</sup> immunolabelling in the spleen. Consistent with the reticular origin hypothesis, it was found that PrP<sup>c</sup> could only be detected in the spleens of SCID/PrP<sup>+/+</sup> irrespective of the PrP<sup>c</sup> status of the bone marrow used in reconstitution.

These results are supported by previous experiments, where adult SCID mice were reconstituted with allogeneic B cells (Yoshida et al., 1995). Using double immunolabelling of FDC and MHC specific markers it was found that only FDCs expressing host MHC antigen could be detected in the spleens of reconstituted SCID mice (Yoshida et al., 1995).



**Fig 1.2 a) FDC ontogeny in the mouse spleen.** BP-3+ Follicular reticular cells present in lymphoid tissue undergo differentiation under the influence of B cell signalling and B cell derived cytokines. FRCs develop into an intermediate stage during the first 2 weeks of birth, eventually gain antigen trapping capabilities and become mature FDCs (Berg, 1995). **b) Signalling required for germinal centre maturation and maintenance.** B cell derived TNF- $\alpha$  and Lt- $\beta$  are necessary for FDC maturation (Endres et al, 1996). IL-6 produced by FDCs is required for germinal centre B cell maturation.

### 1.2.2.2 Signalling required for FDC maturation and maintenance

In adult mice, lymphotoxin  $\beta$  (Lt $\beta$ ) and tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ) are required for maintaining germinal centre FDCs in a mature state by signalling through LT $\beta$  receptor (Endres et al., 1999) and the tumour necrosis factor receptor 1 (TNFR1) (Le Hir et al., 1996; Tkachuk et al., 1998). Recently, the role of B lymphocyte chemoattractant (BLC) has been elucidated. BLC is an FDC derived chemokine that binds the chemokine receptor CXCR5 on B cells. It appears to influence FDC maturation by a positive feedback loop early in development within primary follicles. BLC induces B cell derived lymphotoxin  $\alpha 1\beta 2$  which promotes FDC development and further BLC production which is needed for B cell homing (Ansel et al., 2000). The signalling mechanisms are summarised in Fig 1.2b

<b><i>Chimaeric mouse type</i></b>	<b><i>Bone marrow genotype used in reconstitution</i></b>	<b><i>PrP<sup>c</sup> status in SCID spleen after reconstitution</i></b>
SCID/Prn-p-/-	Prn-p+/+	Negative
	Prn-p-/-	Negative
SCID/Prn-p+/+	Prn-p+/+	Positive
	Prn-p-/-	Positive

**Table 1.2 Evidence in support of a stromal origin for FDCs.** Large amounts of PrP<sup>c</sup> can be detected by immunolabelling on FDCs (McBride et al, 1992), and so PrP<sup>c</sup> was used as a marker for FDCs. SCID mice do not possess mature FDCs, and are negative for PrP<sup>c</sup> immunolabelling (Brown et al, 1999). If FDC precursors reside in the bone-marrow, then PrnP+/+ bone marrow should restore PrP<sup>c</sup> immunolabelling associated with FDCs in SCID/PrnP-/- mice. If FDC precursors reside only in lymphoid tissue and are of reticular origin then PrnP+/+ bone marrow will not restore PrP<sup>c</sup> immunolabelling in SCID/PrnP-/- mice. Consistent with the latter prediction, PrP<sup>c</sup> immunolabelling could only be restored in SCID mice that have a functional Prnp gene. Bone marrow irrespective of PrP<sup>c</sup> status allows the maturation of pre-existing PrPc expressing FDCs in SCID/PrnP+/+ mice  
This table was compiled using data from Brown et al, 1999

### **1.2.3 The role of FDCs in scrapie pathogenesis**

The study described above which supported the reticular origin hypothesis of FDCs, also helped to elucidate the role of FDCs in peripheral scrapie pathogenesis. Following intraperitoneal challenge with ME7, high levels of infectivity were found only in SCID/PrP<sup>+/+</sup> (i.e mice possessing a functional Prn-p gene) irrespective of the Prn-p status of the bone marrow donor (Brown et al., 1999). Replication was never observed in SCID/PrP<sup>-/-</sup> (Brown et al., 1999). This study demonstrates the central role for FDCs in ME7 peripheral pathogenesis and rules out the possibility that PrP<sup>c</sup> expressing B cells may be involved in ME7 replication. The data is summarised in Table 1.3.

Further support for FDC involvement in peripheral scrapie pathogenesis comes from studies using TNF- $\alpha$  deficient mice, IL-6 deficient mice (Mabbott et al., 2000a) and LT $\beta$ R-Ig treated mice (Mabbott et al., 2000b). As described previously, TNF- $\alpha$  <sup>-/-</sup> mice lack mature FDCs and germinal centre structure. After peripheral challenge, TNF- $\alpha$  <sup>-/-</sup> mice fail to replicate scrapie in their spleens and show a decreased susceptibility to scrapie. IL-6 <sup>-/-</sup> mice on the other hand, possess FDCs but germinal centre B cells are absent. These mice develop scrapie after a similar incubation period to wildtype mice (Mabbott et al., 2000a), again demonstrating that ME7 replication is dependent on mature FDCs. Finally, treatment of mice with a fusion protein composed of lymphotoxin  $\beta$  receptor and human immunoglobulin (LT $\beta$ R-Ig) shortly before or shortly after peripheral challenge with ME7, prolongs the incubation period significantly (Mabbott et al., 2000b). This fusion protein has been demonstrated previously to de-differentiate FDCs by binding Lt $\beta$  before it reaches FDCs (Mackay and Browning, 1998). Signalling via the Lt $\beta$  receptor is required for FDC maintenance in a mature state.

Despite the accumulating evidence supporting the central role for FDCs in ME7 scrapie replication after peripheral challenge, pathogenesis studies involving the

RML strain of scrapie have shown that infectivity may be replicated by B cells. Irradiated Prn-p <sup>-/-</sup> mice reconstituted with Prn-p <sup>+/+</sup> foetal liver cells and challenged intraperitoneally with RML, replicate scrapie efficiently in their spleens (Blattler et al., 1997). It should be noted that infectivity levels in the spleens of irradiated Prn-p <sup>+/+</sup> mice reconstituted with Prn-p <sup>-/-</sup> foetal liver cells were not measured. This evidence contradicts the study carried out by Brown et al where Prn-p <sup>+/+</sup> bone marrow could not restore the capacity of SCID/Prn-p <sup>-/-</sup> mice or irradiated Prn-p <sup>-/-</sup> mice to replicate the ME7 strain of scrapie (summarised in Table 1.3). It suggests that bone marrow derived cells can replicate RML. It is possible that RML can target B cells directly whereas ME7 cannot since the two strains have come from different scrapie sources and may behave in distinct ways. Later studies by Klein et al, (1998) have shown that the ability to replicate RML in Rag1 <sup>-/-</sup> mice (deficient in T cells, B cells and as a consequence mature FDCs) can be restored irrespective of the Prn-p status of the foetal liver cells (Klein et al., 1998). This result supports work carried out by Brown et al, (1999), which demonstrates that B cells are only required to provide maturational signals to immature FDCs. It is clear that conflicting data has arisen from the peripheral pathogenesis studies with RML. Until a direct comparison of the behaviour of RML and ME7 strains is carried out in both Zurich and NPU labs with the same immunodeficient strains, the differences between these strains may never be resolved.

It is of interest that differences in pathogenesis in CJD mouse models have also been documented. On the one hand SCID mice are refractory to peripheral challenge with the mouse passaged CJD strain Fukuoka-1 (Kitamoto et al., 1991), similar to results found with ME7 (Fraser et al., 1996). On the other hand, lymphotoxin  $\beta$  <sup>-/-</sup> mice deficient in FDCs, are still susceptible to peripheral challenge with low doses of the Fukuoka-2 strain of CJD (Manuelidis et al., 2000). This result is using TNFR1 <sup>-/-</sup> mice deficient in mature FDC. After intraperitoneal challenge with RML, it was found that TNFR1 <sup>-/-</sup> mice are fully susceptible to scrapie (Klein et al., 1997). However it should be noted that no information was given about infectivity or PrP<sup>Sc</sup> levels in the spleens of these

mice. High dose challenge of immunodeficient mice SCID or TNF- $\alpha$  mice may sometimes bypass the need for a peripheral replication phase, probably by direct infection of peripheral nerve endings (Fraser et al., 1996; Mabbott et al., 2000a) as will be discussed in section 1.2.4. It is possible that this occurred in the TNFR1<sup>-/-</sup> mice since they were inoculated with a relatively high dose of scrapie (Klein et al., 1997).

It is important that caution should be taken when making direct comparisons between the mechanisms of peripheral pathogenesis of different strains of TSE. Although it is likely that different TSE strains target distinct cell populations in the periphery, it is clear that the dose of TSE used in the inoculum could directly affect pathogenesis. Nevertheless, the data produced at NPU regarding the peripheral pathogenesis of ME7 is consistent with the hypothesis that FDCs are required for ME7 replication in lymphoid tissue prior to neuroinvasion. Until TSE infectivity dosage and methodology used in TSE peripheral pathogenesis research is standardised, then apparently conflicting results may be obtained.

<b>Scrapie strain</b>	<b>FDC Prn-p status</b>	<b>B cell Prn-p status</b>	<b>Scrapie Replication in spleen</b>
<b>ME7</b>	-/-	+/+	negative
	-/-	-/-	negative
	+/+	-/-	positive
	+/+	+/+	positive
<b>RML</b>	-/-	+/+	positive
	-/-	-/-	negative

**Table 1.3 Cell types involved in peripheral scrapie pathogenesis.** This table summarises data by Brown et al, (1999) and Blattler et al, (1997) using the ME7 and RML strains of scrapie respectively. By reconstituting irradiated mice with bone marrow to create a mismatch in PrP<sup>c</sup> status in bone marrow derived cells and resident cell types, it was found that ME7 requires PrP<sup>c</sup> expressing resident FDCs for replication (Brown et al, 1999). On the other hand, RML replication is dependent on PrP<sup>c</sup> expressing foetal liver cells (Blattler et al, 1997).

#### **1.2.4 The role of the peripheral nervous system in scrapie pathogenesis**

In peripherally inoculated mice, after replication in the spleen infectivity can next be detected in the spinal cord prior to reaching the brain (summarised in Fig 1.1). There is a time lag of two weeks between infectivity reaching a plateau in the spleen and infectivity being detected in the spinal cord. (Kimberlin and Walker, 1978; Kimberlin and Walker, 1979). After replication in the spleen, it is hypothesised that infectivity reaches the spinal cord via peripheral nerves such as the splanchnic nerve that innervates the spleen. It is possible that the lack of detection of infectivity during the two weeks is due to infectivity travelling through peripheral nerves to the spinal cord. Consistent with this hypothesis is the finding that replication is first detected in the thoracic region of the spinal cord after replication in the spleen, the region that gives rise to the splanchnic nerve (Kimberlin and Walker, 1980). Recent studies tracing the deposition of PrP<sup>Sc</sup> in the peripheral nervous system (PNS) of hamsters after oral challenge with the hamster passaged scrapie strain 263K, have demonstrated PrP<sup>Sc</sup> deposits in the enteric and autonomic ganglia of the splanchnic and vagus nerve circuitry (McBride et al., 2001).

In certain situations, it is possible that infectivity may access the central nervous system (CNS) without the need for prior FDC-dependent replication within lymphoid tissues. This possibility has been highlighted by the observation that using high doses of ME7, 100% of peripherally inoculated SCID mice (lacking mature FDCs and normally resistant to scrapie infection using ME7) succumb to scrapie challenge without any replication of scrapie in their spleens (Fraser et al., 1996). A few scrapie cases have also been observed after peripheral challenge of mature FDC deficient TNF $\alpha$ -/- mice (Mabbott et al., 2000a). As discussed previously, the majority of peripherally challenged TNF $\alpha$ -/- mice resist ME7 scrapie infection (Mabbott et al., 2000a). Although infectivity bioassays were not carried out for the spleens of these mice, no PrP<sup>Sc</sup> could be detected using Western blotting, suggesting that neuroinvasion occurred by an FDC-independent mechanism (Mabbott et al., 2000a). Although the involvement of other cell types such as myeloid cells in scrapie replication or transport cannot be excluded, it is

very likely that direct infection of peripheral nerve endings occurred in these cases.

The role of FDC independent mechanisms in scrapie pathogenesis is the subject of Chapter 6 and is discussed in more detail there. It should be emphasised that it is likely that in some situations FDC dependent and FDC independent mechanisms can be used simultaneously by the scrapie agent. Subtle differences in the concentration of the inoculum used during the experiment may result in direct infection of peripheral nerve endings being favoured as opposed to FDC dependent replication.

### **1.2.5 The effect of age on scrapie peripheral pathogenesis**

The age of the mouse at the time of peripheral inoculation with scrapie is known to affect the length of the incubation period and susceptibility to scrapie (Outram et al., 1973). This project concerns itself with elucidating the cellular and molecular mechanisms that are responsible for the observed differences in susceptibility to scrapie in neonatal mice compared to adult mice. It follows up a series of experiments carried out by Outram et al during the 1970s at the Animal Breeding Research Organisation, Edinburgh which have not until now been pursued any further.

Using doses that were 100% lethal to adult mice, a wide distribution of incubation periods was observed following peripheral inoculation of neonatal mice with a 1% dilution of scrapie (Outram et al., 1973). On the one hand peripherally inoculated adult mice succumb to scrapie after a predictable incubation period. On the other hand, a proportion of mice challenged neonatally, survived peripheral inoculation whereas some mice had significantly prolonged incubation periods compared to adult mice (Outram et al., 1973). Mice challenged after 8 days old, succumbed to scrapie after a normal incubation period demonstrating that mice of this age were fully susceptible to scrapie.

Paradoxically, a proportion of neonatally challenged mice were observed to develop scrapie after significantly short incubation periods compared to adults (Outram et al., 1973). This effect was exaggerated if neonatal mice were challenged with higher scrapie doses (10%), whereby the proportion of mice with shorter incubation periods was larger (Outram et al., 1973).

Although the neonatal data appears conflicting two possible explanations could account for these observations. Mice that survive peripheral scrapie challenge and those with prolonged incubation periods, may lack a mature cell population which could be undergoing differentiation after birth (Outram et al, 1973). The lymphoreticular system and in particular FDCs are still undergoing development during the first two postnatal weeks (Berg and Dijkistra, 1995) and therefore may be unable to replicate scrapie (discussed in detail in Chapter 4). FDC are not fully mature until 2-4 weeks after birth as defined by their antigen trapping capability. This is when the primary follicles appear. Therefore, the time at which the FDC matures coincides with the normalisation of the scrapie incubation period to that of the adult.

Splenectomy of neonatal mice has been used to investigate the role of the spleen in scrapie pathogenesis in neonatal mice. As described in section 1.2.1 adult splenectomy increases the incubation period of scrapie after peripheral challenge with scrapie (Clarke and Haig, 1971; Fraser and Dickinson, 1970; Fraser and Dickinson, 1978). If mice are splenectomised at 0-1 days after birth and inoculated i.p with ME7 immediately after splenectomy, there is no effect on incubation period length (Fraser and Dickinson, 1978). This suggests that in the proportion of neonatally challenged mice that succumb to scrapie, the spleen is not involved in neuroinvasion. It is possible that other pathogenic routes may be more efficient at this age.

When splenectomy is carried out neonatally (0-1 days old) and mice are inoculated peripherally at day 5 after splenectomy, then an increase in the incubation period is observed when compared to sham operations (Fraser and

Dickinson, 1978). This suggests that by 5 days old, there is a greater dependency on the spleen to support scrapie replication. The effect of splenectomy on incubation period in mice between the ages of 1-5 days old was not investigated. Therefore the precise age at which the spleen becomes involved in pathogenesis is still unclear.

The neonatally challenged mice that develop scrapie after unusually short incubation periods compared to adults, suggest that a distinct pathogenic route can be used during early life in mice. One possibility is that infectivity is taken up by developing peripheral nerve endings. There is a great deal of evidence demonstrating that the peripheral nervous system is undergoing maturation during the first two weeks of life (Lichtman, 1977; Lichtman et al., 1999). It is plausible that due to the high degree of PNS innervation (Brown et al., 1976) and the structural immaturity of axons (Mirsky et al., 1999) at birth, that scrapie infectivity could be taken up more efficiently by the PNS in neonatal mice. These possibilities are explored in more detail in Chapter 6.

## 1.3 Aims and Hypotheses of the project

The experiments carried out in neonatal mice to study the pathogenesis of scrapie have offered important insights into the systems that may be controlling the entry of scrapie into the CNS. Work carried out in neonatal mice has shown that the pathogenesis is different to that of the adult. Peripheral inoculation of C57BL mice with the ME7 strain of scrapie produced a population of survivors and a proportion of mice with short incubation periods. Two hypotheses have been developed to test the possible mechanisms underpinning the paradoxical observations of both short incubation periods and the presence of survivors in peripherally challenged neonatal mice.

### 1.3.1 Hypothesis 1

It is predicted that PrP<sup>c</sup> and the presence of mature FDCs are critical for the onset of ME7 scrapie replication in the lymphoreticular system of neonatal and young mice. It is therefore postulated that the immaturity of FDCs within the spleen results in survival or prolongation of incubation periods following peripheral challenge with scrapie.

#### 1.3.1.1 Testing of Hypothesis 1

The first object of this stage of the project is to determine the earliest age at which PrP<sup>c</sup> can be detected in the neonatal spleen and to verify the age at which FDCs reach morphological and functional maturity. The age at which these events occur may be related to the differences in susceptibility to scrapie in neonatal mice compared to that of the adult. The data presented in Chapters 4 and 5 describes the maturation of the neonatal spleen architecture using a panel of antibodies against PrP<sup>c</sup> and germinal centre cells. The timing of maturation is related to the development of scrapie susceptibility in neonatal mice described in Chapter 3.

### **1.3.2 Hypothesis 2**

The second hypothesis predicts that peripherally challenged neonatal mice can succumb to scrapie after short incubation periods as a result of an FDC-independent mechanism of scrapie replication.

#### 1.3.2.1 Testing Hypothesis 2

This hypothesis will be tested by inoculating SCID mice of various age groups, peripherally with scrapie. SCID mice provide a model to investigate the role of developing non-lymphoid compartments (e.g peripheral nervous system, central nervous system and myeloid cells) in peripheral scrapie pathogenesis, without the complications of a maturing lymphoreticular system. If the hypothesis is correct, then it is predicted that peripherally challenged neonatal SCID mice will develop scrapie after very short incubation periods, in contrast with adult SCID mice that are known to be resistant to peripheral scrapie challenge. These experiments are described in Chapter 6.

# Chapter 2

## Methodology

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## 2.1 Aims

The aim of this chapter is to describe the methodology applied during the course of the project (these are listed in section 2.3). The composition of all solutions is described in detail in Appendix A. Fine-tuning of these methods for application to the experiments described in subsequent chapters had to be carried out. These are described in section 2.3 and the outcomes of the optimisations are discussed.

## 2.2 Experimental Rationale

A major part of the thesis was to repeat the original neonatal experiments described in Chapter 3, to verify whether the phenomenon could be re-created and also to gain more information regarding the ability of the developing spleen to support scrapie replication. The most convenient way to test this is by Western blotting for the scrapie specific marker, PrP<sup>sc</sup>. The method used had already been established for lymphoid tissue [Farquhar, 1994 #38]. However, before screening was commenced validation of the previously published protocol was carried out using scrapie affected brain tissue initially, followed by trials on spleen. The quality of immunolabelling of Western blots using different anti-PrP antibodies was compared. In addition, comparisons of target protein detection systems were compared to determine the most sensitive and reliable technique (see section 2.3.12.4 and 2.3.12.5).

In order to determine whether the detection of PrP<sup>c</sup> and maturation of spleen FDCs was in any way correlated to the maturation of scrapie susceptibility in young mice, the age at which PrP<sup>c</sup> could first be detected in the developing spleen was established by immunocytochemistry. When the project was started, a reliable method of detecting PrP<sup>c</sup> in spleens of mice had already been published (McBride et al., 1992). The main drawback to this method was that it involved the use of paraffin embedded spleen. If the cell specificity of PrP<sup>c</sup> immunolabelling was to be determined, the FDC specific markers would have to have the ability to immunolabel paraffin embedded spleen sections. However these markers only label cells in frozen tissue sections. A major part of the project involved developing a suitable double immunolabelling method for PrP<sup>c</sup> and FDCs on the same spleen section (see section 2.3.13.9.4). Before this could be done, a reliable method for detecting PrP<sup>c</sup> on frozen spleen sections had to be developed. This was hampered by

non-specific background problems when using the anti-PrP antibody, 1B3. However, these problems were eventually resolved by employing a fluorescent-based immunodetection system (see section 2.3.13.8)

A central part of the hypothesis to be tested was that the maturation of FDCs played a major role in the development of scrapie susceptibility in young mice. Characterisation of germinal centres was carried out using a panel of antibodies against FDCs and B cells (see section 2.3.13.4, 2.3.13.5 and 2.3.13.7). In order to gain more information regarding the maturation of FDC functionality, immune complex trapping assays were used. A variety of methods were compared (see section 2.3.13.6) in order to find the most convenient for use in mice of various age groups. The optimal method defined in this chapter was later applied to testing mice of various age groups to determine the age of onset of FDC functionality (see Chapter 4, section 4.4.3)

Finally, in order to support the PrP<sup>c</sup> studies, some trials were conducted to determine the earliest age at which PrP mRNA could be detected within the spleen. This was done in collaboration with Neil McLennan of the National Creutzfeldt Jacob Disease (CJD) Surveillance Unit, Western General Hospital, Edinburgh, who had recently developed an *in situ* hybridisation method for detecting PrP mRNA in human and mouse brain. In addition, the technique had been recently applied to the detection of PrP mRNA with gut ileum. Since the method worked well on peripheral as well as CNS tissues, it was decided to apply it to PrP mRNA detection in mouse spleen. These trials are described in section 2.3.15.

## 2.3 Materials and Methods

### 2.3.1 Mice

All mice used in this project were bred in the Neuropathogenesis Unit, Institute for Animal Health, Edinburgh (see Table 2.1 below for details). Mice of 17 days old and younger were randomised and allocated a mother until the age of 18 days old when they were weaned. All mice were ear-punched for identification purposes after weaning.

C57BL, C3H, CB20, PrP null and TNFR1<sup>-/-</sup> mice were housed in cages of six under specific pathogen free conditions (SPF) and given food and water ad libitum. The C57BL mouse strain is used routinely at NPU for studying the role of the LRS in scrapie peripheral pathogenesis and was used in experiments described in Chapter 3. C3H mice possess the same *Sinc* genotype as C57BL mice and were used in some preliminary spleen immunocytochemistry trials when the project was started, since insufficient numbers of C57BL mice were available immediately. However, since the role of the LRS had been well characterised in the C57BL/ME7 model, C57BL mice were used in subsequent immunocytochemistry experiments to establish the earliest age at which PrP<sup>c</sup> could be detected in the developing spleen. PrP<sup>-/-</sup> mice were used as a negative control in these experiments for PrP<sup>c</sup> immunolabelling. PrP<sup>-/-</sup> mice were generated by insertion of an MT/Neo cassette into exon 3 of the PrP gene in 129/Ola mice (Manson et al., 1994).

Inbred Severe Combined Immunodeficient Mice (SCID), on the other hand were housed in individual ventilated cages (IVC) in order to protect the mice from risk of infection. These mice have an autosomal recessive mutation that prevents the formation of functional B and T lymphocytes and consequently possess non-functional FDCs (Bosma et al., 1993). Although SCID mice are relatively resistant to peripheral scrapie challenge at moderate doses (Fraser et al., 1996), in this project they have been used to investigate the role of non-lymphoid systems in scrapie pathogenesis (see Chapter 6). It should be noted at this point that at least 15% of a given population of SCID mice can produce low levels of serum immunoglobulin and are referred to as 'leaky'. In experiments involving SCIDs, blood samples were taken at the time of death. Sera was removed and screened by ELISA for the presence of serum immunoglobulin samples to determine the immunological status of the mouse (see section 2.3.9 and 2.3.10)

Mouse Strain	<i>Prn-p</i> genotype	<i>Sinc</i> genotype
C57BL	<i>Prn-p</i> <sup>a</sup>	<i>Sinc</i> <sup>s7/s7</sup>
C3H	<i>Prn-p</i> <sup>a</sup>	<i>Sinc</i> <sup>s7/s7</sup>
SCID	<i>Prn-p</i> <sup>a</sup>	<i>Sinc</i> <sup>s7/s7</sup>
CB20	<i>Prn-p</i> <sup>a</sup>	<i>Sinc</i> <sup>s7/s7</sup>
PrP -/-	<i>Prn-p</i> <sup>-/-</sup>	<i>Sinc</i> -/-

**Table 2.1 Summary of *Sinc* and *Prn-p* genotypes of mouse strains used in the project.**

### 2.3.2 Scrapie strains

The ME7 strain of scrapie was used for all scrapie challenges referred to in this thesis. ME7 was originally isolated from the spleen of a Suffolk sheep affected with natural scrapie (Zlotnik and Rennie, 1963). Passage into mice was by intragastric challenge of Moredun random bred mice. The agent was then passaged intracerebrally (i.c) into Moredun mice, then 9 times through inbred C57BL mice at NPU.

The mouse-passaged ME7 strain of scrapie has been well characterised and produces prominent neuronal vacuolation. Its has been used extensively in the study of peripheral pathogenesis in adult mice. These studies have shown that after intraperitoneal inoculation, ME7 neuroinvasion is dependent on an intact LRS, and in particular fully functional FDCs (Fraser et al, 1970; Fraser et al, 1978; Fraser et al, 1987; Fraser et al, 1996; (Brown et al., 1999; Mabbott et al., 2000a; Mabbott et al., 2000b). This strain is therefore the most appropriate strain to use in defining the role of the developing LRS in the maturation of scrapie susceptibility.

### **2.3.3 Preparation of $10^{-2}$ dilutions of brain homogenate**

All procedures were carried out in a safety cabinet according to standard methods employed at NPU. Scrapie infected and non-infected mouse brain homogenates were prepared on the same day. In order to avoid any possibility of cross contamination during preparation of the homogenates, the normal brain inocula were prepared first within a safety cabinet pre-treated with 2% sodium hypochlorite. This eliminated the possibility of any cross contamination from TSE containing aerosols that may have been present in the safety cabinet. This procedure is standard practise between inoculation or injection procedures that have taken place within the cabinet. The homogenisers are discarded after a single use to prevent possible cross-contamination occurring through residual infectivity that might be left in homogenisers after decontamination procedures.

The brains used for the inocula preparation were removed aseptically from terminal C57BL mice infected with ME7 and also from non-infected mice for use as a negative control. The terminal and control brains were stored at  $-20^{\circ}\text{C}$  until required and full details of the source and destination of each brain kept. The brains allocated for this experiment were weighed using a torsion balance and pooled into a 20ml capacity new pre-sterilised Griffith homogeniser. Homogenisation was carried out using sterile physiological saline to give an initial concentration of  $10^{-1}$ . This homogenate was transferred to a sterile container and diluted to give a final concentration of  $10^{-2}$ . 1 ml aliquots were dispensed into sterile glass bijoux using a 1 ml syringe and stored at  $-20^{\circ}\text{C}$  until required for inoculation.

### **2.3.4 Inoculation of mice**

#### **2.3.4.1 Intraperitoneal (i.p)**

20 $\mu\text{l}$  of a  $10^{-2}$  dilution of scrapie affected brain homogenate was inoculated using a 26 gauge needle into the ventral abdominal cavity. In some experiments, age matched mice were inoculated with a  $10^{-2}$  dilution of brain homogenate from uninfected mice, as a negative control.

There were particular technical difficulties in the inoculation of neonatal mice of 0-1 day old and a major risk was that inoculum could leak from the injection site. This was due

primarily to the fragility of the skin, but also due to an increase in abdominal pressure when holding the neck of the mouse in preparation for inoculation. This problem was further aggravated if the mouse had recently been fed and the stomach was filled with milk whereby there was a risk that when the needle was inserted into the peritoneal cavity and withdrawn, an increase in abdominal pressure could potentially result in leakage of the inoculum

The problem was avoided by supporting the neck with gentle pressure in order to avoid pulling the abdomen taut. The needle was inserted just below the liver in the central abdominal region (Outram et al, 1972). After inoculation the needle was removed carefully and the abdomen was sprayed with a 'new-skin' product Novoseal™ in order to prevent any inoculum seepage. This product formed a thin transparent seal over the injection site.

#### **2.3.4.2 Subcutaneous (s.c)**

Neonatal and older mice were anaesthetised using halothane. Using a 26 gauge needle mice were inoculated into the scruff of the neck with 20 µl of a 10<sup>-2</sup> dilution of scrapie brain homogenate. A slight inflation of the skin could be observed at the site of inoculation when the injection was performed correctly. Mice surplus to requirements were used in practise attempts of s.c injections. Anaesthetised mice were culled by cervical dislocation before they regained consciousness, immediately after a practise attempt.

#### **2.3.4.3 Intracerebral (i.c)**

Neonatal and older mice were anaesthetised under halothane. A needle guard was applied to a 26-gauge needle in order to prevent any more than 2 mm of needle penetrating the brain. The needle was inserted into the right hand side of the skull through the parietal bone and into the cerebral cortex. All mice were inoculated with 20µl of a 10<sup>-2</sup> dilution of scrapie affected brain homogenate.

#### **2.3.4.4 Intravenous (i.v) inoculation of preformed immune complexes**

Mice were anaesthetised using halothane, prior to intravenous inoculation of pre-formed rabbit peroxidase anti peroxidase (PAP) immune complexes (Dako, Z0113). Using a 27 gauge needle, 50 µl of PAP (Dako) was inoculated into the tail vein. This was a technically difficult procedure particularly in young mice. Since I was not fully competent to carry out these injections, they were performed by Diane Ritchie, NPU.

Mice were culled at 3 d.p.i since immune complexes should have been trapped by spleen FDCs after this length of time. Spleens were removed and stored frozen in liquid nitrogen until required.

#### **2.3.5 Determination of Incubation period**

Incubation period is defined as the time elapsed (in days) between the day of inoculation and the clinical endpoint of the disease (Dickinson et al, 1968). At NPU the policy is that all mice are clinically monitored by experienced animal technicians. Scoring of mice begins 120 days after inoculation. Inoculated mice are assessed weekly on the same day, by experienced animal technicians. Each mouse is given a rating as follows: “unaffected,” “possibly affected” and “definitely affected”. The endpoint is defined in one of 4 ways: 1) the day on which the mouse receives a second consecutive “definite” rating. 2) The day on which a mouse receives a “third definite” rating within four consecutive weeks. 3) The day on which a mouse is killed in extremis or 4) The animal is found dead in its cage after receiving a “definite” rating at time of scoring the previous week. This system has been used for many years at NPU, and has been applied to a wide range of TSE experiments. The scoring system is designed to maximise confidence of clinical diagnosis and at the same time minimising suffering of animals.

### **2.3.6 Statistical analysis of incubation period**

Advice was sought from Dr Jill Sales (Napier University, Edinburgh) regarding the statistical analysis of scrapie incubation period data. Since the data could not be assumed to be normally distributed, the Mood's Median Test was chosen as the most appropriate non-parametric test. It is similar to the Kruskal-Wallis test. However, The Mood's Median test is more robust against outliers. The overall median for the experiment is calculated, and the numbers in each sample less than or equal to the overall median are determined. A Chi-squared test of no association is carried out in order to test the hypothesis that the medians are equal.

### **2.3.7 Aseptic removal of spleens from scrapie inoculated mice**

Scrapie infected and control mice were sacrificed 70 days post infection (d.p.i) by cervical dislocation. The following procedures were carried out in a safety cabinet. Using new sterile dissection equipment, the fur was removed from the abdomen region and the exposed muscle surface passed through a bunsen flame. This was to minimise bacterial contamination of dissection scissors as the peritoneal cavity was dissected. Further dissection was carried out to expose the spleen, which was subsequently removed. The spleens were halved using a sterile disposable scalpel and one half snap frozen and stored in liquid nitrogen for Western blotting analysis. The other half was frozen at  $-20^{\circ}\text{C}$  for future bioassays once the Western blots had been carried out.

### **2.3.8 Removal and processing of brains from scrapie inoculated mice**

Mice that have reached clinical endpoint are culled on a routine basis on a specified day each week. For practical reasons, trained animal technicians carry out culling and removal of tissues, since all clinically affected animals have to be dealt with on the same day.

Brains were removed by an animal technician from endpoint scrapie cases and immersion fixed for at least 48 hours in 10% formol saline (BDH) at room temperature prior to trimming and processing. Experienced pathology staff carried out trimming and processing of brains in this project. Brains were cut at four standard coronal levels. This is necessary so that scoring of the brains and scrapie diagnosis based on the presence of

vacuolation in specific brain areas can be carried out. It also allows better penetration of processing reagents. Coronal sections were processed into paraffin wax using a TP1050 (Reichert Jung/Leica) tissue-processing centre. Once processed the tissues were embedded in paraffin wax within metal moulds (Tissue Tek).

### **2.3.9 Removal and processing of blood samples**

This procedure was used for experiments described in Chapter 6. Blood samples were taken from SCID and CB20 control mice in order to ensure that the SCID mice had not become leaky (see section 2.3.1 for further explanation). Blood was removed by cardiopuncture from a clinically scrapie affected mouse and stored overnight at 4 °C until a clot had formed. Sera were separated by spinning the blood sample at 13,000rpm for 5 minutes. Sera were then frozen until required for immunoglobulin testing by ELISA (see below)

### **2.3.10 Screening of SCID mice for serum immunoglobulin**

#### **2.3.10.1 ELISA**

Sera were diluted to 1/100 in ELISA coating buffer and applied to a 96 well plate. Samples were incubated overnight at 4 °C. In order to prevent cross contamination of samples, every second column was left blank. Included in every screening were a positive control sera sample from an immunocompetent CB20 mouse and a negative control sera sample from a non-leaky SCID mouse.

The following day, sera were flicked out and 100µl/well of ELISA blocking buffer was added. Plates were incubated at room temperature for 30 minutes in a humidity chamber and then washed three times using ELISA wash buffer. Rabbit anti mouse –HRP (Dako) was diluted to 1/100 in ELISA coating buffer and applied to coated wells for 2 hours at room temperature in a humidity chamber. Plates were washed three times in ELISA wash buffer. 100µl/well of ELISA chromagen was applied to the plates for 10 minutes and the reaction terminated using 3M HCL. Plates were read at 490nm using an ELISA plate reader. SCID sera samples positive for immunoglobulin were discarded from incubation period analysis. 17/249 (6.8%) cases were positive for serum immunoglobulin.

### **2.3.10.2 FDC and B cell immunocytochemistry**

If it had not been possible to obtain blood samples from scrapie affected SCID mice., spleens were screened for the presence of FDCs and B cells using immunohistochemistry. This was done by immunolabelling frozen spleen sections with B220 antibody against B cells and FDC-M2 against FDCs (see section 2.3.11.3). If the spleens tested positive for either of these markers or both, then the cases were disregarded from incubation period analysis. 4/26 cases where no sera had been obtained tested positive for both B220+ cells and FDC networks. These cases were therefore excluded from incubation period analysis.

### **2.3.11 Antibodies/Lectins**

#### **2.3.11.1 Anti-PrP antibodies**

1B3, a polyclonal antiserum, was raised by inoculating rabbits with PrP<sup>Sc</sup> fibrils from the brains of Sinc<sup>s7</sup> mice terminally affected with ME7 (Farquhar et al, 1989; (Farquhar et al., 1993); Farquhar, 1994. This antibody recognises 4 distinct sites along the PrP sequence including one site in the N-terminus region. See Fig 2.1 and Table 2.2 for recognition sites.

6H4, a monoclonal antibody, was raised by inoculating PrP<sup>-/-</sup> mice with bovine recombinant PrP prepared in Freund's adjuvant (Korth et al., 1997) and was purchased from Prionics, Zurich. This antibody recognises one site in Helix 1 of PrP<sup>c</sup>. (see Table 2.2 and Fig 2.1 for recognition sites).

Antiserum	Position in mouse PrP sequence	Mouse antigenic sequence
1B3	14-36	AMWTDVG....GGWNTGG
	83-102	PHGGGWG....QWNKPS
	119-139	AVVGGGL.....SRPMMH
	188-212	HTVTTT.....RVVEQM
6H4	144-152	DWEDRYRE

**Table 2.2. Antigenic amino acid sequences for anti-PrP antibodies.** Compiled from (Farquhar et al, 1989) and (Korth et al, 1997). Sequences were derived by PEPSCAN analysis

10	20	30	40	50	60
MANLGYWLLA	<u>LFVTMWTDVG</u>	<u>LCKKRPKPGG</u>	<u>WNTGG</u>	SRYPG	QGSPGGNRY P
		<b>1B3</b>			PQGGTWGQPH
70	80	90	100	110	120
GGGWGQPHGG	SWGQPHGGSW	<u>GQPHGGGWGQ</u>	<u>GGGTHNQWNK</u>	<u>PSKPKTNLKH</u>	VAGAAAAGAV
		<b>1B3</b>			
130	140	150	160	170	180
<u>VGGLGGYMLG</u>	<u>SAMSRPMIHF</u>	<u>GNDWEDRYR</u>	<u>ENMYRYPNQV</u>	<u>YRPPVDQYSN</u>	<u>QNNFVHDCVN</u>
<b>1B3</b>		<b>6H4</b>			
190	200	210	220	230	240
ITIKQHTVTT	<u>TTKGENFTET</u>	<u>DVKMMERVVE</u>	<u>QMCVTQYQKE</u>	SQAYYDGRRS	SSTVLFSSPP
	<b>1B3</b>				
250					
VILLISFLIF	LIVG	<b>C-terminal</b>			

**Fig 2.1 . PrP<sup>c</sup> sequence and antibody recognition sites.** The mouse PrP amino acid sequence was obtained from Swissprot accession number P04925 (<http://ca.expasy.org/sprot/>). Antibody recognition sites have been mapped according to information found in the following publications: (Farquhar et al, 1989) and (Korth et al , 1997)

#### **2.3.11.2 FDC-M1**

FDC-M1 (rat anti-mouse  $\gamma$ 2b) was a gift from Dr Marie Kosco-Vilbois (Serono, Switzerland). It is also known as 4C11. The epitope recognised by FDC-M1 has yet to be sequenced. However many studies have shown that this antibody localises on the surface of FDCs and their dendritic processes (Brown et al., 2000; Gray et al., 1990; Kosco et al., 1992; Ritchie et al., 1999).

Tingible body macrophages (TBMs) appear also to stain positive with this antibody. However, they become positive upon ingestion of extraneous dendritic processes (Kosco et al., 1992).

#### **2.3.11.3 FDC-M2**

The rat anti-mouse antibody was also a gift from Dr Marie Kosco-Vilbois (Kosco-Vilbois et al., 1997). The epitope recognised by this antibody has yet to be determined. In contrast to FDC-M1, this antibody does not stain TBMs.

#### **2.3.11.4 Anti- CR1 antibody (clone 8C12)**

The anti-CR1 antibody (rat anti- mouse  $\gamma$ 2a) recognises an epitope present on the mouse 190kDa complement receptor 1 protein (also known as CD35). It was purchased from Pharmingen (Kinoshita et al, 1988)

#### **2.3.11.5 Peanut Agglutinin (PNA)**

PNA was purchased from Vector Laboratories. It is a lectin which binds preferentially to galactosyl ( $\beta$  1-3) N-acetylgalactosamine residues (T-Antigen) on germinal centre B cell glycoproteins (Rose et al., 1980).

## **2.3.12 Western Blotting Analysis of PrP<sup>Sc</sup>**

### **2.3.12.1 Differential extraction of PrP<sup>Sc</sup> from scrapie affected brain**

Snap frozen brains were homogenised in 5 mls 10% brain lysis buffer (BLB) and 20µl each of Phenylmethylsulphonyl Fluoride (PMSF) and 20µl N-ethylmaleimide (NEM) added. Homogenates were reground with 5ml BLB and homogenisers washed out with a further 10mls BLB. The homogenates were decanted into capped 40ml tubes and spun in JA-20 centrifuge for 30 mins at 13,500 rpm. The pellets were discarded and the supernatants decanted into polycarbonate tubes and topped up with BLB. After weighing and balancing, the tubes were spun in a 70 Ti rotor at 46,000 rpm for 2.5 hours. Pellets were subsequently triturated and vortexed in 50µl 1M, pH8.5 Tris-HCl and 3 mls deionised water. 6 mls of 15% Iodine High Salt Buffer (IHSB) were then added to the resuspended pellet and the solution incubated with shaking at 37 °C for 30 minutes. The samples were then divided into two. One half was treated with 10mg/ml PK at 37 °C for 30 mins with shaking and the reaction stopped with 20µl PMSF. The remaining halves were held at 4°C with 20µl PMSF to prevent protein degradation. 500µl aliquots of 20% sucrose /IHSB were added to SW41 polyallomer tubes and the samples overlaid onto the sucrose cushions. Samples were spun at 100,000 rpm in a SW41 rotor for 1.5 hours, 40K at 4°C. The following day the pellets were triturated again in 50µl of 1M Tris-HCl pH 8.5 and 3 mls deionised water followed by vortexing. 6 mls of 10% IHSB were added and samples vortexed and incubated with shaking for 30 mins at 37°C. Samples were overlaid onto 20% sucrose/IHSB cushions in SW41 polyallomer tubes and spun for 1.5 hrs at 40K. Pellets were drained overnight, resuspended in deionised water and aliquoted to the required brain equivalents. The brain PrP<sup>Sc</sup> extracts were stored at -70 °C until required for Western blotting.

### **2.3.12.2 Differential extraction of PrP<sup>Sc</sup> from scrapie affected spleen**

Snap frozen tissues were weighed and pulverised in pre-cooled Thomas AA size homogenisers. Pulverised tissue was then homogenised in 2 mls of 0.2M potassium

chloride with 20 $\mu$ l each of 100mM PMSF and 100mM NEM, both in propan-1-ol. The homogenates were centrifuged at 1500 rpm for 10 minutes at 4°C in order to clear the protein homogenate of any spleen capsule. The supernatants were decanted and centrifuged at 50,000 rpm for 30 minutes at 4°C in polyallomer tubes (Beckmans). The resultant pellets were resuspended in 2ml of 100mM Tris-HCl at pH7.4 and reground in Dounce homogenisers (Wheaton). The homogenate was divided into two equal parts. 4 $\mu$ l of a 5mg/ml proteinase K (Sigma) was added to one tube to give a final concentration of 50 $\mu$ g/ml and the contents incubated with shaking at 37°C for 60 minutes. 20 $\mu$ l of 100mM PMSF and 100mM NEM was added to the other tube and held at 4°C for 60 minutes. 1 ml of 2% Sarkosyl, 20 $\mu$ l PMSF, 2 $\mu$ l  $\beta$ -Mercaptoethanol, were added to each tube before incubation for 60 minutes at 37°C with shaking. The contents of each tube were layered onto a cushion of 20% sucrose in 50mM Tris-HCl pH7.4. The samples were then centrifuged in polyallomer tubes at 50,000 rpm for 2 hours at 4°C. The supernatants were discarded and the pellets were dried under a hand drier to ensure removal of all residues. The dried pellets were immediately stored at -70°C for Western Blotting.

### **2.3.12.3 Preparation and Loading of PrP<sup>Sc</sup> Extracts**

Frozen brain or spleen PrP<sup>Sc</sup> extracts were resuspended in SDS sample buffer to equal masses (calculated from the wet weight). Samples were heated to 98°C for 45 minutes. After heating it was found that solid protein aggregates had become firmly attached to the bottom of the centrifuge tube and the SDS buffer had condensed at the tops of the tubes. The pellets were allowed to cool for 10 minutes on the bench and then using a pipette, the pellets were gently resuspended in the SDS buffer in order to achieve a completely homogenous suspension. The contents were centrifuged for 10 minutes at 13,000 rpm in order to remove solid protein aggregates still present in solution. The supernatants were loaded onto a 10 lane 12% SDS Mini-Gel (Bio-Rad) and run for 35 minutes at 200V (constant), 0.06A. The gel was placed onto a PVDF membrane (Immobilon P) which had been pre-soaked in transfer buffer and blotted in a Semi-Dry transfer cell at 9V, 0.12A (constant) for 60 minutes. Membranes were dried using a hand drier. The Western blots were usually immunolabelled immediately. However, they could be stored in boxes containing silica gel until required.

#### **2.3.12.4 Immunolabelling of blots**

A major issue during the course of the project was the long-term availability of the antibody of choice for mouse PrP immunolabelling, 1B3 (see section 2.3.11.1) At the start of the project, the anti-PrP monoclonal antibody, 6H4 (see section 2.3.11.2) became available commercially. In an attempt to look for an alternative antibody, a small comparative study was undertaken to determine the validity of using 6H4 to screen the spleens of mice involved in the scrapie pathogenesis study described in Chapter 3.

The PVDF membranes were rehydrated in methanol, rinsed in water and then Western Wash buffer. The membranes were blocked using Western blocking agent for 60 mins at room temperature with shaking. 1B3 was applied at a 1/5000 dilution and 6H4 at 1/20000. The blot was pre-washed in Western Wash Buffer and then washed 4 times for 8 minutes each. The alkaline phosphatase conjugated goat anti rabbit secondary antibody (Jackson) was applied at a dilution of 1/10,000 in Western Wash buffer in order to detect 1B3 labelling. An alkaline phosphatase conjugated rabbit anti-mouse secondary antibody was applied at a dilution of 1/10000. Both secondary antibodies were incubated for 90 minutes at room temperature. The membrane was pre-washed with TBS/Tween and then washed 4 times for 8 minutes each.

#### **2.3.12.5 Detection of target proteins on immunoblots**

##### **2.3.12.5.1 BCIP/NBT**

Binding of phosphatase conjugated secondary antibodies was detected by the formazan reaction using BCIP/NBT (Sigma) as a substrate. Membranes were incubated for 3-5 minutes with this substrate, followed by washing with tap water.

##### **2.3.12.5.2 Enhanced Chemiluminescence (ECL)**

Peroxidase conjugates were detected using the ECL method. 500µl each of Supersignal West Dura reagents (Pierce) were mixed and applied to a sheet of glass to form a small puddle. The membrane was placed horizontally onto the puddle ensuring that no bubbles had formed and allowed to incubate for 5 minutes. The membrane was then placed between two acetate sheets in a developing cassette and exposed to photosensitive film for

30 seconds to 1 minute to obtain a variety of exposures.

## **2.3.13 Spleen Immunocytochemistry**

### **2.3.13.1 Preparation of frozen tissue**

Snap frozen spleens were embedded in Optimal Cutting Temperature compound and cryostat sections were cut at  $-20\text{ }^{\circ}\text{C}$ , fixed on electrostatically charged glass slides (Superfrost Plus\*) and then air dried overnight. These slides were chosen because sections adhere more strongly to them. The slides were stored at  $-20\text{ }^{\circ}\text{C}$  in airtight boxes containing silica gel to minimise humidity or used immediately. Frozen slides were brought slowly up to room temperature before opening the boxes. Tissue sections were fixed for 10 minutes in acetone when ready to use.

### **2.3.13.2 Preparation of PLP fixed tissue**

Spleens fixed in periodate-lysine-paraformaldehyde (PLP) overnight at  $4^{\circ}\text{C}$  to preserve PrP antigenicity. Spleens were paraffin-embedded and  $6\mu\text{m}$  sections were cut using a microtome. Sections were dried on Superfrost glass slides for two days at  $37\text{ }^{\circ}\text{C}$ . The tissues were rehydrated in graded alcohols and pre-treated by hydrated autoclaving at  $121\text{ }^{\circ}\text{C}$  for 15 mins to further enhance PrP staining (Kitamoto et al., 1991). Sections were washed in water post-autoclaving. Tissue preparations were immunolabelled according to section 2.3.13.8

### **2.3.13.3 Enzymatic quenching**

If the immunocytochemical method was to involve a peroxidase based enzyme reaction then, sections were immersed in 250 mls of a 1% peroxide/methanol solution for 10 mins.

### **2.3.13.4 FDC-M1 labelling**

Frozen spleen sections were prepared as in section 2.3.13.1. Endogenous peroxidases present in the tissue were quenched as described in section 2.3.13.3. Tissue sections were rinsed twice for 5 minutes each in ICC wash buffer 2. Irrelevant binding sites were blocked using  $100\ \mu\text{l}$ /section of 20% normal mouse serum.  $100\mu\text{l}$ /section of the appropriate FDC specific antibody. FDC-M1 was applied at 1/400 for 1 hour. Sections

were washed three times in ICC wash buffer 2 and the biotinylated rat anti-mouse antibody applied at 1/600 for 60 minutes at room temperature. Peroxidase conjugated avidin-biotin complex (Dako) was applied for 45 minutes at room temperature. The peroxidase substrate Nova Red (Vector) was prepared according to the kit instructions and applied to each section for 6 minutes each. Slides were rinsed immediately in tap water and counterstained using haematoxylin. Sections were dehydrated through graded alcohols and mounted in DPX according to section

#### **2.3.13.5 FDC-M2 /CR1 labelling**

Frozen spleen sections were prepared as in section 2.3.13.1. Irrelevant binding sites were blocked using 100 µl/section of 20% normal mouse serum. The appropriate FDC specific antibody was diluted to the a previously determined optimal concentration in ICC wash buffer 1 and 100µl/section applied for 1 hour at room temperature (FDC-M2 at 1/1600 and anti-CR1 antibody at 1/400). Sections were washed three times in ICC wash buffer 1 and the biotinylated rat anti-mouse antibody applied at 1/600 for 60 minutes at room temperature. The red fluorophore, Streptavidin conjugated-Cy3 (Jackson) was diluted to 1.6 µg/ml and applied to each section for 30 minutes at room temperature. Slides were washed in ICC wash buffer 1 three times for five minutes each and mounted immediately in Dako fluorescent mounting media (see section 2.3.13.13). Sections were visualised using an epifluorescent microscope (Nikon E800)

#### **2.3.13.6 FDC functionality assays**

##### **2.3.13.6.1 In vitro immune complex trapping assay**

Spleen sections from uninfected C57BL mice were prepared according to section 2.3.13.1. Frozen spleens from mice immunised with sheep red blood cells (SRBC) were donated by Dr Neil Mabbott, NPU, for use as positive control tissue for immune complex trapping. These mice possess large well developed germinal centres and had been previously shown to trap immune complexes.

Spleens sections from immunised and non-immunised mice were incubated with 100µl/section of preformed immune complexes. Mouse peroxidase anti peroxidase ( PAP) and mouse alkaline phosphatase anti-alkaline phosphatase (APAAP) were compared for



efficacy. These immune complexes were diluted to 1/10 in the presence or absence of a source of complement. In these experiments 20% normal mouse serum was compared to purified complement. Sections were incubated for 1 hour at room temperature and washed with the appropriate ICC wash buffer. Sections were developed using Vector Red in the case of APAAP incubated sections or DAB in the case of PAP incubated sections.

Sections were rinsed in tap water and counterstained with haematoxylin (see section 2.3.13.12) then mounted in DPX according section 2.3.13.13

#### 2.3.13.6.2 In vivo immune complex trapping assay

Spleens were removed from mice inoculated i.v with preformed Rabbit PAP immune complexes (see section 2.3.4.4). Spleen sections were prepared according to section 2.3.13.1. In order to amplify the signal of any trapped immune complexes, spleen sections were quenched using 1% hydrogen peroxide as described in section 2.2.13.3. Sections were incubated for 1 hour with PAP diluted 1/10 in ICC wash buffer 2 followed by washing three times for 5 minutes each in ICC wash buffer 2. PAP was re-applied for 1 hour at a 1/10 dilution. Followed by washing three times for 5 minutes each. Immunolabelling was detected by incubation with the chromagen DAB for 6 minutes. Sections were mounted according to 2.3.13.13.

#### 2.3.13.7 Germinal centre B cell labelling

Frozen sections were prepared according to section 2.3.13.1. Biotinylated PNA(Vector, B-1075) was diluted to 10µg/ml in ICC wash buffer 1 and then 100µl/section applied for 1 hour at room temperature within a humidity chamber. PNA had to be used as fresh as possible since quality of labelling declines 2 weeks post-reconstitution of lyophilised PNA. Slides were washed three times for five minutes each in ICC wash buffer 2. Peroxidase conjugated ABC complex was applied for 45 minutes at room temperature. Slides were washed again in ICC wash buffer 2. Labelling was developed using DAB (BDH, Dorset) for 6 minutes. Sections were counterstained in haematoxylin and mounted according to section 2.3.13.12 and 2.3.13.13 respectively.

#### 2.3.13.8 PrP<sup>c</sup> labelling

Frozen and paraffin embedded spleen sections were prepared as in sections 2.3.11.1 and

2.3.11.2 respectively. If the labelling was to involve the use of peroxidase conjugates, then endogenous peroxidases were quenched as described in section 2.3.11.3. Irrelevant binding sites blocked using 100  $\mu$ l/section of 20% normal goat serum for 15 mins at room temperature.

In the case of unfixed, frozen tissue, problems were encountered in achieving optimal PrP<sup>c</sup> labelling. A number of detection methods were employed (summarised table 2.3) in order to obtain the most optimal. 100 $\mu$ l/section of anti-PrP antibody was applied at the dilutions shown in Table 2.3. As a negative control, adjacent sections were incubated with normal rabbit serum at the same concentration as the anti-PrP antibody. Sections were incubated overnight at room temperature. The following day slides were washed three times for 5 minutes each in ICC wash buffer 1 in the case of phosphatase and fluorescent conjugates, or ICC wash buffer 2 in the case of peroxidase conjugates.

The appropriate secondary goat anti-rabbit antibody conjugate (Jackson) was applied at the dilution shown for 1 hour at room temperature in a humidity box. Sections were washed three times for 5 mins each in the appropriate ICC wash buffer. If necessary a 1/100 dilution of enzyme-ABC conjugate (Dako) was applied for signal amplification in chromagen reactions. Again these are indicated in Table 2.3. In order to optimise PrP<sup>c</sup> labelling, a range of chromagens and fluorescent dyes were tested.

Fixation	Primary Antibody (dilution)	Secondary Antibody Conjugate (dilution)	Streptavidin-enzyme conjugate	Chromagen
PLP	1B3 (1/1000)	Biotin-Goat anti-rabbit (1/600)	phosphatase	Vector Red
Frozen	1B3 (1/400)	Biotin-Goat anti-rabbit (1/600)	phosphatase	Vector Red
Frozen	1B3 (1/400)	Biotin-Goat anti-rabbit (1/600)	peroxidase	DAB
Frozen	1B3 (1/400)	Biotin-Goat anti-rabbit (1/600)	Cy3(1.8µg/ml)	-
Frozen	1B3 (1/400)	FITC-goat anti rabbit (1/200)	-	-
Frozen	1B3 (1/1000)	Alexa 488-Goat anti rabbit (10µg/ml)	-	-
Frozen	6H4 (1/500)	Biotin- Rabbit anti mouse (1/600)	peroxidase	DAB

**Table 2.3. Optimisation of PrP<sup>c</sup> labelling in spleen sections.** PLP fixed, paraffin embedded and frozen spleen sections were compared. Most of the optimisation was carried out on frozen sections since this was the tissue of choice for future double immunolabelling. The two anti-PrP antibodies, 1B3 and 6H4 were also compared for their efficacy at immunolabelling PrP<sup>c</sup> on frozen spleen sections. Various chromagens and fluorescent dyes were also compared for their effectiveness.

### **2.3.13.9 PrP<sup>c</sup>/FDC double immunolabelling on frozen sections**

#### **2.3.13.9.1 Experiment 1**

The double labelling was carried out in two stages as follows. In order to block non-specific binding sites normal goat serum was applied to acetone fixed cryostat sections at a 1/20 dilution for 15 minutes. 1B3 was applied at a dilution of 1/400 overnight at 4 C. Slides were washed three times using ICC wash buffer 1. Normal rabbit serum was applied to adjacent sections at a 1/400 dilution. FITC conjugated goat anti rabbit antibody was used at a 1/200 dilution, which had been previously determined as optimal single labelled adult sections. Sections were washed three times in ICC wash buffer 1. For the second stage, FDC-M1 was applied at a concentration of 1/400 for 1 hour at room temperature. Following incubation, the antibody was washed off using ICC wash buffer 1. Biotinylated mouse anti rat conjugate was applied at a 1/600 dilution for 1 hour and then washed off using ICC wash buffer 1. Streptavidin conjugated alkaline phosphatase was

applied for 45 minutes and washed using ICC wash buffer 1. Vector Red was applied and the reaction terminated after 6 minutes. Slides were mounted in Dako fluorescent mounting media.

#### 2.3.13.9.2 Experiment 2

In order to improve labelling and reduce the intensity of the staining reproduced by Vector Red, it was decided to investigate the validity of using indocarbocyanine dyes. Cy3 was selected due to the emission spectra being sufficiently separated from FITC emission. Experiment 1 was repeated, this time streptavidin conjugated Cy3 (Jackson) was applied after biotinylated mouse anti-rat, at a dilution of 1.6 µg/ml for 30 mins at room temperature. Slides were washed three times for five minutes each following washing and mounted in Dako Fluorescent mount according to section 2.3.13.13.

#### 2.3.13.9.3 Experiment 3

The signal intensity emitted from FITC conjugates was found to be inadequate using the microscopic equipment available at the time. An alternative green fluorescent conjugate had to be found in order to carry on with the double immunolabelling trials, crucial to the development of the thesis.

Alexa 488 produced by Molecular Probes provided a stable conjugate similar to FITC, but more photostable, thus enabling improvement of PrP<sup>c</sup> labelling. The company only supplied goat anti rabbit conjugates but no anti- rat conjugates, therefore the chosen conjugate would only be able to be used with 1B3 , which was raised in rabbits. The remaining part of this section describes the method in detail.

The double labelling was carried out in two stages using adult C57BL spleens sections and age matched PrP<sup>-/-</sup> spleens sections as negative control for PrP<sup>c</sup> labelling. In order to block non-specific binding sites normal mouse serum was applied to acetone fixed cryostat sections at a 1/20 dilution for 15 minutes. FDC-M1 was applied at a dilution of 1/400 for 1 hour at room temperature. Adjacent sections were incubated with normal rat serum at a 1/400 dilution. Slides were washed three times using ICC wash buffer 1. Biotinylated mouse anti-rat antibody (Jackson) was applied at a 1/600 dilution for 1 hour and slides washed in ICC wash buffer 1. Streptavidin conjugated Cy3<sup>TM</sup> (Jackson) was applied at 1.8µg/ml dilution for 30 minutes at room temperature. Slides were washed in

ICC wash buffer 1, three times for 5 minutes each. Irrelevant binding sites were blocked using normal goat serum at a 1/20 dilution. 1B3 was applied at a concentration of 1/1000 overnight at room temperature. Adjacent sections were incubated with normal rabbit serum at 1/1000. The following day, the antibody was washed off using ICC wash buffer 1. Alexa 488 conjugated goat anti rabbit (Molecular Probes Inc) was applied at a dilution of 10µg/ml (previously found to optimal for PrP<sup>c</sup> labelling) for 1 hour at room temperature. Slides were washed using ICC wash buffer 1 and mounted directly from buffer according to section 2.3.13.13.

#### 2.3.13.9.4 Experiment 4

In this method, the order in which FDC-M1 and 1B3 were applied was reversed, whereby 1B3 was applied first followed by FDC-M1. Double labelling was again carried out in two stages using adult C57BL spleens sections and age matched

Prn-p/- spleens sections as negative control for PrP<sup>c</sup> labelling. In order to block non-specific binding sites normal goat serum was applied to acetone fixed cryostat sections at a 1/20 dilution for 15 minutes. 1B3 was applied at a dilution of 1/1000 overnight at room temperature. Adjacent sections were incubated with normal rabbit serum at a 1/1000 dilution. The following day, slides were washed three times for 5 minutes each using ICC wash buffer 1. Alexa 488 conjugated goat anti rabbit (Molecular Probes Inc) was applied at a dilution of 10µg/ml (previously found to optimal for PrP<sup>c</sup> labelling) for 1 hour at room temperature. Slides were washed three times for 5 minutes each in ICC wash buffer 1. Irrelevant binding sites were blocked using normal mouse serum at a 1/20 dilution. FDC-M1 was applied at a 1/400 dilution for 1 hour at room temperature. Adjacent sections were incubated with normal rat serum at 1/400. Slides were washed three times for 5 minutes each in ICC wash buffer 1. Biotinylated mouse anti-rat antibody (Jackson) was applied at a 1/600 dilution for 1 hour and slides washed in ICC wash buffer 1. Streptavidin conjugated Cy3<sup>TM</sup> (Jackson) was applied at 1.8µg/ml dilution for 30 minutes at room temperature. Slides were washed in ICC wash buffer 1, three times for 5 minutes each. Slides were mounted directly from buffer as described in section 2.3.13.13

#### 2.3.13.10 PrP<sup>c</sup>/FDC-M2 double immunolabelling

This was carried out exactly as described in section 2.3.13.9.4, however FDC-M1 was substituted for FDC-M2. FDC-M2 (see section 2.3.11.3) was applied at a dilution of 1/1600.

#### **2.3.13.11 PrP<sup>c</sup>/CR1 double immunolabelling**

This was carried out exactly as described in section 2.3.13.9.4, however FDC-M1 was substituted for anti-CR1 antibody. Anti-CR1 (see section 2.3.11.4) was applied at a dilution of 1/400.

#### **2.3.13.12 Counterstaining of sections**

Immunolabelled sections were immersed in haematoxylin for 2 minutes. Rinsed in tap water and the colour intensified using Scott's Tap Water for 2 minutes. Sections were given a final rinse in tap water before dehydration

#### **2.3.13.13 Mounting of tissue**

Sections labelled using non-immunofluorescent chromagens were permanently mounted in non-aqueous DPX. Sections were first dehydrated after the final rinse in tap water, by sequential immersion in graded alcohols. Sections were removed from 100% xylene and mounted immediately in DPX.

Section labelled using fluorescent markers were removed from buffer after the final wash, and mounted in Fluorescent Mounting Media (Dako).

### **2.3.14 Microscopy**

#### **2.3.14.1 Confocal**

A significant amount of time was spent developing double labelling for use with a confocal system. A confocal microscope was not available at NPU until the end of the project; therefore facilities had to be sought elsewhere. Initially, trials were made using a BioRad confocal microscope at the Roslin Institute, however the Leica confocal system offered by the University of Edinburgh included up-to-date software where image analysis could be carried out. Therefore, all the results presented in this thesis were produced using the Leica TCS NT microscope.

#### **2.3.14.2 Co-localisation analysis of double immunolabelled spleens**

Once the desired confocal image was captured on screen, then a line could be drawn through the area of interest which demonstrated co-localisation. The software would calculate pixel intensity values for both red and green labelling separately. These values are plotted against their relative positions along the line.

#### **2.3.14.3 Light Microscopy**

Images of sections were made using a Polaroid camera linked to a Nikon E800 microscope.

#### **2.3.14.3 Fluorescent microscopy**

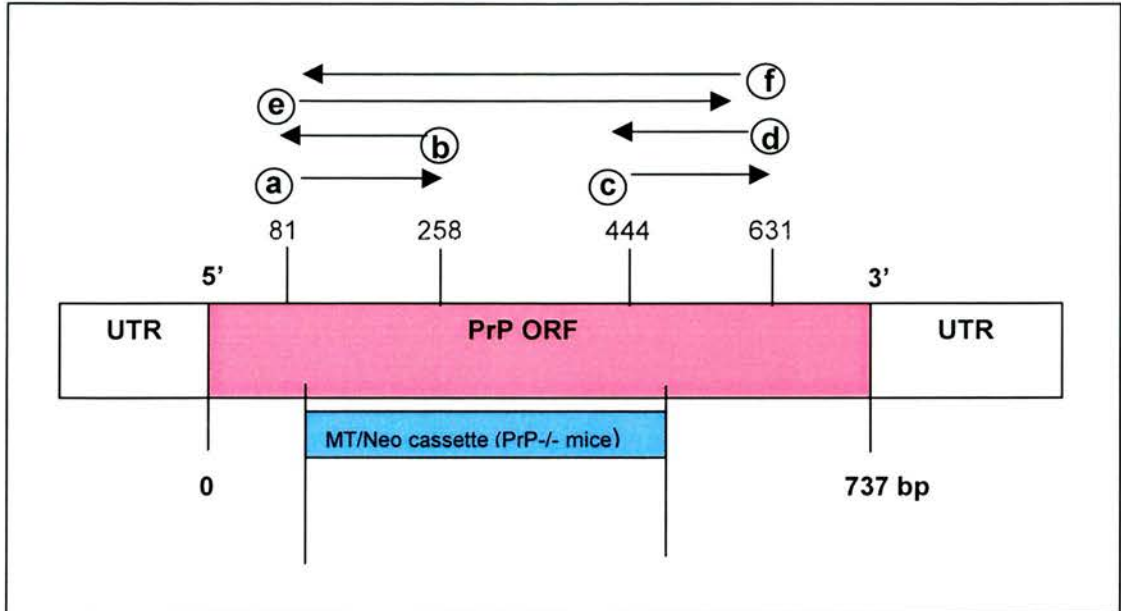
Pilot studies carried out at the beginning of the project were taken using a video camera linked to Nikon E400 epifluorescent microscope since this was the only fluorescent microscopy system available at the time. Image printouts were obtained from a digital printer linked to the video. The transfer of digital information from the video to the printer was poor and resulted in dull, poor quality fluorescent images.

Single labelled fluorescent images shown in this thesis were taken using a Nikon E400 epifluorescent microscope linked to a Hamamatsu ORCA II™ Dual Scan Cooled CCD digital camera (Model C4742-98) via a standard C-Mount. The CCD chip cools down to – 50 °C resulting in a greater sensitivity required for fluorescent microscopy. Images were edited and saved as TIFF files using ImageProPlus™ software. This fluorescent imaging system became available at NPU in January 2000.

### **2.3.15 PrP mRNA In situ hybridisation**

The *in situ* hybridisation method and probes used in this study were developed by Neil McLennan of the National Creutzfeldt Jacob Disease (CJD) Surveillance Unit, Western General Hospital, Edinburgh. The method was originally developed to analyse PrP mRNA in human CNS from individuals of different age groups (McLennan et al., 2001) but was applied to PrP mRNA detection in human gut ileum (Shmakov et al., 2000). In situ hybridisation procedures were carried out as a collaborative effort with Neil

McLennan. Tissue harvesting, fixation and processing were carried out at NPU.



**Fig 2.2** Positions of digoxigenin (DIG)-labelled cRNA probes relative to the Prn-p open reading frame (ORF).

This diagram illustrates the PrP open reading frame (ORF) in exon 3 of the Prn-p gene. Probes a, b, c and d are each approximately 180 nucleotides in length. Probes e and f were shortened to approximately 150 nucleotides using limited alkaline hydrolysis. Probes b, d and f represent the antisense probes. Probes a, c and e represent the sense probes. In order to maximise sensitivity probe cocktails composed of all three separate probes were used in the experiments. Note that the MT/Neo cassette used by Manson et al, 1994 to create the Prn-p<sup>-/-</sup> 129/Ola mice does not cover the entire ORF sequence. Parts of the antisense probes may be able to anneal to the 3' end of the ORF

### **2.3.15.1 Preparation of tissue**

The relative efficacy of the fixatives 4% paraformaldehyde and 10% formol saline were compared for PrP mRNA in situ hybridisation studies. Brain and spleen tissue were harvested from Prn-p +/+ and Prn-p-/- mice and immersion fixed in the fixatives for 48 hours. Fixed brains and spleens were processed into paraffin according to section 2.3.8, followed by paraffin embedding. 6µm sections were cut on a microtome and floated onto RNase-free glass slides (Superfrost Plus, BDH).

### **2.3.15.2 Rehydration of brain and spleen sections**

Sections were dewaxed and rehydrated using the following regime:

Histoclear	two immersions for 5 mins each
99% ethanol	two immersions for 1 minute each
90% ethanol	1 min
80%	1 min
70%	1 min
DEPC water	5 mins
0.2M HCL	15 mins
chloroform	5 mins
2x SSC	5 mins

### **2.3.15.3 Proteinase K treatment**

Sections were rinsed for 5 minutes in pH 7.5 50mM Tris-HCl then treated with proteinase K using a 10 µg/ml<sup>-1</sup> PK solution for 60 mins at 37 °C. After the treatment, tissues were washed in 1X PBS and fixed in 2 mg/ml glycine for 15 mins, in order to terminate the proteinase reaction. Sections were washed in DEPC treated water 3 times for 5 mins each, followed by a 5 minute wash in PBS and for 2 mins in 2X SSC. Finally, the sections were rinsed for 2 mins in DEPC treated water and rinsed in 100% ethanol and dried at room temperature.

#### **2.3.15.4 Prehybridisation treatment**

A short while before preparing the ISH prehybridisation solution, Herring sperm DNA was denatured at 97 °C for 5 mins and chilled on ice to prevent strand re-annealment. The denatured Herring sperm DNA (250 µg/ml<sup>-1</sup>) was then added to the ISH prehybridisation solution (see Appendix A) together with 250 µg/ml<sup>-1</sup> yeast tRNA. The solution was vortexed well and 40µl applied to coverslips. The sections were placed on top of the coverslips containing the prehybridisation mix and incubated for 2 hours at 42 °C in a Hybaid Omnislide incubator.

#### **2.3.15.5 Hybridisation**

The ISH hybridisation solution was prepared (see Appendix A), vortexed well and divided in two. The cRNA sense and antisense probes were added the solution to make a final concentration of 500ng/ml<sup>-1</sup>. (see fig 2.2 for details of probes).

The lid of the eppendorf was pierced prior to incubation of the mixture to 99 °C for 5 mins. The mix was chilled immediately after heating and 25 µl of the hybridisation solution applied to glass coverslips. The sections were covered with the coverslips, sealed using cow gum and allowed to dry briefly.

Sections were heated for 5 minutes at 80 °C then at 55 °C overnight in a Hybaid incubator.

#### **2.3.15.6 Post-hybridisation**

In order to remove the coverslips, the sections were rinsed in 5X SSC. 45µl of RNase A solution was applied to each section and covered with a plastic coverslip. Sections were incubated in Rnase A solution for 30 mins at 37 °C in a humidity box. This time the Hybaid incubator was not used in order to prevent contamination with RNase A.

Sections were washed in RNase wash solution (see Appendix A) 5 times for 5 mins each at 37 °C. The treatment of sections with RNase A in high salt buffer results in digestion of unbound single-stranded RNAs leaving RNA:RNA duplexes untouched, thus greatly

reducing background staining.

Sections were then washed thoroughly using the following regime:

5X SSC

2X SSC        10 mins at 66 °C

0.2X SSC      60 mins at 66 °C

0.2X            5 mins at room temperature

#### **2.3.15.7 Immunological detection of DIG labelled probes**

Sections were rinsed in DIG buffer 1 for 5 minutes and blocked in DIG buffer 2 for 1 hour. Sections were incubated with anti-DIG- alkaline phosphatase antibody diluted 1/500 for 90 mins at room temperature. Sections were rinsed in DIG-buffer 1, twice for 30 mins each, followed by a 5 minute wash in DIG-buffer 3. Sections were then incubated in DIG-buffer 4 containing NBT/BCIP (50µl/section) overnight in the dark at room temperature in a Hybaid chamber.

#### **2.3.15.8 Washing and mounting of sections**

The coverslips were removed and sections rinsed in X1 TE and mounted in Aquamount. The presence of DIG-labelled probes was visualised microscopically and images taken using a Polaroid camera according to section 2.3.14.3.

## 2.4 Results/Discussion

### 2.4.1 Comparison of 1B3 and 6H4 for use in Western blotting

For Western blotting, the first issue to be addressed was which anti-PrP antibody to use. When the project was started, 1B3 was the antibody of choice at NPU for PrP immunocytochemistry, however was in limited supply. The monoclonal antibody, 6H4 became commercially available shortly after the start of the project and was reported to detect PrP<sup>Sc</sup> on brain immunoblots (Klein et al., 1997; Korth et al., 1997) and spleen (Klein et al., 1998). It was decided to compare the quality of labelling produced by the two antibodies on immunoblots before proceeding with experimental tissue.

Since there are high levels of PrP<sup>Sc</sup> in the brains of terminally infected animals, immunoblotting of brain derived PrP<sup>Sc</sup> was carried out first. It was reasoned that this would be a good starting point from which to validate and practice the Western blotting technique in order to achieve a high level of efficiency and reliability, before testing the method on spleen.

PrP<sup>Sc</sup> was extracted from brains according to section 2.3.12.1 and Western blotted according to section 2.3.12.3. The membrane was cut in half and one half incubated with 1B3 and the other half with 6H4 according to section 2.3.12.4. The labelling was detected according to section 2.3.12.5.1.

It can be seen from Fig 2.3 that both antibodies detect brain derived PrP<sup>Sc</sup> satisfactorily. Both antibodies label the four major bands produced after electrophoresis in denaturing conditions: 18-20, 23-25, 27-30 and 33-35 kDa which is in agreement with previous studies (Farquhar et al., 1989). 1B3 produces slightly more background. This could be because it has two more recognition sites than 6H4.

Since 6H4 had worked well on brain PrP<sup>Sc</sup> trials, spleen derived PrP<sup>Sc</sup> extracts were tested using this antibody. It can be seen from Fig 2.3, that 6H4 when applied to spleen PrP<sup>Sc</sup>

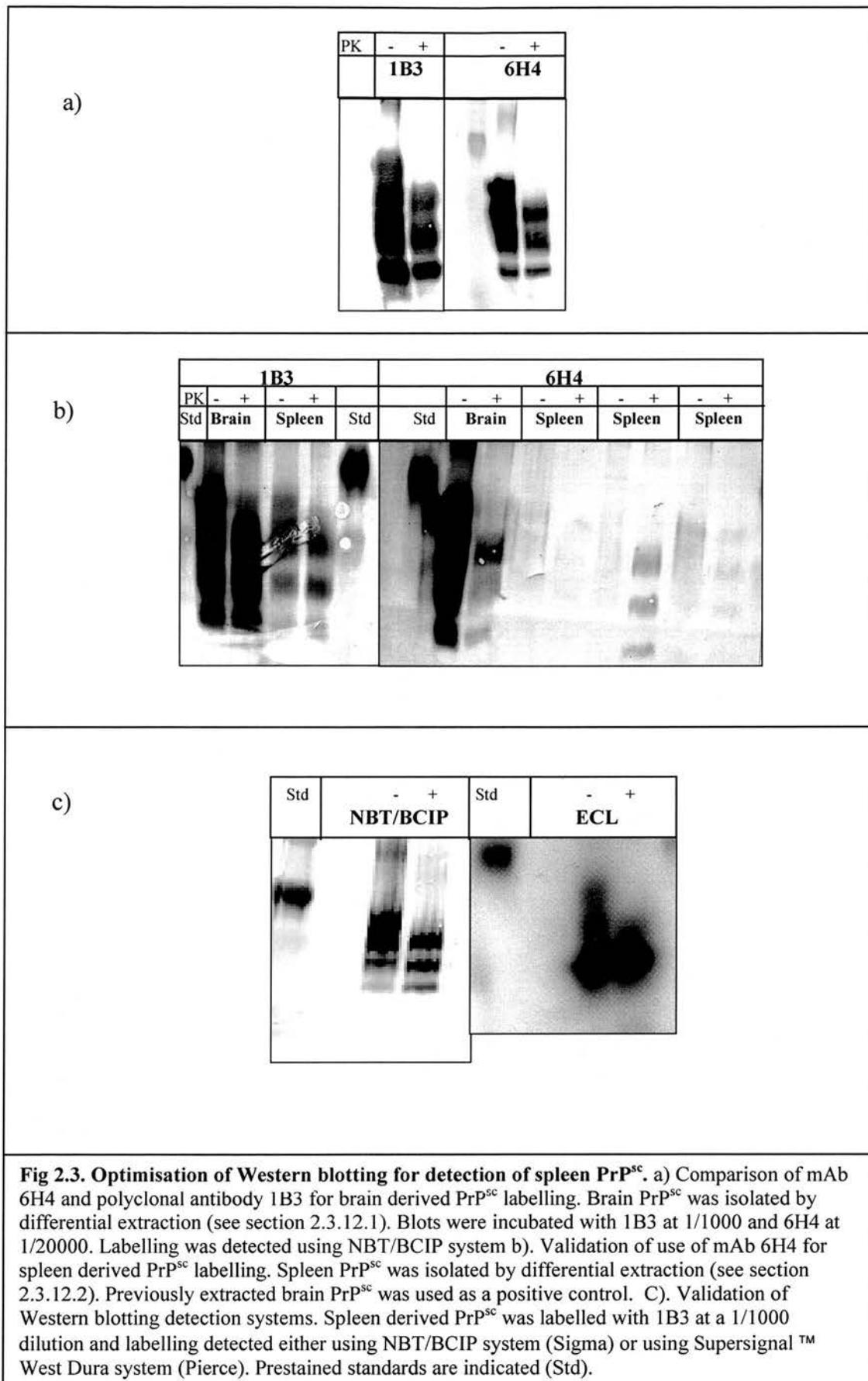
extracts, produces poor quality labelling compared to 1B3. It appeared to label PrP bands from proteinase K (PK) treated samples as opposed to the non-PK treated sample halves. This could have been due to loss of some of the PK untreated sample when the purified homogenates were split into two aliquots for PK treatment. Additionally, the PK treatment might increase the immunogenicity of PrP and enhanced immunolabelling. It is possible that further optimisation of the Western blotting protocol for mAb 6H4 may have resulted in more efficient labelling. However, it was decided that **1B3 should be used since it appeared to be more reliable than 6H4 for spleen derived PrP<sup>Sc</sup> detection in Western blots.**

#### **2.4.2 Comparison of NBT/BCIP and ECL for use as detection systems for Western blotting**

Enhanced chemiluminescence is the detection system of choice for western blotting and has been used widely in PrP<sup>Sc</sup> detection. It is a multi-step procedure and is highly sensitive. The NBT/BCIP system on the other hand is a simple one step procedure and has been described previously for the detection of spleen PrP<sup>Sc</sup> on immunoblots. The two methods were compared for their efficiency at detecting PrP<sup>Sc</sup>.

Spleen PrP<sup>Sc</sup> extracts were Western blotted and immunolabelled with 1B3. The ECL system produced a high background when the blot was exposed to photographic film. This was more than likely to be due to the secondary antibody dilution. When the blot was visualised using a Kodak image analyser, the background was no longer present and what appeared to be PrP<sup>Sc</sup> bands were present. The triple banding pattern could not be clearly seen due to the intensity of the staining. However, the characteristic shift in mobility of the PK treated sample could be clearly seen.

From this pilot study it could be concluded that with further refinements, the ECL system could be used for spleen PrP<sup>Sc</sup> detection. **However, due to the time constraints of the project it was decided to continue with the original protocol published by Farquhar et al. This system proved to be the simplest and most reliable.**



## **2.4.3 Optimisation of immune complex trapping assays**

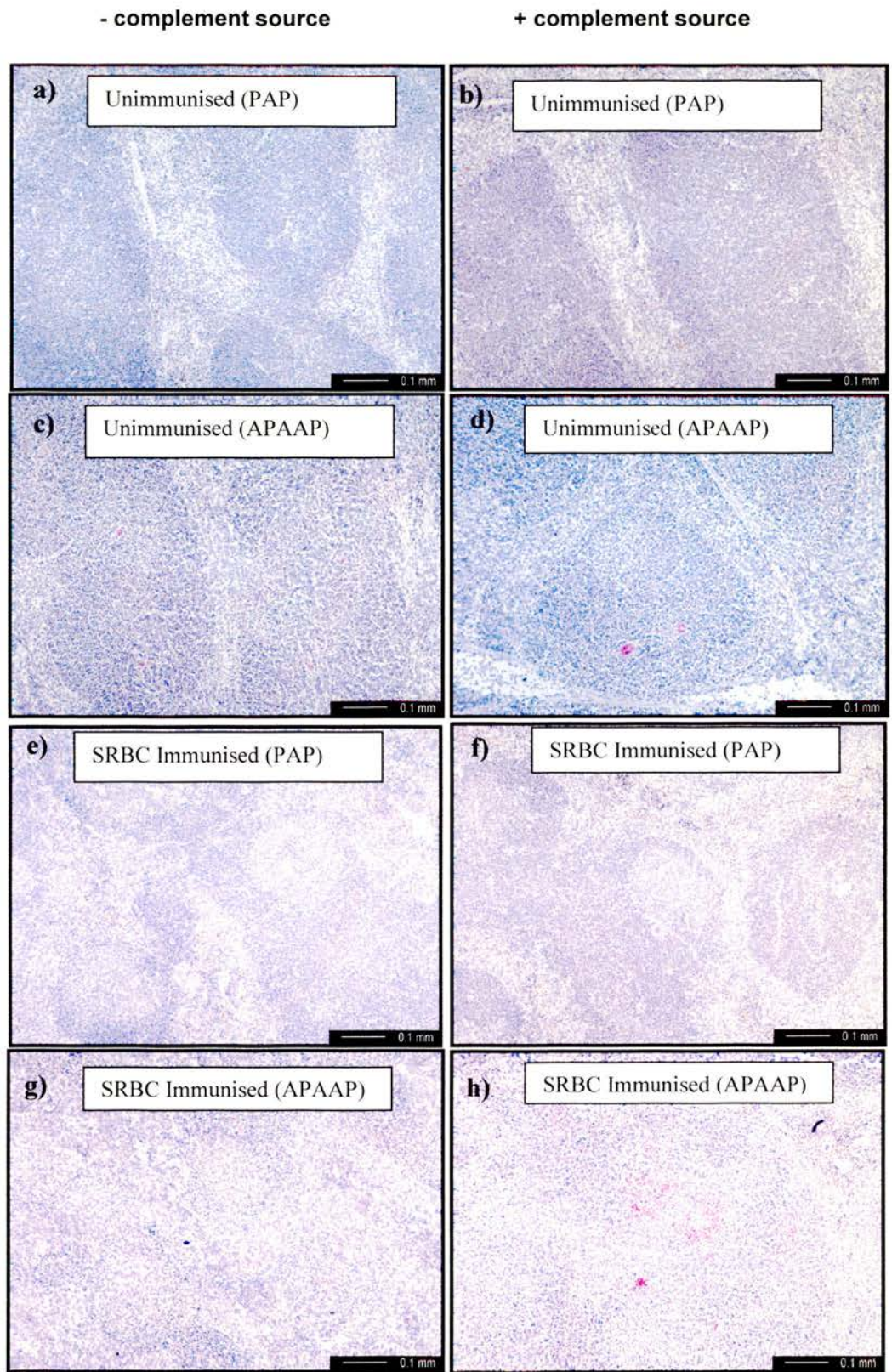
### **2.4.3.1 Validation of *in vitro* immune complex trapping**

In Chapter 4, the ontogeny of FDC function in the developing mouse spleen is addressed as an issue which may have a bearing on the development of scrapie susceptibility. A method for assaying immune complex trapping, which would work on the mouse spleens of various age groups, had to be obtained.

Using the *in vitro* immune complex trapping assay employed by Yoshida et al, 1993 (explained in Chapter 4 section 4.1.2), no evidence of immune complex trapping could be found in the naïve animal even when spleen sections were incubated with APAAP or PAP in the presence of fresh mouse serum (FMS) (see Fig 2.4a-d). The only situation in which immune complex trapping could be found was in the spleen of SRBC-immunised mice (Fig 2.4h). In these cases, FDC associated immune complex trapping could be demonstrated only when sections were incubated with APAAP in the presence of FMS. No trapping could be found when PAP was used as the immune complex (Fig 2.4f). Although tests were repeated several times, the reasons for this difference could not be established.

Since it was not possible to obtain results using the *in vitro* assay, the method could not be applied to studying the age of onset of immune complex trapping. Although it would have been possible to immunise the animals with SRBC, it would have taken at least 3 weeks before the animals were fully immunised and ready for the spleens to be removed for the immune complex trapping assay. This would have defeated the purpose of the study which was to analyse of mice of particular age groups for their immune complex trapping capabilities at that age.

Another method had to be found in order to carry on with the study which would be suitable for all the age groups.



**Fig 2.4: Optimisation of the *in vitro* immune complex trapping technique.** The abilities of two preformed immune complexes (PAP and APAAP) to demonstrate immune complex trapping function in spleen FDCCs of immunised and unimmunised mice were compared. Sections in the lefthand panel were incubated without a source of complement (FMS) and sections on the right hand panel were incubated with FMS.

#### **2.4.3.2 Validation of *in vivo* immune complex trapping following i.v injection**

An alternative to the *in vitro* immune complex trapping assay is the *in vivo* immune complex trapping assay, where immune complexes are injected into the rodent through the tail vein. The *in vivo* method has several disadvantages compared to the *in vitro* approach, namely that it requires the use of extra animals. Stocks of spleens from unimmunised C57BL mice of various age groups had been built up during the course of the project, and could have been used for the *in vitro* assay without having to inject and cull extra animals. In addition, i.v injections are technically difficult. Young mice have small and fragile tail veins making the procedure even more problematic. For these reasons, the *in vitro* trapping assay would have been ideal.

In order to avoid unnecessary injections inoculation of older mice were carried out first, beginning with 11 week old mice and ending at the youngest age group where no immune complex trapping could be detected in the spleen.

The spleens of 11 week old i.v injected mice were used to optimise the staining method. It was found that by applying two separate layers of PAP, good immunolabelling of trapped immune complexes could be obtained (see Table 2.4). This method was applied to the study of immune complex trapping ontogeny in developing mice (see Chapter 4)

Method	PAP dilution	No. PAP layers	Incubation time/layer	No. positive spleens/ No. spleens tested	Comments
1	1/10	1	90 mins	5/5	Weak labelling
2	1/10	2	1= 60 mins 2= 60 mins	2/2	Strong labelling. No background
3	1/10	2	1= 60 mins 2= 60 mins	2/2	Strong labelling. High background
4	1/10	1	overnight	2/2	Strong labelling. High background

**Table 2.4. Development of immunocytochemistry for detecting PAP immune complexes after intravenous administration.** This table summarises the method variations applied to spleen sections from mice previously inoculated intravenously with PAP. The optimal method was once which demonstrated clear immunolabelling of PAP but with low overall background staining. Method 2 gave the best immunolabelling with the lowest background (see section 2.3.13.6.2 for protocol)

#### 2.4.4 Optimisation of PrP<sup>c</sup> immunocytochemistry

A working method for detecting PrP<sup>c</sup> was sought which could be applied to detecting PrP<sup>c</sup> within the spleens of developing mice. Trials were carried out on adult spleen, which had been either PLP fixed or frozen. Sections were immunolabelled with 1B3 according section 2.3.13.8. Immunolabelled frozen sections were developed using either a peroxidase based ABC method or a phosphatase based method. The quality of the staining was found to be poor and background was high especially in peroxidase sections (see Fig 2.5 a and b). Satisfactory results were obtained on the other hand with PLP fixed paraffin embedded sections which had been developed using the Vector Red phosphatase based method. Nevertheless, paraffin sections cannot be used for labelling with FDC specific markers. Since, double immunolabelling studies were to be carried out using such markers; a better method for PrP<sup>c</sup> labelling on frozen sections had to be found.

It was found that when 1B3 was linked to the fluorescent marker Alexa 488, good immunolabelling was obtained with limited background (Fig 2.5e). The only drawback

with this method was that general morphology could not be analysed since visualisation of labelling was by fluorescent microscopy. Nevertheless, the use of this marker solved the problem of detecting PrP<sup>c</sup> on frozen sections and could obviously be applied to double immunolabelling studies.



## **2.4.5 Optimisation of PrP<sup>c</sup> and FDC double immunolabelling**

### **2.4.5.1 Validation of Vector Red as a chromagen**

Adult frozen spleen sections were prepared and immunolabelled according to section 2.3.13.9.1. Using a Nikon E800 epifluorescent microscope, it was possible to observe co-localisation of PrP<sup>c</sup> and FDC-M1, however it was found that the colour intensity produced by Vector Red coupled to FDC-M1 antigen was very high and was diminishing the amount of green fluorescence produced by FITC.

It was found that the quality of the image produced from the video digital printer was extremely low. Green fluorescence emitted by FITC was also very difficult to visualise using the digital video output. This was due to the ultraviolet light quenching FITC. Nevertheless, the quality of labelling appeared greater when visualised under the microscope compared with the digital output. Due to the high colour intensity associated with Vector Red, other red fluorophores were tested.

### **2.4.5.2 Order of application of primary antibodies for double immunolabelling**

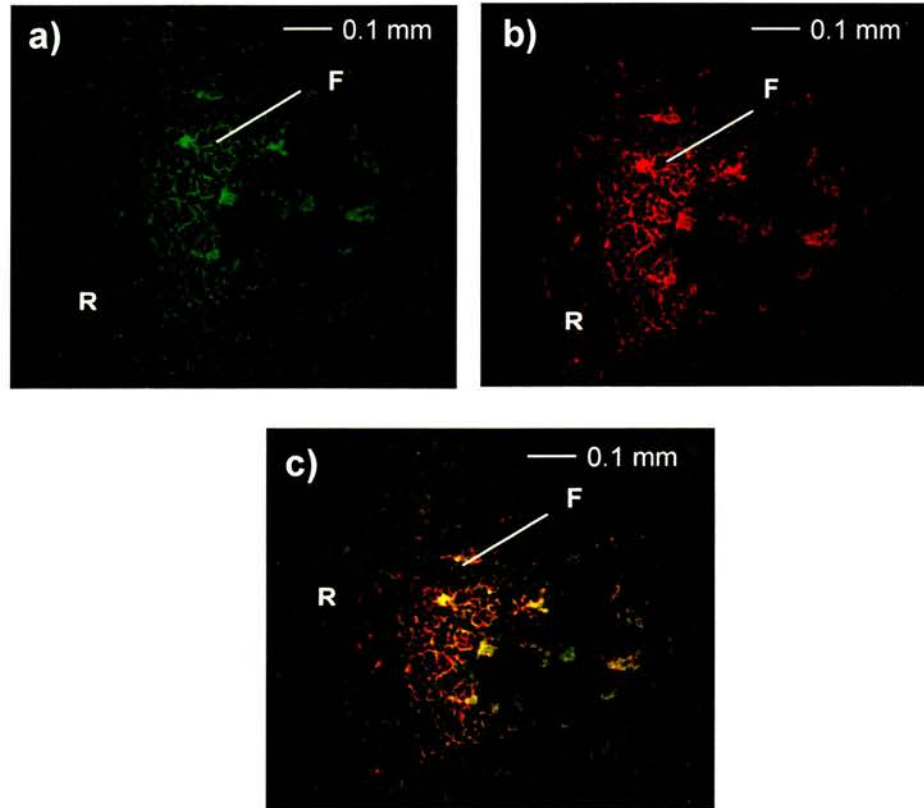
It was found that by using streptavidin conjugated Cy3<sup>TM</sup> (Jackson) to label FDCs and Alexa<sup>TM</sup> 488 (Molecular Probes) to label PrP<sup>c</sup>, then sufficient colour intensities were produced which could withstand quenching after confocal analysis.

During the optimisation of spleen double immunolabelling, it was discovered that the order in which the primary antibodies were applied to the spleen affected the degree of non-specific co-localisation of markers observed using the confocal microscope. Prn-p<sup>-/-</sup> mice were used as a negative control for PrP<sup>c</sup> labelling and should not demonstrate any PrP<sup>c</sup> staining after incubation with 1B3. When Prn-p<sup>-/-</sup> sections are double labelled with PrP<sup>c</sup> and FDC markers, then no co-localisation should be encountered. In the present study it was found that if FDC-M1 is applied first to the spleen section, followed by incubation with 1B3 overnight (see section 2.3.13.9.3), then 1B3 appears to adhere to the FDC-M1 either in a specific or non-specific way resulting in co-localisation of the two markers. Therefore when the double labelled Prn-p<sup>-/-</sup> section is observed under the confocal microscope (see Fig 2.6a), green PrP<sup>c</sup> labelling can be observed when the Prn-p<sup>-/-</sup> section is scanned using the green channel only. The labelling pattern resembles the red

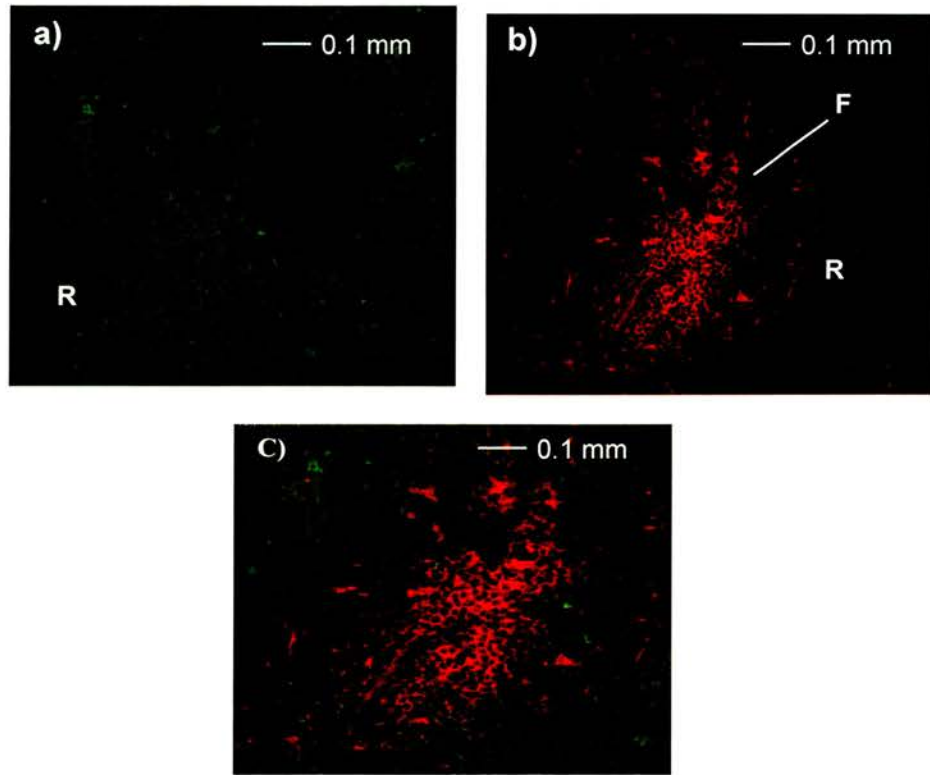
FDC-M1 staining especially in areas where the FDC-M1 is intense (Fig 2.6b). When the images produced by the green and red channels are merged (Fig 2.6c) then an orange hue is observed where the labelling for both markers overlap and appear to co-localise.

There is no non-specific labelling in the Prn-p <sup>-/-</sup> section if 1B3 was applied first, followed by FDC-M1 (see Fig 2.7c). Since 1B3 is applied to the section overnight, a possible explanation for the co-localisation observed in the Prn-p <sup>-/-</sup> spleen is that 1B3 adheres non-specifically to FDC-M1.

There does not appear to be a problem with fluorophore cross talk. If there was cross talk then a section single labelled with 1B3 and detected using a green fluorophore should produce a signal when viewed through the red filter and this was found not to be the case.



**Fig 2.6 Double labelling optimisation: Experiment 3.** A Prn-p <sup>-/-</sup> spleen section was double immunolabelled according to section 2.3.13.8.3 whereby **FDC-M1** (red labelling) was applied first (see Fig 2.6 a.) followed by **1B3** (green labelling) shown in Fig 2.6 b). The composite image is shown in 2.6 c) and the yellow labelling demonstrates co-localisation of markers. Abbreviations: F= follicle; R= red pulp. Scale bar=0.1 mm.



**Fig 2.7 Optimisation of double immunolabelling- Experiment 4.** A Prn-p <sup>-/-</sup> spleen section was double immunolabelled according to section 2.3.13.8.4 whereby IB3 was applied first (see Fig 2.7a.) followed by FDC-M1 (red labelling) shown in Fig 2.7b). The composite image is shown in 2.7c. Since the spleen section is from a Prn-p <sup>-/-</sup> mouse, no PrP<sup>c</sup> labelling can be observed and therefore no co-localisation occurs. Abbreviations: F= follicle; R= red pulp. Scale bar=0.1 mm.

#### **2.4.6 Comparison of 4% Paraformaldehyde and formol saline as fixatives for *in situ* hybridisation of PrP mRNA**

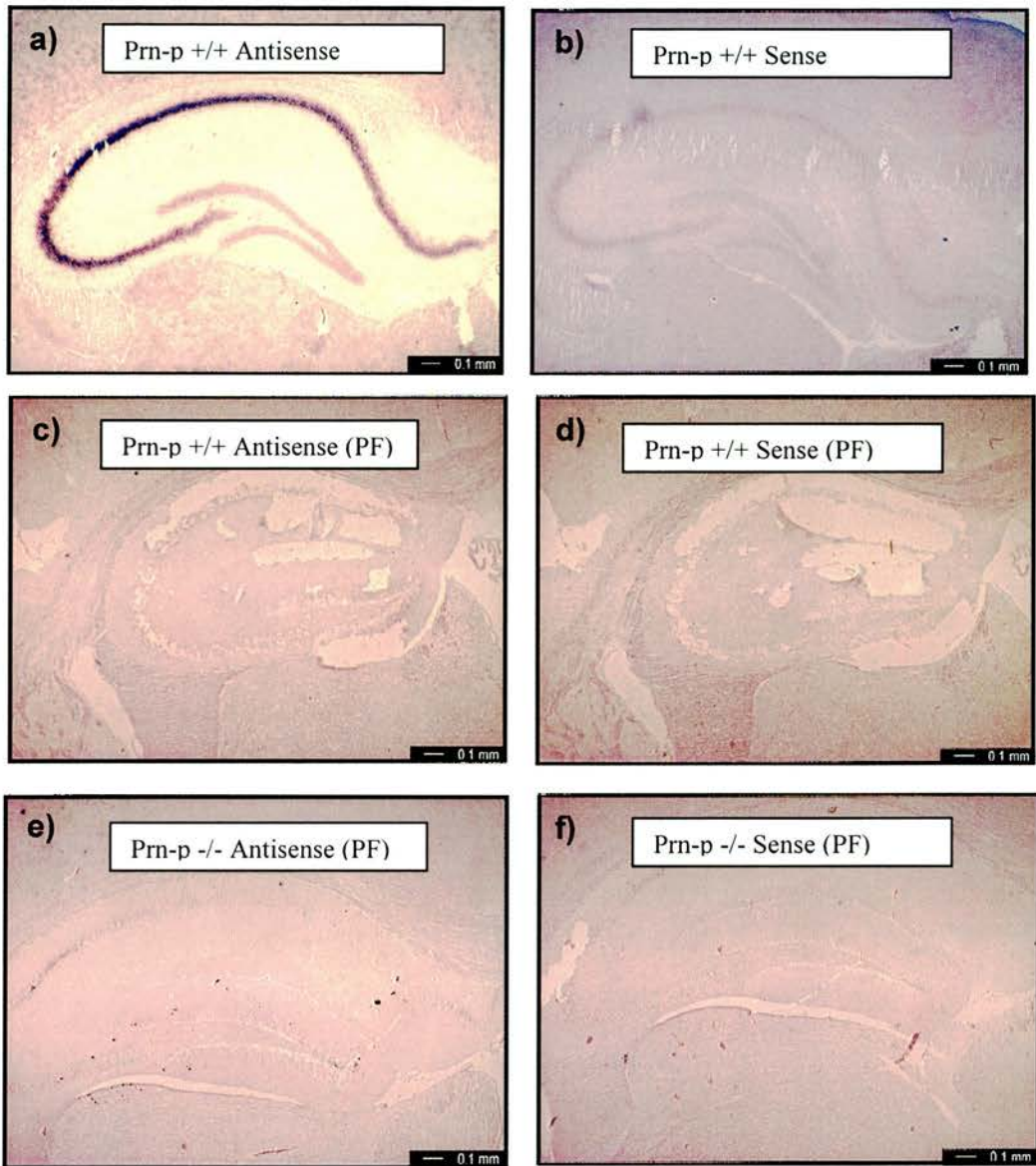
Fixative was the first variable to be studied in the optimisation of PrP mRNA *in situ* hybridisation in spleen. Since brain has high levels of PrP mRNA, trials were carried out on brains fixed either with 10% formol saline or 4% paraformaldehyde. Since this was the first attempt, Neil McLennan (National CJD Surveillance Unit, Edinburgh) provided formol fixed Prn-p *+/+* brain sections, which were known to contain high levels of PrP mRNA. It should be noted that no Prn-p *-/-*, formol fixed brain sections were included in this trial.

It can be seen from Fig 2.8a that PrP mRNA labelling can only be observed in the formol fixed Prn-p *+/+* brain incubated with anti-sense probes (Fig 2.8a). No labelling can be observed in the adjacent section incubated with the sense strands (Fig 2.8b). No labelling can be observed in the 4% paraformaldehyde fixed brain sections (Fig 2.8c and d). In addition the quality of the sections is poor compared to the formol fixed tissue. It is clear that 10% formol saline is superior to 4% paraformaldehyde in the fixation of brain for *in situ* hybridisation. Since brain was to be used as a positive control in any trials with spleen, it was decided to continue the tests using formol fixed spleen.

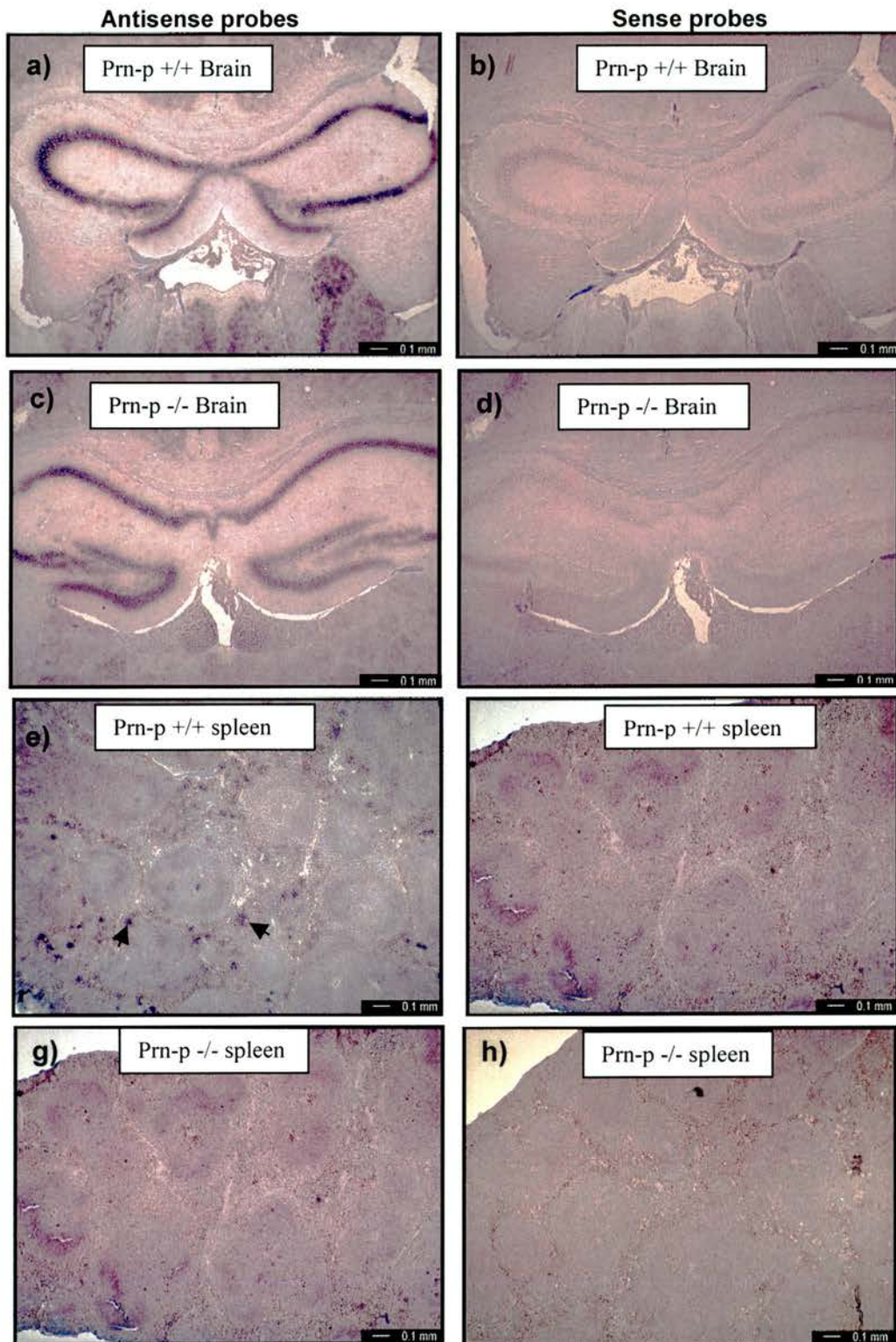
In this trial, Prn-p *-/-* formol fixed brains were included. Fig 2.9a demonstrates that PrP mRNA can be detected in the formol fixed wildtype brain when incubated with the antisense probes, as expected. No labelling can be detected in the adjacent section hybridised with sense probes (Fig 2.9b). Unexpectedly, PrP mRNA labelling could be observed in the Prn-p *-/-* brain section incubated with antisense probes (Fig 2.9c). Manson et al, 1994, inserted an MT/Neo cassette into the Kpn I site in Exon 3 of the PrP gene at codon 95. The insert extends to position 535 in the ORF (see Fig 2.2). It is therefore possible that Neo/PrP fusion mRNA transcripts can be produced (Bueler, 1992), however Manson et al did not report any. It is possible that the antisense probes could hybridise to any fusion transcripts produced since they extend beyond the Neo cassette 3' end. This could be a possible explanation for the presence of labelling in the Prn-p *-/-* brain section, since no labelling can be detected in the adjacent section incubated with the sense probes

(Fig 2.9 d).

In the formal fixed spleen sections, some labelling can be observed which appears to be specific for PrP mRNA. It is observed only in the sections incubated with antisense probes and not in sections incubated with sense probes or in any of the Prn-p/- sections (fig 2.9 g and h). The labelling is observed only in the red pulp and appears to be associated with macrophages. The significance of this finding with regards to scrapie pathogenesis has yet to be clarified. Since PrP<sup>c</sup> is abundant on FDCs (Brown et al., 1999; Jeffrey et al., 2000; McBride et al., 1992), then logically PrP mRNA would have been expected in the follicles. It could be that PrP mRNA within FDCs has a high turnover compared to macrophage PrP mRNA and may be easier to detect within the latter cell type. Indeed, these trials will have to be repeated with larger group sizes since only 2 spleens per group were tested.



**Fig 2.8 Comparison of the fixatives 4% paraformaldehyde and formol for efficacy in PrP mRNA *in situ* hybridisation studies.** All images shown are mouse brain hippocampus a) Prn-p $+/+$ , formol fixed, antisense probes. b) Prn-p  $+/+$ , formol fixed, sense probes. c) Prn-p $+/+$ , 4% paraformaldehyde fixed, antisense probes. d) Prn-p $+/+$ , paraformaldehyde fixed, sense probes. e) Prn-p $-/-$ , paraformaldehyde fixed, antisense probes. f) Prn-p $-/-$ , paraformaldehyde fixed, sense probes.



**Fig 2.9** *In situ* hybridisation of PrP mRNA in formal fixed brain and spleen from adult mice. The left panel shows sections incubated with antisense probes and the right hand panel shows corresponding adjacent sections incubated with sense probes a) Positive control, Prn-p $+/+$  brain. b) Prn-p  $+/+$  brain incubated with sense probes. c) Prn-p $-/-$  brain incubated with antisense probes. d) Prn-p $-/-$  brain incubated with sense probes. e) Prn-p  $+/+$  spleen incubated with antisense probes. Macrophages within red pulp stain positively and are indicated by black arrows. f) Prn-p  $+/+$  spleen incubated with sense probes. g). Prn-p  $-/-$  spleen incubated with antisense probes. h). Prn-p  $-/-$  spleen incubated with sense probes.

## 2.5 Summary/Future Work

This primary aim of this chapter was to optimise immunocytochemical and molecular biological techniques before applying them to the experiments described in the next three chapters.

A double immunolabelling method to detect PrP<sup>c</sup> and FDCs on the same frozen tissue section, which could be applied to frozen spleen sections from mice of various age groups, was not available at the beginning of the project. A suitable method was developed successfully during the course of the PhD. Although attempts were made to quantitate the amount of co-localisation using line profile analysis, the results were by no means absolute. Due to software limitations, it was not possible using the Leica system to analyse all areas of co-localisation within the entire microscopic field. An LSM 5 Pascal Zeiss Confocal microscope recently acquired by NPU and the software offers the possibility of co-localisation quantitation for the entire field of view. This application might provide a way of quantifying PrP<sup>c</sup> during spleen development and at the same time allow analysis of spatial differences of PrP<sup>c</sup> labelling within the spleen during ontogeny. This might be an area of study that could be pursued in the future.

A good level of competency had to be achieved in Western blotting of spleens for PrP<sup>sc</sup> detection. Half-spleens from mice challenged peripherally with scrapie as neonates were to be immunoblotted to determine the presence or absence of PrP<sup>sc</sup> (explained in greater detail in Chapter 3). These experiments were to be crucial to the development of the thesis, but only limited amounts of spleen were available for each scrapie case and all the available spleen had to be used to ensure a correct result. Hence it was major priority of the project to ensure that I could carry out the differential extractions and Western blotting with a high degree of confidence that a true positive or negative result was obtained. A substantial amount of time was spent practising the techniques. Variables such as antibody and detection systems were compared in order to find the best method for screening spleen tissue generated in experiments described in Chapter 3.

Attempts were made to detect PrP mRNA in spleen, despite the known difficulties in

detecting mRNA in spleen due to the high levels of nucleases. There is no information in the literature regarding the earliest age at which PrP mRNA can be detected during spleen ontogeny. Such information could help support PrP protein data produced during the course of this thesis. An *in situ* hybridisation method that could detect PrP mRNA in human gut ileum had been developed at the National CJD Surveillance Unit. A collaborative effort was undertaken to determine whether the method could be applied to PrP mRNA detection in spleen. Since this method only became available towards the end of the project, the study on adult spleen tissue remains incomplete. The results produced did appear to be promising, and some labelling was found in the red pulp and appeared to be associated with macrophages. However, group numbers were low. And it would be of interest to repeat the experiments on adult spleen tissue with larger group sizes. If consistency is achieved, the next stage would be to investigate PrP mRNA spleens from younger mice, with the overall aim of determining the earliest age at which PrP mRNA transcripts can be detected.

# Chapter 3

## The Effect of Age on Scrapie Pathogenesis in the Developing Mouse

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# Chapter 3

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## 3.1 Aims

The aims of this chapter were twofold. Firstly to determine whether the previously described effect of age on peripheral scrapie pathogenesis could be repeated using currently available mouse and scrapie strains. Secondly, to determine whether the spleens of neonatal mice are capable of supporting scrapie replication following peripheral challenge.

## 3.2 Experimental rationale

Brain homogenates from the terminal ME7 cases and non-infected control mice were prepared according to section 2.3.3. Groups of mice of various age groups were inoculated i.p with a  $10^{-2}$  dose (see section 2.3.4) of ME7 brain homogenate or normal brain homogenate according to the plan in Table 3.1.

Age (days)	Number of mice inoculated with ME7	Number of mice inoculated with normal brain
0-1	42	6
10	42	6
14	42	6
30	42	6

**Table 3.1 Inoculation plan for C57BL mice of various age groups.** 42 mice per age group were inoculated i.p with 20  $\mu$ l of a  $10^{-2}$  ME7 (see section 2.3.4.1). 12 Age matched controls were inoculated i.p with 20  $\mu$ l of a  $10^{-2}$  normal brain homogenate

Of the scrapie inoculated groups (shown in Table 3.1), 12 mice from each age group were sacrificed 70 days post inoculation. Of the mice injected with normal brain, 6 mice were sacrificed at 70 days post inoculation. After peripheral challenge with scrapie, it is well documented that at 70 days post injection (d.p.i), infectivity has reached a plateau in the spleen (Eklund et al., 1967; Kimberlin and Walker, 1978) and that high levels of PrP<sup>sc</sup> can be detected by Western blotting in this tissue (Mabbott et al., 2000). Spleens were therefore removed at this time point (see section 2.3.7) and cut in half. One half was stored for Western blot analysis for PrP<sup>sc</sup> (see section 2.3.12) and the other half was kept for possible bioassays to be carried out at a future date.

Due to the small spleen masses available for Western blotting, the entire spleen half was used in the Western blot analysis to ensure that a false negative was not

obtained. Hence, it was not possible to repeat extractions if there had been problems with the Western blotting or the extraction procedure itself. All the immunoblots presented in section 3.3.3.1- 3.3.3.5 were produced after a single attempt.

The remaining 30 mice per age group were allowed to progress until the defined endpoint (see section 2.3.5) in order to determine the incubation period. The non-parametric Mood's Median Test (see section 2.3.6) was used to determine differences in the median incubation period between age groups. These results are discussed, together with data from experiments carried out previously by Outram et al in section 3.3.1-3.3.3.

## 3.3 Results/Discussion

### 3.3.1 Experiment 1

#### 3.3.1.1 The effect of age on the spread of incubation periods after intraperitoneal challenge

The data to be discussed in this section were generated from experiments carried out in the Neuropathogenesis Unit during the period of 1969 – 1973 by George Outram, Hugh Fraser and Alan Dickinson, studying the effect of age on scrapie pathogenesis (described in section 1.2.5). Although some of the data was published in Nature in 1973 (Outram et al., 1973) a great deal of work remains unpublished. Since the purpose of the thesis was to revisit the age related phenomena revealed by these studies, it was appropriate to re-analyse the raw data from the original experiments in order to obtain more detailed information about the effect of age on scrapie pathogenesis. As described in section 2.3.6, The Mood's Median Test was chosen as the most appropriate statistical tool for data analysis.

In this series of experiments, a standard  $10^{-2}$  dose of ME7 was inoculated i.p into C57BL mice of various age groups. Fig 3.1 shows the spread of incubation periods for each age group challenged with scrapie, including mice that survived scrapie challenge.

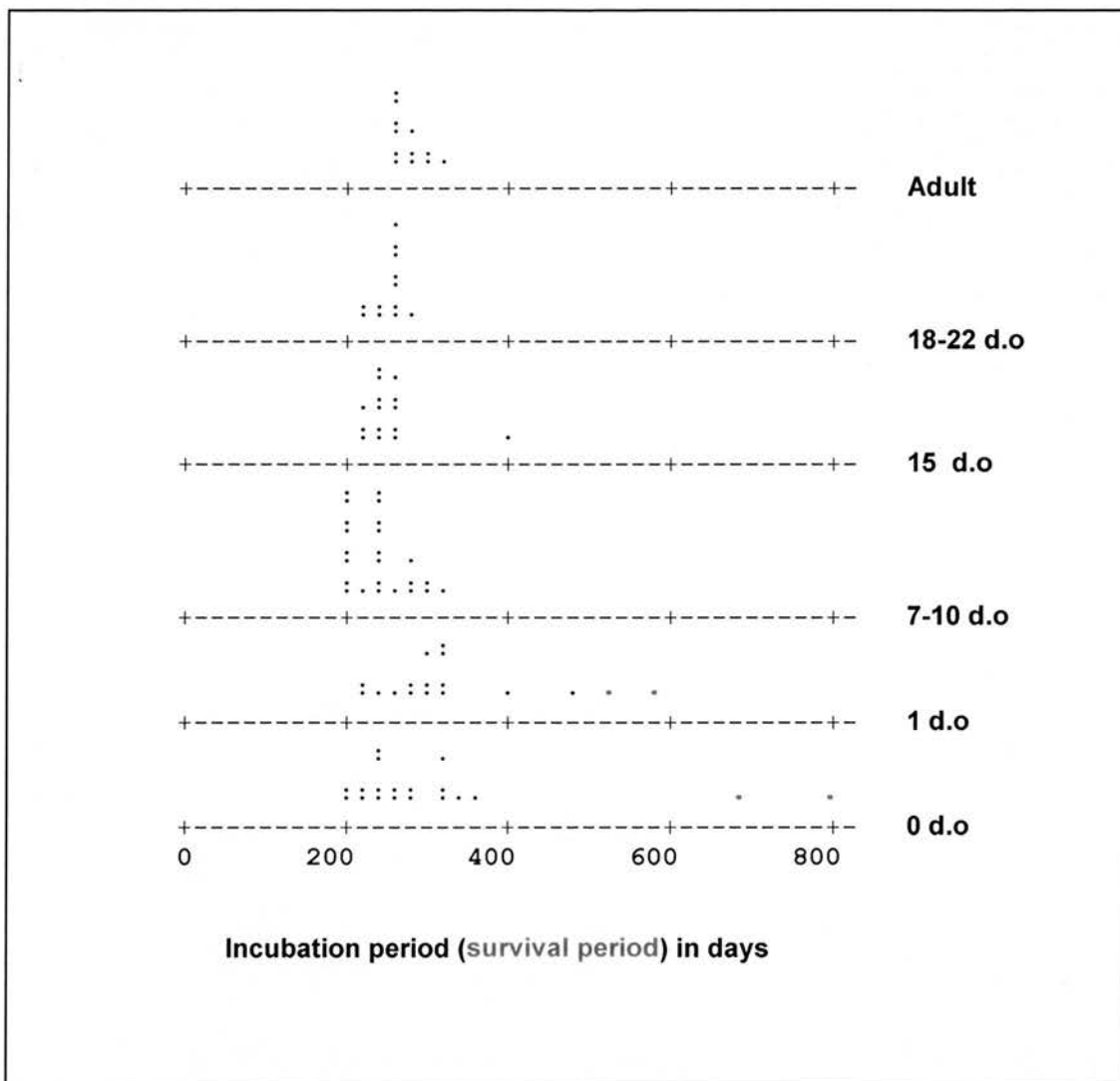
In the neonatally challenged group, a proportion of mice (20% of total inoculated) survived scrapie challenge. In this experiment 0 day olds and 1 day olds were treated as separate groups. It can be seen from Fig 3.2a and b, that mice inoculated as 1 day olds have a longer median incubation period (307 days) compared to 0 day olds (261 days); resulting in a difference of 46 days

The wide dispersion of incubation periods in the neonatally challenged groups and the presence of survivors differs from the incubation period pattern in older mice. When the median incubation periods of neonatal mice are compared with older

mice the shift in average incubation period is more pronounced and statistically significant. Using the Mood's median test, there is a statistically significant 38 day prolongation in incubation period ( $p=0.037$ ) between mice inoculated at 1 day old and adults (see Fig 3.2a and b).

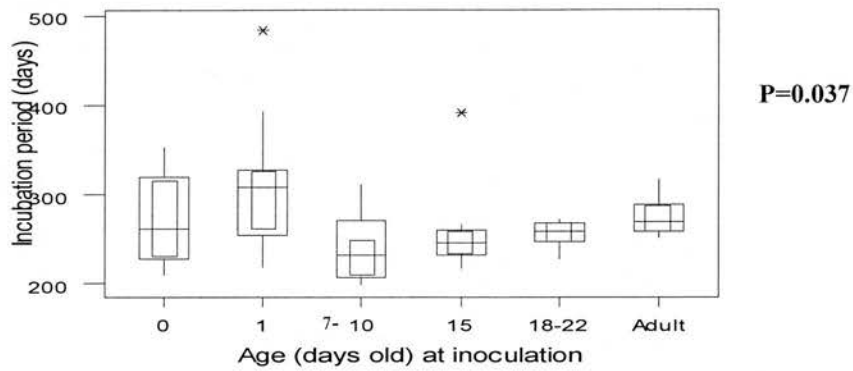
Survivors **are not** found in mice challenged at 7 days old or older (see Fig 3.1a). Interestingly, there appears to be a step-wise increasing trend in incubation period length for older mice (see Fig 3.2a). Mice challenged between the ages of 7 and 10 days old have the shortest median incubation period of 230 days. Adults, on the other hand have the longest (see Fig 3.2a and b). The reasons for this gradual lengthening of incubation period is unclear. However possible explanations are discussed in more depth in section 3.4.2.

**In general, the data demonstrate that early neonatal are more resistant to peripheral scrapie challenge compared to older mice and have a better chance of survival.**



**Figure 3.1 Experiment 1: The effect of age on scrapie incubation period (old data).** Panels of mice of the following age groups: 0, 1, 7-10, 15, 18-22, 40 days old were inoculated i.p with 20µl of 10<sup>-2</sup> supernatants (500g for 10 mins) of mouse-brain homogenates from terminal ME7 cases . The dotplots show the spread of incubation periods after challenging mice of various age groups. Terminal cases are represented by black closed circles and survivors in red closed circles.

a)



b)

Age (days old)	n	n ≤ overall median	n ≥ overall median	Median
0	17	8	9	261
1	15	4	11	307
7-10	24	17	7	230
15	15	11	4	244
18-22	12	6	6	257
Adult	12	4	8	269
<b>Overall Median</b>				<b>258</b>

**Figure 3.2 Experiment 1: Statistical Analysis of the effect of age on scrapie incubation period..**  
 a). the box –whisker plots show the median incubation periods (inner middle line) for each age group together with interquartile ranges (top and bottom lines of outer box). Moods median test (see section 2.3.6) was used to compare equality of medians B). Table summarising median values.

## 3.3.2 Experiment 2

### 3.3.2.1 The effect of inoculum dose on the spread of incubation period

The effect of scrapie dose on the spread of incubation periods after peripheral challenge of neonatal mice was also studied by Outram et al, although the data remain unpublished. The data has been re-analysed and dotplots of incubation period spreads are shown in Fig 3.3a. In the adult, when mice are inoculated i.p with 10-fold dilutions ranging from  $10^{-1}$  to  $10^{-3}$ , there was a significant prolongation of the incubation period ranging from 30-39 days as inoculum is diluted 10-fold. There is a great degree of overlap between incubation periods for mice inoculated with  $10^{-3}$  and  $10^{-4}$  doses. As can be seen in Fig 3.4, survival rates increase dramatically in the adult; survivors are observed in adult mice following inoculation with a  $10^{-4}$  dilution of inoculum only.

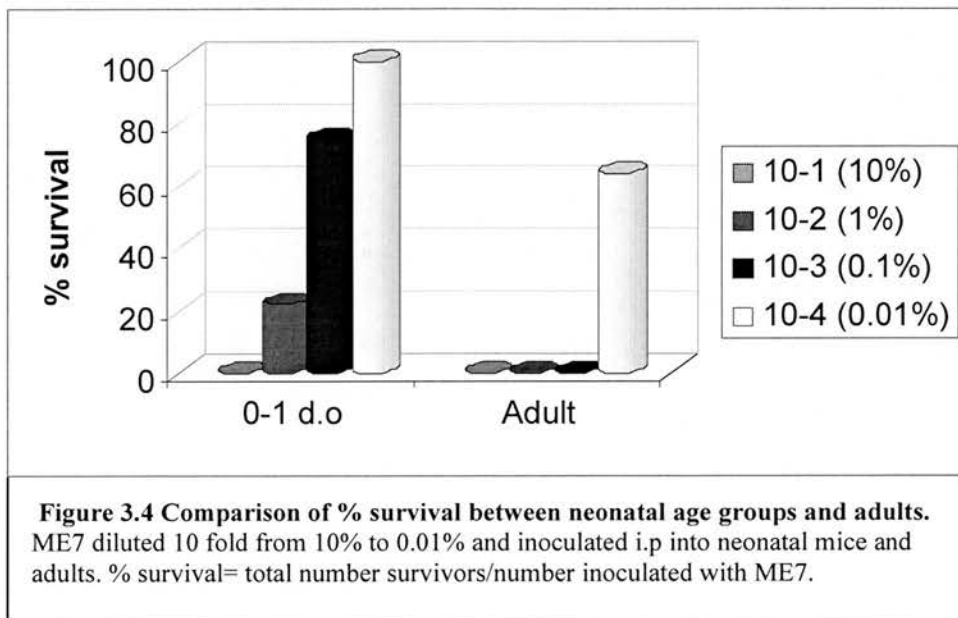
Inoculation of 0-1 d.o mice using a high dose of scrapie ( $10^{-1}$ ), resulted in 100% of mice dying with relatively short incubation periods (see Fig 3.4). In contrast, when neonatal mice were inoculated with doses ranging from  $10^{-2}$  to  $10^{-4}$  then survival occurred. This differs from the adult in that survivors are only obtained following inoculation with a  $10^{-4}$  dilution of infectivity.

In order to quantify relative susceptibilities of neonates and adults to scrapie inoculation the  $LD_{50}$  titre was calculated for both age groups (see Fig 3.3b). For neonatal mice, the  $LD_{50}$  titre calculated using the Reed-Muench method was  $10^{2.6}$ . When mice are inoculated as adults, the  $LD_{50}$  was  $10^{3.9}$ . This constitutes a log difference in the dose of scrapie required to achieve 50% mortality between neonatally injected and adult inoculated mice. **The main conclusion that can be drawn from this study is that neonatal mice are less susceptible to scrapie challenge compared to adults, since the neonates require a higher concentration of scrapie to produce 50% mortality.**

The bypassing of resistance of neonatal mice to peripheral scrapie challenge, bears similarities with the situation in SCID models of scrapie pathogenesis.

SCID mice possess an autosomal recessive mutation that results in an immunodeficiency status which prevents the formation of functional B cells, T cells and mature FDCs (Bosma et al., 1983). If these mice are challenged i.p with  $10^{-2}$  or  $10^{-3}$  doses of ME7, then mice completely resist infection and 100% survival occurs and this is associated with a lack of functional FDCs (Brown et al, 1999). However, if SCID mice are challenged with a very high  $10^{-1}$  scrapie dose, then resistance is broken and 100% incidence of disease is found (Fraser et al., 1996).

One hypothesis to explain this observation is that the infectious agent may be taken up directly by peripheral nerves following high dose i.p inoculation without the need for an FDC dependent amplification phase, resulting in a shorter incubation period. Indeed elegant studies have shown that direct inoculation of the sciatic nerve with scrapie results in a shortening of the incubation period (Kimberlin et al., 1983). The range of incubation periods produced after intraneural inoculation of 139A into *Sinc*<sup>s7</sup> mice was 143-149 days. These incubation periods are similar in length to incubation periods produced after i.c inoculation of mice. Extraneural inoculation produced the longest incubation periods ranging from 186-198 days. Interestingly, intraneural infection was established with low efficiency. The sciatic nerves had to be damaged either by crushing or demyelination in order to readily establish infection whereby the majority (65%) of short incubation period cases when the sciatic nerve was crushed 2.5 hours before inoculation (Kimberlin, 1983).



### 3.3.3 Experiment 3

#### 3.3.3.1 The effect of age on the spread of incubation periods after intraperitoneal scrapie challenge

The aim of this chapter was to determine the reproducibility of the neonatal effect shown in Fig 3.1. In order to test this the experiment was set up again under the same conditions to determine whether it is possible to achieve the same phenomenon using strains of mouse and scrapie currently in use at NPU. This experiment has been named Experiment 3.

Due to time constraints, it was decided to inoculate 4 age groups: 0-1 d.o (neonatal), 10 d.o, 14 d.o and 30 d.o (adult), instead of all the age groups used in the Experiment 1. To try to achieve a similar spread of incubation periods in neonatally challenged mice, large numbers of neonatal mice were required. 0-1 day old mice were treated as one group instead of being grouped separately in order to limit the amount of time setting up the experiment and litters were inoculated as they became available.

The spread of incubation periods, together with survival periods are illustrated in Fig 3.6. Similar to the situation in Experiment 1, survivors were observed in this experiment. Although the proportion of survivors appears to be greater in this experiment compared to Experiment 1 ( 60% as opposed to 20%), it is difficult to analyse this difference statistically, since certain conditions, such as inoculum preparation differed between experiments.

With regards to the terminal cases of neonatally challenged mice, it can be seen from Fig 3.7a there is a significant prolongation of median incubation period (14 days) between neonatally challenged and adult mice ( $p < 0.0001$ ). However, in this study the dispersion of the incubation periods is not as pronounced as that observed in Experiment 1.

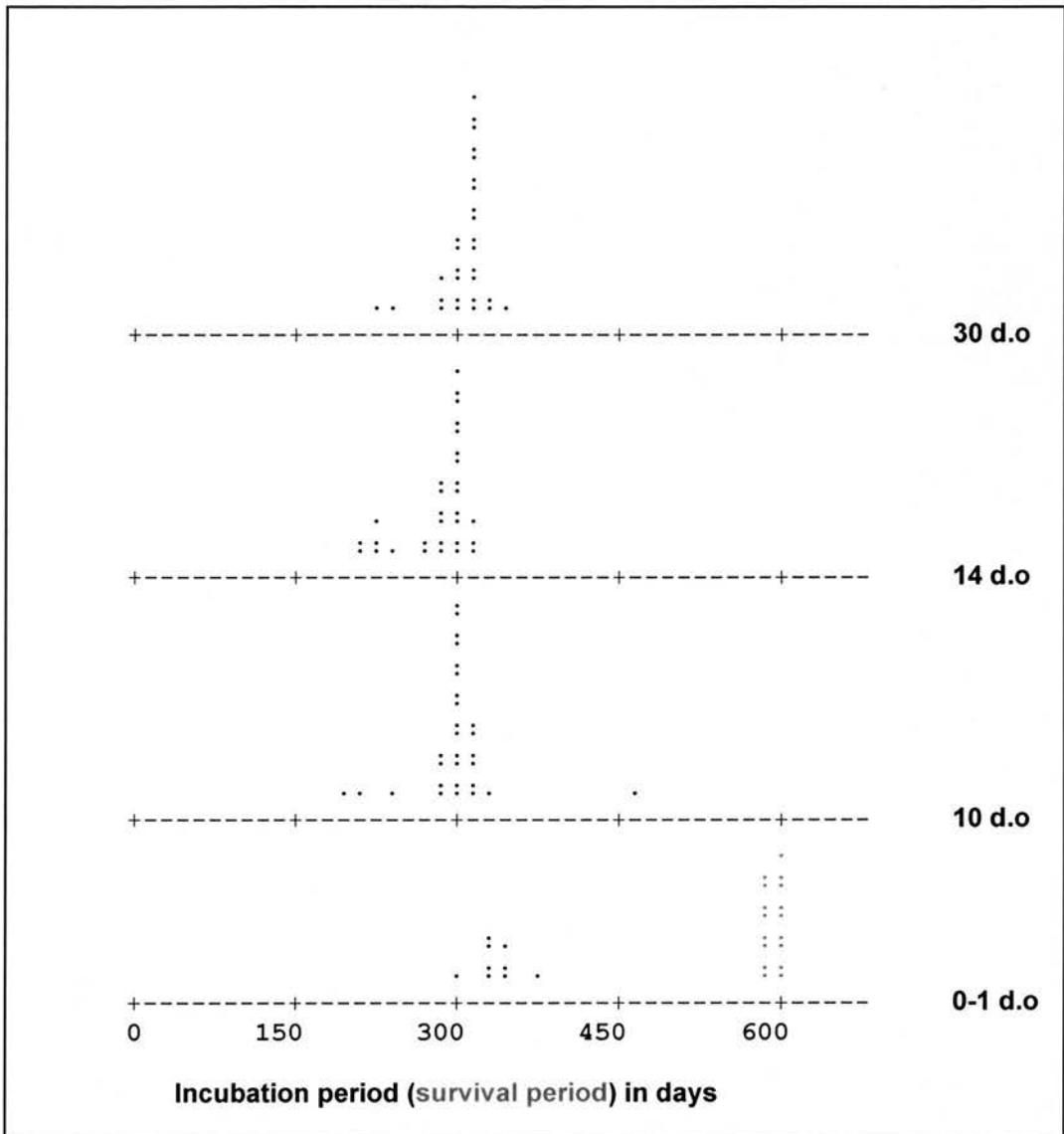
With regard to the incubation period prolongation in the neonatally challenged group, possible reasons for this could be that the titre of inoculum was lower than expected. The median incubation period for i.p inoculated adult group in Experiment 3 is 315 days, which is longer than the expected incubation period of 269 days (Outram et al., 1973). This may suggest that the inoculum was of low titre. Moreover, if the survival rates are observed for Experiment 3 (see Fig 3.5) it can be seen that 60% of mice survive scrapie challenge compared to Experiment 1 where 20% survive. If the titration experiment (Experiment 2) is considered (see Fig 3.3), then a 60% survival rate would be achieved if a dilution between  $10^{-2}$  and  $10^{-3}$  was inoculated into mice.

Similar to the situation in Experiment 3, the 10 and 14 day old mice have shorter incubation periods compared to the adult and neonatal mice (see outliers in Fig 3.6a). In fact the majority of terminal cases in these age groups have incubation periods less than the overall median (see Fig 3.6b). As hypothesised in section 3.3.2.1, this could be due to chance uptake of inoculum by peripheral nerves. However, if the inoculum titre is in fact low as previously discussed, then the

presence of short incubation periods contradicts the dose response data shown in Fig 3.3. No outliers with short incubation periods are observed at low dose of  $10^{-3}$  to  $10^{-4}$ . This anomaly is therefore difficult to explain in the context of previously established data.

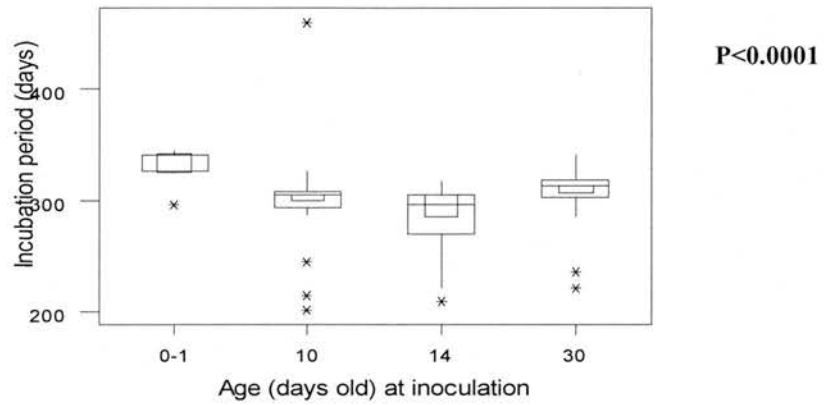
Overall, this study demonstrates that the neonatal effect in scrapie pathogenesis can be reproduced whereby the average incubation period is longer than in older age groups. In fact, the phenomenon demonstrated in the current experiment is more pronounced than in previous experiments since the % survival after neonatal challenge is markedly increased compared to Experiment 1 (see Fig 3.7).

The remainder of this chapter deals with verifying the earliest age at which scrapie replication in the spleen can be sustained.



**Figure 3.5 Experiment 3: The effect of age on scrapie incubation period (new data).** Panels of mice of the following age groups were inoculated i.p with 20 $\mu$ l of 10<sup>-2</sup> dilution of brain homogenate from terminal ME7 cases. The dotplots show the spread of incubation period and survival times after peripheral scrapie challenge. Terminal cases are represented by the closed black dots and survivors by closed red dots.

a)



b)

Age (days old)	n	n ≤ overall median	n ≥ overall median	Median
0-1	8	1	7	327
10	29	21	8	306
14	30	26	4	296
30	29	9	20	313

**Figure 3.6 Experiment 3: Statistical analysis of the effect of age on scrapie incubation period**  
a). box –whisker plots show the median incubation periods (inner middle line) for each age group together with interquartile ranges (top and bottom lines of outer box). Statistical analysis was carried out using the Mood’s Median Test. b). Summary of median scrapie incubation period values for each age group.

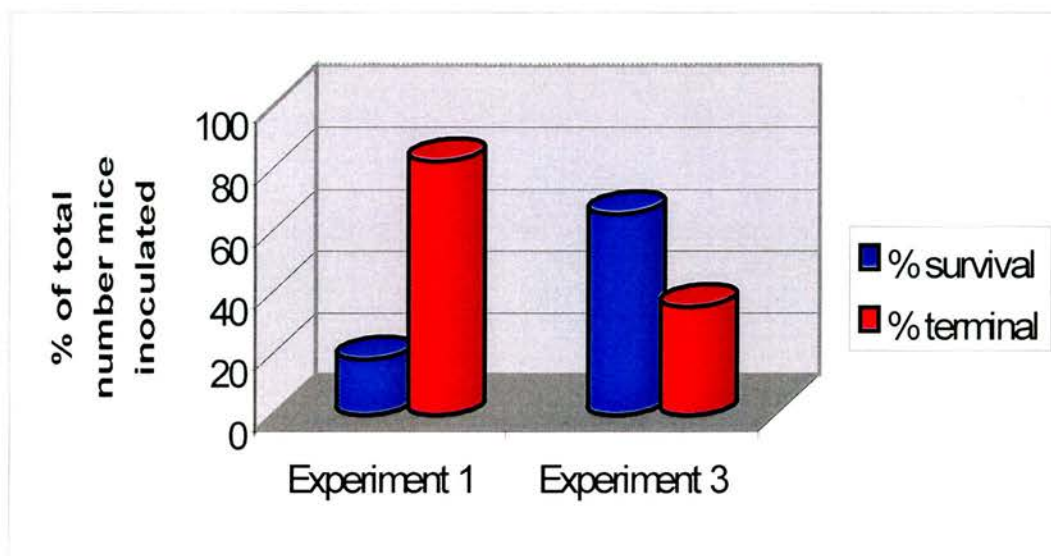


Figure 3.7 Comparison of survival rates between neonatal age groups from Experiment 1 and 3. Neonatal (0-1 d.o) mice were inoculated with a  $10^{-2}$  dilution of brain from a scrapie affected mouse

### **3.3.3.2 Detection of PrP<sup>sc</sup> 70 days post infection in the spleens of mice inoculated at 10 days old with scrapie**

Groups of 12 mice randomly selected from each age group, were culled 70 days post injection and the spleens removed. Approximately quarter of the incubation period had elapsed at this stage and it has been shown that replication of infectivity in the spleen plateaus (Eklund et al., 1967) and PrP<sup>sc</sup> levels in the spleen are high (Farquhar et al., 1994; Mabbott et al., 2000). If any replication had occurred then it should be possible to detect PrP<sup>sc</sup> after this time.

Only 4 spleen PrP extractions could be carried out in one day due to space constraints in ultracentrifuge rotors. In each extraction run, positive and negative controls were always included. Therefore two experimental samples only could be included in any extraction procedure. The positive control was included to ensure that the PrP extraction procedure had been efficient. The negative control, as well as demonstrating that protease resistant PrP<sup>sc</sup> cannot be found after intraperitoneal challenge with normal brain, ensured that no cross contamination had occurred

intraperitoneal challenge with normal brain, ensured that no cross contamination had occurred during ultracentrifugation. All PrP<sup>Sc</sup> extracts (with and without PK treatment) from one extraction run were loaded onto the same gel and subsequently immunoblotted. The throughput of experimental samples was therefore low. However it was important to ensure that the efficiency of the PrP extraction was controlled for and that false positives or negatives were not obtained.

Since the other half of each spleen had been kept for future bioassays, only one spleen half was available for Western blotting. Some trials were carried out using a 1/4 of a spleen or less. It was found that PrP extraction from spleens of smaller masses resulted in inconsistencies in achieving a positive result. To decrease the chance of getting a false negative, it was decided to carry out extractions on the entire spleen that was available for each case.

Immunoblotting was carried out successfully on all PrP<sup>Sc</sup> extracts from mice inoculated as 10 day olds. PrP<sup>Sc</sup> could be detected at high levels in 11/12 spleen extracts (see Fig 3.8 a-f for immunoblots and Table 3.2 for a summary of results for individual mice). Spleen 4 (see Fig 3.8b) had barely detectable levels of PrP<sup>Sc</sup>. There are several reasons for this, such as inefficient extraction of proteins or poor immunolabelling. However, the positive control spleen (see Fig 3.8b +C) taken from a terminal animal did have strong immunolabelling and since this spleen was extracted under the same conditions as Spleen 4 it can be concluded that the low immunolabelling is a true reflection of the amount of PrP<sup>Sc</sup> actually present. The reasons for this are unclear. However, if the spread of the incubation periods are considered, it can be seen that there is one case with a very long incubation period of 460 days (see Fig 3.5- 10 day olds). This could reflect a delay in the initiation of replication in the spleen during the early stages of the incubation period in a less susceptible mouse. This may affect the deposition of PrP<sup>Sc</sup> in the spleen, resulting in the observed low PrP immunolabelling. It will be of interest to determine the infectivity levels in this PrP<sup>Sc</sup> low spleen, since a recent publication has demonstrated that in the brain at least, accumulation of PrP<sup>Sc</sup> (detected by

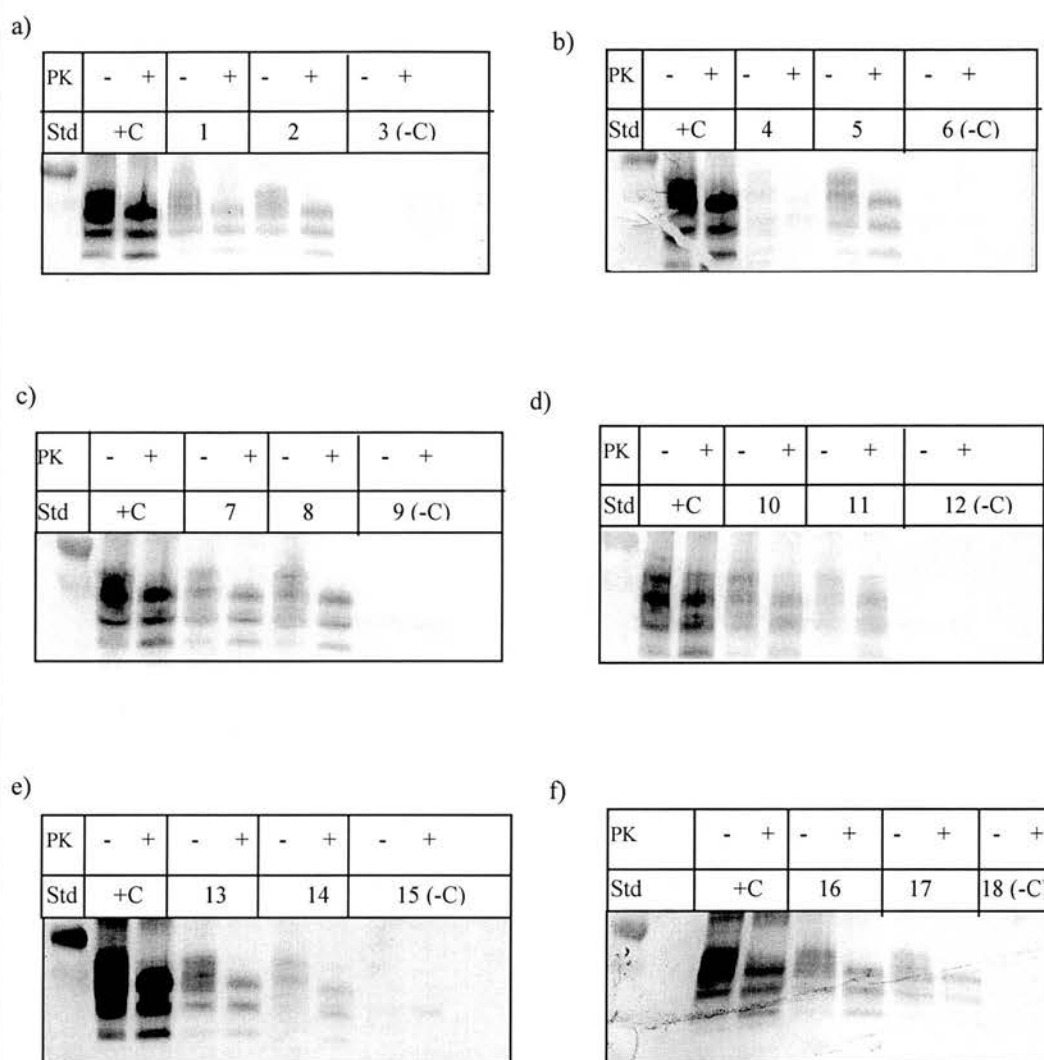
Western blotting and histoblots) lags behind the development of infectivity titres (Jeffrey et al., 2001).

All other spleen samples taken from mice inoculated at 10 days have similar intensities for PrP immunolabelling. The intensities of these extracts were subjectively lower than the intensities of all the positive control spleens ( see +C, Fig 3.9 a-f) taken from terminal scrapie cases. The reasons for this are unclear, since infectivity should have reached a plateau at 70 d.p.i. At this stage conclusions cannot be made about whether this reflects a lower titre of infectivity. Again this may correlate with observations by Jeffrey et al, 2001, where PrP<sup>sc</sup> lags behind infectivity levels.

The Western blotting data suggests that the lymphoreticular system in the 10 day old can support scrapie replication since PrP<sup>sc</sup> can be detected in the spleens of mice inoculated as 10 day olds. A major barrier to forming definite conclusions about the ability of young mice to sustain scrapie replication is that it is not known at what point replication of scrapie commences after inoculation.

Hence it is plausible that if the 10 day old mouse does not have full replicative capacity at the time of inoculation then it may be acquired during the subsequent days/weeks post injection. If residual inoculum is still available, then it may be replicated once the replication sites have reached a critical level of maturity or number.

In Chapter 1, evidence was given supporting the hypothesis that in adult mice, PrP<sup>c</sup> expressing mature FDCs in the spleen are required for ME7 scrapie replication peripheral challenge (Brown et al., 1999). One of the hypotheses to be tested in this thesis is that neonatal mice lack PrP<sup>c</sup> expressing FDCs and are therefore more resistant to peripheral scrapie challenge. Since it is predicted that neonatal mice lack functional FDCs, it follows that by 10 days old, availability of PrP<sup>c</sup> expressing FDCs must be established, as susceptibility to scrapie in this age group is similar to adults. These predictions are explored in Chapters 4 and 5.



**Figure 3.8: The detection of PrP<sup>Sc</sup> 70 d.p.i in the spleens of mice challenged i.p at 10 day old with ME7.** PrP<sup>Sc</sup> was isolated by differential extraction (see section 2.3.12.2) and Western blotted (see section 2.3.12.3-2.3.12.5) . A prestained Kaleidoscope (BioRad) standard was loaded onto Lane 1 (Std). +C denotes the positive control for PrP<sup>Sc</sup> labelling which is the spleen extract of a terminally infected adult mouse, inoculated i.p as an adult. Samples 1-18 represent 1/2 spleen extracts taken from individual mice culled 10 weeks post inoculation. Extracts were split and either treated with PK (+) or left untreated (-).(-C) denotes negative controls, which are the spleens of mice challenged intraperitoneally as 0-1 day olds with normal brain homogenate.

Blot	Case	Inocula	Age at challenge (days)	Clinical status	Brain pathology	Spleen PrP <sup>Sc</sup> status
a	1	ME7	10	Negative	Negative	Positive
	2	ME7	10	Negative	Negative	Positive
	3	NB	10	Negative	Negative	Negative
b	4	ME7	10	Negative	Negative	Positive
	5	ME7	10	Negative	Negative	Positive
	6	NB	10	Negative	Negative	Negative
c	7	ME7	10	Negative	Negative	Positive
	8	ME7	10	Negative	Negative	Positive
	9	NB	10	Negative	Negative	Negative
d	10	ME7	10	Negative	Negative	Positive
	11	ME7	10	Negative	Negative	Positive
	12	NB	10	Negative	Negative	Negative
e	13	ME7	10	Negative	Negative	Positive
	14	ME7	10	Negative	Negative	Positive
	15	NB	10	Negative	Negative	Negative
f	16	ME7	10	Negative	Negative	Positive
	17	ME7	10	Negative	Negative	Positive
	18	NB	10	Negative	Negative	Negative

**Table 3.2 70 days post infection: case profiles of mice inoculated at 10 days old with ME7.** This table summarises the clinical and pathological details for each mouse challenged intraperitoneally at 10 days old with ME7. The case number refers to the number allocated for spleen samples in Fig 3.8a-f). These include age matched mice challenged intraperitoneally with ME7 infected brain homogenate (ME7) or with normal brain homogenate as control (NB). Clinical status and brain pathology are expressed as positive or negative depending on whether mice showed classical scrapie symptoms or scrapie associated cerebral vacuolation respectively. Spleen PrP<sup>Sc</sup> status is expressed as positive if PK resistant PrP<sup>Sc</sup> was detected by immunoblotting or negative if no immunolabelling of PK resistant PrP<sup>Sc</sup> was detected (see Fig 3.8a-f for immunoblots).

### **3.3.3.3 Detection of PrP<sup>sc</sup> 70 days post infection in the spleens of mice inoculated at 0-1 day old with scrapie**

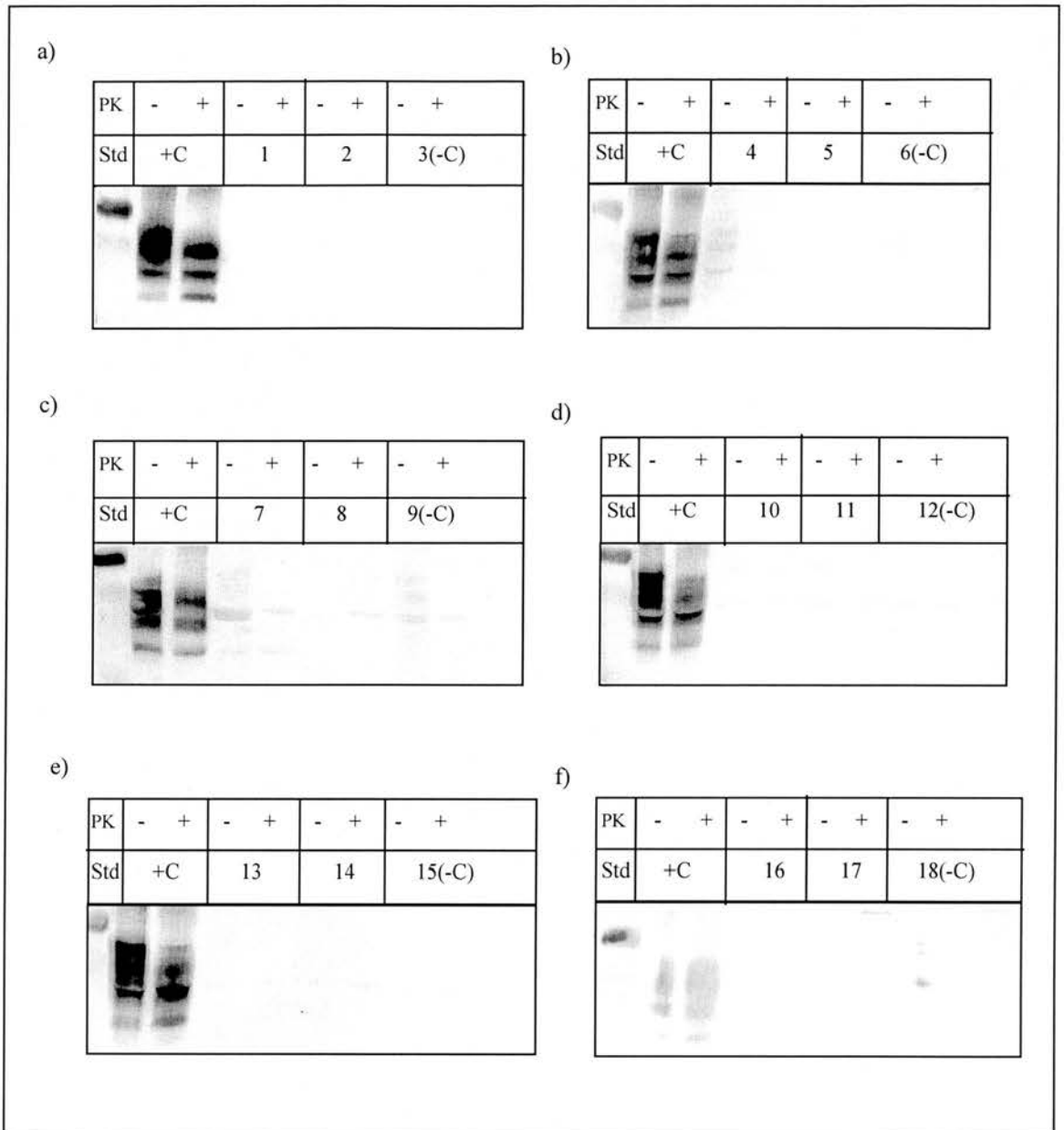
As can be observed from Fig 3.9 a-f, 0/12 spleens tested for PrP<sup>sc</sup> showed the presence of the disease specific isoform of PrP. Fig 3.9b sample 4 and Fig 3.9c sample 7 appeared to show triple bands typical of PrP in the PK untreated lanes. However, the portion of the sample that had been PK treated and loaded into the adjacent lane showed no detectable levels in both cases. This suggests that the faint triple bands observed could have been due to slight precipitation of PrP<sup>c</sup> during sarkosyl treatment. Precipitated PrP<sup>c</sup> would have been sedimented during the final ultracentrifugation step of the extraction procedure, thus resulting in immunolabelling by the 1B3 polyclonal antibody. This antibody recognises both disease specific and cellular PrP, therefore if any PrP<sup>c</sup> is present in the extract then it would be detected. Since PrP<sup>c</sup> is fully degraded by proteinase K, then this may explain the absence of banding in the PK treated lane.

These results show that during the 70 days post inoculation, the spleens of neonatal mice have not yet been capable of supporting PrP<sup>sc</sup> synthesis. This was an unexpected result since one third of the population of neonatally mice inoculated developed scrapie, it would be reasonable to assume that one third of the group randomly selected for Western blotting would demonstrate PrP<sup>sc</sup> in their spleens.

**Nevertheless, the Western blotting results suggest that neonatal mice have an impaired ability to replicate scrapie during the first 70 days of the incubation period.** However, bioassay results will be required to determine whether or not replication has taken place and these studies are currently on going.

If the data for the 10 day old mouse and the neonatal data are considered together, a possible conclusion that can be drawn is that full replicative function is achieved between the periods of 0-10 days after birth. This result is consistent with the incubation period data where full adult susceptibility to peripheral scrapie

challenge is achieved by this age (see Fig 3.5).



**Figure 3.9** The detection of PrP<sup>Sc</sup>70 d.p.i, in the spleens of mice challenged i.p at 1 day old with ME7.PrP<sup>Sc</sup> was isolated by differential extraction and Western blotted. A prestained Kaleidoscope standard (Biorad) was loaded onto Lane 1 (Std). +C denotes the positive control for PrP<sup>Sc</sup> labelling which is the spleen extract of a terminally infected adult mouse, inoculated i.p as an adult. Samples 1-18 represent 1/2 spleen extracts taken from individual mice culled 10 weeks post inoculation. Extracts were split and either treated with PK(+) or left untreated (-). (-C) denotes negative controls, which are spleens taken from mice challenged as 0-1 day olds with normal brain homogenate.

Blot	Case	Inocula	Age at challenge (days)	Clinical status	Brain pathology	Spleen PrP <sup>sc</sup> status
a	1	ME7	1	Negative	Negative	Negative
	2	ME7	1	Negative	Negative	Negative
	3	NB	1	Negative	Negative	Negative
b	4	ME7	1	Negative	Negative	Negative
	5	ME7	1	Negative	Negative	Negative
	6	NB	1	Negative	Negative	Negative
c	7	ME7	1	Negative	Negative	Negative
	8	ME7	1	Negative	Negative	Negative
	9	NB	1	Negative	Negative	Negative
d	10	ME7	1	Negative	Negative	Negative
	11	ME7	1	Negative	Negative	Negative
	12	NB	1	Negative	Negative	Negative
e	13	ME7	1	Negative	Negative	Negative
	14	ME7	1	Negative	Negative	Negative
	15	NB	1	Negative	Negative	Negative
f	16	ME7	1	Negative	Negative	Negative
	17	ME7	1	Negative	Negative	Negative
	18	NB	1	Negative	Negative	Negative

**Table 3.3** 70 days post infection: case profiles of mice inoculated at 0-1 days old. This table summarises the clinical and pathological details for each mouse challenged intraperitoneally at 0-1 day old with ME7. The case number refers to the number allocated for spleen samples in Fig 3.9a-f. These include age matched mice challenged intraperitoneally with ME7 infected brain homogenate (ME7) or with normal brain homogenate as control (NB). Clinical status and brain pathology are expressed as positive or negative depending on whether mice showed classical scrapie symptoms or scrapie associated vacuolation respectively. Spleen PrP<sup>sc</sup> status is expressed as positive if PK resistant PrP<sup>sc</sup> was detected by immunoblotting or negative if no immunolabelling of PK resistant PrP<sup>sc</sup> was detected (see Fig 3.9a-f for immunoblots).

#### **3.3.3.4 Detection of PrP<sup>sc</sup> in the spleens of terminal mice inoculated at 0-1 day old with scrapie**

8/27 mice showed clinical signs of scrapie and whole spleens were removed for western blot analysis (see Fig 3.10). 7/8 of the mice showing clinical signs had scrapie associated pathological lesions in the brain (see Table 3.4). Western blot analysis of the corresponding spleens reflected this result, whereby 7/8 spleen extracts tested positive for PrP<sup>sc</sup> presence (see Fig 3.10). The spleen that was negative for the characteristic triple banding pattern came from the mouse that was negative for scrapie cerebral pathology. However, there were two low molecular weight bands in both PK untreated and PK treated lanes. This spleen was enlarged and weighed 180mg. The mass of the spleen used for the initial extraction was 100mg and could have had a deleterious effect on the ultracentrifugation process since the method had been optimised for spleens of approximately 30 mg equivalent weights. The splenomegaly suggests that the mouse could have had an infection or a tumour, which may have led to a clinical misdiagnosis of scrapie. Since the spleen had been frozen, it was not possible to carry out a pathological examination of the spleen. There was no evidence of any lymphocytic infiltrations of the brain.

Nevertheless, to ensure that a false negative result had not been obtained after western blotting, the extraction was repeated using a fraction of the remaining half of the spleen, to verify that the unusually high load used for the extraction could have had an effect on the outcome of the immunoblotting. After loading 15mg equivalents of the remaining spleen, no PrP<sup>sc</sup> could be detected. It can be concluded that this case was definitely scrapie negative and was not included in any statistical analysis of incubation periods.

After inoculation of neonatal mice, it is evident that replication can be supported in the spleen but the precise period of time before replication commences cannot as yet be determined. Neonatal mice are obviously less susceptible to infection and the ability to support replication is impaired; no PrP<sup>sc</sup> can be found in the spleens of 12 mice randomly selected for analysis, 70 days post infection- a stage

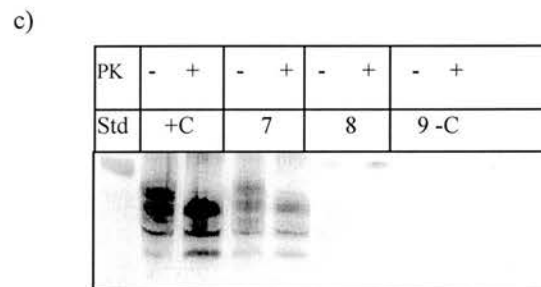
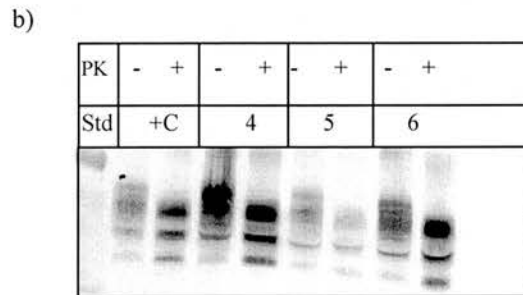
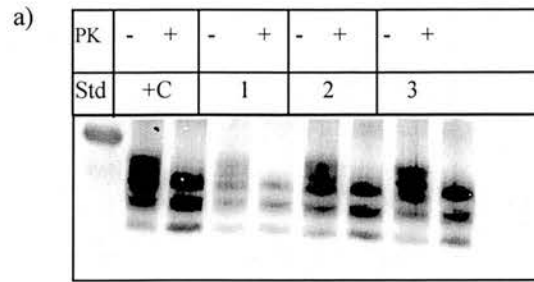
in the incubation period where replication should have occurred. In addition, less than a 1/3 of the total group of mice inoculated developed scrapie and demonstrated PrP<sup>sc</sup> deposition in their spleens. 2/3 of the total inoculated group survived scrapie challenge and were culled approximately 600 days after inoculation without clinical or pathological signs of scrapie (see section 3.3.3.5).

The following explanation could account for this. If there was inefficient sequestration and/or disposal of infectivity, then infectious agent may have still been available for replication in the spleen once the relevant cell types had matured.

Experiment 2 highlighted the low efficiency with which scrapie infection takes hold after i.p challenge of neonates. 10 fold dilutions of infectivity results in an incremental increase in the number of survivors after neonatal challenge. However, challenge of adult mice only results in survival with a 10<sup>-4</sup> dilution. It suggests that alternative route(s) of entry into the CNS are utilised in the absence of a mature LRS, but that these routes are not as efficient as an LRS routed entry into the CNS. Such a route could be the peripheral nervous system (this possibility is explored in more detail in Chapter 5).

If the PNS is being used as a direct access into the CNS without the need for LRS amplification, then it could be predicted that no PrP<sup>sc</sup> deposition would occur in the spleen. This is clearly not the case as shown by the immunoblots in Fig 3.10. However, the possibility that retrograde spread of infectivity from the CNS to the peripheral lymphoid system cannot be excluded.

Until, these possibilities are explored in more detail the main conclusion that can be drawn from this series of experiments is that **neonatal mice have an impaired ability to replicate the scrapie agent.**



**Figure 3.10 The detection of PrP<sup>sc</sup> in the spleens of terminal mice challenged i.p at 1 day old with ME7.** PrP<sup>sc</sup> was isolated by differential extraction and Western blotted. A prestained Kaleidoscope standard (BioRad) was loaded onto Lane 1. +C denotes the positive control for PrP<sup>sc</sup> labelling which is the spleen extract of a terminally infected adult mouse, inoculated i.p as an adult. Samples 1-9 represent 1/2 spleen extracts taken from individual terminally affected mice or control mice. Extracts were split and either treated with PK (+) or left untreated (-). -C denotes the negative control, which is the spleen taken from a 1 day old mouse challenged i.p with normal brain.

Blot	Case	Inocula	Age at challenge (days)	Incubation period (days)	Clinical status	Brain pathology	PrP <sup>sc</sup> status
a	1	ME7	1	296	Positive	Positive	Positive
	2	ME7	1	327	Positive	Positive	Positive
	3	ME7	1	327	Positive	Positive	Positive
b	4	ME7	1	327	Positive	Positive	Positive
	5	ME7	1	338	Positive	Positive	Positive
	6	ME7	1	345	Positive	Positive	Positive
c	7	ME7	1	366	Positive	Positive	Positive
	8	ME7	1	376	Positive	Negative	Negative
	9	NB	1	-	Negative	Negative	Negative

**Table 3.4 Case profiles of terminal mice inoculated at 0-1 days old.** This table summarises the clinical and pathological details for each mouse challenged intraperitoneally at 10 days old with ME7. The case number refers to the number allocated for spleen samples in Fig 3.10. These include age matched mice challenged intraperitoneally with ME7 infected brain homogenate (ME7) or with normal brain homogenate as control (NB). The incubation period corresponding to each case is shown. Clinical status and brain pathology are expressed as positive or negative depending on whether mice showed classical scrapie symptoms or scrapie associated vacuolation respectively. Spleen PrP<sup>sc</sup> status is expressed as positive if PK resistant PrP<sup>sc</sup> was detected by immunoblotting or negative if no immunolabelling of PK resistant PrP<sup>sc</sup> was detected (see Fig 3.10 for immunoblots).

### **3.3.3.5 Detection of PrP<sup>sc</sup> in the spleens of survivor mice inoculated at 0-1 days old with scrapie**

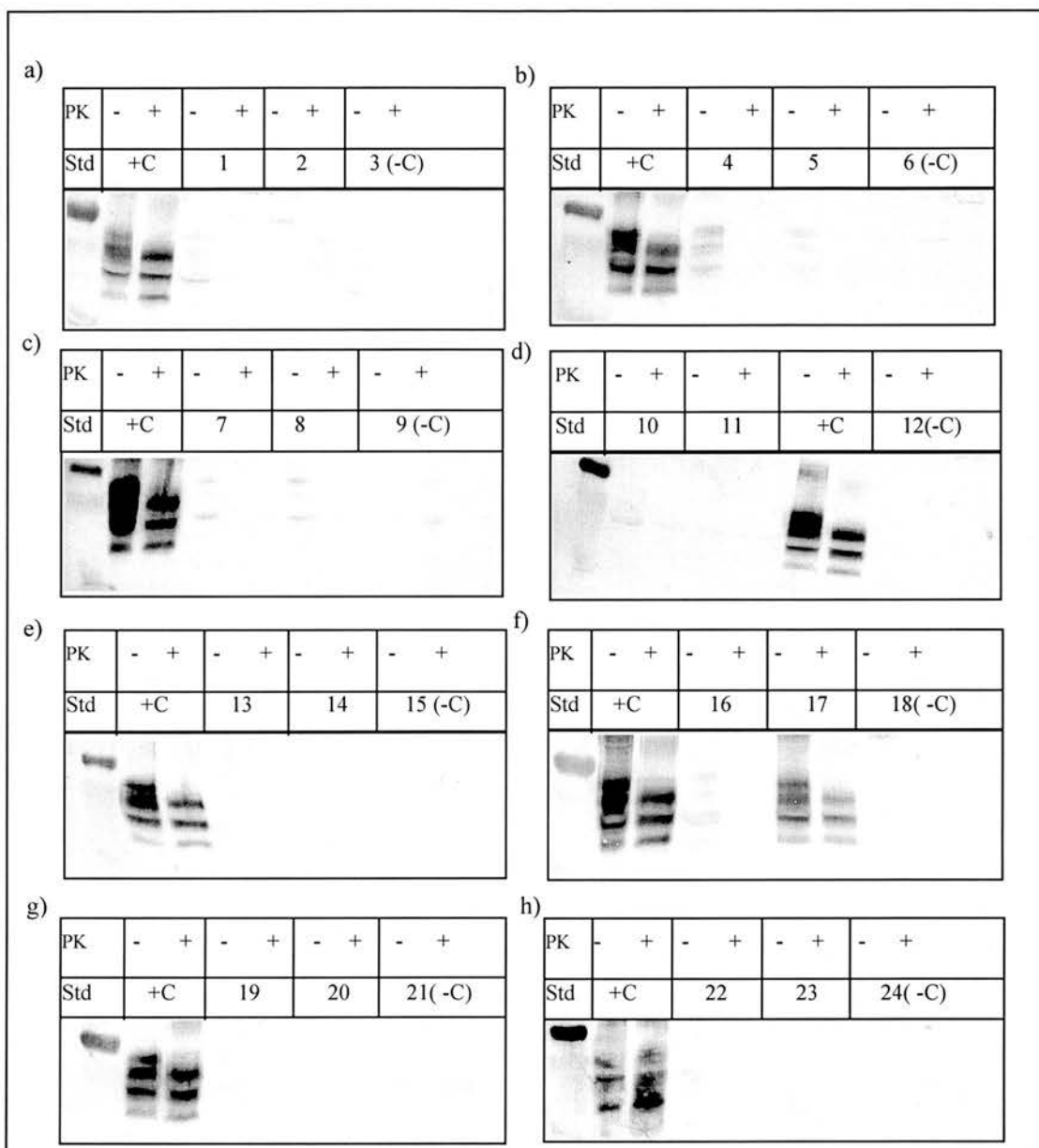
In order to avoid losing mice to natural causes or intercurrent infection, a defined end-point of 600 days was chosen. This period of time was long enough to determine whether any mice had survived scrapie infection. After 600 days, the chances of mice dying as a result of old age increases greatly. Although it could be argued that the incubation period of scrapie in neonatally inoculated mice may be longer than the lifespan of the mouse, it is clear from the results about to be discussed that this is not the case for the majority of the mice in this study.

The spleens of survivor mice were tested for the presence of PrP<sup>sc</sup>. Table 2.5 illustrates that 17/28 mice were clinically negative at the end of the experiment and hence survived scrapie challenge. Fig 3.11 a-h shows that 1/17 of the spleens of the survivors tested positive for PrP<sup>sc</sup> in their spleens. Again each gel represents single attempts at extraction of PrP<sup>sc</sup> and immunoblotting.

High levels of PrP<sup>sc</sup> are present in Sample 17 (see Fig 3.11.f) . It can be seen from Table 3.5, that the clinical and cerebral pathology were both negative. The intensity of the immunolabelling suggests that replication in the spleen may have reached a plateau. Replication in the brain commences around this time. Under normal circumstances i.e. i.p inoculation of adults, replication in the brain would commence 70 days post inoculation (Kimberlin and Walker, 1979). In this case, replication could have been occurring at an extremely low rate in the spleen and may have recently commenced in the brain at the point of culling. If this were the case then vacuolation would not have appeared until 90 days later. It is possible that this mouse could have developed scrapie if it had lived longer. From the Western blotting analysis, this appears to be the only case where the incubation period would have been significantly prolonged.

The immunoblot represented in Fig 3.11h did not label optimally. This could have been due to lack of coverage of the PVDF membrane during the overnight

incubation period with primary antibody. There does not appear to be a problem with the transfer of proteins since the protein standard in lane 1 has transferred well. There was still a fair degree of transfer of PrP<sup>Sc</sup> from the positive control so it can be concluded that the spleen extracts from the survivor spleens are truly negative.



**Figure 3.11** The detection of PrP<sup>Sc</sup> in the spleens of survivor mice challenged i.p at 0-1 days old with ME7. Survivors were defined as mice that had survived until at least 580-600 days post challenge. PrP<sup>Sc</sup> was isolated by differential extraction and Western blotted. A prestained Kaleidoscope standard (Biorad) was loaded onto Lane 1. +C denotes the positive control for PrP<sup>Sc</sup> labelling which is the spleen extract of a terminally infected adult mouse, inoculated i.p as an adult. Samples 1- 24 represent \_ spleen extracts taken from individual mice inoculated with either ME7 or normal brain homogenate. Extracts were split and either treated with PK (+) or left untreated (-). (-C) denotes negative controls, which are spleens taken from a 1 day old mouse challenged i.p with normal brain.

Blot	Case	Inocula	Age at infection (days old)	Survival period (days)	Clinical Status	Brain pathology	Spleen PrP <sup>sc</sup> status
a	1	ME7	1	595	Negative	Negative	Negative
	2	ME7	1	595	Negative	Negative	Negative
	3	NB		-	Negative	Negative	Negative
b	4	ME7	1	595	Negative	Negative	Negative
	5	ME7	1	595	Negative	Negative	Negative
	6	NB		-	Negative	Negative	Negative
c	7	ME7	1	595	Negative	Negative	Negative
	8	ME7	1	595	Negative	Negative	Negative
	9	NB		-	Negative	Negative	Negative
d	10	ME7	1	595	Negative	Negative	Negative
	11	ME7	1	595	Negative	Negative	Negative
	12	NB		-	Negative	Negative	Negative
e	13	ME7	1	592	Negative	Negative	Negative
	14	ME7	1	592	Negative	Negative	Negative
	15	NB		-	Negative	Negative	Negative
f	16	ME7	1	592	Negative	Negative	Negative
	17	ME7	1	592	Negative	Negative	Positive
	18	NB		-	Negative	Negative	Negative
g	19	ME7	1	592	Negative	Negative	Negative
	20	ME7	1	592	Negative	Negative	Negative
	21	NB		-	Negative	Negative	Negative
h	22	ME7	1	592	Negative	Negative	Negative
	23	ME7	1	592	Negative	Negative	Negative
	24	NB		-	Negative	Negative	Negative

**Table 3.5 Survivors: case profiles of mice inoculated at 0-1 d.o.** This table summarises the clinical and pathological details for each mouse challenged intraperitoneally at 10 days old with ME7. The case number refers to the number allocated for spleen samples in Fig 3.11. These include age matched mice challenged intraperitoneally with ME7 infected brain homogenate (ME7) or with normal brain homogenate as control (NB). The incubation period corresponding to each case is shown. Clinical status and brain pathology are expressed as positive or negative depending on whether mice showed classical scrapie symptoms or scrapie associated vacuolation respectively. Spleen PrP<sup>sc</sup> status is expressed as positive if PK resistant PrP<sup>sc</sup> was detected by immunoblotting or negative if no immunolabelling of PK resistant PrP<sup>sc</sup> was detected (see Fig 3.11 for immunoblots).

### 3.4 Summary

The main aim of this chapter was to repeat scrapie pathogenesis studies in neonatal mice to determine whether an age-dependent susceptibility could be observed using current strains of mice and scrapie in use today. After neonatal challenge a high proportion of mice resist scrapie infection. A small proportion succumb to scrapie with a small but significant prolongation of the incubation period. This is consistent with previous data documented by Outram et al, 1972, whereby a decreased susceptibility after peripheral challenge of neonates was also found.

At 70 d.p.i, detectable levels of PrP<sup>Sc</sup> are found in spleens of mice challenged at 10 days old. PrP<sup>Sc</sup> could not be detected in the spleens of neonatally challenged mice at this same time point. However, not all neonatal mice are resistant to scrapie and terminally affected animals challenged as neonates have high levels of PrP<sup>Sc</sup> present in the spleen. **Overall, this suggests that neonatal mice have an impaired ability to replicate scrapie.**

### 3.5 Future work

It will be of great interest to carry out bioassays of the corresponding spleen halves from neonatally challenged mice. Due to the time constraints of the project these could not be carried out within the allocated time. This will allow firmer conclusions to be drawn about the ability of neonatal mice to support scrapie replication.

## Chapter 4

# The maturation of germinal centres in the mouse spleen

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## 4.1 Introduction

Scrapie incubation period and pathogenesis are unaffected by ionising radiation suggesting that a long-lived radioresistant cell is involved in scrapie pathogenesis (Fraser and Farquhar, 1987). The candidate cell that fits these criteria is the follicular dendritic cell (FDC). FDCs have also been implicated in TSE pathogenesis due to the high accumulations of abnormal PrP found in association with FDCs in mice infected experimentally with scrapie [(Brown et al., 1999; Jeffrey et al., 2000; McBride et al., 1992; Ritchie et al., 1999) and CJD (Kitamoto et al., 1991)]. Above all, PrP<sup>c</sup> expressing FDCs as opposed to B cells are required for replication of scrapie after peripheral infection (Brown et al., 2000; Mabbott et al., 2000)

The relative resistance of neonatal mice compared to adult mice to peripheral scrapie challenge was described in Chapter 3. A proportion of neonatal mice survive scrapie infection after i.p inoculation (Outram et al., 1973). The ability of mice to survive scrapie challenge disappears in the first week of life and by the age of 10 days old, susceptibility to scrapie is similar to the adult. The purpose of this chapter is to investigate the possible reasons for this phenomenon.

The main hypothesis to be tested in this chapter is that neonatal mice lack a mature cell population involved in scrapie pathogenesis, which may undergo differentiation after birth and restore susceptibility to scrapie after certain postnatal age. The intrinsic requirement for FDCs as opposed to B cells in ME7 scrapie pathogenesis after the challenge of adult mice (Brown et al., 1999), suggests that the maturation of FDCs may affect neonatal scrapie pathogenesis with the ME7 strain.

Neonatal mice have a decreased ability to mount T-dependent antigen responses and this ability increases during the first 2-8 weeks of life depending on the antigen, adjuvant and strain of mouse used. Immaturity of B cells (Marshall-Clarke et al., 2000; Sartori et al., 1999) and the inability of mice to trap immune complexes (Holmes et al, 1984) suggests that FDCs are in an immature state during the first

month of life. Electron microscopy studies (Villena et al., 1983) and immune complex trapping studies in rat lymphoid tissue support this hypothesis (Imai et al., 1986).

In this chapter, the maturation of FDCs within germinal centres of the C57BL mouse will be studied to determine any correlations with the onset of scrapie susceptibility in young mice.

#### **4.1.1 Follicular dendritic cell function**

As described in section 1.2.2, FDCs are a unique non-lymphoid cell type found only in lymphoid follicle germinal centres. The long dendritic arms of FDCs form close contacts with follicular B cells and it is this close contact which is central in the role of FDCs as presenters of native antigen to B cells, thus aiding in the generation of memory. For the remainder of this section, the mechanisms by which immune complexes are trapped on FDCs are described. In addition, current knowledge regarding the age at which FDCs reach functional maturity in rodents and become capable of trapping immune complexes in lymphoid tissue is also discussed.

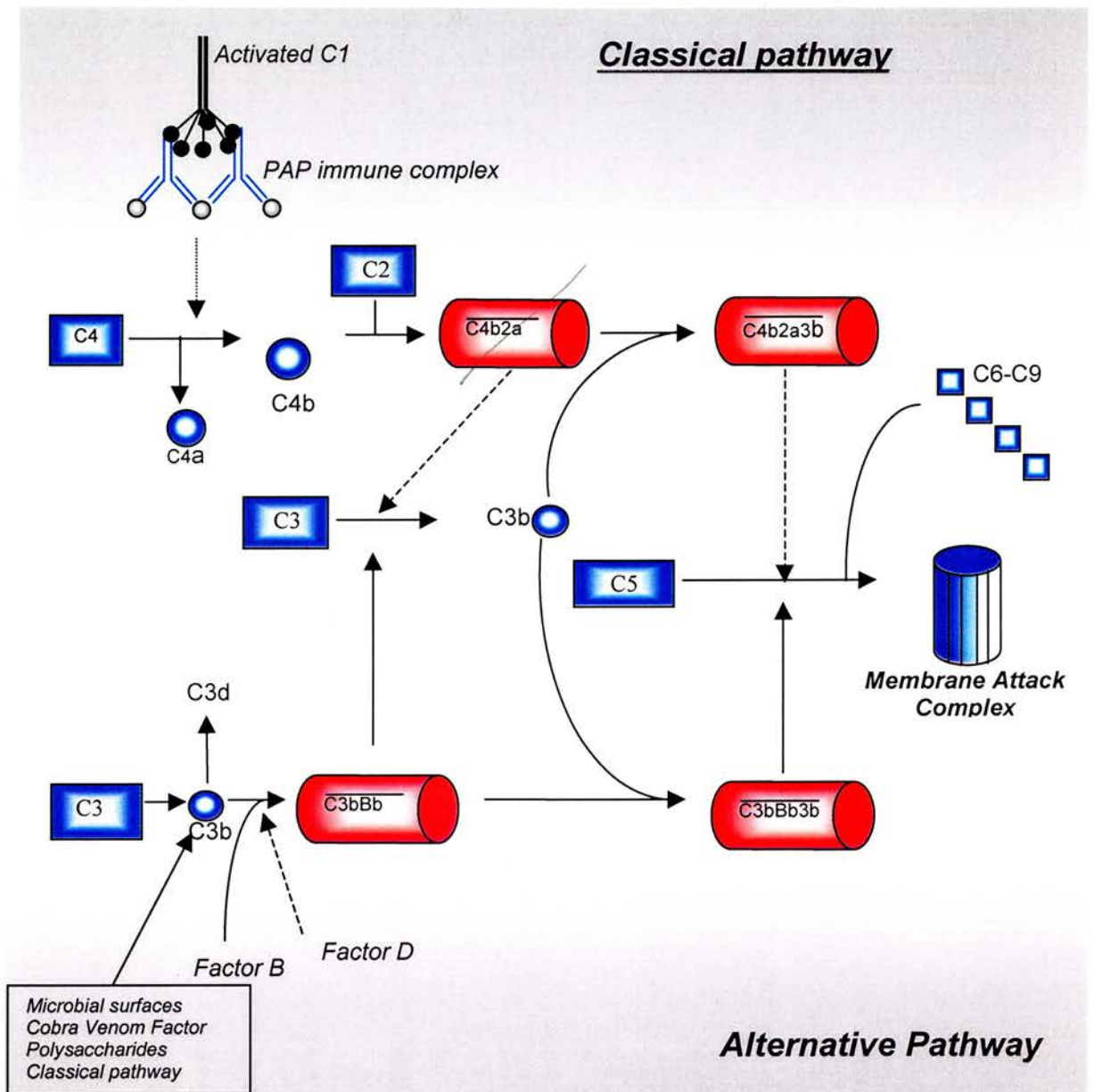
Since a major component of immune complexes is antibody, hypotheses were developed based on knowledge of the effector function of antibodies, to predict the mechanisms by which immune complexes become trapped in lymphoid follicles. Antibodies forming part of an immune complex can either be retained directly by Fc receptors or the immune complex can be opsonised and trapped by complement receptors. The roles of IgG receptors in immune complex trapping have been characterised. Fc $\gamma$ RII (CD32) appears to have a role in immune complex trapping in immunised mice. The lack of detectable Fc $\gamma$ RII levels in un-immunised mice (Van den Berg et al., 1995) suggests that other trapping mechanisms are involved in naïve animals. Fc $\gamma$ RI is not found on FDCs.

Immune complexes can activate complement and be retained indirectly through the deposition of C3 breakdown products onto antibody molecules. Complement containing immune complexes are subsequently retained by complement receptors.

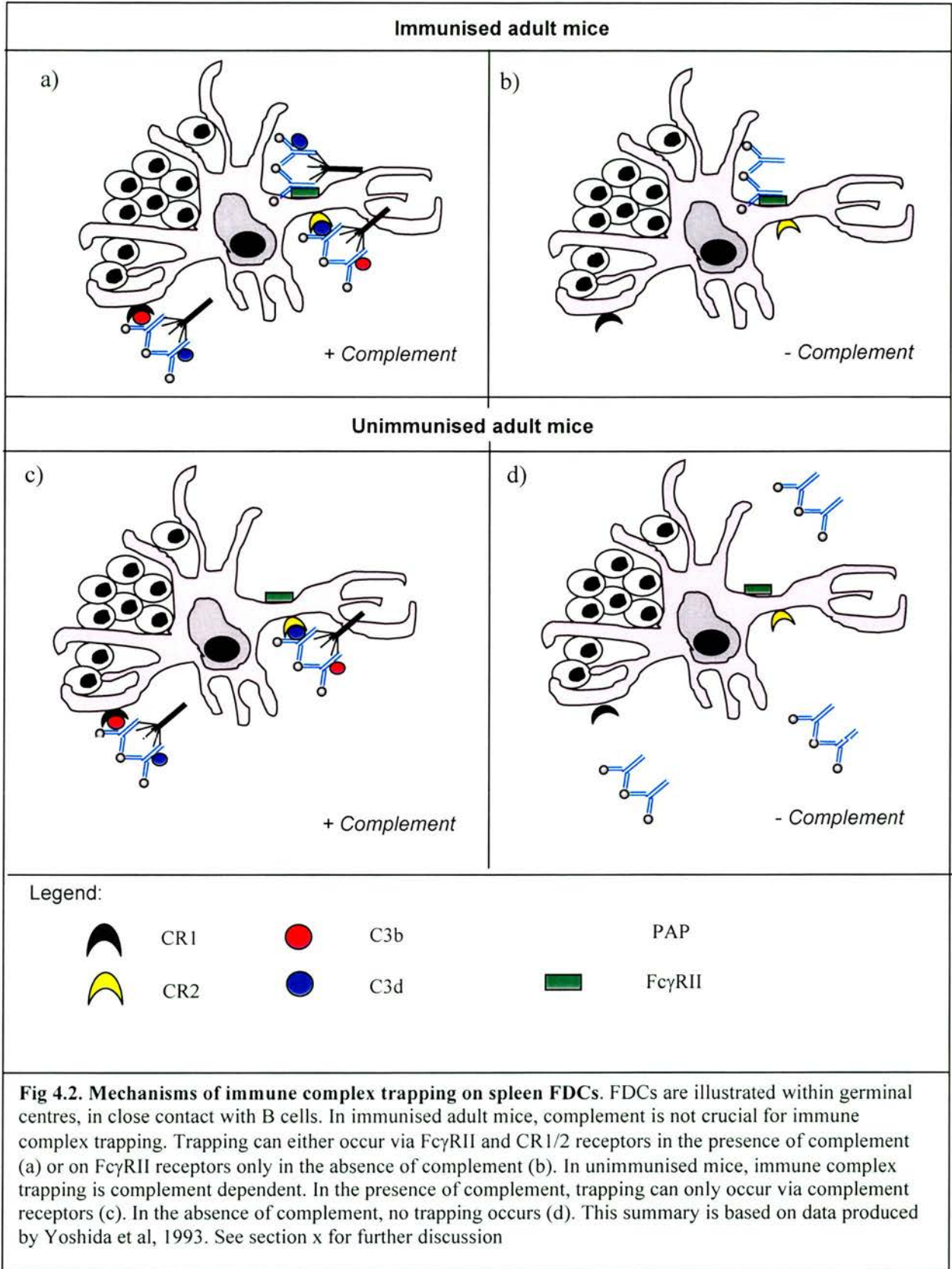
#### **4.1.2 The role of complement in immune complex trapping**

Studies have shown the central role of complement in immune complex trapping. Cobra venom factor (CoVF) is a powerful C3 depleting agent. It does so by activating the alternative pathway of complement (see Fig 4.2 for summary of the complement cascade). Administration of CoVF prevents or strongly inhibits immune complex trapping in mice (Papamichail et al., 1975). The fact that anti-C3 mAb also prevents immune complex trapping supports the hypothesis that C3 is ultimately required for immune complex trapping (Van den Berg et al., 1995).

Previous studies by Yoshida et al, have demonstrated the requirement for complement in immune complex trapping particularly in non-immunised mice, highlighting the importance of complement as a first line defence in the absence of specific antibody (Yoshida et al., 1993). The *in vitro* immune complex trapping technique was applied using fresh mouse serum (FMS) as a source of complement (Yoshida et al., 1993). In the absence of a source of complement, immune complex trapping was demonstrated in the spleens taken from mice immunised with sheep red blood cells, and this trapping strongly co-localised with the Fc $\gamma$ RII receptor (see Fig 4.2b). In the presence of FMS, immune complex labelling co-localised to a greater extent with CR1/2 labelling, although the Fc $\gamma$ RII and CR1/2 labelling overlapped to substantially (see Fig 4.2a). On the other hand, in non-immunised adult mice, immune complex trapping could only be demonstrated on spleen sections incubated with FMS (see Fig 4.2c) but no trapping was observed in the absence of it (see Fig 4.2d) (Yoshida et al., 1993). **The findings by Yoshida et al highlight the requirement for complement in immune complex trapping in non-immunised mice.**



**Fig 4.2. Summary of complement activation pathways leading to immune complex clearance.** The classical pathway is activated by immune complexes. In this example, peroxidase molecules bound and crosslinked by anti-peroxidase IgG. IgG molecules are in turn cross-linked by C1 molecules. The activated C1, catalyses the formation of C3 convertase of the classical pathway (C4b2a). The breakdown product C3b, forms the C5 convertase (C4b2a3b) by binding to the C3 convertase. C3b can also bind to the immune complex itself, thus allowing it to attach to CR1 receptors on FDCs and erythrocytes and be cleared from the circulation. A further breakdown product of C3b is C3d, which specifically binds to CR2 receptors on FDCs. The alternative pathway C3 convertase catalyses the production of more C3b. It is also incorporated into the C5 convertase (C3bBb3b) which is involved in MAC formation. Adapted from Cellular and molecular Immunology ( Abbas et al, 1995).



The precise mechanism of the immune complex trapping was further investigated by Yoshida et al. 1993 using a panel of monoclonal antibodies raised against CR1, CR1/2 (an antibody specifically against CR2 does not exist) and Fc $\gamma$ RII receptors. Blocking of CR1 with anti-CR1 mAb significantly inhibited immune complex trapping on FDCs. However the mAb against CR1/2 completely inhibited trapping. Although a mAb against CR2 alone does not exist, it can be deduced that most of the immune complex trapping on FDCs can be attributed to CR2 receptor, which binds C3d (Yoshida et al., 1993).

#### **4.1.3 The maturation of FDC function**

The age at which FDCs become fully functional in mice is an important question in scrapie pathogenesis since it has been deduced that mature FDCs are the target cell after peripheral scrapie challenge (Brown et al, 1999; Mabbott et al, 2000). Most rodent studies concerned with the ontogenic development of immune complex trapping have been carried out in the rat using either intravenous or direct injection into the footpads (Horie, et al ,1999; Imai et al., 1986). In the rat, the first primary follicle is observed 28 days after birth and this coincides with PAP immune complex trapping. However, further unpublished material by the same authors suggests that immune complex trapping can be detected from 3 weeks old. One study investigating the ontogeny of immune complex trapping in mice has been carried out (Holmes et al., 1984). In this study BALB/c mice of various postnatal age groups were immunised passively by injection of human serum albumin into the footpad followed by anti human serum albumin sera the next day. Spleens from neonatally injected and mice injected at 14 days old did not demonstrate any trapping in the spleen. However immune complex trapping could be detected in mice injected at 21 days old. The main difference between this study and recent immune complex trapping studies is the length of time between passive immunisation and removal of tissue. In recent experiments, the removal of tissue after passive immunisation with preformed immune complex (such as PAP) is carried out 2-3 days later. In the experiment by Holmes et al, 1984 this was done after 7 days. Since the mice inoculated at 21 days

would have been 28-29 days when culled, it is not clear at precisely what age the trapping occurred.

These experiments have been repeated in this project to verify the effect of age on immune complex retention in C57BL mice. Standard immune complex trapping techniques have been used whereby mice were given i.v injection of PAP and tissue removed 2-3 days later

## 4.2 Aims

In order to gain insights into the development of mouse spleen germinal centres, in particular spleen FDCs, and determine any correlations between the maturational state of FDCs and the onset of scrapie susceptibility in developing mice, the aim of this chapter is two fold. Firstly an immunocytochemical approach will be employed to study germinal centre structure development in the spleens of C57BL mice. Secondly, and in parallel to the morphological studies, the development of the functional ability of FDCs will also investigated.

## 4.3 Experimental Rationale

In order to investigate the maturation of germinal centres in the mouse spleen, immunocytochemical labelling of FDCs, B cells and macrophages was carried out. Spleens of C57BL mice of various age groups were harvested according to the plan shown in Table 4.1 and frozen. Frozen sections were prepared according to section 2.3.13.1. Sections were labelled using FDC-specific markers FDC-M1 and FDC-M2 (see section 2.3.13.5. B cells were labelled with the pan B cell marker B220 and germinal centre B cells were labelled with the lectin PNA (see section 2.3.13.7).

Age (days)	No mice
1	6
4	6
8	6
10	6
14	6
26	6
30	6

**Table 4.1 Experimental plan for investigation of spleen germinal centre development .** The spleens of C57BL mice of the above age groups were harvested and frozen in liquid nitrogen. Frozen sections were prepared and labelled with markers for FDCs and B cells.

Although FDC-M1 has been cited in the literature as being a maturational marker for FDCs (Berg and Dijkistra, 1995), the epitope for FDC-M1 has not yet been sequenced and its function has not been determined. For these reasons, it was necessary to determine the maturational status of FDCs in the developing spleen, based on their functional capacity. A well established method of determining FDC functionality is by testing their ability to trap immune complexes. As described in section 2.4.3, this can be done by either an *in vitro* or *in vivo* approach. The *in vitro*

immune complexes in the presence of a complement source (Yoshida et al., 1993). It has the advantage that stored spleen tissue can be used and no extra animals need be injected or culled. The *in vivo* method on the other hand is more complicated since it requires a high degree of skill in intravenous injections (i.v) and also requires animals for inoculation. Hence, the two methods were tested for their efficiency at immune complex trapping in adult mouse spleens (see sections 2.3.13.6). The most efficient method was found to be i.v injection of immune complexes and so this approach was chosen to study the ontogeny of immune complex trapping.

The study was carried out using C57BL and TNFR1 <sup>-/-</sup> mice as controls. TNFR1 <sup>-/-</sup> mice do not have functional, mature FDCs and hence do not form germinal centres within the spleen (discussed in more detail in Chapter 1). However, they do have immature primary follicles containing B and T cells (Le Hir et al, 1996). Hence, they serve as negative controls for FDC specific trapping of immune complexes. Mice of various age groups were intravenously inoculated with 50 µl neat PAP and the spleens harvested 3 days later (see section 2.3.13.6.2). The inoculation summary is shown in Table 4.2.

Age (days)	No. mice injected	
	C57BL	TNFR1 <sup>-/-</sup>
21	6	6
26	6	6
54	6	6
77	6	6

**Table 4.2 Inoculation plan for i.v inoculation of C57BL and TNFR1<sup>-/-</sup> mice with immune complexes.** 6 mice per age group were anaesthetised and inoculated with 50  $\mu$ l PAP. Spleens were harvested 3 days later and frozen. Spleen sections were prepared and immunolabelled according to section 2. 3.13.6. It should be noted that mice younger than 21 days old were not included in this study due to difficulties associated with i.v inoculation of young mice as a result of tail size.

Immune complexes are trapped and retained by either complement receptors or Fc receptors. In un-immunised mice it has been shown that complement receptors as opposed to Fc receptors are crucial for immune complex trapping (discussed in detail in section 4.1.2). For these reasons, the ontogeny of complement receptors was studied using fluorescence immunocytochemistry, to determine any correlation between the onset of immune complex trapping and the age at which complement receptors can first be detected. Since complement activation is known to occur in neonatal rodents the availability of functional complement components could not have been a limiting factor in the trapping of immune complexes during ontogeny (personal communication with Sepo Meri, Finland).

The spleens of C57BL mice described in Fig 4.4 were used in this study. Frozen sections were cut (see section 2.3.13.1) and labelled with mAb 8C12 specific for complement receptor 1 (CR1) according to section 2.3.13.5.

## 4.4 Results and Discussion

### 4.4.1 The ontogeny FDC-M1 labelling in the developing spleen

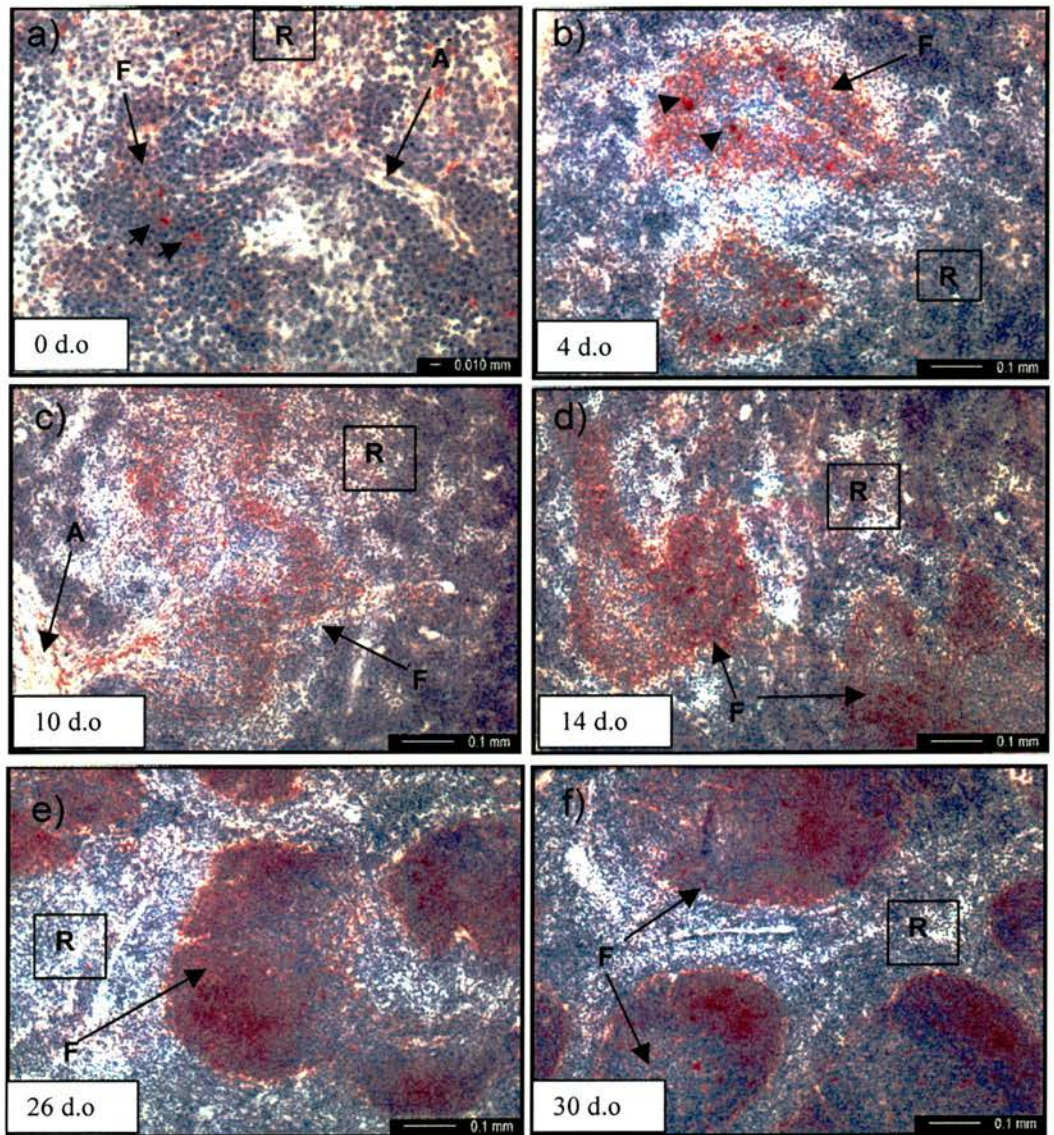
FDC-M1+ cells were observed consistently in all age groups tested. FDC-M1+ cells were observed from 0 days after birth in the neonatal spleen (see Fig 4.3a). Labelling was punctate and was found around blood vessels within immature primary B cell follicles. (see adjacent section labelled with B220 – Fig 4.4a). In the four day old mouse, punctate labelling could still be observed. However, staining was found distributed around the edge of the immature white pulp, again corresponding with B220+ areas (see Fig 4.3b). By 26 days after birth, the FDC networks are more apparent and the staining pattern for B cell follicles and FDC networks is more adult-like (see Fig 4.3 e and f).

The pattern of B220 labelling in neonatal and young mice is consistent with previously reported patterns observed in TNFR1<sup>-/-</sup> mice (Le Hir et al., 1996) and also neonatal rat lymphoid tissue (Dijkstra, 1983). TNFR1<sup>-/-</sup> mice are devoid of FDCs and have irregular B cell patterns within the follicles (Le Hir et al., 1996) The irregularities are probably due to the prevention of B cell polarisation due to a lack of mature FDCs. By 26 d.o, B cell follicles resemble the adult mouse spleen ( see Fig 4.4e and f)

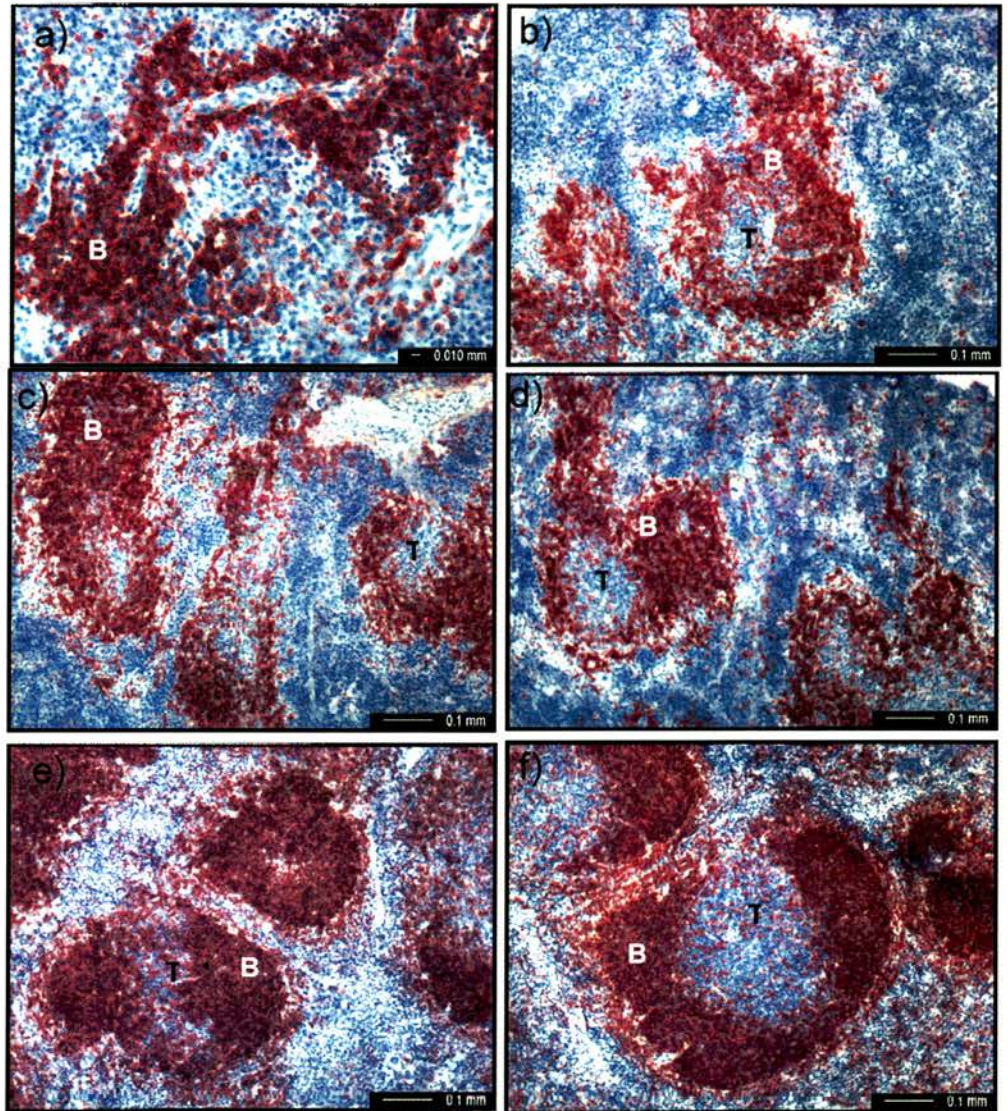
Detection of FDC-M1+ cells in neonatal mice was not expected since it has been referred to as a maturity marker for FDCs and reputedly distinguishes FDCs from immature follicular reticular cells (FRCs) (Berg and Dijkstra, 1995). The finding of possible immature FDCs in neonatal spleens has relevance in the context of the ongoing debate concerning the origins of follicular dendritic cells. As discussed in section 1.2.2.1, there are two main hypotheses regarding the tissue origin of rodent FDCs. On the one hand, a bone marrow origin is postulated, predicting that FDC precursors exist in the bone marrow and have the capacity to populate secondary

lymphoid tissue postnatally (Szakal et al., 1995). On the other hand, the reticular cell hypothesis postulates that immature follicular reticular cells present perinatally in secondary lymphoid tissue eventually mature into follicular dendritic as a result of cellular and molecular cues present in the lymphoid tissue environment (Kapasi et al., 1998).

Although this study does not rule out the possibility of bone marrow derived FDCs populating lymphoid tissue postnatally, it does suggest that immature forms of FDCs do exist in neonatal secondary lymphoid tissue from which mature FDCs may derive. This is in agreement with a recent study which demonstrated that human fibroblast marker (AS02) could recognise human follicular dendritic cells in adult secondary lymphoid tissue, whereby AS02 co-localised with CD21+ FDCs in adult frozen tonsil and spleen sections (Bofill et al., 2000).



**Fig 4.3 Ontogeny of FDC-M1 in the developing spleen.** Frozen spleen sections from C57Bl mice of various age groups with FDC-M1 antibody. Labelling was detected using a standard ABC method linked to NovaRed (Dako) and sections counterstained with haematoxylin. Age groups indicated in white boxes. Images taken using a Nikon E800 microscope linked to a Kodak polaroid camera. Scale bars are indicated. A= arteriole, R= red pulp, F=follicle. Small arrow heads indicate areas of punctate FDC-M1+ cells.



**Fig 4.4 Spleen follicle development.** Frozen spleen sections from C57BL mice of various age groups were labelled with B220. Sections are adjacent to those in Fig 4.3a-f. Labelling was detected using the ABC method using NovaRed as a substrate. Sections were counterstained with heamatoxylin.

#### **4.4.2 The ontogeny of FDC-M2 labelling in the developing spleen**

FDC-M2 is another FDC specific marker which became available during the course of the project (Kosco-Vilbois et al., 1997). It differs from FDC-M1 in that it does not label tingible body macrophages within germinal centres. The onset of FDC-M2 labelling occurs between 10 and 14 days old (see Fig 4.5 and summary in Fig 4.4).

Although FDC-M2 stains the blood vessels of 10 day old mice very strongly (see Fig 4.5 a) and b), FDC-M2+ cells within follicles do not appear until 14 days old. Unlike FDC-M1+ cells, the FDC-M2+ cells appear as well defined networks in the 14 day old mouse spleen. 5/5 spleens from 14 day old mice demonstrate FDC-M2 reactivity in follicles compared to 0/6, 10 day old spleens (see Fig 4.4). The morphology of the FDC-M2 labelling at 14 days old is very similar to the adult (see Fig 4.5c and e).

The appearance FDC-M2+ cells at 14 days old may suggest that the FDCs are reaching functional maturity. However, because the epitope recognised by FDC-M2 has yet to be defined, it makes the formation of conclusions regarding the functionality of the FDC-M2 positive cells difficult.

The targeting of FDC-M1 and FDC-M2 labelling within the spleens of 14 day old is very different. FDC-M1 staining covers the whole of the white pulp area, whereas FDC-M2 targets a discrete area of the white pulp. This is of interest since it could suggest the existence of distinct populations of FDCs, perhaps at differing maturational stages.

Age (days old)	No. spleens FDC-M2+/ No spleens tested	% FDC-M2+ spleens
10	0/6	0
14	5/5	100
30	5/5	100

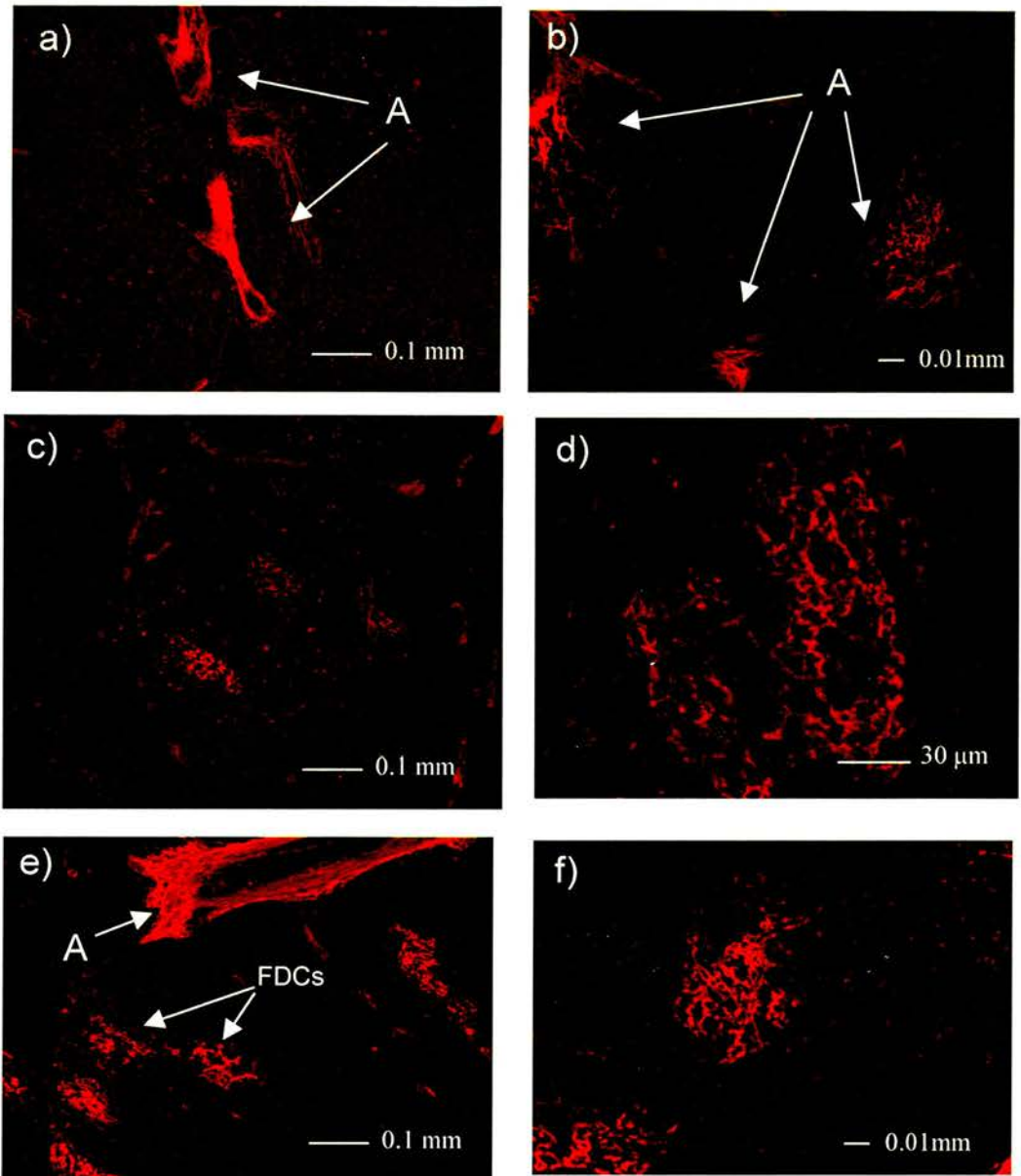
**Table 4.3 Summary of FDC-M2 ontogeny in the mouse spleen.** a).The table shows the number of individual spleens tested for each age group and the number of spleens showing FDC-M2+ cells within the follicles.

#### 4.4.3 Determination of the age at which the spleen can support immune complex trapping

As expected, all TNFR1<sup>-/-</sup> mice inoculated i.v with immune complexes demonstrated no immune complex trapping in spleen (Fig 4.7 b, d, and f. Results summarised in Fig 4.6) since these mice do not possess functional FDCs. This demonstrates the requirement of fully functional FDCs or immune complex trapping.

All 63 day old C57BL mice (6/6) were inoculated intravenously with PAP and immune complex trapping can be observed in all mice inoculated. The staining was defined and intense, with many follicles demonstrating PAP immune complexes (see Fig 4.7 a. All 26 d.o mice were inoculated successfully with PAP however, there were 2 deaths due to complications as a result of anaesthesia hence only 4 spleens could be tested. PAP labelling could be detected in the spleen follicles of all 26 day old mice tested (4/4), however in contrast with the situation in the 63 day old mice, the staining was more diffuse and covered a wider area of the follicle (see Fig 4.7c).

It is more difficult to draw conclusions regarding the capacity of 21 day old mice to trap immune complexes. Only one mouse from a group of 6 was successfully



**Fig 4.5 The ontogeny of FDC-M2 in the developing mouse spleen.** Frozen spleen sections labelled with FDC-M2 and detected using Cy3 (a). X10 mag. 10 day old spleen. b). X20 mag, 10 day old spleen. c) X10 mag, 14 day old spleen d) X40 mag, 14 day old spleen. e) X10 mag 40 day old spleen. f) X20 mag 40 day old spleen  
 A= arterioles FDCs= FDC-M2+ networks

inoculated with PAP. The difficulties associated with i.v inoculations usually arise from the size of the tail and the colour of the mouse. The tails of the 21 day old C57BL group were small and very fragile, making it difficult to insert the needle properly. Albino mice tend to be easier to inject since the tail is more transparent than black mice. Despite this, all 6 mice from the TNFR1<sup>-/-</sup> groups were inoculated. The reasons for this are not clear since TNFR1<sup>-/-</sup> are of the same genetic background as C57BL mice and hence are of similar size and colour.

Although only one 21 day old C57Bl mouse was inoculated, it was decided that the spleen should nevertheless be screened for immune complex trapping. It was possible that trapping of PAP may have occurred and would therefore suggest that mice of this age are actually capable of trapping immune complexes. No PAP immune complexes could be found in the spleen of the inoculated 21 day old mouse (see Fig 4.7e). Age matched controls were also negative. At this stage it was decided that younger mice would not be inoculated with PAP in the light of the difficulties experienced with the 21 day old C57BL group.

Overall, the data suggests that by 26 days old, the mouse spleen is capable of trapping immune complexes. Conclusions regarding the precise age at which immune complex retention occurs in C57BL mice cannot be made since only one mouse was successfully inoculated at 21 days old. Studies carried out in BALB/c mice have suggested that 21 day old mice are capable of trapping immune complexes in the spleen and lymph nodes after passive immunisation via the footpad (Holmes et al., 1984). However, spleens from mice injected at 21 days old were not removed until 29 days old, hence the precise age at which trapping occurred is not known precisely. If BALB/c mice can trap immune complexes at 26 days old, then it is possible that it could have occurred shortly before the cull date of 29 days after birth and not prior to 26 days old. Nevertheless, immune complexes were not encountered in the spleens of mice immunised at 14 days old. Thus taken together, the data from

studies by Holmes et al suggest that onset of the ability to retain immune complex in the spleen occurs between 21 and 29 days old in BALB/c mice.

Most other studies looking at the age of onset of immune complexes have been carried in the rat model, probably because the larger tail vein facilitates inoculation. Immune complex trapping occurs in the popliteal lymph nodes of rats inoculated with preformed PAP at 28 days old into the footpad. No retention could be observed in the lymph nodes of 24 day old rats (Imai et al., 1986). However, trapping of PAP has been reported to occur in the spleens of rats injected at 21 days old (Dijkistra et al., 1982). In both studies the trapping of immune complexes correlated with the development of primary follicles. If the previous studies are considered as a whole, they suggest that there age differences of up to 1 week between laboratories with regards to the earliest age at which immune complexes can be retained in lymphoid tissue. A possible explanation could be that exposure of experimental animals to environmental antigens varies between laboratories. Type of antigen and route of exposure may affect the maturation rates of FDCs in different lymphoid organs at such a critical point in development.

It is clear that the ontogeny of immune complex trapping study should be repeated, but due to time constraints towards the end of the project this could not be carried out. Intravenous inoculation of C57BL mice presented grave technical difficulties, which impeded the progress of the study and eventual conclusions. Further studies could involve use of albino mouse strains as opposed to C57BL mice, although the reason for using C57BL mice was to gain more information regarding FDC development for the mouse strain of choice in peripheral scrapie pathogenesis studies. Additionally, footpad injections could also be used as an alternative to intravenous studies since a good level of spleen retention occurs (Holmes et al., 1984) and they should be more straightforward in young mice compared to i.v injections.

Age (days)	No.mice positive/No. mice tested	
	C57BL	TNFR1-/-
21	0/1	0/6
26	4/4	0/6
63	6/6	0/6

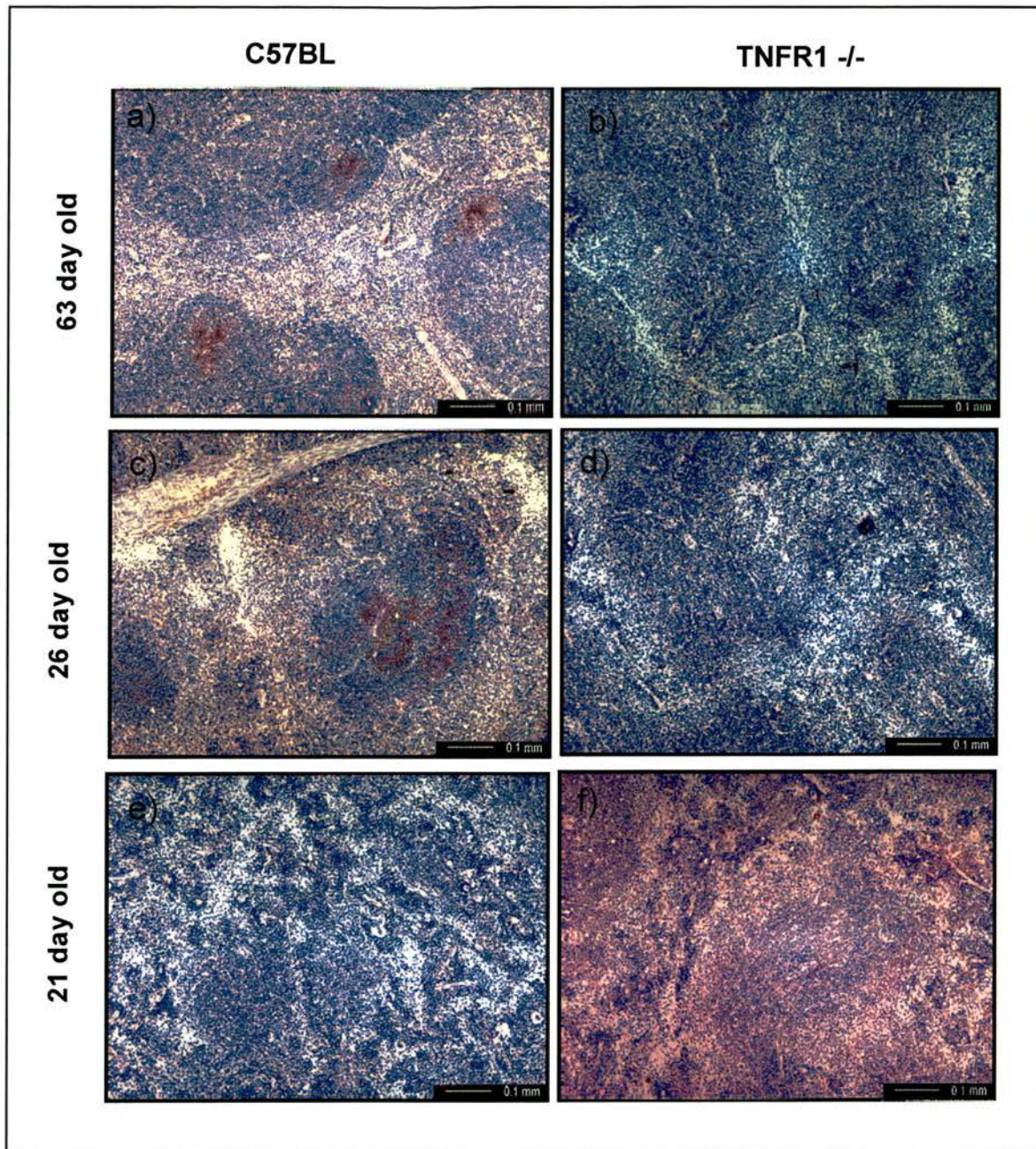
**Fig 4.6 Summary of results from i.v inoculation of developing mice with immune complexes.** C57BL and TNFR1-/- mice were inoculated with PAP at various postnatal age groups. All 26 d.o and 63 d.o mice were inoculated successfully. Problems were encountered with i.v injections of 21 d.o C57BL (1/6 mice injected successfully). PAP complexes could be detected in the 26 d.o and 63 d.o C57BL mice. No complexes were detected in TNFR1-/- mouse spleens from any age group.

#### **Onset of PNA+ germinal centre B cell in the developing spleen of unimmunised mice**

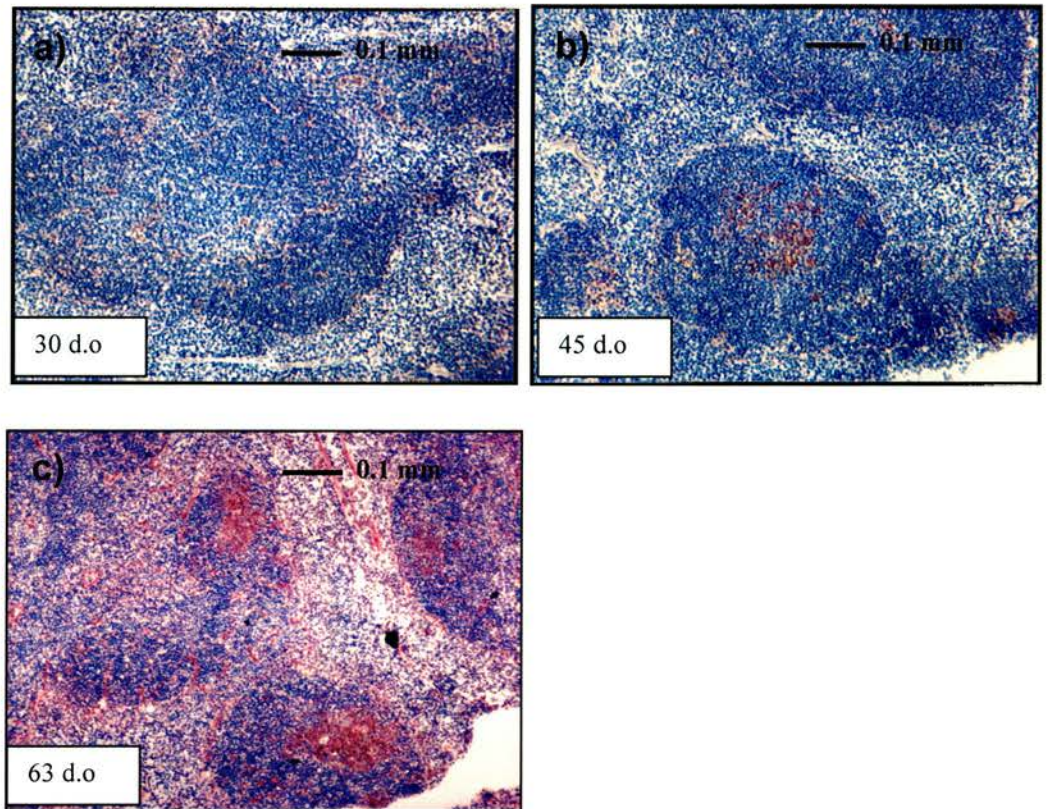
The PNA binding feature of germinal centre B cells has long been used to distinguish them from other lymphoid tissue residing B cells (Coico et al., 1983). PNA+ germinal centre B cells were first detected between 30 and 45 days old in unimmunised mice (see Fig 4.8). At 30 days old, 0/2 spleens have germinal centres however by 45 days 2/5 spleens have them. PNA+ germinal centres do not form until at least 3 weeks after the onset of immune complex trapping ability. This delay is expected and agrees with past studies which have shown that germinal centre development is dependent on antigen retention abilities of FDCs (Szakal et al., 1990). Nevertheless, other studies in rat have suggested that germinal centre reactions can be observed earlier in development. One study has demonstrated the presence of germinal centre B cells at the ultrastructural level in the mesenteric lymph nodes of unimmunised rats as early as 15-18 days after birth (Villena et al., 1983). However, germinal centres in cervical and thoracic lymph nodes were observed later. Other studies have shown germinal centre reactions to be present in the spleens of 28 day old rats using the absence/low levels of IgM labelling on

germinal centre B cells as a distinguishing factor (Dijkstra and Dopp, 1983). Germinal centres have also been detected in the popliteal lymph nodes of 35 day old rats (Imai et al., 1986). In the last two studies, rats had been passively immunised with immune complexes. All studies used morphological characteristics to distinguish the germinal centre B cells from other B cells; PNA was not applied as a distinguishing marker. Again it is possible that the route, type and amount of environmental antigenic stimulation and the type of lymphoid tissue studied may affect the number of germinal centres observed. **In general most studies have highlighted the requirement for functional FDCs before germinal centre formation occurs, regardless of the age at which germinal centres can first be observed.**

In order to try to explain why immune complex trapping occurred at the particular age observed, more information was required regarding the status of the receptors involved in immune complex trapping. The central role of complement receptors in immune complex trapping has been highlighted (Fang et al., 1998; Yoshida et al., 1993) especially in un-immunised mice (Yoshida et al., 1993). Section 4.4.4 deals with the ontogeny of CR1 in the developing spleen to determine whether any correlations exist between the onset of immune complex trapping and the detection of CR1.



**Fig 4.7 The onset of ability to trap immune complexes in the spleen.** Mice of various age groups were inoculated i.v with 50 $\mu$ l PAP. Spleens were harvested 3 days post-injection. Frozen sections were prepared and trapped PAP immune complexes were detected as described in section 2.3.13.6.2. Left-hand panel show spleens from 63, 26, and 21 day old C57BL mice. The right hand panel shows spleens from 63, 26 and 21 day old TNFR1<sup>-/-</sup> control mice. Spleen images were taken using a Nikon E800 microscope linked to a polaroid camera. The scale bar represents 0.1mm.



**Fig 4.8 The detection of PNA positive germinal centre B cells in the developing spleen.** Splens from C57BL mice were labelled using biotinylated PNA according to section 2.3.13.7. Germinal centre B cells were visualised using DAB and sections were counter stained using haematoxylin. a) spleen from 30 day old mouse, X10 mag. b) spleen from 45 day old mouse, X10 mag. c) spleen from 63 day old mouse, X10 mag. All images taken using a Polaroid digital camera linked to to Nikon E800.

#### **4.4.4 The ontogeny of complement receptor labelling**

Fluorescent immunocytochemistry was used to detect CR1. As can be seen from Fig 4.10 b, labelling can be detected from 14 days old in the mouse spleen (2/5 spleens test positive). By 18 days old all spleens test positively for CR1 (4/4).

An increase in the levels of the appropriate receptor for immune complex trapping occurs between 14 and 18 days after birth. If it is assumed that immune complex trapping occurs between the age of 21-29 days after birth (Holmes et al., 1984), there appears to be a short lag between the detection of CR1 immunolabelling in spleen and the onset of immune complex trapping ability.

There are several possible explanations for this. Firstly, it may suggest that a co-receptor not present at this stage may be required for trapping. The dependency for trapping in unimmunised mice seems to rest with CR1 and CR2 receptors. Blocking of Fc $\gamma$ RII, FDC-M1, and ICAM-1 on spleen sections does not seem to affect the capacity of the follicles to trap immune complexes in un-immunised mice (Yoshida et al., 1993). The ontogeny of CR2 was not investigated in this project since a specific antibody against CR2 alone is unavailable. If the onset of this receptor was delayed slightly relative to CR1 then this may explain the apparent delay in immune complex trapping onset reported by Holmes et al. The proposed differences between the 26 day old and 21 day old mice are summarised in Fig 4.11.

Another hypothesis is that the FDCs of 21 d.o mice are still structurally immature and hence unable to physically capture and retain antigen on the surface. Indeed, the difference between the immune complex labelling between the 26 d.o mouse and the 63 d.o mouse in terms of intensity and pattern was evident. At 26 d.o the labelling is not confined to the apex but is diffuse and covers a wide area of the follicle. The diffuse nature of the immune complex staining may suggest morphological differences between the dendritic processes of FDCs of young mice (i.e. 21-26 d.o) and older mice (i.e. 63 d.o). It would have been of interest to determine the precise

age at which the FDC processes become adult-like in the mouse. During the course of the project, the possibility arose to collaborate with Dr Martin Jeffrey, VLA, Edinburgh in an ultrastructural investigation of spleen FDC development in mice of various age groups. A previous publication by Jeffrey et al, (2000) had shown that mature mouse spleen FDCs residing within the light zone of the follicles have long cytoplasmic extensions. They could be distinguished from FDCs residing within the dark zone, which have less pronounced cytoplasmic extensions (Jeffrey et al., 2000). The ultrastructural study of mouse spleen FDCs during development would therefore have been an ideal method of determining when the change in morphology of cytoplasmic extensions occurred in the mouse spleen. However, problems were encountered trying to locate germinal centres within the white pulp of 4-5 week old mice. Germinal centres may have been easier to locate in immunologically stimulated mice. However, the purpose of the study was to investigate the development of FDCs in non-immunised mice of specific age groups. In order to investigate germinal centre presence in mice of specific age groups, a quick method of immune stimulation for inducing large germinal centres would have been required. Previous studies have shown that it takes at least 1 week to develop large germinal centres post-immunisation (Szakal et al., 1990). Hence, it would not have been possible to determine when FDCs showed mature characteristics during natural development.

Nevertheless, ultrastructural studies of developing rat lymph nodes demonstrate that FDCs characterised by a “rough nuclear surface” and “cytoplasmic membranous foldings”, first appear around the time of the first germinal centre reaction at 15-18 days old. (Villena et al., 1983). In another, the transition between immature and mature FDCs with labyrinthine structures, occurred between 28 and 35 days after birth (Imai et al., 1986).

The role of complement in scrapie pathogenesis has recently been investigated for the first time and shown to affect pathogenesis (Mabbott et al, 2001). Specific targeting of C3 by administration of CoVF resulted in a significant incubation period prolongation of 24 days compared to untreated animals. CoVF does not affect early classical pathway components such as C1, C4, C2 or membrane attack complex

components. In addition significant lengthening of incubation period is observed in mice deficient for C1q genes (classical pathway activation abrogated) and double knockout mice deficient in Factor B /C2 (Mabbott et al, 2001). In this knockout production of the alternative pathway C3 convertase is affected due to a lack of Factor B. In addition the production of the classical pathway convertase is abrogated by the deletion of C2.

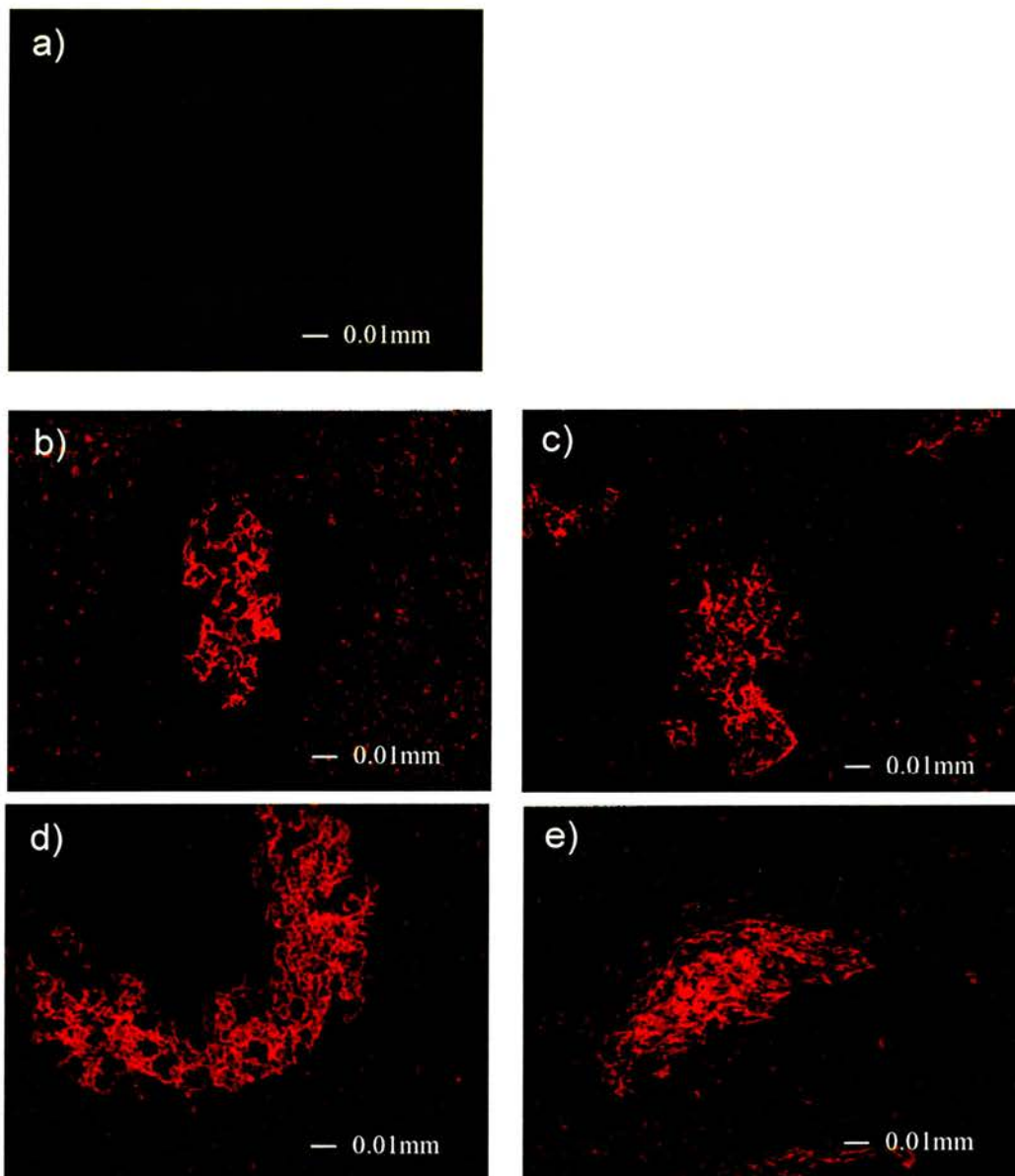
This study has demonstrated that complement fixation and possibly complement receptors have a role in scrapie pathogenesis in the early stages of infection (i.e. the first few days). The fact that scrapie is not completely prevented in the knockout mice suggests that neither the classical or alternative pathways are essential for scrapie pathogenesis but may aid in initial targeting of infectivity/PrP<sup>sc</sup> to the lymphoid follicles. The most likely explanation is that PrP<sup>sc</sup> may activate complement directly through the alternative pathway as opposed to the classical pathway, since anti-PrP<sup>sc</sup> antibodies have yet to be found in infected animals.

In the light of the published data on the role of complement in scrapie pathogenesis and the present data on FDC maturation, a possible reason why a proportion of neonatal mice resist scrapie infection, may be due to either a lack of complement receptors with which to trap infectivity or an impaired complement system. This may in turn affect localisation of infectivity to follicles and coupled with the immaturity of FDC networks, may then delay scrapie replication or prevent it completely. It is of interest that young mice become fully susceptible to scrapie around the time in which complement receptor 1 labelling in spleen is observed.

a)

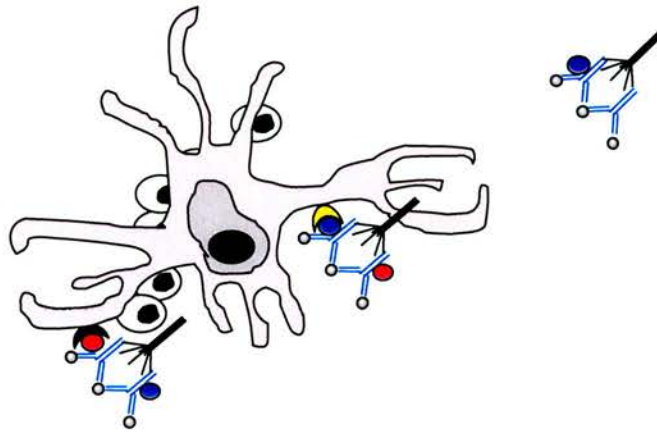
Age (days old)	No. spleens CR1+/ No spleens tested	% CR1+ spleens
10	0/5	0
14	2/5	40
18	4/4	100
26	3/3	100
30	5/5	100

**Table 4.4 Summary CR1 expression onset in the developing mouse spleen.** The table shows the number of individual spleens tested for each age group and the number of spleens showing CR1 labelling within spleen follicles. b). The graph summarises the increase in CR1+ cells as the mouse matures

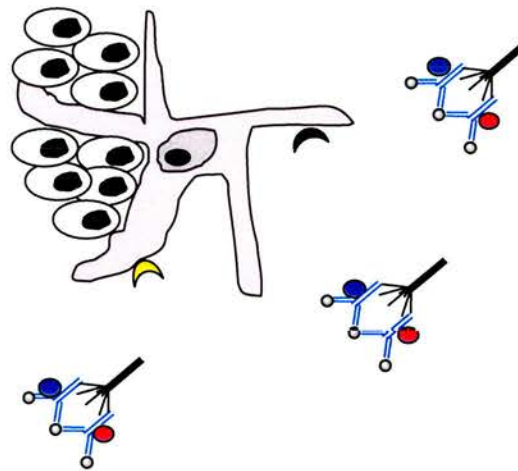


**Fig 4.10 The ontogeny of CR1+ cells in the developing mouse spleen.** Frozen spleen sections from unimmunised C57BL mice of various age groups were labelled with mAb 8C12 against CR1 according to section 2.3.13.5. Labelling was detected using streptavidin linked to Cy3. Images were taken using a Hamamatsu Orca II digital camera linked to a Nikon E400 epifluorescent microscope. a). 10 d.o b) 14 d.o c). 18 d.o, d) 26 d.o, e) 40 d.o. All images were observed under a X20 objective.

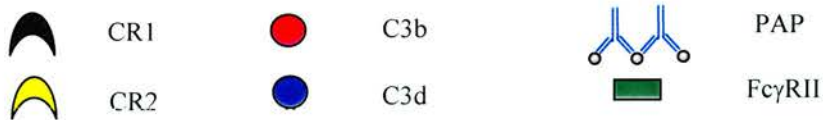
**Unimmunised 27 day old mouse**



**Unimmunised 21 day old mouse**



**Legend:**



**Fig 4.10. Mechanisms of immune complex trapping on spleen FDCs in developing naive mice.** In the unimmunised 27 d.o mouse, the presence of complement receptors and a mature dendritic network may contribute to immune complex trapping. In the 21 day old mouse, although detectable levels of complement receptors are present to trap immune complexes, the dendritic network may not be fully formed yet and retention of immune complexes may not be possible.

## 4.5 Summary/ Future Work

FDC-M1<sup>+</sup> cells are detected from birth, however a mature phenotype displaying dendritic networks does not become apparent until 14-26 days after birth. FDC-M2<sup>+</sup> cells on the other hand cannot be detected until 14 days after birth. FDC functionality studies suggest that FDCs have gained full antigen trapping capabilities by 26 days after birth. The precise age at which this function commences in C57BL mice was not established in this project due to technical difficulties associated with i.v injections. Nevertheless, previous studies in other rodent models suggest that this occurs between 21 and 29 days after birth. Hence, the FDC specific markers (FDC-m1 and FDC-M2) can detect non-functional forms of FDCs, but FDC-M2 may detect FDCs at a critical stage in maturation since the detection of FDC-M2<sup>+</sup> cells coincides with CR1 detection. Detectable levels of CR1 appear much before the onset of immune complex trapping ability and may suggest that the dendritic networks are not able to trap immune complexes due to their structural immaturity. However since CR2 ontogeny was not investigated, it is possible that this is upregulated later in FDC development than CR1.

The failure to determine the precise age of onset of immune complex trapping ability in the spleens of C57BL mice after i.v injection should be re-addressed. This could be done using albino mice, since it is easier to visualise the tail veins due to their lighter skin colour. In addition, the use of footpad injection may offer another way of successfully injecting immune complexes into mice.

## Chapter 5

# The detection and cell association of PrP<sup>c</sup> in the developing mouse spleen

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## 5.1 Introduction

As described in greater detail in Chapter 1, various *in vivo* approaches have demonstrated that PrP<sup>c</sup> expressing FDCs are critical for replication of scrapie and eventual neuroinvasion after peripheral challenge. (Brown et al., 1999; Mabbott et al., 2000a; Mabbott et al., 2000b). Accumulation of infectivity occurs in the spleens of SCID mice with a functional PrP gene, irrespective of the PrP status of the donor bone marrow derived cells. However, if the SCID mice do not possess the PrP gene, then susceptibility to scrapie cannot be restored, irrespective of the PrP status of the donor bone marrow. Since FDCs are not replaced by bone marrow grafting in adult SCID mice (Brown et al., 1999; Yoshida et al., 1995), it revealed the requirement for mature PrP<sup>c</sup> expressing FDCs as opposed to donor bone marrow derived cells (Brown et al., 1999). The only requirement for the bone marrow was to support the maturation of the already present immature FDCs in the SCID spleen.

In support of these data, a failure to replicate scrapie in spleen is also associated with cytokine pathway deficiencies, which support FDC maturation. For example TNF $\alpha$  deficient mice are resistant to scrapie challenge because signalling through the TNFR1 receptor pathway is required for FDC maturation (Mabbott et al., 2000a).

The role of PrP<sup>c</sup> expressing mature FDCs is therefore well characterised at least in the FDC dependent ME7/C57BL scrapie model. However, the requirement for PrP<sup>c</sup> expressing FDCs in neonatal scrapie pathogenesis has not been investigated. In addition, the ontogeny of PrP<sup>c</sup> within the mouse spleen and how this might affect scrapie susceptibility in young mice is also unknown.

### 5.1.1 The developmental onset of PrP mRNA

Most ontogenic studies involving PrP have centred largely on the developmental regulation of Prn-p gene expression. The earliest reported age for PrP mRNA expression has recently been demonstrated as being embryonic day 9 (E9) (Miele, 1999). This is earlier than the previously reported finding of E13.5 (Manson et al., 1992). During the period E8.5 to E9, PrP mRNA is transcriptionally activated

whereby PrP mRNA is readily detectable using RT-PCR. The anatomical location of PrP mRNA was determined using whole mount–in situ hybridisation (WM-ISH) and was found to be restricted to the neuroepithelium of the developing CNS (Miele, 1999). Transcripts could only be detected on E9.5 using WM-ISH.

Very little information regarding the developmental regulation of the Prn-p gene in spleens is available. The levels of spleen PrP mRNA in adult mice are very low compared to those in adult mouse brain (Miele, 1999; Robakis et al., 1986) and there is no detectable PrP mRNA in fetal rat spleens using *in situ* hybridisation (Tanji et al., 1995). There are high levels of PrP mRNA in other non-neuronal tissue such as seminiferous tubules, uterus, amnion and mesodermal layer of the yolk sac in adult rats (Tanji et al., 1995). In the lungs, kidney and heart of foetal rats, no detectable levels of PrP mRNA could be found (Tanji et al., 1995). However, Manson et al, (1992) have shown that Prn-p gene expression can be detected in embryonic nephrons at E16.5 in embryonic mice.

These studies have shown that there is wide variation in the level of PrP mRNA both prenatally and postnatally. Indeed, mouse brain PrP mRNA levels increase 8 fold postnatally (Miele, 1999) and there is differential timing of Prn-p gene expression within regions of hamster brain (Mobley et al, 1988). Although there are no quantitative studies in spleen, it could be extrapolated that the spleen is also subject to developmental increases in the expression of the Prn-p gene, perhaps at a different developmental stage than the brain and other tissues. If this is the case then it could lend support to the hypothesis that low levels of PrP<sup>c</sup> might be responsible for the long incubation periods observed in neonatal mice. However, one such study that set out to investigate this possibility found, paradoxically that in neonatal hamsters, incubation periods were in fact reduced from 125 to 87 days after i.p inoculation (McKinley et al., 1988). Although these data could at first sight lead the rejection of the hypothesis, many studies have shown that direct infection of peripheral nerves can occur without the need for prior replication in lymphoid tissues, leading to very short incubation periods (Kimberlin et al, 1983). The hamster model is in itself an

excellent model for studying the role of peripheral nerves in scrapie pathogenesis due to the neurotropism of hamster adapted scrapie strains such as 263K. An alternative hypothesis for the observation of these short incubation periods in neonatal hamsters is that direct uptake of infectivity by peripheral nerve endings occurs with greater ease in neonatal rodents compared to adults. This could be due to immaturity of peripheral nerve structure in younger animals. This hypothesis is tested in mice in Chapter 6.

### **5.1.2 Developmental onset of PrP<sup>c</sup>**

Age related expression of PrP<sup>c</sup> has been recently investigated in human peripheral blood (Politopoulou et al., 2000). It was found that PrP<sup>c</sup> is significantly increased on lymphocyte surfaces in the elderly (mean age of 68 years) compared to children (mean age of 6 years) and adults (mean age of 33 years). There were no age related PrP<sup>c</sup> differences on monocytes between children and adult groups. Within the elderly group, higher levels of PrP<sup>c</sup> could be found on CD3+ T cells compared with CD19+ B cells. CD8+ T cells in turn had higher levels than CD4+ cells. Unfortunately, PrP<sup>c</sup> levels on human FDCs from various age groups were not investigated in this study.

## 5.2 Aims

The main aims of this chapter are therefore to determine the earliest age at which PrP<sup>c</sup> can be detected immunocytochemically in the developing spleen and to verify the cell association of PrP<sup>c</sup> during ontogeny. This data would aid in the interpretation of the effect of age on scrapie pathogenesis. In Chapter 3, it has been established using incubation period analysis and immunoblotting, that neonatal mice are less susceptible to scrapie than older mice. Full adult susceptibility is achieved around the age of 10 days old.

The hypothesis to be tested is that the decreased susceptibility of neonatal mice is due to a lack of PrP<sup>c</sup> within the neonatal spleen. This chapter investigates the prediction that the onset of detectable levels of PrP<sup>c</sup> occurs between 7-10 days old; the age when scrapie susceptibility resembles the situation in the adult mouse.

## 5.3 Experimental Rationale

In order to investigate the age related detection of PrP<sup>c</sup> in mouse spleen and cell specificity of the labelling, immunocytochemistry and confocal analysis of double labelled spleen sections were employed.

When C57BL mice became available a method for detecting PrP<sup>c</sup> on frozen spleen sections was developed which would work well on the spleens of mice of various age groups (see Table 5.1). Spleen sections were immunolabelled with 1B3 according to table 2.3 (Method 6). Results are shown in section 5.3.2.

The optimisation of immunocytochemistry for double immunolabelling of spleen sections for PrP<sup>c</sup> and FDCs is described in section 2.3.13.9. Double immunolabelled sections were analysed by confocal microscopy in order to determine areas of co-localisation between PrP<sup>c</sup> and FDC markers. Line profile analyses were made of areas of co-localisation as described in section 2.3.14.2. This was to aid in the characterisation of areas of co-localisation and determine any possible spatial differences in PrP<sup>c</sup> and FDC labelling during development.

Age (days)	No mice	
	C57BL	Prn-p/-
1	6	6
4	6	6
8	6	6
10	6	6
14	6	6
26	6	6
30	6	6

**Table 5.1 Experimental plan for investigation of PrP<sup>c</sup> onset in unfixed frozen spleen sections.** The spleens of C57BL and Prn-p/- mice of the above age groups were harvested and frozen in liquid nitrogen. Frozen sections were prepared and labelled with the polyclonal antibody 1B3 against PrP.

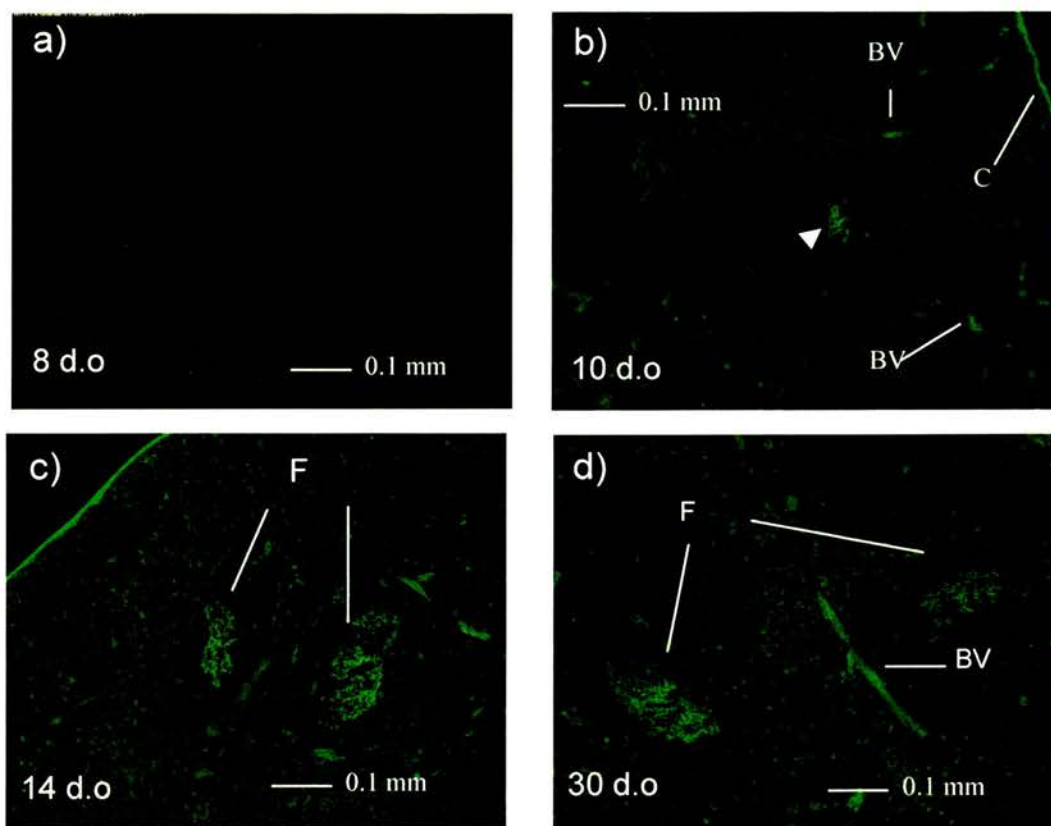
## 5.3 Results and Discussion

### 5.3.1 The age at which PrP<sup>c</sup> can first be detected in the developing spleen using frozen tissue

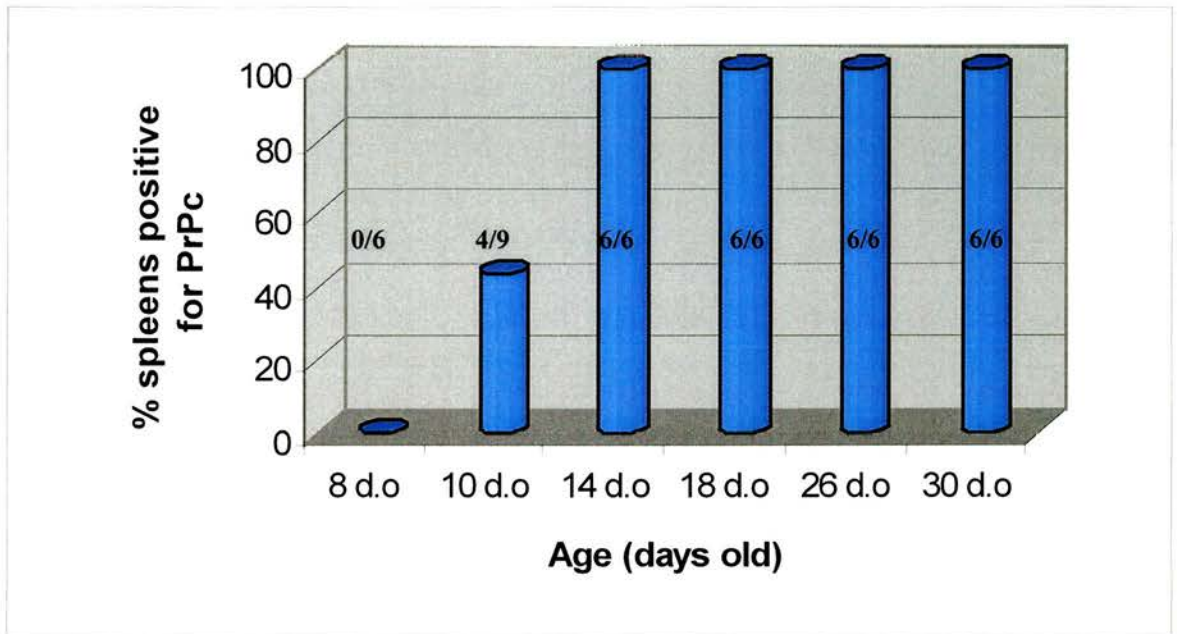
PrP<sup>c</sup> can first be detected within spleen follicles at 10 days after birth, in 4/9 spleens tested. No PrP<sup>c</sup> can be detected in 8 day old spleens (0/6 tested). The polyclonal antibody 1B3 tends to produce a relatively high background throughout the red pulp in frozen sections. Although the immunolabelling technique was optimised to such an extent that red pulp cross reactivity was minimised, high levels of immunolabelling was found to be associated with the spleen capsule and blood vessels. This was observed in mice of 10 days old and above, but was not seen in mice of 8 days old and younger.

Blood vessel labelling is not observed in sections incubated with normal rabbit serum and so suggests that the labelling is due to PrP<sup>c</sup>. However, blood vessel staining is not observed after light fixation of spleen vibratome sections with PLP (Ritchie, 1999). PLP preserves the antigenicity of PrP<sup>c</sup> and strong PrP<sup>c</sup> immunolabelling can be detected in vibratome spleen sections. Hence, this staining may be attributed to cross reactivity between 1B3 and non-PrP epitopes present on blood vessels.

The detection of PrP<sup>c</sup> within the spleen follicles of 10 day old mice correlates well with the development of scrapie susceptibility and is therefore an intriguing result. However, caution must be taken in forming the conclusion that scrapie susceptibility is purely dependent on PrP<sup>c</sup> and this is discussed in more detail in Chapter 7.



**Fig 5.1 The detection of PrP<sup>c</sup> in the developing mouse spleen using frozen spleens sections.** Frozen spleen sections from 8, 10, 14 and 30 day old C57Bl mice were immunolabelled with 1B3 and PrPc labelling detected using the Alexa 488 system. All images were taken using a Hamamatsu Cooled Digital Camera Orca II model (magnification= X10). a). 8 day old spleen: no PrPc+ cells. b). 10 day old spleen: PrPc+ cells indicated by small arrow head, within developing follicle. C). 14 day old spleen: PrPc+ cells within follicle d). 30 day old spleen: PrPc+ cells within follicle. Abbreviations are as follows: C= capsule; BV= bloodvessel; F=follicle.



**Fig 5.2 PrP<sup>c</sup> detection in frozen spleen sections.** The graph summarises the number of individual spleens tested for each age group and the total number of spleens with PrP<sup>c</sup> labelling within the spleen follicles.

### 5.3.3 The cell specificity of PrP<sup>c</sup> expression in the developing spleen

#### 5.3.3.1 Double immunolabelling of spleen sections with 1B3 and FDC-M1

Alexa 488 had been reported to be a high quality, stable fluorophore suitable for use in double immunolabelling protocols (Panchuk-Voloshina, et al, 1999). Using the 1B3/Alexa 488 system for PrP<sup>c</sup> detection, PrP<sup>c</sup>+ cells can be observed from 10 days after birth within a discrete area of spleen white pulp (see Fig 5.1b). The PrP<sup>c</sup>+ area is very small compared to the adult (see Fig 5.1d).

Confocal imaging shows clearly that PrP<sup>c</sup> staining co-localises with FDC-M1+ cells, due to the presence of yellow labelling (see Fig 5.3a, white arrow head) where PrP<sup>c</sup> and FDC-M1 labelling overlap. Co-localisation analysis was used to characterise the double immunolabelling in more detail. By plotting the intensity of the immunolabelling detected through the green and red channels versus the position of the labelling along a user defined line, an immunolabelling intensity profile can be obtained. In the case of a double immunolabelled sample, characterisation of areas of co-localisation can be obtained by superimposing the profiles of the two channels. In the case of the 10 day old spleen, the labelling detected through the green and red channels match each other closely between XY positions 121-181µm (see Fig 5.4a). Nevertheless, the area of co-localisation is surrounded largely by PrP<sup>c</sup> negative/FDC-M1+ cells. This can be seen in Fig 5.4a, where 4 peaks of medium to high intensity can be observed in the red channel indicating PrP<sup>c</sup>negative/FDC-M1+ cells (indicated by black arrows). These are distant to the area of PrP<sup>c</sup> staining.

As expected, PrP<sup>c</sup> labelling could not be observed in the 10 d.o Prn-p/- spleen. However, the overall intensity of the FDC-M1 labelling is lower than the intensity of the C57BL spleen. (see pixel intensity analysis 5.4b). Further study will be required to determine whether this is a significant finding. However, it should be noted that the Prn-p -/- mice are on a different genetic background to C57BL mice and may have a different rate of FDC maturation.

In the 14 d.o spleen, the PrP<sup>c</sup> labelling pattern is similar to the pattern observed in the 10 d.o spleen (see Fig 5.3 c), whereby the majority of PrP<sup>c</sup> co-localises with FDC-M1 between XY positions 99 to 140  $\mu$ m. There are many single labelled FDC-M1+ cells not expressing PrP<sup>c</sup> (see Fig 5.5a arrows). Again the age matched PrP null spleen, does not contain any PrP in the spleen (see Fig 5.3d) and this is confirmed by the line profile analysis. The intensity of FDC-M1 labelling in the 14 d.o PrP null spleen is similar to the C57BL spleen.

In the adult, the PrP<sup>c</sup> labelling pattern is rather different compared to the 10 and 14 day old mice. Most of the PrP<sup>c</sup> labelling within the follicle co-localises with FDC-M1+ cells as can be seen in Fig 5.3e. When the co-localisation profile is observed (see Fig 5.6a), no single labelled FDC-M1+ cells can be observed.

The data presented here suggest that there may be quantitative differences between the amount of PrP<sup>c</sup> present in young spleens (e.g 10-14 day old), compared to the adult. This is based on the finding that the area covered by the immunolabelling appears to be smaller than the adult. Possible ways of quantifying PrP<sup>c</sup> levels are discussed in section 5.5

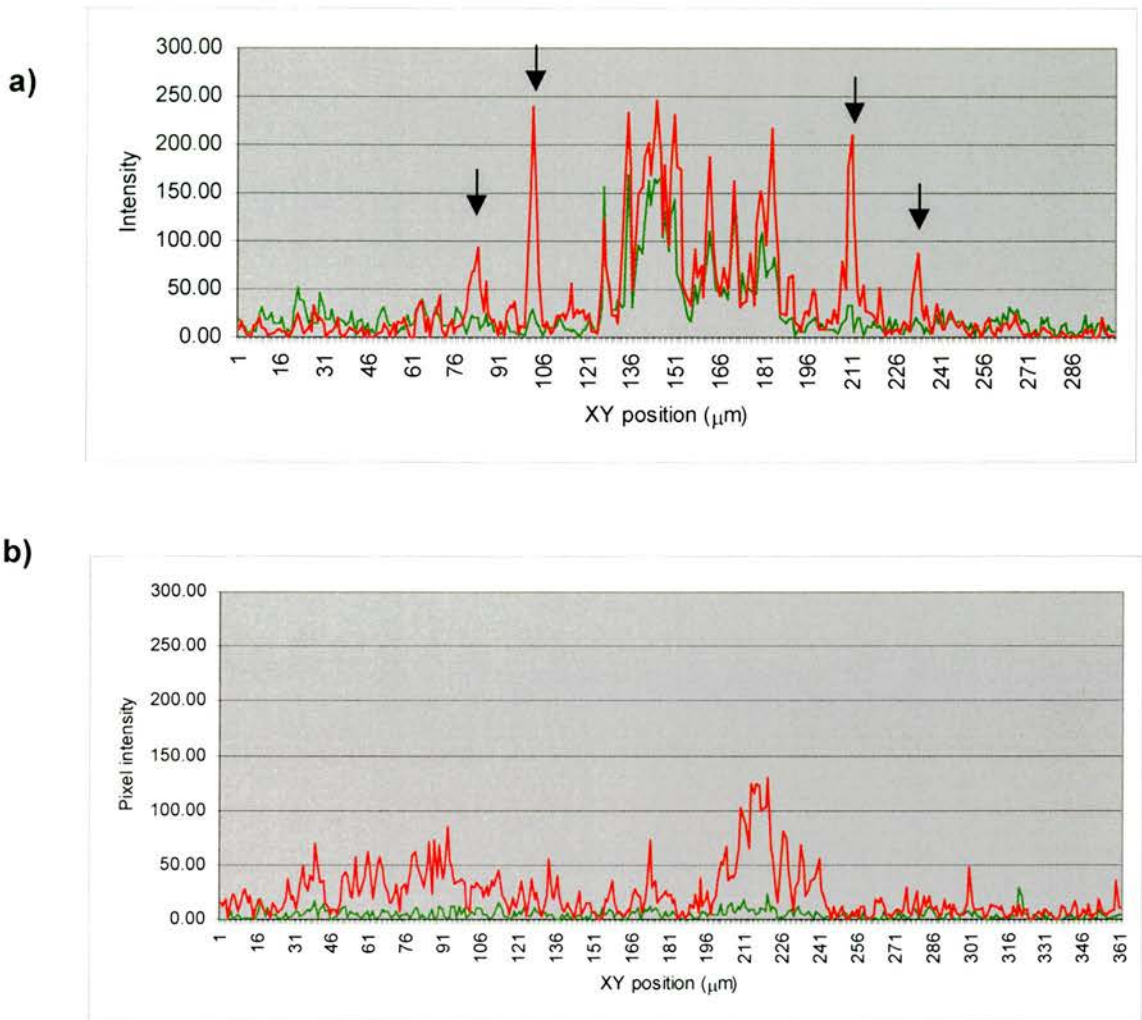
This co-localisation of PrP<sup>c</sup> with only a small number of FDC-M1+ cells in younger spleens, may suggest that these PrP<sup>c</sup> expressing FDCs are at a different maturational state than the surrounding single labelled FDC-M1+ cells within the follicle. The existence of PrP<sup>c</sup> expressing FDCs in 10 day old mice, which are not fully functional suggested by the morphological studies described in Chapter 4, suggests that immature FDCs may be capable of sustaining scrapie replication after peripheral challenge.

As discussed previously, SCID mice lack mature FDCs and cannot support scrapie replication despite the presence of a functional Prn-p gene. SCID mice do not possess FDC-M1+ cells in their spleens. However, FDC-M1+ cells become detectable 10-21 days post reconstitution with bone marrow. Consequently, PrP<sup>c</sup>

becomes detectable 14-21 days after reconstitution and is associated with FDCs. It is not known whether these FDCs are functional at 14 days post reconstitution or whether they can support scrapie replication, since most scrapie challenges are carried out at 28 days post reconstitution. These studies have assumed that mature FDCs are required for scrapie replication, however in the light of the PrP<sup>c</sup> immunocytochemistry studies and FDC maturation studies in this project, it is possible that infectivity may replicate on immature FDCs with PrP<sup>c</sup> being the limiting factor.

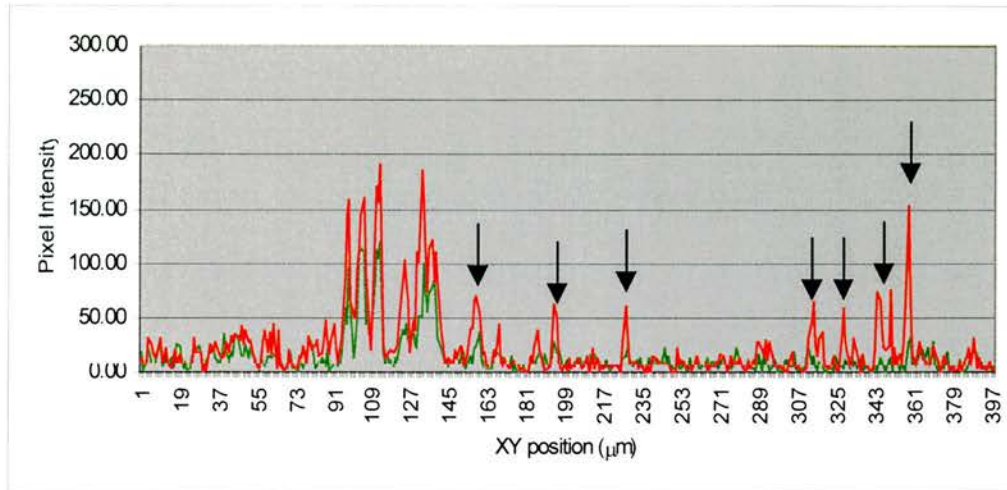
An alternative explanation, which cannot be excluded from the interpretation of these results is that infectivity may reside in other cell types (e.g macrophages) until FDCs have reached a suitable maturational level. This possibility is discussed in more detail in Chapter 7.



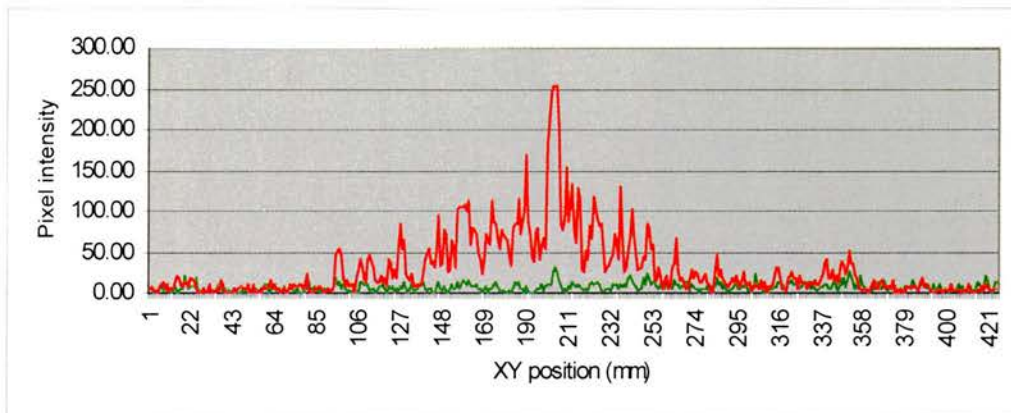


**Fig 5.4 Colocalisation analysis of PrP<sup>c</sup> and FDC-M1 immunolabelling in the spleen of a 10 day old C57BL mouse.** Characterisation of an area of co-localisation (yellow labelling) within a follicle of a spleen from a 10 d.o C57BL mouse (same image as Fig 5.3a) was carried out by drawing a line of approximately 300  $\mu\text{m}$  through the area of interest. The same process was repeated for the age matched PrP null spleen (same image as 5.3.b). The pixel intensities for PrPc (green) and FDC-M1 (red) labelling were calculated using Leica image analysis software, and the values plotted along the Y-axis. The XY position of PrPc and FDC-M1 immunolabelling along the line is plotted on the X-axis. Graphs a and b show the profiles obtained for the 10 day old C57BL and PrP null mouse respectively. Areas of co-localisation are indicated by close matching of XY positions for green and red labelling. Black arrows indicate areas of single labelled FDC-M1+ cells.

a)

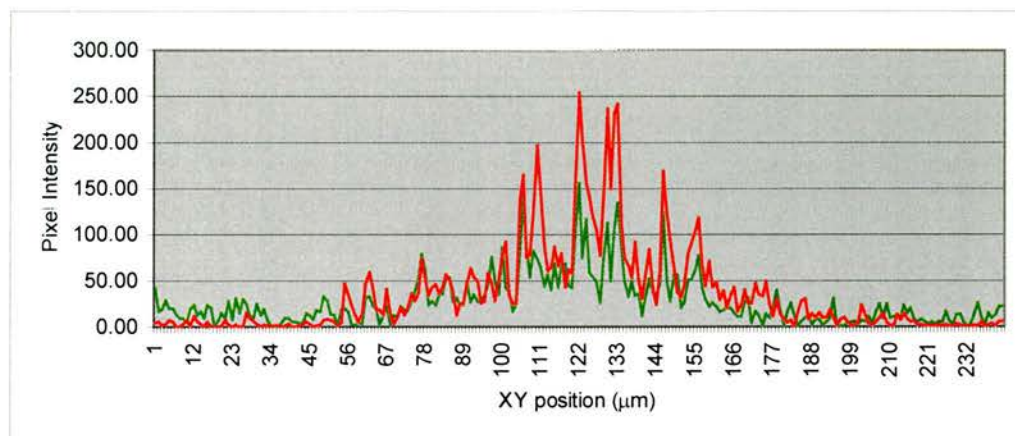


b)

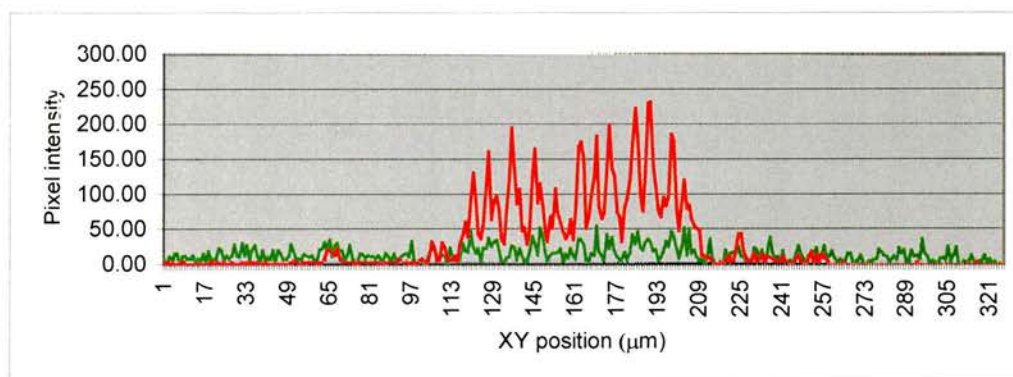


**Fig 5.5 Co-localisation analysis of a 14 day old spleen follicle double labelled for PrP<sup>c</sup> and FDC-M1.** Characterisation of an area of co-localisation (yellow labelling) within a spleen follicle from a 10 day old C57BL mouse (same image as Fig 5.3c) was carried out by drawing a line of approximately 300 $\mu$ m through the area of interest. The same process was repeated for the age matched PrP null spleen (same image as 5.3d). The pixel intensities for PrP<sup>c</sup> (green) and FDC-M1 (red) labelling were calculated using Leica image analysis software and the values plotted along the y-axis. The XY- position of PrP<sup>c</sup> and FDC-M1 labelling along the line is plotted on the X-axis. Graphs a and b show the profiles obtained for the 10 day old C57BL and PrP null mouse respectively. Areas of co-localisation are indicated by close matching of XY positions for green and red labelling. Black arrows indicate areas of single labelled FDC-M1+ cells.

a)



b)



**Fig 5.6 Co-localisation analysis of adult spleen follicle double immunolabelled for PrP<sup>c</sup> and FDC-M1.** Characterisation of an area of co-localisation (yellow labelling) within a spleen follicle from a 35 day old C57BL mouse (same image as Fig 5.3e) was carried out by drawing a line of approximately 300µm through the area of interest. The same process was repeated for the age matched PrP null spleen (same image as 5.5f). The pixel intensities for PrP<sup>c</sup> (green) and FDC-M1 (red) labelling were calculated using Leica image analysis software and the values plotted along the y-axis. The XY- position of PrP<sup>c</sup> and FDC-M1 labelling along the line is plotted on the X-axis. Graphs a and b show the profiles obtained for the 10 day old C57BL and PrP null mouse respectively. Areas of co-localisation are indicated by close matching of XY positions for green and red labelling. Black arrows indicate areas of single labelled FDC-M1+ cells.

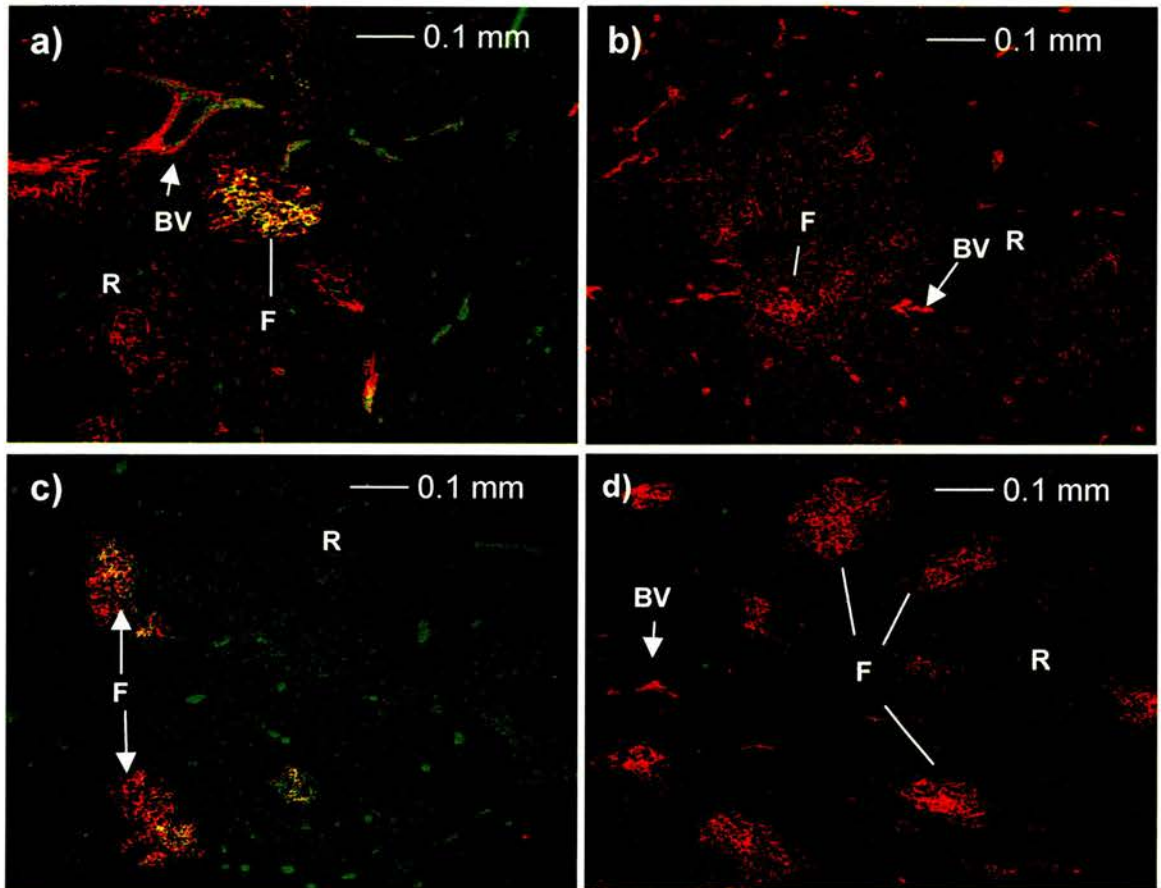
### **5.3.3.2 Double immunolabelling of spleen sections with 1B3 and FDC-M2**

In order to gain more information regarding the cell specificity of PrP<sup>c</sup>, double immunolabelling was carried out using another FDC specific marker, FDC-M2.

FDC-M2 can only be detected from 14 days old onwards in the developing spleen (see Chapter 4). PrP<sup>c</sup> labelling co-localises with FDC-M2 to a much greater extent than PrP<sup>c</sup> and FDC-M1 at this age (see Fig 5.7a). This is due to the fact that FDC-M2 does not cover the whole follicle area as does FDC-M1 in this age group. No PrP<sup>c</sup> immunolabelling can be observed in the corresponding Prn-p <sup>-/-</sup> section

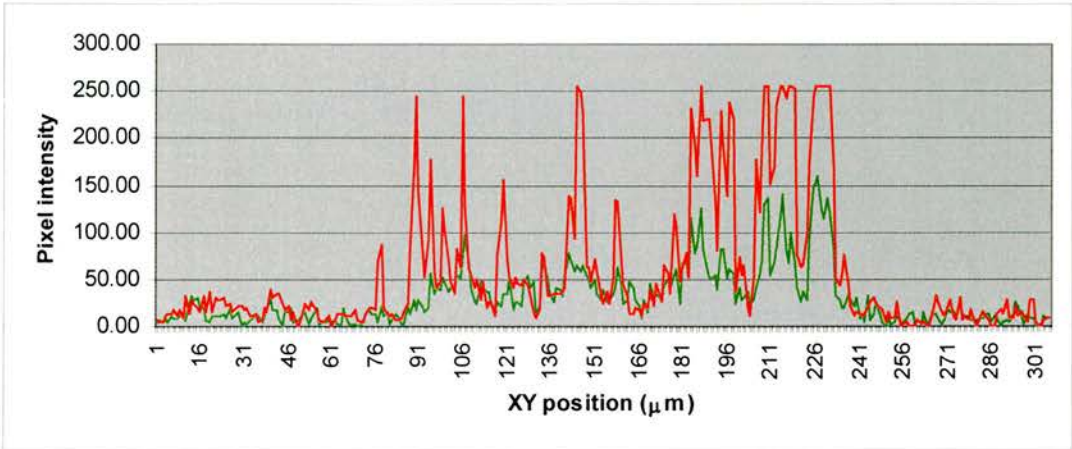
It can be seen in Fig 5. 7a that the intensity of PrP<sup>c</sup> labelling is not as great as FDC-M2. Despite this, the line profile analysis of PrP<sup>c</sup> and FDC-M2 follow each other closely between positions 181µm and 230 µm (Fig 5.8a).

It is interesting that the onset of FDC-M2 labelling follows closely the onset of detectable levels of PrP<sup>c</sup> in the developing spleen follicles; FDC-M2+ cells can be detected from between 10 and 14 days old, PrP<sup>c</sup>+ cells can be detected from 10 days old. Since most of the FDC-M2 labelling co-localises with the PrP<sup>c</sup> labelling, it may correspond to areas of FDC-M1 staining which co-localises with PrP. This in turn may lend support to the hypothesis that the PrP<sup>c</sup>+ FDCs in young mouse spleens represent FDCs at a higher maturational level than the surrounding single labelled FDC-M1+ cells

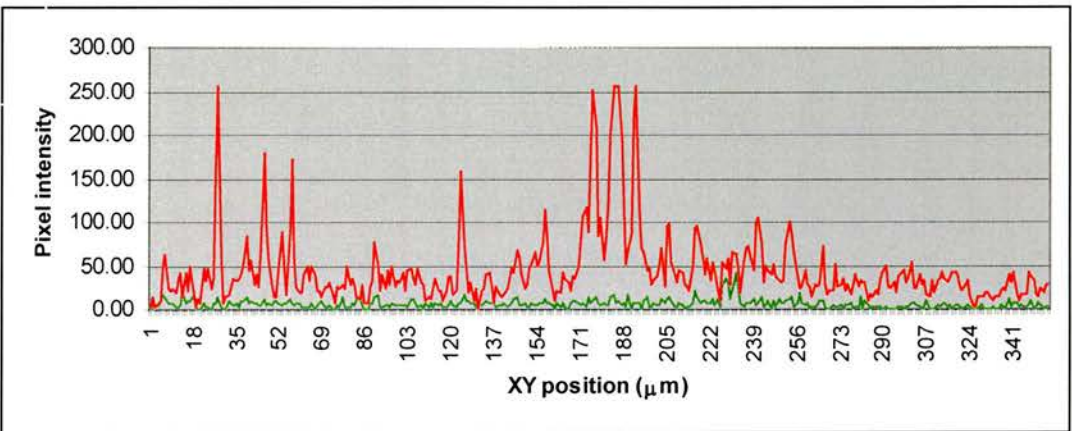


**Fig 5.7 Confocal imaging of spleen sections from 14 and 30 day old mice double immunolabelled for PrP<sup>c</sup> and FDC-M2.** Frozen spleen sections of 14 and 30 day old C57BL (panels a,c, respectively) and Prn-p <sup>-/-</sup> mice (b,d, respectively) were immunolabelled with 1B3 and FDC-M2 antibody as described in section 2.3.13.10. PrP<sup>c</sup> labelling was detected using goat anti rabbit conjugated Alexa 488<sup>TM</sup> (green). FDC-M2 labelling was detected using Streptavidin conjugated Cy3<sup>TM</sup> (red). PrP<sup>c</sup>+ /FDC-M2+ cells are indicated by yellow areas where green and red labelling overlap. 10 d.o mice were not included since it had been established that they did not have FDC-M2+ networks. All sections were scanned with a Leica confocal microscope and images stored on optical disk. Abbreviations are as follows: R= red pulp; F= follicle; BV= blood vessels

a)

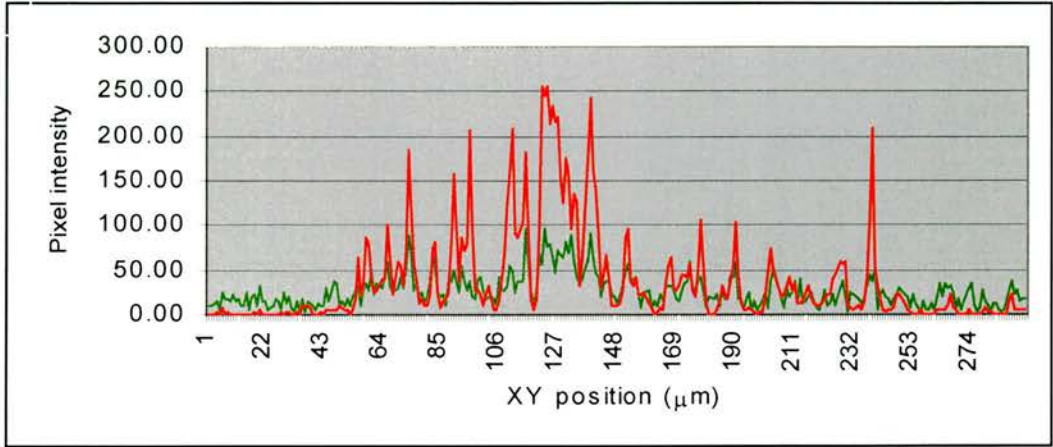


b)

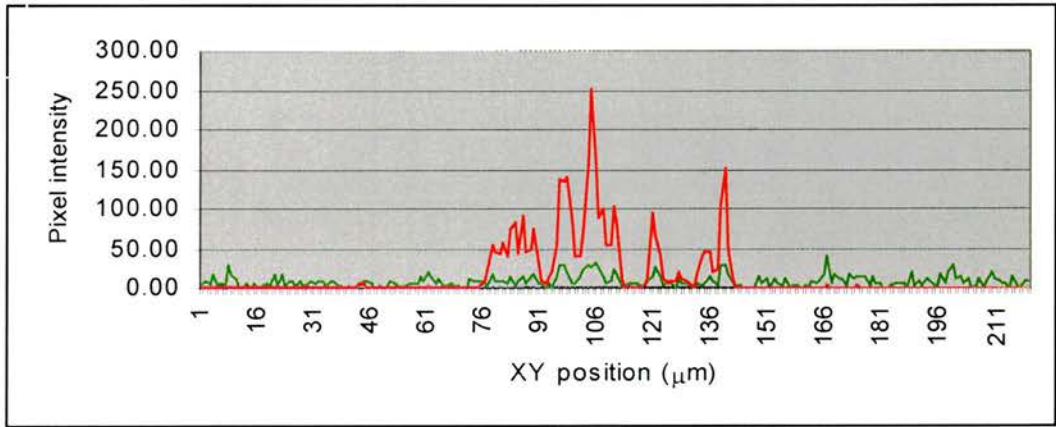


**Fig 5.8 Co-localisation analysis of a 14 d.o spleen follicle double immunolabelled for PrP<sup>c</sup> and FDC-M2.** Characterisation of an area of co-localisation (yellow labelling) within a spleen follicle from a 14 day old C57BL mouse (same image as Fig 5.7a) was carried out by drawing a line of approximately 300 $\mu$ m through the area of interest as shown in panel a. The same process was repeated for the age matched PrP null spleen (same image as 5.7b). The pixel intensities for PrP<sup>c</sup> (green) and FDC-M2 (red) labelling were calculated using Leica image analysis software and the values plotted along the y-axis. The XY- position of PrP<sup>c</sup> and FDC-M2 labelling along the red line indicated on the images is plotted on the X-axis. Areas of co-localisation are indicated by close matching of XY positions for green and red labelling. Black arrows indicate areas of single labelled FDC-M2+ cells.

a)



b)

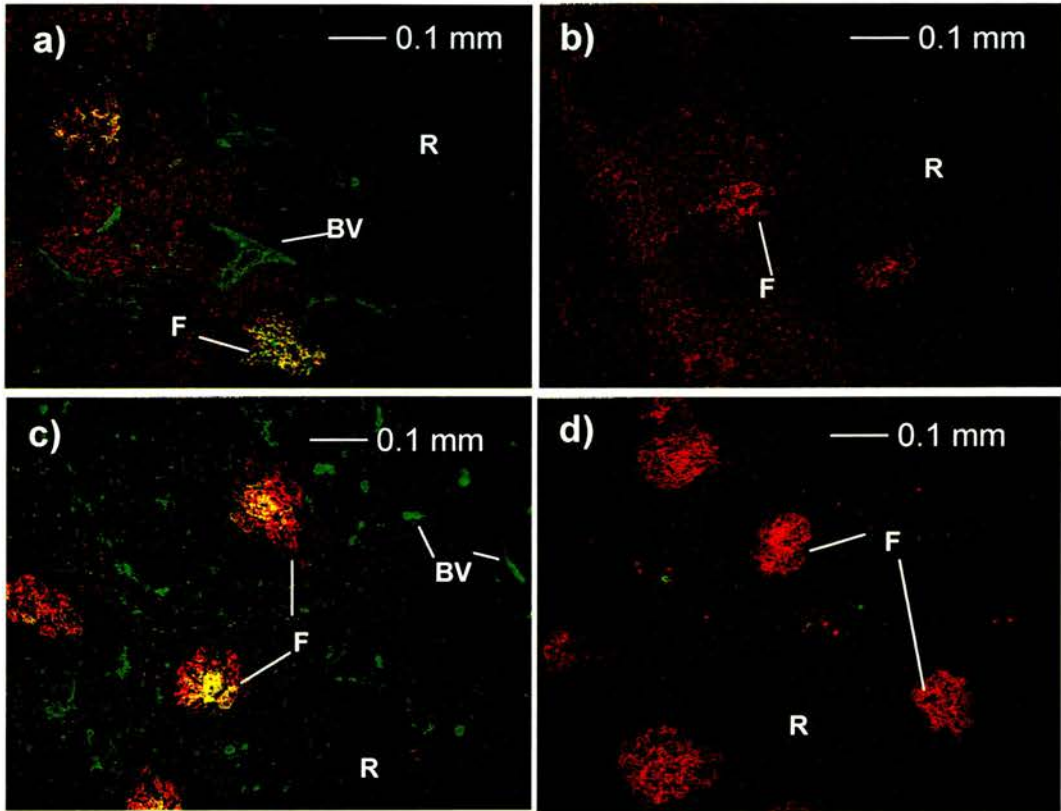


**Fig 5.9 Co-localisation analysis of a 35 d.o spleen follicle double immunolabelled for PrP<sup>c</sup> and FDC-M2.** Characterisation of an area of co-localisation (yellow labelling) within a spleen follicle from a 35 day old C57BL mouse (same image as Fig 5.7c) was carried out by drawing a line of approximately 300µm through the area of interest as shown in panel a. The same process was repeated for the age matched PrP null spleen (same image as 5.7d). The pixel intensities for PrP<sup>c</sup> (green) and FDC-M2 (red) labelling were calculated using Leica image analysis software and the values plotted along the y-axis. The XY- position of PrP<sup>c</sup> and FDC-M2 labelling along the red line indicated on the images is plotted on the X-axis. Areas of co-localisation are indicated by close matching of XY positions for green and red labelling. Black arrows indicate areas of single labelled FDC-M2+ cells.

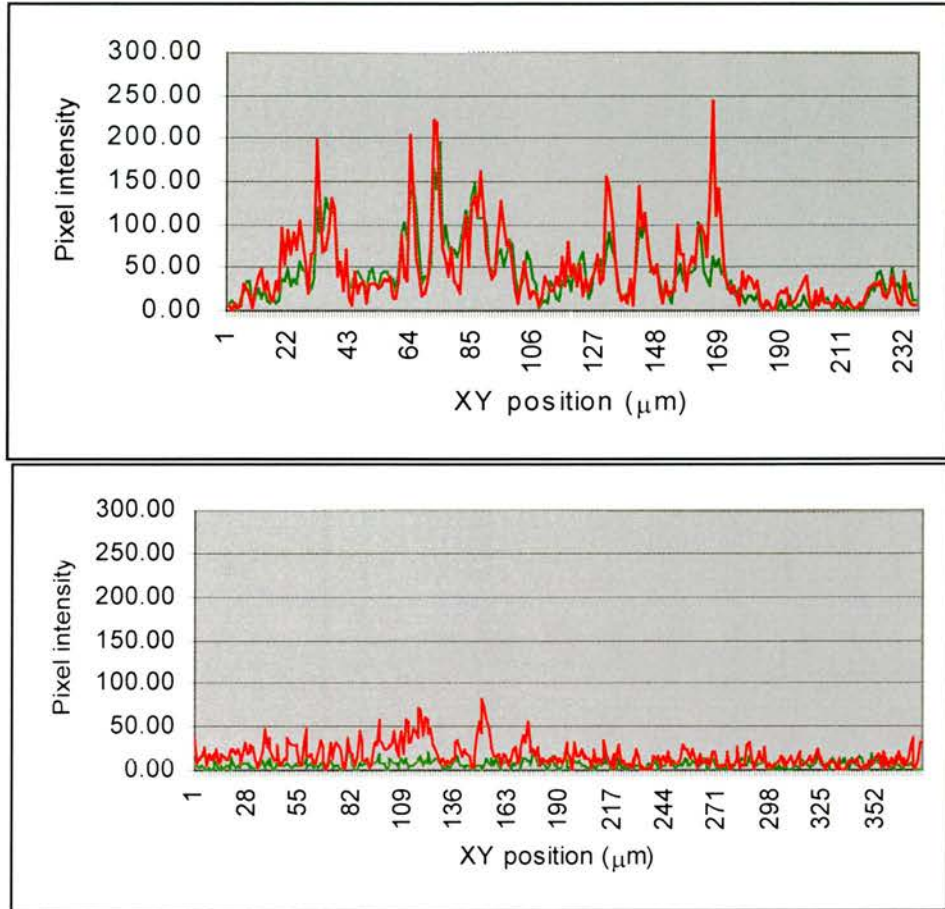
### 5.3.3.2 Double immunolabelling of spleen sections with 1B3 and anti-CR1 antibody

CR1<sup>+</sup> cells can be detected from 14 days old in the mouse spleen (see Chapter 4). It can be seen from Fig 5.11a, that CR1 colocalises with PrP<sup>c</sup> cells with a similar labelling pattern to FDC-M2 cells (see Fig 5.8a). Most of the CR1<sup>+</sup> cells co-localise with PrP<sup>c</sup> in 14 day old mice (see Fig 5.12a), whereby the intensity profile lines for both red and green channels follow each other closely. Again this is unlike the labelling pattern associated with FDC-M1 where only a small proportion co-localise with PrP<sup>c</sup> immunolabelling.

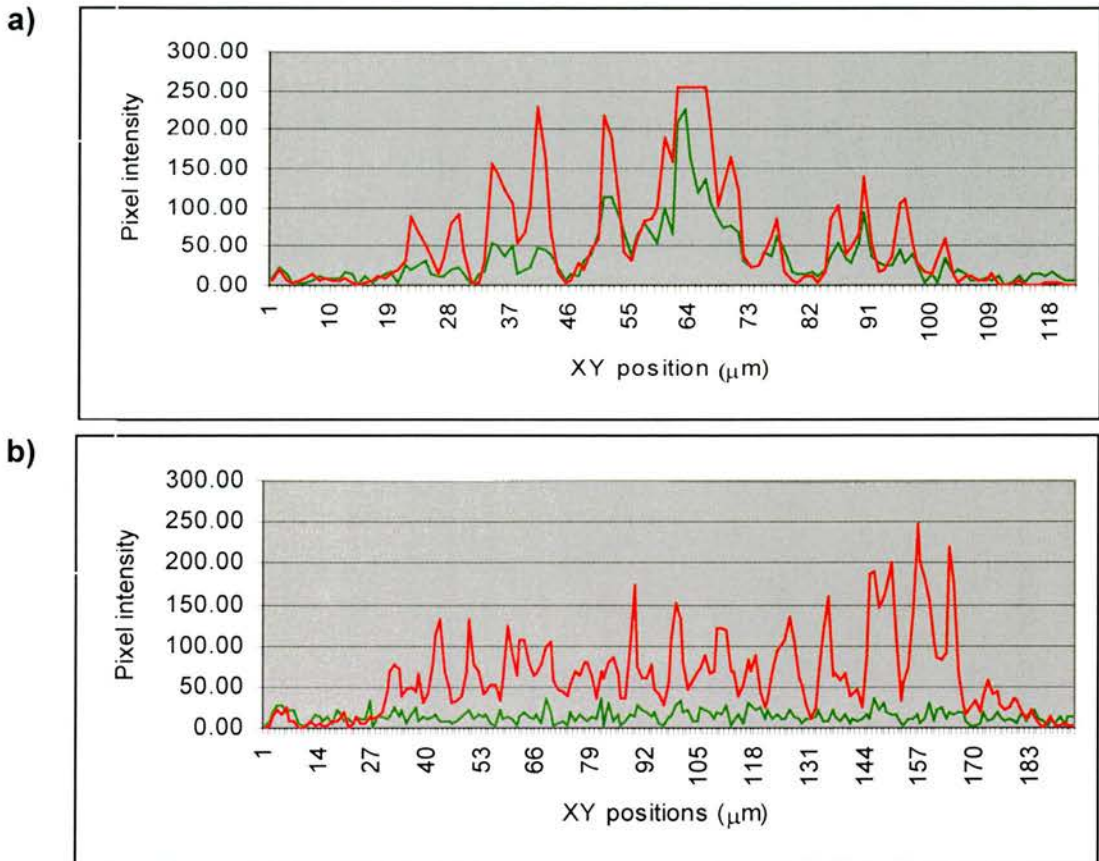
Since CR1 is an important molecule involved in complement dependent immune complex trapping (see Chapter 4) by mature FDCs, it lends further support to the idea that FDCs exist within the developing spleen at different maturational levels. The detection of PrP<sup>c</sup> a few days prior to CR1 and FDC-M2, may mark an important phase in FDC maturation or it may be intrinsically involved in the maturation process.



**Fig 5.10** Confocal imaging of spleen sections from 14 and 30 day old mice double immunolabelled for PrP<sup>c</sup> and CR2. Frozen spleen sections of 14 and 30 day old C57BL (panels a,c,e respectively) and PrP null mice (b,d,f respectively) were immunolabelled with 1B3 and CR2 antibody as described in section 2.3.13.11. PrP<sup>c</sup> labelling was detected using goat anti rabbit conjugated Alexa 488™ (green). CR2 labelling was detected using Streptavidin conjugated Cy3™ (red). PrP<sup>c</sup>+ /CR2+ cells are indicated by yellow areas where green and red labelling overlap. All sections were scanned with a Leica confocal microscope and images stored on optical disk. Abbreviations are as follows: R= red pulp; F= follicle; BV= blood vessels.



**Fig 5.11 Co-localisation analysis of a 14 d.o spleen follicle double immunolabelled for PrP<sup>Sc</sup> and CR1.** Characterisation of an area of co-localisation (yellow labelling) within a spleen follicle from a 14 day old C57BL mouse (same image as Fig 5.10a) was carried out by drawing a line of approximately 300µm through the area of interest as shown in panel a. The same process was repeated for the age matched PrP null spleen (same image as 5.10b). The pixel intensities for PrP<sup>Sc</sup> (green) and CR1 (red) labelling were calculated using Leica image analysis software and the values plotted along the y-axis. The XY- position of PrP<sup>Sc</sup> and CR1 labelling along the red line indicated on the images is plotted on the X-axis. Areas of co-localisation are indicated by close matching of XY positions for green and red labelling. Black arrows indicate areas of single labelled CR1+ cells.



**Fig 5.12 Co-localisation analysis of a 35 d.o spleen follicle double immunolabelled for PrP<sup>c</sup> and CR1.** Characterisation of an area of co-localisation (yellow labelling) within a spleen follicle from a 35 day old C57BL mouse (same image as Fig 5.10c) was carried out by drawing a line of approximately 300µm through the area of interest as shown in panel a. The same process was repeated for the age matched PrP null spleen (same image as 5.10d). The pixel intensities for PrP<sup>c</sup> (green) and CR1 (red) labelling were calculated using Leica image analysis software and the values plotted along the y-axis. The XY- position of PrP<sup>c</sup> and CR1 labelling along the red line indicated on the images is plotted on the X-axis. Areas of co-localisation are indicated by close matching of XY positions for green and red labelling. Black arrows indicate areas of single labelled CR1+ cells.

## 5.4 Summary

To summarise the data presented in this chapter, PrP<sup>c</sup> can be detected in the spleen using immunocytochemistry from the age of 10 days old in unfixed, frozen sections. Confocal analysis of double immunolabelled frozen spleen sections was used to determine the cell association of PrP<sup>c</sup> staining. Similar to the situation in the adult spleen, the cell type was confirmed to be FDCs. At 10 days old, only a small number of FDC-M1+ cells co-localised with PrP<sup>c</sup> labelling. The majority of FDC-M1+ cells were PrP<sup>c</sup> negative. By adulthood, the spatial differences between PrP<sup>c</sup> and FDC-M1 labelling had changed dramatically and co-localisation of both markers was augmented. On the other hand, PrP<sup>c</sup> co-localises to a greater extent with FDC-M2 and CR1 at 14 days old after birth compared to FDC-M1.

## 5.5 Future Work

The main aim of this chapter was to determine the age at which PrP<sup>c</sup> could first be detected in the developing spleen and the cell association. The next step would be to quantify the levels of PrP<sup>c</sup> on spleen FDCs. The main difficulty with this is isolating intact FDCs using flow cytometry due to their long dendritic processes. A possible solution to this would be to compare PrP<sup>c</sup> levels using FACS analysis of whole spleen suspensions, between irradiated and non-irradiated mice. Irradiation would destroy mitotic cells and radioresistant long lived cells such as FDCs would be left behind in the irradiated spleen. Differences in mean fluorescence intensity may be attributed to FDCs.

The expression of PrP mRNA during postnatal spleen development also warrants investigation. Pilot studies were carried out at the end of the project using a recently available technique. Promising results were obtained in adult spleen whereby DIG labelled probes were found in association with red pulp macrophages (see section 2.4.9). This study could be expanded to include younger mice to examine the possibility that mice younger than 10 d.o could be expressing the Prn-p gene.

## Chapter 6

# The involvement of non-lymphoid systems in scrapie pathogenesis

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## 6.1 Introduction

It is hypothesised that following peripheral inoculation with scrapie infectivity can gain access to the peripheral nervous system (PNS) after an FDC dependent amplification phase with consequential entry into the CNS. This hypothesis is supported by studies carried out in hamster and mouse scrapie models. Early experiments have indicated that infectivity enters the thoracic region of the spinal cord at position T4-T7 prior to entering the brain (Kimberlin and Walker, 1980). This region shows earliest PrP<sup>Sc</sup> deposits after i.p inoculation of hamsters with 263K (Baldauf et al., 1997). The point of entry is consistent with spread of infectivity via the splanchnic nerve.

Direct intraneural inoculation of 139A scrapie into mouse sciatic nerve produced incubation periods, which were on average 47 days shorter than those in i.p inoculated groups (Kimberlin et al., 1983). The spread of infectivity in the intraneurally injected groups was very similar to that in i.c injected groups and infectivity reached the brain and spinal cord after 8 weeks, a significantly shorter time compared to i.p injected mice (Kimberlin et al., 1983). A recent study tracing the deposition of PrP<sup>Sc</sup> in PNS after oral challenge of hamsters has found PrP<sup>Sc</sup> deposits in the enteric and autonomic ganglia of the splanchnic and vagus nerve circuitry early in the incubation period (McBride et al., 2001). This supports the hypothesis that at least after oral scrapie challenge, the enteric nervous system is a portal of entry into the splanchnic and vagus nerve circuits.

There is accumulating evidence to suggest that scrapie neuroinvasion can occur without prior lymphoid tissue amplification and it is hypothesised that direct infection of the PNS occurs in these situations. SCID mice, deficient in T and B cells and as a consequence FDCs, are resistant to moderate doses (1%) of i.p inoculated ME7. However at high doses (10%), all peripherally inoculated SCID mice succumb to infection (Fraser et al., 1996). Similar findings have been reported in other immunodeficient mouse lines that are normally resistant to peripheral scrapie challenge. For example in TNF- $\alpha$  gene ablated mice, which lack FDCs and germinal

centre B cells, 3/8 intraperitoneally inoculated mice succumbed to scrapie without PrP<sup>Sc</sup> accumulation in their spleens (Mabbott et al., 2000). The remaining mice survived scrapie challenge. Although bioassays of these spleens were not carried out; the lack of PrP<sup>Sc</sup> in spleens at endpoint strongly suggests a pathway of neuroinvasion which does not require prior FDC amplification of infectivity.

In previous chapters, the possible reasons behind the resistance of neonatal mice to peripheral challenge were explored. The hypothesis that the onset of susceptibility was correlated to the development of FDCs and detection of PrP<sup>c</sup> on these cells was tested. Although a proportion of neonatally challenged mice resist scrapie challenge, unpublished experiments by George Outram, NPU have demonstrated that some peripherally inoculated neonatal mice can develop scrapie after comparatively short incubation periods. Most of these experiments were carried out by challenging inbred mice of the VL strain i.p with ME7 and it was discovered that high proportion of short incubation periods are found following s.c inoculation of neonates (Outram, Unpublished observations). 60% of 0-4 day old mice inoculated s.c with 1% ME7 brain homogenates had short incubation periods ranging from 150-230 days. This decreased to 30% in 8 day old mice and even further to 10% in adult mice.

The apparent high efficiency with which a proportion of neonatal mice can succumb to peripheral scrapie challenge bears certain similarities to changes in susceptibility of neonatal mice to the neurotropic alphavirus, Semliki Forest Virus (SFV). Infection of neonatal mice results in a fulminant panencephalitis resulting in rapid death; on the other hand peripheral inoculation of weaned mice is not lethal, although a focal, subclinical infection is established within the CNS (Amor et al., 1996; Oliver et al., 1997). This change in pathogenesis occurs very precisely at 14 days old and mice of this age show a reduced viral dissemination within the brain compared to those challenged at 12 days old (Oliver et al., 1997). There is a concomitant decrease in mortality at this point from 60% in 12 day old mice to 0% in 14 day old mice. Intriguingly, this change in susceptibility correlates with the developmental maturation of the CNS, which is still ongoing in the first 2 weeks of life, involving

axonogenesis, synaptogenesis and myelination. Neonatal susceptibility to SFV is not related to an immature immune system since adult SCID mice show a focal as opposed to fulminant brain infection (Amor et al., 1996). The explanation for these findings is thought to be that infectivity is taken up through a non-specific mechanism for example by endocytosis, which occurs with greater frequency in the developing postsynaptic membranes. By 14 days old the maturation of the CNS is complete.

## 6.2 Aims

The aim of this chapter is to study the role of non-lymphoid systems in neonatal scrapie pathogenesis by using immunodeficient SCID mice. If the short incubation periods observed in neonatal mice after peripheral challenge are due to direct infection of immature peripheral nerves then it is predicted that as the mice mature then the efficiency of scrapie infection will decrease due to the maturation of the peripheral nerve network. SCID mice offer an experimental model with which to test this hypothesis without the confounding effects of a developing immune system on scrapie pathogenesis.

## 6.3 Experimental Rationale

The hypothesis to be tested in this chapter is documented in section 1.3.2. It is predicted that if direct infection of immature peripheral nerves occurs in peripherally challenged neonatal mice, then the mice should succumb to scrapie after very short incubation periods. The resultant incubation periods should be of similar length to those obtained after direct i.c inoculation. If the immaturity of the PNS affects the efficiency of scrapie infection in neonatal mice, it is predicted that the efficiency of infection should decrease in older mice, concomitant with the maturation of the PNS during the first few weeks of life.

In order to test these predictions, SCID (BALB/c, CB17 *scid/scid*) and control immunocompetent CB20 *+/+* congenic BALB/c mice of various age groups were inoculated via the i.p or s.c route with a  $10^{-2}$  dilution of ME7 as described in section 2.3.4. i.c inoculated mice were included as controls to demonstrate that any differences in incubation period length after peripheral challenge of mice was due to maturation of cells in the periphery and not in the CNS. The experimental plan is shown in Table 6.1. SCID and CB20 mice were housed as described in section 2.3.1.

Age (d.o)	No. SCID mice to be inoculated			No. CB20 mice to be inoculated		
	i.p	i.c	s.c	i.p	i.c	s.c
1	35	14	38	29	13	19
14	23	21	44	19	13	17
30-40	28	17	29	19	13	18

**Table 6.1 Inoculation plan for SCID and CB20 mice of various age groups.**

Intracerebral and subcutaneous injections were carried out under anaesthesia as described in section 2.3.4.2 and 2.3.4.3. s.c injection of neonates, was done without anaesthetic.

Mice were allowed to incubate scrapie up to clinical disease (see section 2.3.5). Brains were removed from clinically ill animals for scrapie pathology assessments as described in section 2.3.8. In addition, blood samples were taken and spleens removed from terminal cases in order to screen for leakiness of the SCID phenotype. Sera samples were prepared as described in section 2.3.9 and used for ELISA testing for immunoglobulin presence (2.3.10.1). If blood could not be taken then spleens were assessed for lymphoid structure using B220 and FDC markers (2.3.10.2).

Any SCID scrapie cases, which were leaky as judged by either the presence of immunoglobulin or B220+ cells and FDC networks were removed from incubation period analysis. In addition any SCID or CB20 cases which had no scrapie pathology in the brain despite being suspected of having scrapie symptoms were also removed from further incubation period analysis.

These checks ensured that only true scrapie cases in confirmed immunodeficient mice would be studied. The incubation periods for pathology confirmed non-leaky SCID cases were used to analyse spread of incubation periods for each age group tested compared to age matched immunocompetent CB20 mice.

## 6.4 Results and Discussion

### 6.4.1 The effect of age on intracerebral inoculation of SCID and CB20 mice

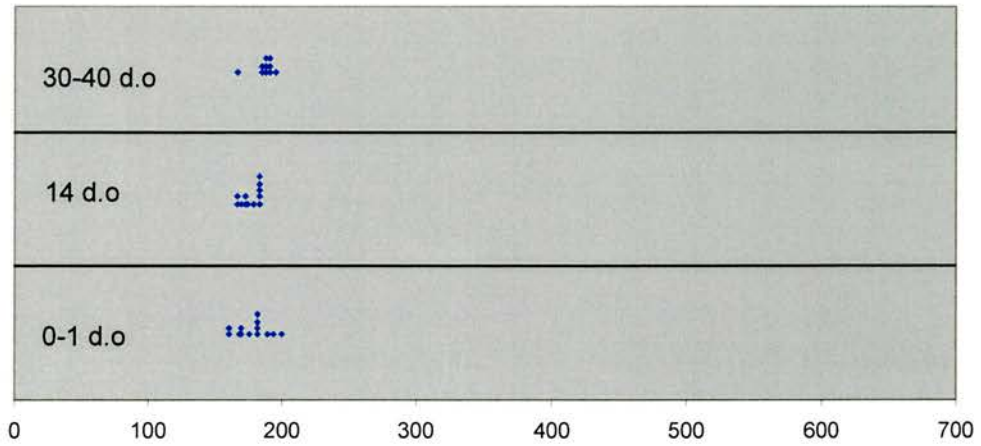
SCID and CB20 mice of all ages succumbed to scrapie after i.c inoculation with  $10^{-2}$  dilution of ME7. It can be observed from Fig 6.1a and b that there was no effect of age or mouse strain on the incubation period patterns. This is consistent with previous findings firstly, that i.c inoculation of adult SCID mice produces 100% mortality and incubation periods of similar length to immunocompetent CB20 mice (Fraser et al., 1996) and secondly, that neonatal mice are equally susceptible to intracerebral scrapie challenge as adults (Outram et al., 1973). These results confirm that scrapie replication in the brain can proceed without an intact LRS, if infectivity is injected directly into the brain

### 6.4.2 The effect of age on intraperitoneal inoculation of SCID and CB20 mice

Fig 6.2 a and b show the spread of incubation periods after i.p challenge SCID and CB20 mice respectively. After i.p challenge, 2 out of 28 adult SCID mice succumbed to scrapie with incubation periods of 224 and 399 days (see Fig 6.2a). This contrasts with previous observations, since scrapie cases were not encountered after i.p challenge of adult mice with a  $10^{-2}$  dilution of ME7. Only after i.p inoculation with a  $10^{-1}$  high dose of ME7 were scrapie cases found in SCIDs (Fraser et al., 1996). This may suggest that the inoculum titre used in the present study was slightly higher than that used in previous experiments. The majority of SCID mice challenged as adults had to be culled due to intercurrent infection and had no neuropathological indications of scrapie (cases represented by white diamonds in Fig 6.2a). Since the mice were culled before reaching a stage where they could be regarded as survivors (600 days post infection) they cannot be labelled as such. This result highlights the difficulty in carrying out scrapie studies using SCID mice since their susceptibility to intercurrent infection can result in high loss of experimental animals. Since many cases had no evidence of brain lesions at 500 days post inoculation, it is very likely that these mice would have survived scrapie challenge.

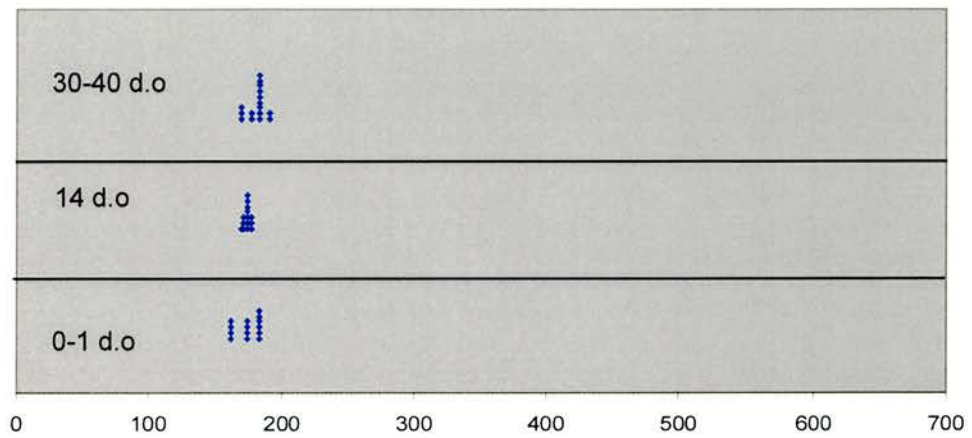
a)

**CB20**



b)

**SCID**



Incubation period (days)

**Fig 6.1** The effect of i.c inoculation of SCID and CB20 mice on spread of incubation periods. CB20 (dotplot a) and SCID (dotplot b) mice of the age groups indicated above were inoculated i.c with 20 $\mu$ l of 10<sup>-2</sup> ME7. Each blue diamond represents an individual pathology confirmed scrapie case.

All CB20 mice challenged i.p as adults succumbed to scrapie with a mean incubation period of  $327 \pm 33$  days as expected (see Fig 6.2a). Inoculation of neonatal mice produced a higher number of scrapie cases than was expected. Five mice were culled without clinical signs of scrapie and have been confirmed as survivors (red diamonds in Fig 6.2b). This contrasts with the low number of scrapie cases (9/27) and the high number of survivors (18/27), after i.p injection of C57BL neonatal mice as discussed in Chapter 3. Nevertheless, the wider spread of incubation periods bears similarities to some experiments involving ME7 inoculation of VL mice (George Outram unpublished data).

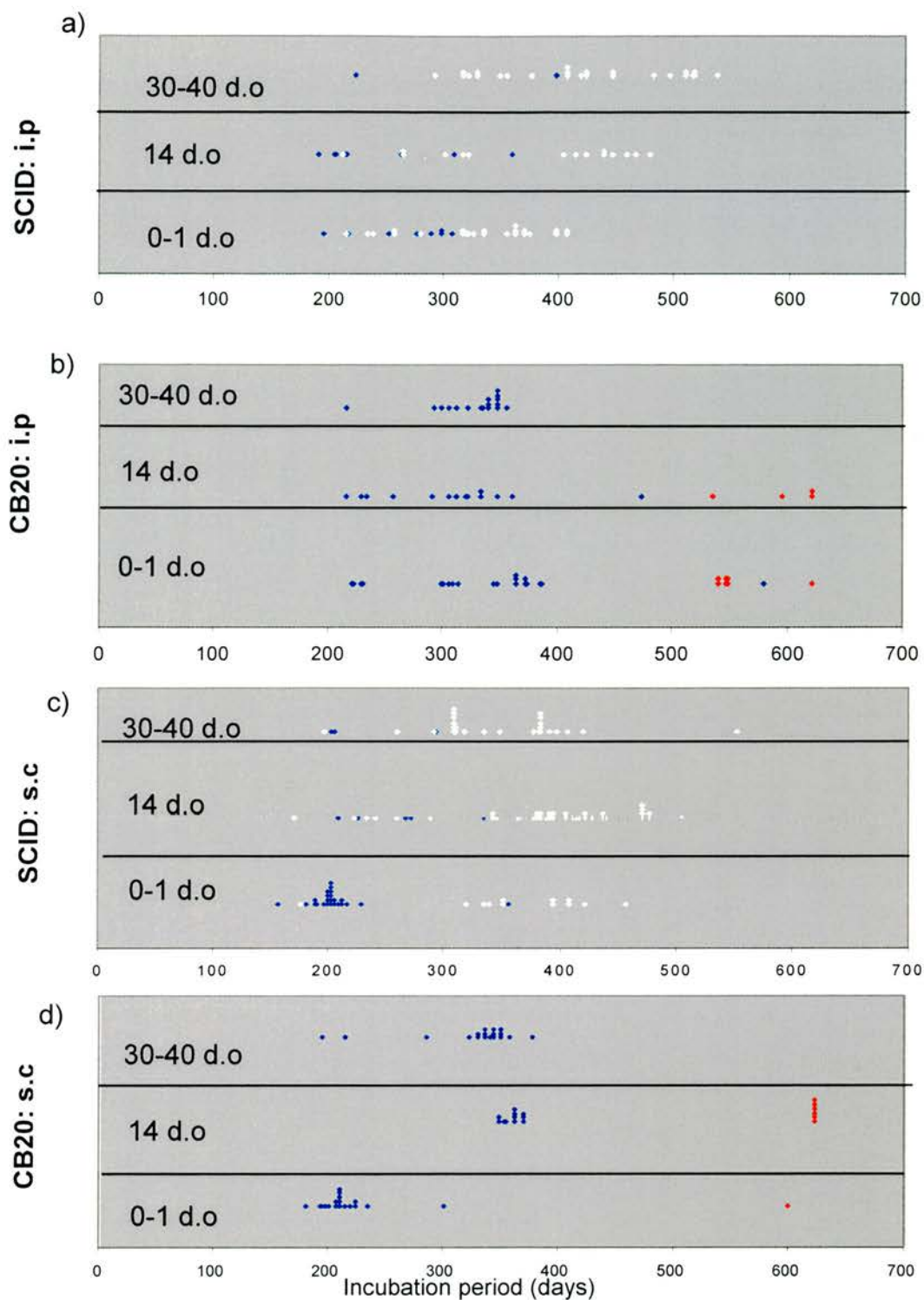
Unexpectedly, 4/19 survivors were observed after i.p inoculation of 14 d.o mice (see Fig 6.2 b). In addition, there was wide variation of incubation periods after challenge of 14 d.o CB20 mice. At this age an adult pattern of incubation periods was observed in the C57BL studies discussed in Chapter 3.

The presence of survivors after i.p challenge of 14 d.o CB20 mice may suggest that the lymphoreticular system matures at a different rate in CB20 mice compared to C57BL mice. Since an immunocytochemical study of FDC development in CB20 mice was not carried out in this project, firm conclusions cannot be made about the FDC status in CB20 mice. This is an obvious area for further investigation.

Intraperitoneal injection of neonatal and 14 d.o SCID mice produced a similar spread of incubation periods (see Fig 6.2a). A few neonatal SCID mice demonstrated short incubation periods ranging from 196-218 days. However, this was not different from 14 day old mice, since scrapie incubation periods of similar length (191-216 days) were observed. This result is not consistent with the prediction that neonatal SCID mice should be more efficient than older mice at infectivity uptake through peripheral nerves. If this were the case then there would be a larger number of short incubation period cases in neonatally challenged mice compared to 14 d.o mice and adults. In this sense, the results from the i.p challenged neonatal SCIDs could lead to

a rejection of the hypothesis. Nevertheless, a possible explanation could be that most of the infectivity is sequestered by macrophages, thus lowering the concentration of infectious agent and decreasing the chance of direct peripheral nerve infection. However, evidence from s.c challenge of neonatal SCIDs and CB20 mice, to be discussed in section 6.4.3, may lend support to the original hypothesis.

However, the results obtained at this stage are consistent with previous findings that under certain circumstances, regardless of age at time of infection, the lymphoid system is not required for scrapie pathogenesis and that other mechanisms exist by which scrapie can enter the CNS (Mabbott et al., 2000).



**Fig 6.2** The effect of age and route of inoculation on scrapie pathogenesis in SCID and CB20 mice. Mice were challenged with a  $10^{-2}$  dilution of ME7 i.p (dotplots a and b) and s.c (dotplots c and d). Each diamond represents individual pathology confirmed scrapie cases. Age groups and inoculation route are indicated on the left-hand side. The legend is as follows:

- ◆ Neuropathologically confirmed scrapie cases.
- ◆ Clinically negative survivors. No neuropathological signs of scrapie detected
- ◇ Intercurrent deaths. No neuropathological signs of scrapie detected

Route	Age (days)	SCID Incubation period (days $\pm$ SD)	CB20 Incubation period (days $\pm$ SD)
<b>i.c</b>	<b>0-1</b>	<b>174 <math>\pm</math> 9</b>	<b>178 <math>\pm</math> 11</b>
	14	174 $\pm$ 2	177 $\pm$ 6
	30	181 $\pm$ 6	187 $\pm$ 7
<b>i.p</b>	0-1	266 $\pm$ 45	334 $\pm$ 81
	14	246 $\pm$ 60	310 $\pm$ 64
	30	311 $\pm$ 123	327 $\pm$ 33
<b>s.c</b>	0-1	205 $\pm$ 34	215 $\pm$ 25
	14	261 $\pm$ 49	361 $\pm$ 8
	30	224 $\pm$ 46	325 $\pm$ 47

**Fig 6.3 Summary of incubation periods after inoculation of SCID and CB20 mice via peripheral and intracerebral route. Mean incubation periods for each inoculation route and recipient age group are shown together with SD values.**

### **6.4.3 The effect of age on subcutaneous inoculation of SCID and CB20 mice**

S.c inoculation of SCID produced strikingly different results compared to i.p inoculation in neonatally challenged mice. 66% of the scrapie cases had very short incubation periods ranging from 150-230 days compared to 9% for i.p challenged neonates (see Fig 6.2c and summary Fig 6.4). Only one s.c inoculated case with a prolonged incubation period of 350 days was observed. 11 mice were culled with suspected scrapie symptoms but scrapie lesions were not detected in their brains (see Fig 6.2c, white diamonds). It is possible that these cases may have become survivors if they had lived long enough. Interestingly, in the present study the proportion of challenged mice demonstrating short incubation periods dropped dramatically from 66% to 10% between the ages of 0-14 days old (see Fig 6.4 -graph). This finding is consistent with the prediction that if PNS immaturity does affect the efficiency of infection after s.c challenge, then the number of mice with short incubation periods should decrease during the first two weeks of life. These results also reflect previous observations that the number of short incubation period cases decreases between the ages of 0-20 days old after s.c challenge of immunocompetent VL mice (George Outram, unpublished results).

Similar to the situation in neonatal SCID mice, there is a high number of short incubation periods after s.c challenge of neonatal CB20 mice (see fig 6.2d and Fig 6.4). The incubation period pattern is similar to that in neonatally challenged SCIDs (compare Fig 6.2c and d) and 95% of challenged mice demonstrate short incubation periods. There is only one survivor and one case with a prolonged incubation period.

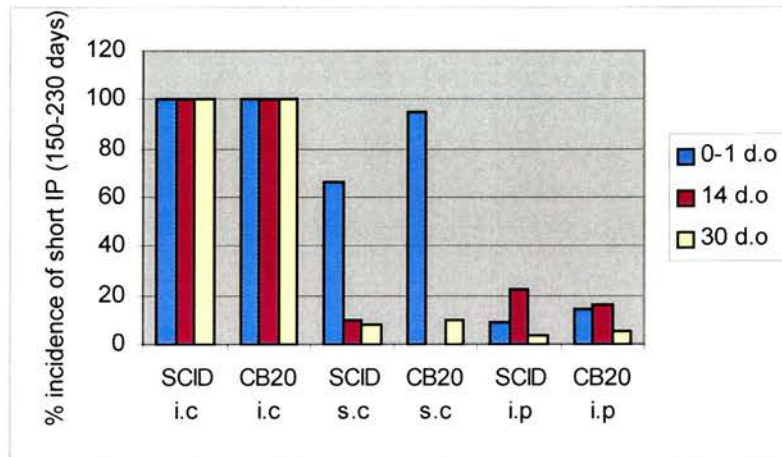
The similarity of incubation period patterns and length of incubation period ( see Fig 6.4) between s.c challenged neonatal SCID and CB20 mice is intriguing. It suggests that the pathogenetic route in both strains is similar and not dependent on an intact immune system. Since the scrapie challenged SCID mice were confirmed to be immunodeficient at disease endpoint by serum immunoglobulin ELISA, the route of entry into the CNS must have occurred without LRS involvement. This, together with the fact that the incubation periods were short and almost within the range

expected following i.c challenge of mice, is further supported by previous intraneural studies where direct inoculation of mouse sciatic nerve produces very short incubation periods (Kimberlin et al., 1983). Although a definite conclusion cannot be reached at this stage about the CB20 neonatal pathogenesis, the similarity between incubation period patterns and the length of incubation periods strongly point towards a direct neural spread.

Unexpectedly, a proportion of CB20 mice inoculated at 14 d.o, were still clinically negative at 620 days post inoculation. These cases were confirmed to be survivors by the absence of any scrapie associated lesions in the brain. This result is unexpected since C57BL mice are fully susceptible at this age. Nevertheless, previous experiments by George Outram using the VL inbred mouse strain have shown that survivors occur after challenge of 8 day old mice, although 14 day old mice were not investigated in that series of experiments. The presence of survivors after i.p and s.c inoculation of 14 d.o CB20 mice suggests differences in the maturation of the lymphoreticular system between CB20 and C57BL mice. This warrants further investigation.

**Overall, the results demonstrate that neonatal mice possess an efficient mechanism by which infectivity can gain access to the CNS quickly after s.c challenge with ME7.** The effect disappears by 14 d.o, although the exact age at which this occurs will require further investigation between the ages of 0 and 14 d.o. Nevertheless, this data bears similarities to observations in the Semliki Forest Virus model discussed in section 6.1 (Oliver et al., 1997). Susceptibility to CNS SFV infection decreases abruptly between the ages of 12-14 days after birth. From the ages of 0-12 days old a fulminant infection was acquired after i.p challenge and death quickly ensued. Mice of 14 days old and older, survived infection completely but developed a subclinical infection status. This change in susceptibility correlates well with the period of time after birth where the CNS is still undergoing development.( i.e axonogenesis , synaptogenesis and myelinogenesis)

Route	Age (d.o)	SCID		CB20	
		No. short IP/ No. injected	% incidence of short IP	No short IP/ No. injected	% incidence of short IP
I/C	0-1	14/14	100	13/13	100
	14	21/21	100	13/13	100
	30	17/17	100	13/13	100
S/C	0-1	25/38	66	17/18	95
	14	4/44	10	0/17	0
	30	3/39	8	2/18	11
I/P	0-1	3/35	9	4/29	14
	14	5/23	22	3/19	16
	30	1/28	4	1/19	5



**Fig 6.4** The effect of age and route of inoculation on the length of the scrapie incubation period in SCID and CB20 mice. Short incubation periods were defined as those falling between the 150-230 day range. The number of short incubation period cases are expressed as a percentage of the total number of mice inoculated.

#### **6.4.4 The potential involvement of maturing peripheral nerves in neonatal scrapie pathogenesis**

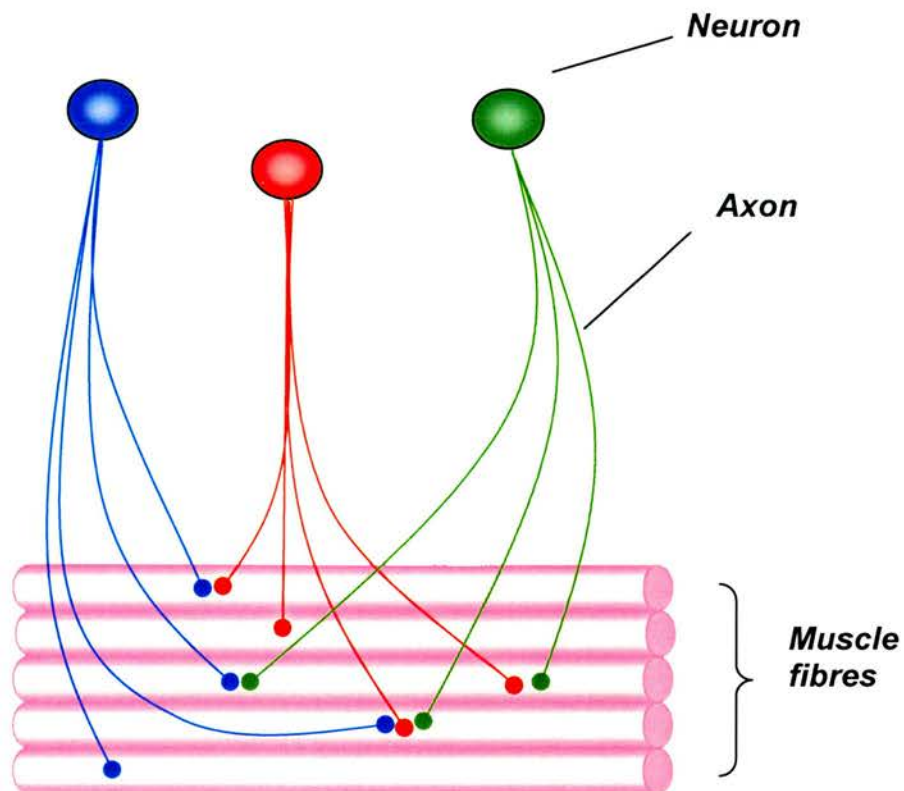
The role of peripheral nerves as an alternative pathway for peripheral scrapie pathogenesis has been the subject of considerable investigation in the past (Kimberlin et al., 1983; Kimberlin and Walker, 1980) and in recent years (Groschup et al., 1999; McBride et al., 2001). The short incubation periods after s.c challenge of neonatal mice in this experiment, resembles the length of incubation periods after direct inoculation of peripheral nerves (Kimberlin et al., 1983). During the embryonic period and early postnatal period, the peripheral nervous system (PNS), like the CNS is also in a state of flux. In the present study it is hypothesised that scrapie uptake occurs more efficiently in the developing PNS. The development of PNS innervation has been studied in a variety of rodent models.

Investigations carried out in rat neuromuscular junctions have shown that at birth, muscle fibres are innervated by between 2-7 different motor axons (Lichtman et al, 1977). This was determined by impaling neonatal muscle fibres with an electrode and pulsing them with a hyperpolarising current. The number of discrete steps in the excitatory post-synaptic potential in response to graded stimulation was taken as a measure of the number of presynaptic nerve fibres innervating each cell. In neonatal mice, up to seven discrete steps could be observed hence the conclusion that there is a higher degree of innervation in the neonatal rodent compared to adults.

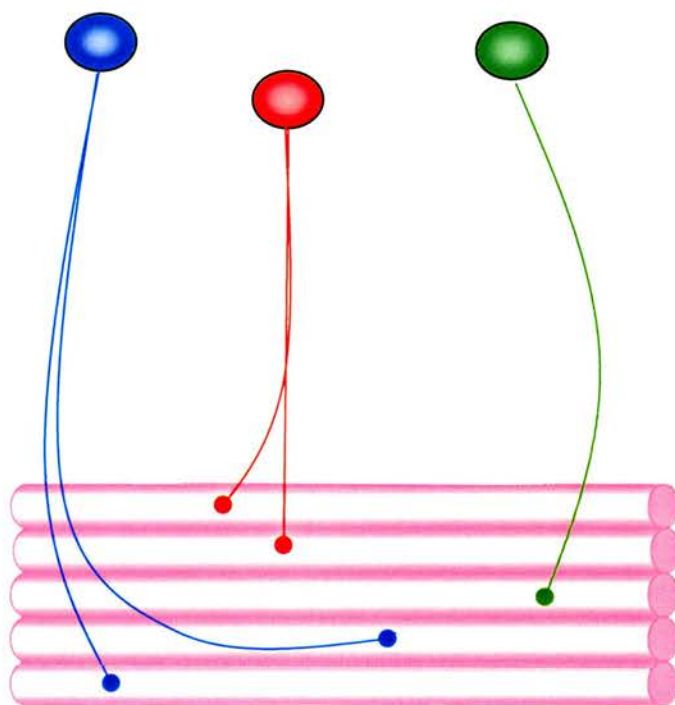
The synaptic endings of each axon closely intermingle at the neuromuscular junction. This is illustrated in Fig 6.5. Further analysis has revealed that the number of multiply innervated soleus muscle fibres decrease dramatically from 91% to 2.5% between 10 and 14 days after birth (Brown et al, 1976). By the end of the second postnatal week, the number of innervating axons has been reduced to one axon per muscle fibre (Brown et al, 1976).

Similar findings have been encountered in the development of the submandibular ganglion in rat. This study also revealed that in adult ganglia many boutons are visible at synaptic junctions. However, few boutons are visible in neonatal rats, although many fine axonal fibres are seen. This suggests that the neonatal neuron receives a smaller number of synaptic contacts but many more axons are available (Lichtman, 1977).

**Neonate**



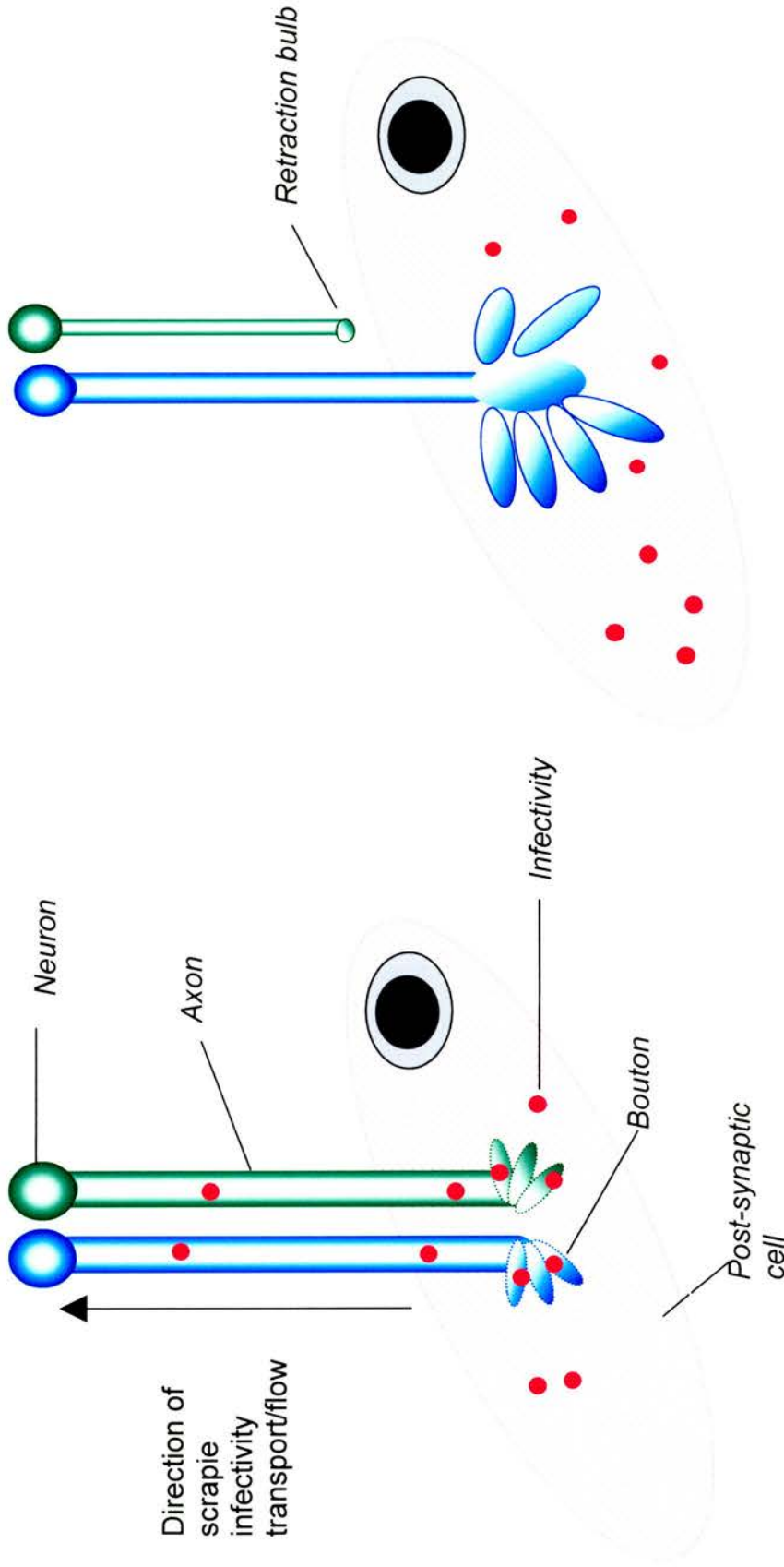
**14 days old**



**Fig 6.5 Synapse elimination during the first two weeks of life.** At birth, muscle fibres are innervated by between 2 and 7 different axons. During the first two postnatal weeks axonal convergence decreases until all but one axon innervates each muscle fibre by 14 days after birth. This occurs by axon removal rather than neuronal loss. If scrapie infectivity is injected at birth then it will have up to a seven times greater chance of entering the PNS due to the high degree of axonal convergence present in neonatal rodents. This schematic illustration was adapted from *Fundamental Neuroscience*, (Lichtman, 1999)

Neonate

14 day old



**Fig 6.7 Hypothetical mechanisms of scrapie infectivity uptake in neonatal mice during PNS synaptogenesis.** At birth, post-synaptic cells are innervated by between 2 and 7 axons. In addition boutons are not well developed and pinocytosis is more frequent in neonates than in older mice. The synaptic endings of the different axons are interspersed amongst each other. Infectivity present in a particular post-synaptic area during the neonatal period will have a greater chance of entering the CNS directly via the PNS due to the multiple innervation of cells at birth and the frequency of pinocytosis by synaptic endings. By 14 days after birth, synapse elimination has reduced the degree of innervation and cells become singly innervated. Surviving axons expand their synaptic connections and occupy more territory whilst the other axons, destined for elimination lose synaptic territory and die back into a retraction bulb. The decrease of innervation and pinocytosis and a greater structural maturity will lessen the chances of uptake of infectivity. This schematic diagram was adapted from Fundamental Neuroscience, 1999

From a scrapie perspective, the high degree of axonal convergence illustrated in Fig 6.5 could mean that scrapie infectivity inoculated at birth will have a greater opportunity to access the PNS compared to 14 day old mice. However, studies have shown that the amount of territory occupied by the synaptic boutons of each axon increases during the first two postnatal weeks (illustrated in Fig 6.6). This developmental change could theoretically cancel out the effect imposed by the greater number of axonal inputs present at birth for scrapie uptake. However, it is possible that the higher frequency of pinocytosis occurring at synaptic junctions at birth coupled with the high degree of convergence could directly affect the efficiency of scrapie infectivity uptake by the developing PNS. This is illustrated in Fig 6.6.

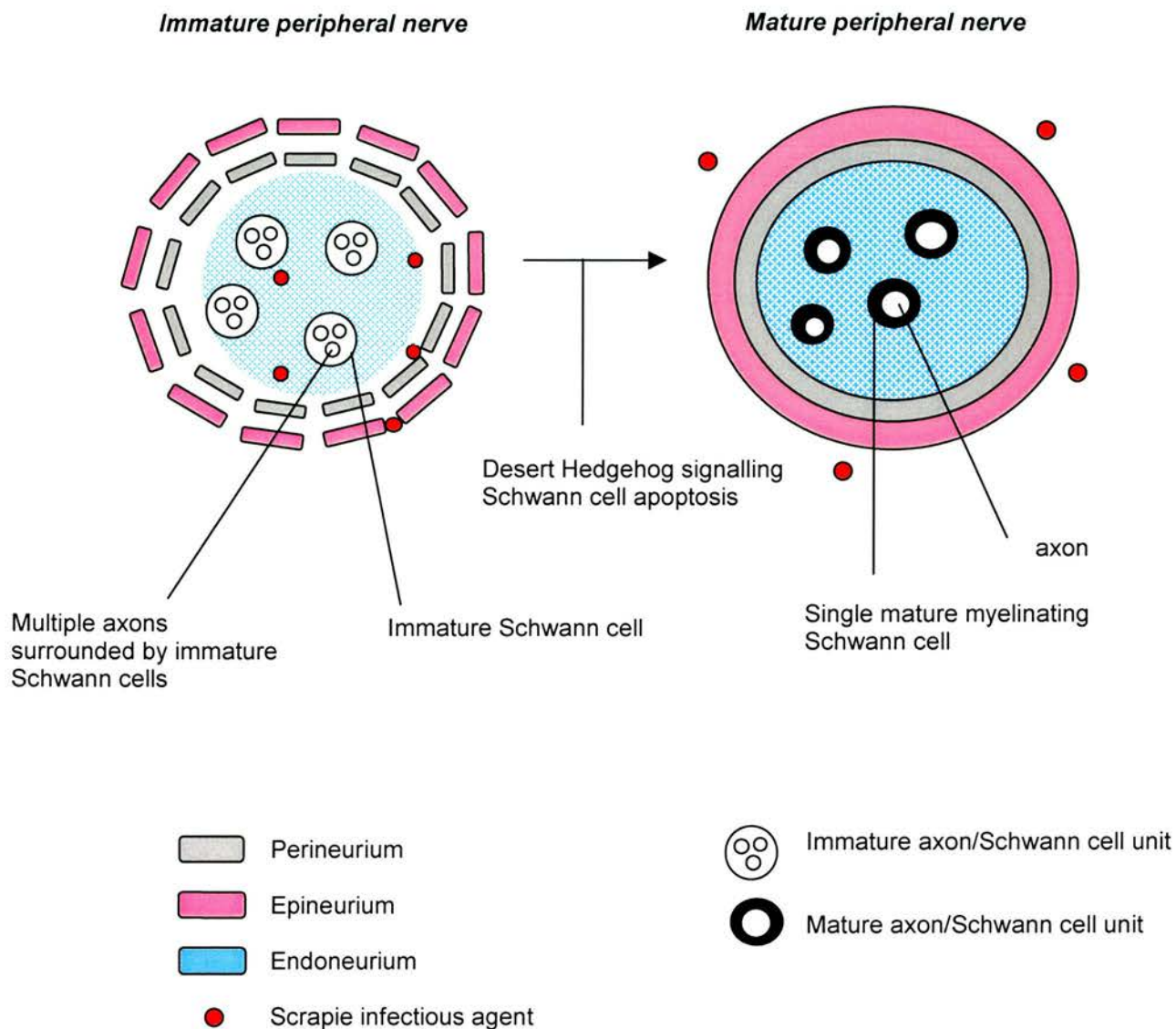
It is also of interest that if the sciatic nerve from adult mice is damaged by mechanical crushing or by lysophosphatidyl choline treatment shortly before scrapie inoculation, then a higher percentage of short incubation period cases are observed compared to uninjured controls (Kimberlin et al., 1983). It has been demonstrated that after nerve crush injury, peripheral nerve axons revert back to an immature state i.e multiple innervation of postsynaptic cells occurs whilst the damage is repaired. The repair process lasts for two weeks whereby muscle fibres become single innervated once more (Brown et al., 1976). This bears similarities to PNS maturation in neonatal mice. Hence, it is possible that after nerve crush injury, the efficiency of scrapie uptake by the PNS is increased due a reversion back to a multiply innervated status.

The structure of peripheral nerves is also undergoing development during the first few weeks of birth (Mirsky et al., 1999). In the mature peripheral nerve, there is a 1:1 interaction ratio between Schwann cell and axon i.e one Schwann cell surrounds and individual axon. There is current evidence to suggest that the 1:1 ratio is achieved by apoptosis of Schwann cells during the early postnatal period. (Grinspan et al., 1996; Trachtenberg and Thompson, 1996). Neonatal Schwann cells demonstrate an increased susceptibility to apoptosis during the first two weeks of life. Apoptosis occurs due to competition for axonally derived neuroregulins

which promote cell survival (Grinspan et al., 1996; Syroid et al., 1996; Trachtenberg and Thompson, 1996). The Schwann cell population is therefore pruned and as the mouse matures the surviving Schwann cells extend their processes to encompass axons into progressively smaller bundles (Jessen and Mirsky, 1999).

Surrounding the Schwann cell/axon units are the endoneurium and perineurium. These components form a multilayered sheath around the developing axon in order to protect the axons from cellular infiltration, infectious agents and unwanted chemical infiltrates (Mirsky et al., 1999; Parmantier et al., 1999). The signalling molecule, Desert hedgehog (Dhh) has been implicated in the development of both the endoneurium and epineurium. In *dhh* <sup>-/-</sup> mice, there is almost no epineurium and the perineurium is thin and highly abnormal (Bitgood and McMahon, 1995). Dhh is produced in Schwann cell precursors from embryonic day 11 until postnatal day 10 (Mirsky et al., 1999) suggesting that the nerve fibres themselves do not have a mature structure until the first two weeks of birth. Therefore the diffusion barrier protecting the peripheral nerves is still compromised during early neonatal life.

If scrapie is inoculated at birth, then it is possible that infectivity may diffuse through the maturing peripheral nerve sheaths and gain access to nerves undergoing synaptogenesis and axonogenesis. By 14 days after birth, the epineurium and perineurium will be fully formed and hence infectivity will have more difficulty gaining access to the axons within the peripheral nerve sheath due to the diffusion barrier in place. These hypotheses are illustrated in Fig 6.7.



**Fig 6.7 Maturation of peripheral nerve sheaths and implications for scrapie pathogenesis.** During the first two weeks of life, the structure of peripheral nerves is still undergoing development. It is hypothesised that infectivity inoculated at birth could diffuse through the immature peripheral nerve sheath as illustrated. This schematic diagram Compiled from data in the following literature: (Mirsky, 1999; Jessen, 1999; Grinspan, 199, Syroid, 1996)

## 6.5 Summary

The results produced in this chapter demonstrate a pronounced age effect on scrapie pathogenesis after subcutaneous challenge of neonatal mice in immunocompetent and immunodeficient mice. After s.c challenge of neonatal SCID and CB20 mice a very high proportion of mice developed scrapie with short incubation periods ranging between 150-230 days. SCID mice inoculated at 14 days old or older did not succumb to scrapie with short incubation periods and were relatively resistant to infection.

It can be concluded that the s.c route in neonates is very efficient compared to older mice. Entry of infectivity into the CNS after s.c challenge of neonates must occur via routes other than the LRS, since the majority of neonatally inoculated SCID mice succumbed to scrapie despite being deficient in mature FDCs. Interestingly, similar proportions of neonatally challenged CB20 mice succumbed to scrapie after short incubation periods. This suggests suggested that similar pathogenic mechanisms were used and were more than likely to be FDC independent.

It is hypothesised that the scrapie infectivity may be taken up directly by peripheral nerve endings. In the neonate it is predicted that scrapie uptake will occur with greater efficiency due to the increased axonal convergence and structural immaturity of peripheral nerves at birth. Consistent with this hypothesis is the finding that neonatal SCID mice succumb to scrapie with short incubation periods and that very few scrapie cases are observed following inoculation of 14 day old SCIDs. Nevertheless, the data does not preclude the involvement of other cell types maturing at the same time (e.g myeloid cells). In addition the possibility of haematogenous spread of infectivity directly to the brain due to the immaturity of the blood brain barrier resulting in short incubation periods cannot be excluded.

## 6.6 Future Work

The route by which infectivity spreads after s.c challenge of neonatal mice is of particular interest and could not be determined in this experiment. However, if the study was repeated then the brains of peripherally challenged mice could be tested for the presence of PrP<sup>sc</sup> at predetermined time points post-inoculation.

Differentiating between PrP<sup>sc</sup> and PrP<sup>c</sup> at early stages during the incubation period using immunocytochemistry is difficult since PrP antibodies cannot distinguish between the two isoforms. However, this problem could be overcome by using the histoblotting technique. This technique is similar to Western blotting for PrP<sup>sc</sup> since it allows the differentiation of PrP<sup>sc</sup> from PrP<sup>c</sup> by proteinase treatment of paraffin embedded tissue sections on nitrocellulose membranes. However it has the advantage of immunocytochemistry, since the precise anatomical location of the PrP<sup>sc</sup> staining can be analysed. The targeting of PrP<sup>sc</sup> in the brain early in the incubation period could yield clues as to the points of entry of infectivity into the brain. This could in turn shed light on the most likely route of spread of infectivity to the brain.

# Chapter 7

## Summary and Discussion

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## **7.1 Introduction**

During the course of this project, experiments carried out during the 1970s and 1980s documenting an age related effect on peripheral scrapie pathogenesis were revisited, with a view to exploring the possible mechanisms giving rise to the phenomenon. After i.p challenge of mice of various postnatal age groups with doses of ME7 that are 100% lethal to adult mice, it was found that a proportion of neonatally challenge mice resisted scrapie. Other neonatally challenged mice from the same group developed the disease after a prolonged incubation period and full adult susceptibility to scrapie was not apparent until between 7-10 days after birth (Outram et al., 1973). Other experiments investigating the effect of age on scrapie pathogenesis after s.c challenge, found that a high proportion of neonatally challenged mice developed the disease after a short incubation period, of similar length to ones expected after i.c injections. The proportion of mice with shorter incubation periods declined after 8-10 days old (Outram, unpublished observations). Although short incubation periods were observed after i.p challenge of neonates, the effect was more marked after s.c inoculation.

The aims of the project were therefore to explore the possible reasons for this wide variation in incubation period and disease outcome in neonatal mice contrasting with the relative uniformity of incubation period length observed in older mice. When the neonatal pathogenesis studies were published, it was suggested that the increase in scrapie susceptibility during the first 7-10 days of life could be attributed to the development of lymphoreticular system during this period of time after birth (Outram et al., 1973). However, the target cell for scrapie during peripheral pathogenesis had not been elucidated. Further studies during the 1970s-1990s helped to identify the FDC as the candidate cell for the replication of infectivity in the periphery and within two years of commencement of this project, the FDC was formally defined as the target cell for scrapie after peripheral challenge (Brown et al., 1999; Mabbott et al., 2000a; Mabbott et al., 2000b). Since there was a substantial body of evidence in support of the role of

FDCs in scrapie pathogenesis in adult mice, it was hypothesised that neonatal mice resist scrapie due to a lack of PrP<sup>c</sup> expressing FDCs.

Paradoxically, previous experiments had shown a proportion of peripherally inoculated neonatal mice to develop scrapie after very short incubation periods ranging between 150-230 days. During the first two weeks of life, the number of cases with short incubation periods decreases. It is hypothesised that due to the immaturity of peripheral nerves at birth, infectivity can be taken up more efficiently by neonatal peripheral nerve endings and reach the central nervous system (CNS) without prior replication in the spleen. For the remainder of this chapter, the results from the experiments carried out during the course of this project will be discussed and put into context with what is known about age related susceptibility of other species to natural TSEs.

## **7.2 The maturation of germinal centres and detection of PrP<sup>f</sup> in the developing mouse spleen: implications for age-dependent susceptibility to peripheral scrapie challenge.**

Since the maturational status of FDCs in adult mice was of importance to scrapie replication (Brown et al., 1999) in this project, a study was carried out in C57BL mice to characterise spleen FDCs during early postnatal development using FDC specific markers and immune complex trapping assays. These results are described in Chapter 4. Using the FDC-M1 antibody, FDC-M1+ cells could be detected from birth within the immature white pulp of spleens from 0 day old mice. The labelling was punctate and did not have a dendritic morphology. In 4 day old mice, the FDC-M1 labelling covered the periphery of the white pulp area and could be found in the B220+ B cell area as judged by immunolabelling of adjacent section with B220. The morphology became similar to that in adults between the ages of 14 and 26 days after birth. By 26 days after birth, the morphology was indistinguishable from that in adult mice.

Further characterisation was carried out using another FDC specific antibody, FDC-M2. The earliest age group at which FDC-M2+ cells could be detected was 14 days after birth. This coincided with the detection of cells expressing the complement receptor CR1 using the anti-CR1 monoclonal antibody. Although the possibility that these cells could

have been B cells cannot be excluded, the morphology of FDC-M2+ cells and CR1+ cells was very similar and occupied the same follicle location. In Chapter 5, it was shown that the PrP<sup>c</sup> can first be detected at 10 days old in spleen frozen sections. PrP<sup>c</sup> labelling is associated with FDC-M1+ cells at 10 days old, and co-localises with FDC-M2+ cells and CR1+ cells at 14 days old.

The neonatal pathogenesis studies described in Chapter 3 have demonstrated the repeatability of the age related scrapie susceptibility phenomenon described in previous studies (Outram et al, 1973; Outram unpublished studies). The experiments carried out in this project demonstrated that a proportion of neonatal mice (18/27) resist neonatal challenge. This is a higher degree of survival than previously observed in experiments by Outram et al, 1972, where 4/32 neonatally challenge mice survived peripheral scrapie challenge.

In the present study, 9/27 mice succumbed to scrapie and the median incubation period of this group was found to be significantly longer ( $p < 0.001$ ) than mice injected at 10 days old or older. In the previous studies by Outram et al, no information regarding the ability of spleens from neonatally challenged mice to support scrapie replication was available since bioassays of the spleens were not carried out. Due to the time constraints of the project, Western blotting of spleen derived PrP<sup>Sc</sup> was used as a marker for scrapie infectivity as this technique is less time consuming than the bioassay. PrP<sup>Sc</sup> could not be detected 70 d.p.i in the spleens of mice challenged i.p as neonates, but could be detected in the spleens of mice challenged i.p at 10 days old or older. This suggests that neonatally challenged mice are resistant to scrapie because they cannot support scrapie replication in the spleen. However, there appeared to be certain exceptions to this conclusion, since a proportion of neonatally challenged mice did succumb to scrapie and these cases contained high levels of PrP<sup>Sc</sup> in the spleens at terminal stages of the diseases. However, these results must be treated with caution as conclusions cannot be drawn as to precisely when replication occurred in the spleens of these mice, since the spleens were taken from terminally affected mice. One possible explanation is that infectivity gained access to the CNS early in the incubation period without prior

amplification in the spleen, but then spread to lymphoid tissue via the PNS after infection of the brain had taken place. Without spleen bioassay information to underpin the PrP<sup>sc</sup> data, it is difficult to build a full picture of scrapie replication kinetics in the spleens of neonatally challenged animals. Bioassays of spleens from neonatally challenged mice are currently underway.

A possible approach for future work is to use SCID mice, which are deficient in mature FDCs, to determine whether infectivity can spread from the CNS to lymphoid tissue after i.c inoculation. Similar to immunocompetent mice, SCID mice succumb to scrapie after i.c challenge without the need for prior lymphoid tissue amplification. Following i.c challenge, SCID mice would then be irradiated and reconstituted with bone marrow in order to induce FDC maturation. If infectivity can travel from the CNS to peripheral lymphoid tissue, then replication in the spleen should be initiated after bone marrow reconstitution of SCID mice, when FDCs become mature.

At the moment the main conclusion that can be drawn from the data is that neonatal mice have an impaired ability to replicate scrapie and therefore resist scrapie challenge. The timing of germinal centre maturation and PrP<sup>c</sup> detection in relation to changes in scrapie susceptibility in developing mice is summarised in Fig 7.1. The shift from relative resistance to peripheral scrapie challenge to an adult-like susceptibility occurs between 7-10 days old. This shift precedes the development of fully functional germinal centres by 11-15 days at least. However it coincides with the presence of immunocytochemically detectable levels of PrP<sup>c</sup> on immature FDCs as shown in Fig 7.1.

It is therefore tempting to suggest that PrP<sup>c</sup> may be the only prerequisite to the maturation of scrapie susceptibility in neonatal mice. However, if PrP<sup>c</sup> was the only factor required for peripheral scrapie pathogenesis, then expression of the PrP<sup>c</sup> at high levels on any lymphoid cell should bypass the need for FDCs in scrapie replication after peripheral challenge. This hypothesis has been tested elsewhere in adult Prn-p <sup>-/-</sup> mice over-expressing PrP<sup>c</sup> on T cells under the control of the *Lck* promoter, on an otherwise

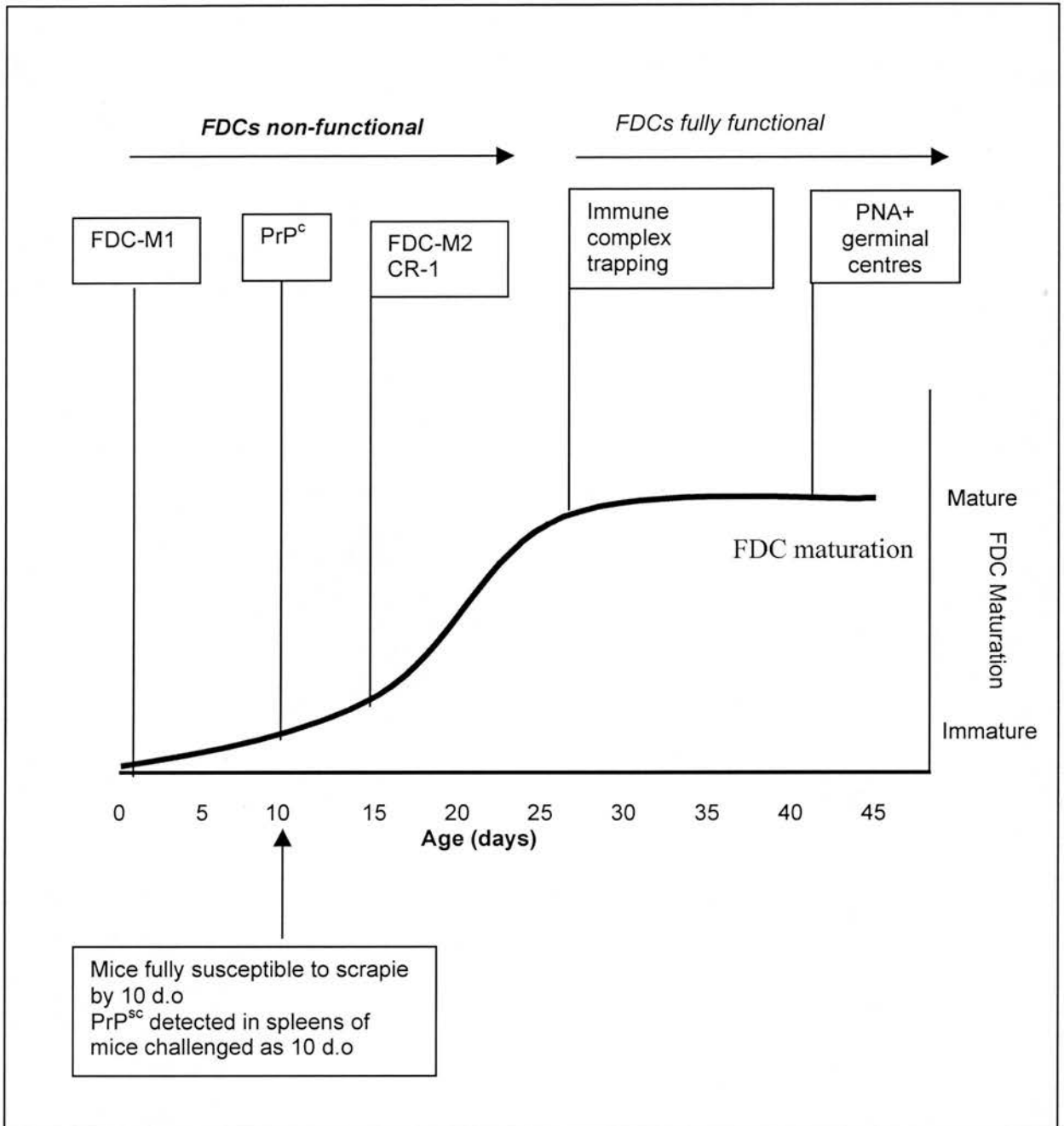
Prn-p<sup>-/-</sup> background (Raeber et al., 1999). It was found that replication could not occur in the spleens of peripherally challenged mice despite there being a 100-fold increase in PrP levels on T cells. Although the possibility that infectivity transport mechanisms were abrogated in the Prn-p transgenic mice cannot be excluded, it indicates that PrP<sup>c</sup> is not sufficient for peripheral pathogenesis and that the processes involved in the eventual amplification of infectivity in spleen are more complex. More importantly, if PrP<sup>c</sup> expression in lymphoid tissues is not sufficient for adult peripheral scrapie pathogenesis in adult mice, then it is unlikely to be enough to explain the maturation of scrapie susceptibility in young mice.

Although the data produced in this project suggests that 10 day old mice are capable of supporting scrapie replication at a time when their FDCs are functionally immature, it is not known if replication occurs immediately in mice of this age or if it begins when the mice are older. In adult mice, there appears to be a lag of 1-2 weeks post inoculation before increasing levels of infectivity can be detected in spleen (Eklund et al., 1967). It is hypothesised that immediately after inoculation, infectivity is sequestered by macrophages and indeed there is some evidence to suggest this occurs (Beringue et al., 2000). However, this apparent lag may be due to the limits of sensitivity of the bioassay. Indeed, the following scenarios could occur immediately after i.p challenge in adults:

- a) all infectivity is cleared possibly by macrophages or other cell types and a lag phase ensues until infectivity eventually reach spleen follicles and is amplified on FDCs.
- b) replication may commence immediately after inoculation but is masked by the inoculum and therefore remains undetected by bioassay.

In the case of neonatally challenged mice that resist scrapie infection completely, it is possible that in the majority of cases, infectivity is cleared and disposed of before sufficiently mature FDCs are available. Sequestration of infectivity immediately after inoculation may also occur in mice challenged at 10 or 14 days old. However, FDCs in mice of this age may reach functional maturity before the inoculum is disposed of. If this is the case, then sequestered infectivity may gain access to these maturing FDCs at a critical stage in their development and replication could carry on as normal when the mice are slightly older.

Overall, the data from this project support the hypothesis that neonatally challenged mice resist scrapie due to the immaturity of FDCs since the onset of scrapie susceptibility after peripheral challenge of young mice correlates well with the detection of PrP<sup>c</sup> on maturing FDCs in the spleen. Although the FDCs are not fully mature at the age when adult susceptibility is achieved, the possible reasons for this have explained in detail above and therefore a rejection of the original hypothesis cannot be merited. A more thorough investigation of the age at which replication commences after i.p challenge of young mice is an obvious area for further investigation. However, this will be hampered by the sensitivity of infectivity assays currently available since any replication at an early stage after inoculation could be masked by infectivity present in the inoculum.



**Fig 7.1 The relationship between FDC maturation and the onset of scrapie susceptibility**

This graph correlates key events during FDC maturation with the onset of scrapie susceptibility in neonatally challenged mice. Red arrows indicate age at time of scrapie challenge. FDC maturation is described by the blue line. Key events during FDC maturation and the age at which they occur are indicated in the boxes. The age at which mice shift from resistance to susceptibility to scrapie challenge is indicated.

### **7.3 The role of non-LRS dependent mechanisms in neonatal scrapie pathogenesis.**

In Chapter 6, the involvement of non-lymphoid systems in peripheral scrapie pathogenesis were explored. Previous studies had shown that after s.c challenge a high proportion of neonatally inoculated mice succumb to scrapie after a short incubation period (Outram unpublished data). The length of the incubation period (often as short as 200 days after s.c challenge) was similar to experiments where direct inoculation of the sciatic nerve was carried out. Although the involvement of the lymphoid system could not be excluded since the experiments were carried out using immunocompetent mice, other studies using immunodeficient mice such as SCID and  $\text{TNF}\alpha^{-/-}$  mice (both deficient in mature FDCs) have shown that occasionally mice succumb to peripheral scrapie challenge (Fraser et al., 1996; Mabbott et al., 2000a). In these mice, scrapie neuroinvasion occurred without prior LRS dependent amplification (Fraser et al., 1996; Mabbott et al., 2000a).

SCID mice provided a useful model to study the role of non- LRS systems in scrapie pathogenesis since they do not possess mature FDCs. Although adult SCID mice resist peripheral scrapie challenge (Fraser et al., 1996; O'Rourke et al., 1994) it was predicted that neonatal SCID mice should succumb to scrapie after short incubation periods if mechanisms of neuroinvasion which were independent of an intact LRS existed in the neonate. After s.c challenge, it was found that a large proportion of neonatally challenged mice developed scrapie after a short incubation period. The number of cases with short incubation periods had decreased dramatically after s.c inoculation of mice of 14 days old or older. It is hypothesised that the infectivity may be taken up more efficiently by developing peripheral nerves as the degree of peripheral innervation of tissue is high in neonates compared to 14 day old mice and adults (Brown et al, 1976; Lichtman et al, 1977). The data obtained after s.c challenge of neonatal SCID mice in this project is consistent with this hypothesis. However, a greater efficiency of infection is not observed after i.p challenge of neonatal SCID mice. The reasons for this difference are as yet unclear, although a possible reason could be that more infectivity is sequestered after i.p challenge compared to s.c.

#### **7.4 Implications of neonatal peripheral scrapie pathogenesis studies for predicting susceptibility of other species to natural TSEs during early life.**

The results presented in this project have shown that in general, neonatal mice are resistant to scrapie after i.p challenge and this has been attributed to a lack of PrP<sup>c</sup> expressing FDCs. Paradoxically, neuroinvasion can occur more readily after s.c challenge of neonates and these mice succumb to scrapie after a short incubation period. In this section, the implications that these findings could have on predicting whether other species such as sheep and humans are susceptible to natural TSEs during prenatal or early postnatal life is explored.

vCJD is a neurodegenerative disorder in humans caused by the BSE agent (Bruce et al., 1997). Symptoms include locomotor incoordination, memory loss and myoclonus. The features that distinguish vCJD from CJD is the distinct neuropathological features and accumulation of PrP<sup>res</sup> in lymphoid tissue such tonsil, lymph nodes, spleen and appendix (Hill, 1999). The pattern PrP<sup>res</sup> immunolabelling has distinct reticular morphology similar to FDC networks. Another intriguing feature of vCJD is the predominantly younger age group which it affects. The mean age of death has been until recently 29 years  $\pm 9.1$ ; the youngest reported case being a 12 year old girl (Verity et al., 2000) and the eldest, a 74 year old man (Lorains et al., 2001). The reason for teenagers and young adults being predominantly affected are unknown. However, the age-dependent susceptibility to peripheral scrapie infection in mice may offer a framework with which to explore the reasons for this phenomenon in humans.

Humans differ from mice in that humans possess FDCs in lymphoid tissue before birth. FDC dendritic networks have been demonstrated in immature human fetal B cell follicles by 22 gestational weeks (g.w) (Timens et al., 1987). By the third trimester FDCs have surface IgM expression (Timens et al., 1987). However fully formed germinal centres are not present until at least 14 days after birth (Barzanji and Emery, 1978) and adult lymphoid morphology is not observed until 1 year after birth (Timens et al., 1987).

In the last month of gestation in sheep (day 120-130 of gestation), immature follicular dendritic cells identified by 5'- nucleotidase activity can be found in the primary follicles

of foetal sheep lymph nodes (Halleraker et al., 1994). This occurs later than the human foetus probably because of the structure of the sheep placenta which impedes the entry of maternal immunoglobulins and exogenous antigen into the foetal sheep bloodstream (Boyd et al., 1976). Sheep FDCs do not demonstrate a mature morphology until around 42 days after birth (Halleraker et al., 1994).

The presence of FDCs prenatally in foetal humans and sheep suggests that they could be susceptible to TSEs before birth. Indeed, there is accumulating evidence to suggest that maternal transmission of scrapie from sheep to lamb embryos can occur and this has been explored using embryo transfer from experimentally infected sheep (Foster et al., 1992). In these experiments scrapie susceptible ewes (*SipsA/sA*) were injected subcutaneously with the experimental sheep scrapie strain SSBP/1 prior to artificial insemination. Embryos were transferred to scrapie resistant recipient ewes (*Sip pA/pA*) and lambing was carried out in decontaminated premises. Six out of twenty surviving lambs developed scrapie with relatively short incubation periods of 2 years suggesting a perinatal infection. The precise mode by which the embryos could have become infected is not known. For example infection may have occurred by the transovarian route and existed within the embryo mass or infectivity could have adhered to the zona pellucida during passage through the oviduct and uterine horn.

Recent unpublished studies by Foster et al, have developed these initial trials further by using immunocytochemistry to detect the presence of PrP<sup>sc</sup> within the vagus of terminally affected lamb progeny. Natural scrapie in sheep produces an intense PrP<sup>sc</sup> immunolabelling within the vagus in terminal stages of the disease. However, infection of sheep with the experimental scrapie strain SSBP/1 does not produce vagus associated PrP<sup>sc</sup> staining (Foster et al., 2001). Thus by screening all progeny of experimentally scrapie infected ewes, then it is possible to distinguish between true experimental infection that could have been acquired prenatally and natural scrapie, that could have been acquired after birth in the field.

In the study, embryos were transferred from ewes experimentally inoculated with SSBP/1, to resistant recipient ewes. Lambs were born naturally or by caesarian derivation. From the caesarian derived progeny, 2/13 lambs developed clinical signs of scrapie after short incubation periods below 750 days and these were confirmed to be

SSBP/1 scrapie by the absence of PrP<sup>Sc</sup> immunolabelling within the vagus using immunocytochemistry. 2/8 progeny born naturally to ewe recipients, developed scrapie after short incubation periods and these were also confirmed to have been infected with SSBP/1 by the lack of PrP<sup>Sc</sup> in the vagus. The fact that progeny born by caesarian derivation developed scrapie after significantly shorter incubation periods suggests that the lambs could have been infected by SSBP/1 *in utero*. Although these experiments are ongoing, the uninfected recipient ewes that gave birth to the progeny have yet to develop scrapie. Again this suggests that the scrapie affected progeny must have acquired the infection during the embryonic phase of their development before embryo transfer to recipients.

Although the possible mechanisms of maternal transmission are not known, the sheep studies suggest that prenatal transmission is possible. The similarities between human and sheep immune system maturation before birth and in particular the presence of FDCs prenatally suggests that sheep scrapie models may offer a clinically relevant experimental system for studying maternal transmission of vCJD or BSE in humans.

## **7.5 Concluding Remarks**

The results presented in this thesis have re-affirmed that the age at the time of peripheral scrapie challenge has a profound effect on scrapie incubation period and susceptibility of mice to scrapie. The results have demonstrated a correlation between the detection of PrP<sup>c</sup> on FDCs and the onset of scrapie susceptibility in neonatal mice. These studies provide a framework with which to form predictions regarding the susceptibility of other species to natural TSEs during development. There is compelling evidence in experimental sheep scrapie models to suggest that maternal transmission of scrapie from ewes to lambs can occur prenatally. Interestingly, other studies have demonstrated the presence of FDCs within spleen and lymph nodes prenatally in foetal sheep. Although the mode of transmission and the cell types involved in pathogenesis in the lamb have yet to be defined, it is possible that foetal lambs are fully susceptible to scrapie before birth. The similarities between the timing of maturation between sheep and human FDCs suggests that human foetuses could be theoretically susceptible to vCJD before birth.

## 7.6 Future Work

In the long term, further exploration of maternal transmission and the susceptibility of humans to vCJD is clearly required using clinically relevant scrapie models and routes of infection. Sheep scrapie models may provide a good experimental system with which to study prenatal susceptibility to vCJD since the maturation of the lymphoreticular system during sheep development closely resembles that of humans. Studies involving the maternal transmission of scrapie in sheep have used the subcutaneous route of inoculation. It will be of interest to study the effect of oral dosing or intravenous inoculation of scrapie on maternal transmission in sheep since these are relevant routes of transmission of TSEs in humans. As yet, bioassays or PrP<sup>sc</sup> analysis of CNS and peripheral lymphoid tissue from foetal or neonatal sheep produced during scrapie maternal transmission experiments have not been carried out. This will be of interest to determine the pathogenesis of scrapie in foetal sheep exposed to the disease through maternal transmission.

Immunohistological investigations of the human and foetal sheep lymphoid tissue development with particular attention to PrP<sup>c</sup> detection should be carried out in parallel to TSE pathogenesis studies, to determine the age at which sheep and humans could become susceptible to natural TSEs.

# Bibliography

- Abbas, A.K., Lichtman, A., Pber, J.S (1994) Cellular and Molecular Immunology. 2<sup>nd</sup> Edition. W.B Saunders Company.
- Amor, S., Scallan, M. F., Morris, M. M., Dysn, H., and Fazakerley, J. K. (1996). Role of immune responses in protection and pathogenesis during Semliki Forest Virus encephalitis. *Journal of General Virology*, 77, 281-291.
- Ansel, K. M., Ngo, V. N., Hyman, P. L., Luther, S. A., Forster, R., Sedgwick, J. D., Browning, J. L., Lipp, M., and Cyster, J. G. (2000). A chemokine driven positive feedback loop organises lymphoid follicles. *Nature*, 406, 309-314.
- Baldauf, E., Beekes, M., and Diringer, H. (1997). Evidence of an alternative direct route of access for the scrapie agent to the brain bypassing the spinal cord. *Journal of General Virology*, 78, 1187-1197.
- Barzanji, A. J., and Emery, J. L. (1978). Germinal centres in the spleens of neonates and stillbirths. *Early Human Development*, 1(4), 363-369.
- Berg, v. d., T.K, and Dijkstra, C. D. (1995). Ontogenesis and Phylogenesis of FDC. Follicular Dendritic Cells in Normal and Pathological Conditions, Landes Company, 35-51.
- Beringue, V., Demoy, M., Lasmezas, C. I., Gouritin, B., Weingarten, C., Deslys, J.-P., Andreux, J.-P., Couvreur, P., and Dormont, D. (2000). Role of spleen macrophages in the clearance of scrapie agent early in pathogenesis. *Journal of Pathology*, 190, 495-502.
- Bitgood, M. J., and McMahon, A. P. (1995). *Hedgehog* and *Bmp* genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo. *Developmental Biology*, 172, 126-138.
- Bofill, M., Akbar, A. N., and Amlot, P. L. (2000). Follicular dendritic cells share a membrane bound protein with fibroblasts. *Journal of Pathology*, 191, 217-226.
- Bosma, G. C., Custer, R. P., and Bosma, M. J. (1983). A severe combined immunodeficiency mutation in the mouse. *Nature*, 301, 1339-1347.
- Boyd, R. D. H., Haworth, C., Stacey, T. E., and Ward, R. H. T. (1976). Permeability of the sheep placenta to unmetabolised polar non-electrolytes. *Journal of Physiology*, 256, 617-634.
- Brown, D. R., Qin, K., Herms, J. W., Madlung, A., Manson, J., Strome, R., Fraser, P. E., Kruck, T., von Bohlen, A., Schulz-Schaeffer, W., Giese, A., Westaway, D., and Kretzshmar, H. (1997). The cellular prion protein binds copper *in vivo*. *Nature*, 390, 684-686.

- Brown, K. L., Stewart, K., Ritchie, D., Fraser, H., Morrison, W. I., and Bruce, M. E. (2000). Follicular dendritic cells in scrapie pathogenesis. *Archives of Virology*, 16, 13-21.
- Brown, K. L., Stewart, K., Ritchie, D., Mabbott, N. A., Williams, A., Fraser, H., Morrison, W. I., and Bruce, M. E. (1999). TSE replication in lymphoid tissue depends on prion protein-expressing-expressing follicular dendritic cells. *Nature Medicine*, 5(11), 1308-1312.
- Brown, M. C., Jansen, J. K. S., and van Essen, D. (1976). Polyneuronal innervation of skeletal muscle in new-born rats and its elimination in maturation. *Journal of Physiology*, 261, 387-422.
- Bruce, M. E., and Dickinson, A. G. (1987). Biological evidence that scrapie agent has an independent genome. *J. Gen. Virol.*, 68, 79-89.
- Bruce, M. E., Will, R. G., Ironside, J. W., McConnell, I., Drummond, D., Suttie, A., McCardle, L., Chree, A., Hope, J., Birkett, C., Cousens, S., Fraser, H., and Bostock, C. J. (1997). Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. *Nature*, 389(6650), 498-501.
- Bueler, H., Aguzzi, A., Sailer, A., R.A., G., Autenried, P., Aguet, M., and Weissmann, C. (1993). Mice devoid of PrP are resistant to scrapie. *Cell*, 73, 1339-1347.
- Carlson, G. A., Kingsbury, D. T., Goodman, P. A., Coleman, S., Marshall, S. T., DeArmond, S. J., Westaway, D., and Prusiner, S. B. (1986). Linkage of prion protein and scrapie incubation time genes. *Cell*, 46, 503-511.
- Caughey, B., Race, R. E., and Chesebro, B. (1988). Detection of prion protein mRNA in normal and scrapie-infected tissues and cell lines. *J. Gen. Virol.*, 69, 711-716.
- Cerny, A., Zinkernagel, R. M., and Groscurth, P. (1988). Development of follicular dendritic cells in lymph node of B-cell depleted mice. *Cell and Tissue Research*, 254, 449.
- Chandler, R.L. (1961). Encephalopathy in mice produced with scrapie brain material. *Lancet* 1, 1378-1379
- Cho, H. J. (1976). Is the scrapie agent a virus? *Nature*, 262(5567), 411-412.
- Cho, H. J., and Greig, A. S. (1975). Isolation of 14-nm virus-like particles from mouse brain infected with scrapie agent. *Nature*, 257, 685-686.
- Cho, H. J., Greig, A. S., Corp, C. R., Kimberlin, R. H., Chandler, R. L., and Millson, G. C. (1977). Virus-like particles from both control and scrapie-affected mouse brain. *Nature*, 267(5610), 459-460.
- Clarke, M. C., and Haig, D. A. (1966). Attempts to demonstrate neutralising antibodies in the sera of scrapie-affected animals. *Vet. Rec.*, 78(19), 647-649.

- Clarke, M. C., and Haig, D. A. (1971). Multiplication of scrapie agent in mouse spleen. *Res. Vet. Sci.*, 12, 195-197.
- Coico, R. F., Bhogal, B. S., and Thorbecke, G. J. (1983). Relationship of germinal centres in lymphoid tissue to immunologic memory. *Journal of Immunology*, 131(5), 2254-2257.
- Collinge, J., Palmer, M.S., Dryden, A.J. (1991). Genetic predisposition to iatrogenic Creutzfeldt-Jakob disease. *Lancet*, 337, 1441-1442.
- Collinge, J., Whittington, M. A., Sidle, K. C. L., Smith, C. J., Palmer, M. S., Clarke, A. R., and Jefferys, J. G. R. (1994). Prion protein is necessary for normal synaptic function. *Nature*, 370(6487), 295-297.
- Creutzfeldt, H.G. (1920). Uber eine eignartige herdformige erkankung des zentralnervensystems. *Z. Gestame Neurol. Psychiatrie*, 57, 247-250.
- Dickinson, A. G. (1976). Scrapie in sheep and goats. *Frontiers of Biology*, 44, 209-241.
- Dickinson, A. G., Meikle, V., and Fraser, H. (1968). Identification of a gene which controls the incubation period of some strains of scrapie agent in mice. *J. Comp. Pathol.*, 78, 293-299.
- Dickinson, A. G., and Meikle, V. M. (1971). Host-genotype and agent effects in scrapie incubation: change in allelic interaction with different strains of agent. *Molec. Gen. Genetics*, 112, 73-79.
- Dickinson, A. G., and Outram, G. W. (1964). Genetical control of the incubation period in mice of the neurological disease, scrapie. *Heredity*, 19, 279-288.
- Dickinson, A. G., and Outram, G. W. (1979). The scrapie replication-site hypothesis and its implications for pathogenesis. Slow transmissible diseases of the central nervous system, S. B. Prusiner and W. J. Hadlow, eds., Academic Press, New York, 13-32.
- Dickinson, A. G., and Outram, G. W. (1988). Genetic aspects of unconventional virus infections: the basis of the virino hypothesis. [Review]. *Ciba Foundation Symposium*, 135, 63-83.
- Dijkistra, C. D., Vam Tilburg, N. J., and Dopp, E. A. (1982). Ontogenic aspects of immune complex trapping in the spleen and popliteal lymph nodes of the rat. *Cell and Tissue Research*, 236, 545-552.
- Dijkistra, C. D., and Dopp, E. A. (1983). Ontogenetic development of T and B lymphocytes and non-lymphoid cells in the white pulp of the rat spleen. *Cell and Tissue Research*, 229, 351-363.
- Eklund, C. M., Kennedy, R. C., and Hadlow, W. J. (1967). Pathogenesis of scrapie infection in the mouse. *Journal of Infectious Diseases*, 117, 15-22.

Endres, R., Alimzhanov, M. B., Plitz, T., Futterer, A., Kosco-Vilbois, M. H., Nedospasov, S. A., Rajewsky, K., and Pfeffer, K. (1999). Mature follicular dendritic cell networks depend on expression of lymphotoxin  $\beta$  receptor by radioresistant stromal cells and of lymphotoxin  $\beta$  and tumour necrosis factor by B cells. *Journal of Experimental Medicine*, 189(1), 159-167.

Fang, Y., Chenguang, X., Yang-Xin, F., Holers, M., and Molina, H. (1998). Expression of Complement receptor 1 and 2 on Follicular Dendritic cells is necessary for the generation of a strong antigen-specific IgG response. *Journal of Immunology*, 160, 5273-5279.

Farquhar, C. F., Somerville, R. A., Dornan, J., Armstrong, D., Birkett, C., and Hope, J. A Review of the detection of PrP<sup>Sc</sup>. *A consultation on BSE with the Scientific Veterinary Committee of the Commission of the European Communities*, Brussels.

Farquhar, C. F., Somerville, R. A., and Ritchie, L. A. (1989). Post-mortem immunodiagnosis of scrapie and bovine spongiform encephalopathy. *J. Virol. Meth.*, 24(1-2), 215-221.

Foster, J., Parnham, D., Goldmann, W., and Hunter, N. (2001). Maternal transmission of scrapie in sheep. .

Foster, J. D., McKelvey, W. A. C., Mylne, M. J. A., Williams, A., Hunter, N., Hope, J., and Fraser, H. (1992). Studies on maternal transmission of scrapie in sheep by embryo transfer. *The Veterinary Record*, April 18, 341-343.

Fraser, H., Brown, K. L., Stewart, K., McConnell, I., McBride, P., and Williams, A. (1996). Replication of scrapie in spleens of SCID mice follows reconstitution with wild-type mouse bone-marrow. *Journal Of General Virology*, 77, 1935-1940.

Fraser, H., and Dickinson, A. (1968). Identification of a gene which controls incubation period of some strains of scrapie agent. *Journal of Comparative Pathology*, 78, 301.

Fraser, H., and Dickinson, A. G. (1970). Pathogenesis of scrapie in the mouse: the role of the spleen. *Nature*, 226, 462-463.

Fraser, H., and Dickinson, A. G. (1970). Studies of the lymphoreticular system in the pathogenesis of scrapie: the role of spleen and thymus. *Journal of Comparative Pathology* 88, 4,563-573

Fraser, H., and Farquhar, C. F. (1987). Ionising radiation has no influence on scrapie incubation period in mice. *Veterinary Microbiology*, 13(3), 211-223.

Gabus, C., Derrington, E., Leblanc, P., Chnaiderman, J., Dormont, D., Swietnicki, W., Morillas, M., Surewicz, W. K., Marc, D., Nandi, P., and Darlix, J. L. (2001). The prion

- protein has RNA binding and chaperoning properties characteristic of nucleocapsid protein NCp7 of HIV-1. *Journal of Biological Chemistry*, 276(22), 19301-19309.
- Gajdusek, D.C and Zigas, V. (1957) Degenerative diseases of the central nervous system in New Guinea: epidemic occurrence of "Kuru" in the native population. *New Engl. J. Med.* 257, 974-978.
- Gambetti, P., Medori, R., Tritschler, H., Leblanc A., Montagna, P., Cortelli, P., Tinuper, P., Monari, L., Tabaton, M., Petersen, R., Autiliogambetti, L. and Lugaresi, E. (1992). Fatal familial insomnia (FFI)- aprion disease with a mutation at codon-178 of the prion protein gene. *J. Neuropath. Exp. Neurol.* 51, 353.
- Gerstmann, J., Straussler, E., and Scheinker, I. (1936). Uber eine eigenartige hereditarfamiliare erkankung des zentralnervensystems zugleich ein betrag zur frage des vorzeitigen lokalen alterns. *Z. Neurol*, 154, 736-762.
- Gesser, R. M., and Koo, S. C. (1996). Oral inoculation with herpes simplex virus 1 infects enteric neurons and mucosal nerve fibres within the gastrointestinal tract in mice. *Journal of Virology*, 70(6), 4097-4102.
- Goldfarb, L.G., Cervenakova, L., Brown, P., Gajdusek, D.C. (1996). Genotype-phenotype correlations in familial spongiform encephalopathies associated with insert mutations. *Transmissible Subacute Spongiform Encephalopathies: Prion Diseases*. L.Court., B. Dodet, eds. Elsevier, Paris, 425-431.
- Gordon, W. S. (1946). *Vet. Rec*, 58, 516.
- Gray, D., Kosco, M., and Stockinger, B. (1990). Novel pathways of antigen presentation for maintenance of B cell memory. *International Immunology*, 3, 141.
- Griffith, J. S. (1967). Self-replication and scrapie. *Nature*, 215, 1043-1044.
- Grinspan, J. B., Marchionni, M. A., Reeves, M., Coulaloglou, M., and Scherer, S. S. (1996). Axonal interactions regulate Schwann cell apoptosis in developing peripheral nerve: neuregulin receptors and the role of neuregulins. *Journal of Neuroscience*, 16(19), 6107-6118.
- Groschup, M. H., Beekes, M., McBride, P. A., Hardt, M., Hainfellner, J. A., and Budka, H. (1999). Deposition of disease-associated prion protein involves the peripheral nervous system in experimental scrapie. *Acta Neuropathologica*, 98, 453-457.
- Halleraker, M., Press, C., and Landsverk, T. (1994). Development and cell phenotypes in primary follicles of foetal sheep lymph nodes. *Cell and Tissue Research*, 275, 51-62.
- Hill, A. F. e. a. (1999). Investigation of variant Creutfeldt-Jakob disease and other human prion diseases with tonsil biopsy samples. *Lancet*, 353, 183-189.

- Holmes, K. L., Schnizlein, C. T., Perkins, E. H., and Tew, J. G. (1984). The effect of age on antigen retention in lymphoid follicles and in collagenous tissue of mice. *Mechanisms of Ageing and Development*, 25, 243-255.
- Horie, K., Chen, D., Hoshi, H. (1999). Development of immune complex trapping: experimental study of lymphoid follicles and germinal centres newly induced by exogenous stimulants in mouse popliteal lymph nodes. *Histol and Histopathol*, 14, 11-21.
- Horn, G., Bobrow, M., Bruce, M., Goedert, M., McLean, A., and Webster, J. (2001). Review of the origin of BSE. <http://www.defra.gov.uk/animalh/bseorigin.pdf>. 1-69.
- Hunter, G. D. (1972). Scrapie: a prototype slow infection. [Review]. *The Journal of Infectious Diseases*, 125(4), 427-440.
- Hunter, N., Dann, J. C., Bennett, A. D., Somerville, R. A., McConnell, I., and Hope, J. (1992). Are Sinc and the PrP gene congruent? Evidence from PrP gene analysis in Sinc congenic mice. *J. Gen. Virol.*, 73, 2751-2755.
- Hunter, N., Foster, J. D., Dickinson, A. G., and Hope, J. (1989). Linkage of the gene for the scrapie-associated fibril protein (PrP) to the SIP gene in Cheviot sheep. *Vet. Rec.*, 124(14), 364-366.
- Hunter, N., Hope, J., McConnell, I., and Dickinson, A. G. (1987). Linkage of the scrapie associated fibril protein (PrP) and *Sinc* using congenic mice and restriction fragment length polymorphism analysis. *Journal of General Virology*, 68, 2711-2716.
- Imai, Y., Dobashi, M., and Terashima, K. (1986). Postnatal development of dendritic reticulum cells and their immune complex trapping ability. *Histology and Histopathology*, 1, 19-26.
- Jakob, A. (1921). Über eigenartige erkankungen des zentralnervensystems mit bemerkenswertem anatomischen Befunde (spastische Pseudosklerose-Encephalmyopathie mit disminierten Degenerationsherden). *Z. Gestame Neurol. Psychiatrie*, 64, 147-228.
- Jaroslav, B. N., and Nossal, G. J. V. (1968). Effects of X-irradiation on antigen localisation in lymphoid follicles. *Austrian Journal of Experimental and Biological Medicine*, 44, 609-628.
- Jeffrey, M., Martin, S., Barr, J., Chong, A., and Fraser, J. R. (2001). Onset of accumulation of PrPres in murine ME7 scrapie in relation to pathological and PrP immunohistochemical changes. *Journal of Comparative pathology*, 124, 20-28.
- Jeffrey, M., McGovern, G., Goodsir, C. M., Brown, K. L., and Bruce, M. E. (2000). Sites of prion protein accumulation in scrapie-infected mouse spleen revealed by immuno-electron microscopy. *Journal of Pathology*, 191(323), 332.
- Jessen, R. J., and Mirsky, R. (1999). Schwann cells and their precursors emerge as major regulators of nerve development. *Trends in Neuroscience*, 22(9), 402-410.

- Kapasi, Z. F., Qin, D., Kerr, W., Kosco-Vilbois, M. H., Shultz, D., Tew, J. G., and Szakal, A. K. (1998). Follicular Dendritic Cell (FDC) Precursors in Primary Lymphoid Tissue. *Journal of Immunology*, 160, 1078-1084.
- Kimberlin, R. H., and Walker, C. A. (1978). Pathogenesis of mouse scrapie: effect of route of inoculation on infectivity titres and dose-response curves. *Journal of Comparative Pathology*, 88(1), 39-47.
- Kimberlin, R. H., and Walker, C. A. (1979). Pathogenesis of mouse scrapie: dynamics of agent replication in spleen, spinal cord and brain after infection by different routes. *Journal of Comparative Pathology*, 89(4), 551-562.
- Kimberlin, R. H., and Walker, C. A. (1980). Pathogenesis of mouse scrapie: evidence for neural spread of infection to the CNS. *J. Gen. Virol.*, 51, 183-187.
- Kimberlin, R. H., and Walker, C. A. (1983). Pathogenesis of mouse scrapie: evidence for direct neural spread of infection to the CNS after injection of sciatic nerve. *J. Neurol. Sci.*, 61, 315-325.
- Kitamoto, T., Muramoto, T., Mohri, S., Doh-Ura, K., and Tateishi, J. (1991). Abnormal isoform of prion protein accumulates in follicular dendritic cells in mice with Creutzfeldt-Jakob disease. *J. Virol.*, 65(11), 6292-6295.
- Klein, M. A., Frigg, R., Flechsig, E., Raeber, A. J., Kalinke, U., Bluethmann, H., Bootz, F., Suter, M., Zinkernagel, R. M., and Aguzzi, A. (1997). A crucial role for B cells in neuroinvasive scrapie. *Nature*, 390(6661), 687-690.
- Klein, M. A., Frigg, R., Raeber, A. J., Flechsig, E., Hegyi, I., Zinkernagel, R. M., Weissmann, C., and Aguzzi, A. (1998). PrP expression in B lymphocytes is not required for prion neuroinvasion. *Nature Medicine*, 4(12), 1429-1433.
- Kocisko, D. A., Come, J. H., Priola, S. A., Chesebro, B., Raymond, G. J., Lansbury, P. T., and Caughey, B. (1994). Cell-free formation of protease-resistant prion protein. *Nature*, 370(6489), 471-474.
- Korth, C., Stierli, B., Streit, P., Moser, M., Schaller, O., Fischer, R., Schulz-Schaeffer, W., Kretzschmar, H., Raeber, A., Braun, U., Ehrensperger, F., Hornemann, S., Glaockshuber, R., Riek, R., Billeter, M., Wuthrich, K., and Oesch, B. (1997). Prion protein (PrP<sup>Sc</sup>)-specific epitope defined by a monoclonal antibody. *Nature*, 390, 74-77.
- Kosco, M. H., Pflugfelder, E., and Gray, D. (1992). Follicular dendritic cell-dependent adhesion and proliferation of B cells in vitro. *The Journal of Immunology*, 148(8), 2331-2339.
- Kosco-Vilbois, M., Bonnefoy, J.-Y., Zentgraf, H., and Gerdes, J. (1997). To 'B' or not to 'B' a germinal centre. *Immunology Today*, 18, 225-230.

- Le Hir, M., Bluethmann, H., and Kosco-Vilbois, M. H. (1996). Differentiation of follicular dendritic cells and full antibody responses require tumour necrosis factor receptor-1 signalling. *Journal of Experimental medicine*, 183(2367-2372).
- Lichtman, J. (1977). The reorganisation of synaptic connexions in the rat submandibular ganglion during post-natal development. *Journal of Physiology*, 273, 155-177.
- Lichtman, J. W., Burden, S. J., Culican, S. M., and Wong, R. O. L. (1999). Synapse Formation and Elimination. *Fundamental Neuroscience*, M. J. Zigmond, F. E. Bloom, S. C. Landis, J. L. Roberts, and L. R. Squire, eds., Academic Press, San Diego, 547-576.
- Lorains, J. W., Henry, C., Agbamu, D. A., Rossi, M., Bishop, M., Will, R. G., and Ironside, J. W. (2001). Variant Creutzfeldt-Jacob disease in an elderly patient. *The Lancet*, 357, 1339-1340.
- Marsh, R.F. and Kimberlin, R.H. (1975). Comparison of scrapie and TME in hamsters: clinical signs, pathology and pathogenesis. *J. Infectious Disease* 13, 104-110.
- Mabbott, N., Williams, A., Farquhar, C., Pasparakis, M., Kollias, G., and Bruce, M. E. (2000a). Tumour Necrosis Factor Alpha-Deficient, but not Interleukin-6-deficient mice resist peripheral infection with scrapie. *Journal of Virology*, 74(7), 3338-3344.
- Mabbott, N. A., Brown, K. L., Manson, J., and Bruce, M. E. (1997). T-lymphocyte activation and the cellular form of the prion protein. *Immunology*, 92(2), 161-165.
- Mabbott, N. A., Mackay, F., Minns, F., and Bruce, M. E. (2000b). Temporary inactivation of follicular dendritic cells delays neuroinvasion of scrapie. *Nature Medicine*, 6(7), 719-720.
- Mabbott, N.A., Bruce, M. E., Botto, M., Walport, M.J., Pepys, M.B (2001) Temporary depletion of complement component C3 or genetic deficiency of C1q significantly delays onset of scrapie. *Nature Medicine*, 7, 485-487.
- Mackay, F., and Browning, J. L. (1998). Turning off follicular dendritic cells. *Nature*, 395, 26-27.
- Manson, J. (1996). Prnp gene dosage, allelic specificity and gene regulation. *Transmissible Subacute Spongiform Encephalopathies: Prion Diseases*, B. D. Louis Court, ed., Elsevier, Paris, 239-245.
- Manson, J., West, J. D., Thomson, V., McBride, P., Kaufman, M. H., and Hope, J. (1992). The prion protein gene - a role in mouse embryogenesis. *Development*, 115(1), 117-122.
- Manson, J. C., Clarke, A. R., Hooper, M. L., Aitchison, L., McConnell, I., and Hope, J. (1994a). 129/Ola mice carrying a null mutation in PrP that abolishes mRNA production are developmentally normal. *Molecular Neurobiology*, 8, 121-127.

- Manson, J. C., Clarke, A. R., McBride, P. A., McConnell, I., and Hope, J. (1994b). PrP gene dosage determines timing but not the final intensity or distribution of lesions in scrapie pathology. *Neurodegeneration*, 3, 331-340.
- Manuelidis, L., Zaitsev, I., Koni, P., Lu, Z. Y., Flavell, R. A., and Fritch, W. (2000). Follicular dendritic cells and dissemination of Creutzfeldt-Jakob Disease. *Journal of Virology*, 74(18), 8614.
- Marshall-Clarke, S., Reen, D., Tasker, L., and Hassan, J. (2000). Neonatal immunity: how well has it grown up? *Immunology Today*, 21(1), 35-41.
- McBride, P. A., Eikelenboom, P., Kraal, G., Fraser, H., and Bruce, M. E. (1992). PrP protein is associated with follicular dendritic cells of spleens and lymph nodes in uninfected and scrapie-infected mice. *Journal of Pathology*, 168(4), 413-418.
- McBride, P. A., Schulz-Schaeffer, W. J., Donaldson, M., Bruce, M., Diringer, H., Kretzschmar, H. A., and Beekes, M. (2001). Early spread of scrapie from the gastrointestinal tract to the central nervous system involves autonomic fibres of the splanchnic and vagus nerve. *Journal of Virology (In Press)*.
- McFarlin, D. E., Raff, M. C., Simpson, E., and Nehlsen, S. H. (1971). Scrapie in immunologically deficient mice. *Nature*, 233, 336.
- McKinley, M. P., Bolton, D. C., and Prusiner, S. B. (1983). A protease-resistant protein is a structural component of the scrapie prion. *Cell*, 35(1), 57-62.
- McKinley, M. P., Mobley, W. C., Coleman, S., Peterson, M., and Prusiner, S. B. (1988). Developmental regulation of scrapie incubation times in neonatal golden hamsters. , 147.
- McLennan, N. F., Rennison, K. A., Bell, J. E., and Ironside, J. W. (2001). In situ hybridisation analysis of PrP mRNA in human CNS tissues. *Neuropathology and Applied Neurobiology*, In press.
- Merz, P. A., Somerville, R. A., Wisniewski, H. M., and Iqbal, K. (1981). Abnormal fibrils from scrapie infected brain. *Acta Neuropathologica*, 54, 63-74.
- Miele, G. (1999). Analyses of gene expression in PrnP<sup>+/+</sup> and PrnP<sup>-/-</sup> mice, , University of Edinburgh, Edinburgh.
- Mirsky, R., Parmantier, E., McMahon, P., and Jessen, K. R. (1999). Schwann cell-derived desert hedgehog signals nerve sheath development. *Annals of the New York Academy of Sciences*, 883, 196-202.
- Moore, R. C., Lee, I. Y., Silverman, G. L., Harrison, P. M., Strome, R., Heinrich, C., Karunaratne, A., Pasternak, S. H., Chishti, M. A., Liang, Y., Mastrangelo, P., Wang, K., Smit, A. F. A., Katamine, S., Carlson, G. A., Cohen, F. E., Prusiner, S. B., Melton, D. W., Trembley, P., Hood, L. E., and Westaway, D. (1999). Ataxia in prion protein (PrP)-deficient mice is associated with upregulation of the novel PrP-like protein Doppel. *Journal of Molecular Biology*, 292(4), 797-817.

Oesch, B., Westaway, D., Walchli, M., McKinley, M. P., Kent, S. B., Aebersold, R., Barry, R. A., Tempst, P., Teplow, D. B., Hood, L. E., and al., e. (1985). A cellular gene encodes scrapie PrP 27-30 protein. *Cell*, 40(4), 735-746.

Oliver, K. R., Scallan, M., Dyson, H., and Fazakerley, J. K. (1997). Susceptibility to a neurotropic virus and its changing distribution in the developing brain is a function of CNS maturity. *Journal of Neurovirology*, 3, 38-48.

Outram, G. W., Dickinson, A. G., and Fraser, H. (1973). Developmental maturation of susceptibility to scrapie in mice. *Nature*, 241, 536-537.

Pan, K. M., Baldwin, M., Nguyen, J., Gasset, M., Serban, A., Groth, D., Mehlhorn, I., Huang, Z., Fletterick, R. J., Cohen, F. E., and al., e. (1993). Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. *Proc. Natl. Acad. Sci., USA*, 90(23), 10962-10966.

Panchuk-Voloshina, N., Haugland, R. P., Bishop-Stewart, J., Bhargat, M. K., Millard, P. J., Mao, F., Leung, W.-Y., and Haugland, R. P. (1999). Alexa Dyes, a series of new fluorescent dyes that yield exceptionally bright, photostable conjugates. *Journal of Histochemistry and Cytochemistry*, 47(9), 1179-1188.

Papamichail, M., Gutierrez, C., Embling, P., Johnson, P., Holbrow, E. J., and Pepys, M. B. (1975). Complement dependence of localisation of aggregated IgG in germinal centres. *Scandinavian Journal of Immunology*, 4, 343-347.

Parmantier, E., Lynn, B., Lawson, D., Turmaine, M., Namini, S. S., Chakrabarti, L., McMahon, A. P., Jessen, K., R, and Mirsky, R. (1999). Schwann cell-derived desert hedgehog controls the development of peripheral nerve sheaths. *Neuron*, 23(4), 713-724.

Pattison, I. H., Hoare, M. N., Jebbett, J. N., and Watson, W. A. (1972). Spread of scrapie to sheep and goats by oral dosing with foetal membranes from scrapie-affected sheep. *Vet. Rec.*, 90, 465-468.

Pearson, G.R., Gruffyddjones, T.J., Wyatt, J.M., Hope, J., Chong, A., Scott, A.C., Dawson, M., Wells, G.A.H. (1991) Feline Spongiform Encephalopathy. *Veterinary Record*, 128 (22), 532.

Politopoulou, G., Seebach, J. D., Schmutz, M., Schwarz, H. P., and Aguzzi, A. (2000). Age-related expression of the cellular prion protein in human peripheral blood leukocytes. *Haematologica*, 85, 580-587.

Prusiner, S. B. (1982). Novel proteinaceous infectious particles cause scrapie. *Science*, 216(4542), 136-144.

Race, R., Ernst, D., Jenny, A., Taylor, W., Sutton, D., and Caughey, B. (1992). Diagnostic implications of detection of proteinase K-resistant protein in spleen, lymph nodes, and brain of sheep. *American Journal of Veterinary Research*, 53(6), 883-889.

- Raeber, A. J., Sailer, A., Hegyi, I., Klein, M. A., Rulicke, T., Fischer, M., Brandner, S., Aguzzi, A., and Weissmann, C. (1999). Ectopic expression of prion protein (PrP) in T lymphocytes or hepatocytes of PrP knockout mice is sufficient to sustain prion replication. *PNAS*, 96, 3987-3992.
- Ritchie, D. L., Brown, K. L., and Bruce, M. E. (1999). Visualisation of PrP protein and follicular dendritic cells in uninfected and scrapie infected spleen. *Journal of cellular pathology*, 4(1), 3-10.
- Robakis, N., Sawh, P., Wolfe, G. C., Rubenstein, R., Carp, R., and Innis, M. (1986). Isolation of cDNA clone encoding the leader peptide of prion protein and expression of the homologous gene in various tissues. *PNAS*, 1986(83), 6377-6381.
- Rose, M. L., M.S.C, B., Wallis, V. J., Forrester, J. A., and Davies, A. J. S. (1980). Peanut lectin binding properties of germinal centres of mouse lymphoid centres. *Nature*, 284, 364-366.
- Saborio, G. P., Permanne, B., and Soto, C. (2001). Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. *Nature*, 411, 810-813.
- Sartori, M., finke, D., Karapetian, O., and Acha-Orbea, H. (1999). Development of T-B collaboration in neonatal mice. *International immunology*, 11(3), 445-451.
- Shmakov, A. N., McLennan, N. F., McBride, P., Farquhar, C. F., Bode, J., Rennison, K. A., and Ghosh, S. (2000). Cellular prion protein is expressed in the human enteric nervous system. *Nature Medicine*, 6(6), 840-841.
- Sigurdson, C. J., Williams, E. S., Miller, M. W., Spraker, T. R., O'Rourke, K. I., and Hoover, E. A. (1999). Oral transmission and early lymphoid tropism of Chronic Wasting Disease PrP<sup>res</sup> in mule deer fawns (*Odocoileus Hemionus*). *Journal of General Virology*, 80, 2757-2764.
- Stamp (1962). *Vet Rec*, 74, 357.
- Stamp, J. T. Scrapie, a review of the problem. *Report of Scrapie Seminar*, Washington, D.C, 187-198.
- Syroid, D. E., Maycox, P. R., Burrola, P. G., Liu, N., Wen, D., Lee, K.-F., lemke, G., and Kilpatrick, T. J. (1996). Cell death in the Schwann cell lineage and its regulation. *PNAS*, 93, 9229-9234.
- Szkal, A. K., and Hanna, M. G. (1968). The ultrastructure and antigen localistaion and viruslike particles in mouse spleen germinal centres. *Experimental Molecular Pathology*, 8, 75.
- Szkal, A. K., Kapasi, Z. F., S.T, H., and Tew, J. G. (1995). Multiple lines of evidence favouring a bone marrow derivation of follicular dendritic cells (FDCs). *Dendrite Cells in Fundamental and cClinical Immunology*, D. S. J. Banchereau, ed., Plenum Press, 267-272.

Szakal, A. K., Taylor, J. K., Smith, J. P., Kosco, M. H., Burton, G. F., and Tew, J. J. (1990). Kinetics of germinal centre development in lymph nodes of young and aging mice. *The Anatomical Record*, 227, 475-485.

Tanji, K., Saeki, K., Matsumoto, Y., Takeda, M., Hirasawa, K., Doi, K., and Onodera, T. (1995). Analysis of PrP mRNA by in situ hybridisation in brain, placenta, uterus and testis of rats. *Intervirology*, 38, 309-315.

Taylor, D. M., McConnell, I., and Fraser, H. (1996). Scrapie infection can be established readily through skin scarification in immunocompetent but not immunodeficient mice. *Journal Of General Virology*, 77(Pt7), 1595-1599.

Timens, W., Rozeboom, T., and Poppema, S. (1987). Fetal and neonatal development of human spleen: an immunohistological study. *Immunology*, 60, 603-609.

Tkachuk, M., Bolliger, S., Ryffel, B., Pluschke, G., Banks, T. A., Herren, S., Gisler, R. H., and Kosco-Vilbois, M. H. (1998). Crucial role for tumour necrosis factor receptor 1 expression on non-hematopoietic cells for B cell localisation within the splenic white pulp. *Journal of Experimental Medicine*, 4, 469-477.

Trachtenberg, J. T., and Thompson, W. J. (1996). Schwann cell apoptosis at developing neuromuscular junctions is regulated by glial growth factor. *Nature*, 379, 174-177.

Van den Berg, T. K., Yoshida, K., and Dijkstra, C. D. (1995). Mechanisms of Immune Complex trapping by follicular dendritic cells. *Current Topics in Microbiology and Immunology*, 201, 49-63.

van Rooijen, N. (1975). Immune complexes in the spleen. *International Archives of Allergy and applied Immunology*, 49, 754-762.

Verity, C. M., Nicoll, A., Will, R. G., Devereux, G., and Stellitano, L. (2000). Variant Creutzfeldt-Jacob Disease in UK children: a national surveillance study. *The Lancet*, 356, 1224-1227.

Villena, A., zapata, A., Rivera-Pomar, J. M., Barrutia, M. G., and Fonfria, J. (1983). Structure of the non-lymphoid cells during the postnatal development of the rat lymph nodes. *Cella and Tissue Research*, 229, 219-232.

Weil, R. (1963). *PNAS*, 49, 480.

Wells, G. A. H., Scott, A. C., Johnson, C. T., Gunning, R. F., Hancock, R. D., Jeffrey, M., Dawson, M., and Bradley, R. (1987). A novel progressive spongiform encephalopathy in cattle. *Vet. Rec.*, 121, 419-420.

Will, R. G., Ironside, J., and Zeidler, M. (1996). A new variant of Creutzfeldt-Jakob disease in the UK. *Lancet*, 347, 921-25.

Yoshida, K., Kaji, M., Takahashi, T., Van den Berg, T. K., and Dijkstra, C. D. (1995). Host origin of follicular dendritic cells induced in the spleen of SCID mice after transfer of allogeneic lymphocytes. *Immunology*, 84, 117-126.

Yoshida, K., Van den Berg, T. K., and Dijkstra, C. D. (1993). Two functionally different follicular dendritic cells in secondary lymphoid follicles of mouse spleen, as revealed by CR1/2 and FcR $\gamma$ II-mediated immune complex trapping. *Immunology*, 80, 34-39.

Zlotnik, I., and Rennie, J. C. (1963). *Journal of Comparative Pathology*, 73, 150-162.

## **Appendix A: Solutions and Reagents**

### Brain Lysis Buffer

Mix 2.5g of N-Lauroyl Sarcosine (Sigma) and 2.5 mls 1M sodium dihydrogen orthophosphate in 250 mls deionised water. Store at 4 °C.

### Denhardt's Solution (X50)

1% each of BSA, Ficoll and polyvinylpyrrolidone

### DIG Buffer 1

Mix 1M Maleic Acid and 1.5M NaCl to make a X10 stock then dilute to X1.

### DIG Buffer 2

1% blocking agent (Roche) in DIG buffer 1

### DIG Buffer 3

0.1M pH9.5 Tris-HCl, 0.1M NaCl, 0.05M MgCl<sub>2</sub>

### ISH chromagen

Mix 10µl of 0.1M levamisole and 20µl of stock NBT/BCIP (Roche) in 1ml of DIG buffer 3.

### ELISA Blocking Buffer

Mix 1g BSA and 50 ul Tween 20 in 100 mls X1 PBS.

### ELISA Chromagen

1 tablet of pH 5, 0.05M phosphate-citrate buffer containing 0.03% sodium perborate (Sigma P4922) and 2 tablets of 10mg O-Phenylendiamine (Sigma P-8287) were dissolved in 100 mls deionised water.

### ELISA Coating Buffer (pH 9.6 ,0.05M)

The contents of 1 capsule of pH 9.6 carbonate-bicarbonate buffer (Sigma 3041) was dissolved in 100 mls deionised water.

### ELISA Wash buffer

Mix 5g BSA (Sigma) and 500ul of Tween 20 (Sigma) in 1l of X1 PBS.

### ICC wash buffer 1

To make 3 litres, mix 22.35g pH 7.6 Tris pre-set crystals, 26.28g sodium chloride, 6g BSA in 3 litres distilled water.

#### ICC wash buffer 2.

To make 2.5 litres, mix 5g BSA in 250mls phosphate buffer. Make up to 2.5 litres using distilled water.

#### Iodine High Salt Buffer (10% IHSB)

Mix 10g potassium iodide together with 1.5g sodium thiosulphate, 1g N-Lauroyl Sarcosine(Sigma) and 1ml of 1M sodium dihydrogen orphosphate (Sigma) in 100ml deionised water.

#### Iodine High Salt Buffer (15% IHSB)

Mix 15g potassium iodide together with 1.5g sodium thiosulphate (Sigma), 1g N-Lauroyl Sarcosine (Sigma) and 1ml of 1M sodium dihydrogen orphosphate in 100ml deionised water.

#### ISH Prehybridisation solution

Mix 500 $\mu$ l Ultrapure 100% formamide (Sigma F5786), 250 $\mu$ l of X50 SSC, 100 $\mu$ l of X50 Denhardt's solution and 100 $\mu$ l DEPC treated water. Heat 250  $\mu$ g/ml<sup>-1</sup> yeast tRNA 10 minutes before required. 250  $\mu$ g/ml<sup>-1</sup> herring sperm DNA was heated to 97 °C for 5 minutes then chilled immediately to prevent re-annealment of strands. It was then added to the prehybridisation solution.

#### ISH hybridisation solution

Mix Prehybridisation solution (see above) with 5% dextran sulphate. Add cRNA probes (see section 2.xx) at 500ng/ml-1 each.

#### NBT/BCIP

Purchased in tablet form and stored -20 °C. One tablet mixed in 10 mls distilled water immediately prior to use

#### NEM

0.313g of NEM dissolved in 25 mls propan-1-ol. Stored in foil wrapped bijoux at 4 °C

#### N-Lauroyl Sarcosine (2%)

2g of N-Lauroyl sarcosine dissolved in 100 mls water.

#### Paraformaldehyde (8%)

To make 500 mls, mix 40g paraformaldehyde with 500 mls distilled water and add 1 ml 5M sodium hydroxide. Store at 4 °C

Paraglutaraldehyde (0.5%/0.5%)

To make 100 mls of fixative, mix 6.25 mls of 8% paraformaldehyde and 2 mls of 25% glutaraldehyde in 50 mls 0.2M phosphate buffer and 41.75 mls distilled water. Stable for 1 day. Store at 4 °C

Periodate lysine paraformaldehyde

To make 100 mls of fixative: mix 75 mls of 0.05M phosphate buffer with 25 mls of 8% paraformaldehyde (mixed solution stable for 2 weeks approximately). Just before required, add 0.108g sodium periodate and 0.69g D L-Lysine (solution stable for 1 day). Store at 4 °C

Phenylmethylsulfonylfluoride (PMSF) (100mM)

0.435g PMSF dissolved in 25 mls propan-1-ol. Stored in foil wrapped bijoux at 4 °C

Phosphate buffer (0.05M)

For 400mls of buffer, mix 324 mls 0.05M disodium hydrogen orthophosphate and 76 mls 0.05M sodium dihydrogen orthophosphate. pH adjusted to 7.4. Store at 4 °C.

Phosphate buffer (0.2M)

Mix 0.2M disodium hydrogen orthophosphate with 0.2M sodium dihydrogen orthophosphate in a ratio of 4:1 and adjust pH to 7.4.

Potassium chloride (0.2M)

1.49g KCL dissolved in 100 mls distilled water. Stored at 4°C for Western blotting purposes.

RNaseA solution

20µl RNase A in 1ml of RNase wash solution

RNase wash solution

10ml of 1M Tris pH8, 2ml of 0.5M EDTA, 100ml 5M NaCl made up to 1l with deionised water.

SDS running buffer (X1)

50 mls of X10 Biorad SDS buffer (Biorad) diluted in 450 mls distilled water.

SDS sample buffer (X5)

To make 25 mls of sample buffer, mix 0.819g Tris, 1.25g SDS, 12.5g sucrose and 250ul β-mercaptoethanol in 25 mls distilled water. Mix reagents slowly in a fume hood. Dilute to X1 using deionised water for pellet resuspension prior to loading on gel.

Sodium dihydrogen orthophosphate (1M)

Dissolve 15.6g Sodium dihydrogen orthophosphate in 100 ml deionised water.

SSC (x20)

Purchased from Boehringer diluted as required.

Sucrose cushion (20%)

40% sucrose solution mixed in equal ratios with 100mM Tris-HCl pH7.4

Sucrose IHSB (20%)

Dissolve 20g of sucrose in 100mls of 10% IHSB

TE buffer

10 mM Tris-HCl (pH 8), 1mM EDTA

Tris-HCl pH7.4, pH 7.5, pH 8, pH 9.5

Trizma pre-set crystals purchased (Sigma), dissolved in appropriate volume of water

Western Blocking Agent (10%)

Mix 2g BSA in 20 mls distilled water.

Western Chromagen

Dissolve 1 NBT-BCIP (Sigma) tablet in 10 mls of distilled water

Western Transfer buffer

To make 1 litre, dissolve 2.93g glycine, 5.810g Tris, 0.375g SDS in 200mls methanol.  
Make up to 1 l with distilled water.

Western Wash buffer

Mix pH7.6 Tris pre-set crystals in 1l distilled water.