STUDIES OF RUMINANT PESTIVIRUS FETOPATHOGENICITY WITH SPECIAL REFERENCE TO THE NERVOUS SYSTEM

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LIST OF ABBREVIATIONS.

APAAP: Alkaline phosphatase anti-alkaline phosphatase.

BD: Border disease.

BF: Buffered formalin.

BVD-MD: Bovine virus diarrhoea-Mucosal disease.

CAII: Carbonic anhydrase isoenzyme II.

CK: Creatine kinase.

cm: Centimetre.

CNS: Central nervous system.

DAB: 3,3'-diaminobenzidine tetrachloride.

DIC: Differential interference contrast

EDTA: Ethylenediaminetetracetic acid.

GABA: Gamma amino benzoic acid.

GC: Galactocerebroside.

GDPH: L-glycerol 3-phosphate dehydrogenase.

GFAP: Glial fibrillary acidic protein.

GSA-B4: Griffonia simplicifolia B4.

HE: Haematoxylin and eosin.

IPX: Immunoperoxidase.

LFBCV: Luxol fast blue cresyl violet.

MAG: Myelin associated glycoprotein.

MBP: Myelin basic protein.

mg: Milligram.

ml: Millilitre.

MSB: Martius scarlet blue.

nm. Nanometer.

NS-1: Nervouse sytem antigen-1

PAP: Peroxidase-anti-peroxidase.

PAS: Periodic-acid-Schiff.

PI: Persistently infected.

PLP: Proteolipid protein.

PML: Progressive multifocal leucoencephalopathy.

PNS: Peripheral nervous system.

SSEA-1: Stage specific embryonic antigen-1.

TBS: Tris buffered saline.

TCID: Tissue culture infectious doses.

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DECLARATION

I certify that this Thesis is a record of my own work carried out under the guidance of Dr J T Done, Mr G A H Wells and Mr C J Randall. Where use has been made of the work of others it has been acknowedged.

Some of the information used in Chapters two, three, four and five has been published previously:

Chapter two, Roeder, Jeffrey and Cranwell, Veterinary Record (1986) 118, 44-48;

Chapter three, Jeffrey and Roeder, Research in Veterinary Science (1987) 43, 22-27;

Chapter four, Jeffrey and Hogg, Veterinary Record (1988) 122, 89-90;

Chapter five, Jeffrey and Wells, Veterinary Bulletin (1987) 57, 247-266.

All pathological studies reported in articles published in Veterinary Record and Research in Veterinary Science were performed by myself; virology was performed by Dr P L Roeder or the Virology Department CVL. All literature cited in Veterinary Bulletin was personally read.

SUMMARY

This thesis presents morphologic studies of naturally acquired Bovine virus diarrhoea-mucosal disease (BVD-MD) virus infections of cattle and naturally acquired and experimental Border disease (BD) virus infection of sheep. The distribution and cellular localisaton of selected immunohistochemical central nervous system glial cell markers is described for the normal sheep. Using two of these markers and a monoclonal antibody to BVD-MD virus a double immunoenzyme labelling technique was used to determine the cellular localisation of BD virus for the persistently viraemic fetal and neonatal sheep. The ultrastructural features of the peripheral nervous system (second muscular branch of sciatic nerve) of a viraemic fetus and neonate is also described.

Field studies of BVD-MD virus infection and BD virus infection showed that lesions of hydranencephaly, porencephaly, cerebellar dysplasia and atrophy, and retinopathy occur following pestivirus infections of both cattle and sheep. The infection status (presence of virus or serum neutralising antibody) of affected animals suggests a relationship between the gestational age of infection and the nature of lesions.

Animals infected early in gestation are viraemic and show either no significant histopathological changes or, mild retinopathy and/or mild granuloprival cerebellar dysplasia. These lesions are probably caused by virus induced degeneration of proliferating neuroblasts. Viraemic sheep may also show central nervous system hypomyelinogenesis (classical BD). Ultrastructural observations suggest that a deficiency of myelin may also be a feature of the peripheral nervous system of newborn lambs persistently infected with BD virus.

Animals with cystic cerebral or cerebellar cavitation are infected around midgestation and are invariably non-viraemic, most show a serological response. One experimentally infected lamb with porencephaly was shown to have viral persistence in white matter of the central nervous system but virus was not demonstrated in viscera or blood. These features suggest that cavitating lesions of the central nervous system following pestivirus infection may have an immunological basis.

Immunohistochemical markers of glial cells are widely used in neurobiology generally and have been particularly useful in elucidating glial cell differentiation pathways. Such markers are potentially useful for studies of the effect of pestivirus on the developing central nervous system. The results of experimental immunoperoxidase studies of selected glial cell markers show that glial fibrillary acidic protein, carbonic anhydrase II, myelin basic protein and galactocerebroside are conserved in the sheep. Glial fibrillary acidic protein is expressed in cells which were defined by topography and morphology as protoplasmic astrocytes, fibrous astrocytes, Bergmann glial cells, a sub-population of ependymal cells (including possibly tanycytes), amphicytes of spinal ganglia and some Schwann cells. In the 90 day ovine fetus glial fibrillary acidic protein expression was also seen in sub-pial glia. The processes of some of these cells were continuous with myelin basic protein expressing myelin sheaths. Such cells are morphologically and biochemically similar to transitional cells.

In the neonatal lamb brain myelin basic protein is expressed in myelin sheaths and in the cytoplasm of presumed myelinating oligodendroglia. These cells are found throughout the neuraxis and in all funiculi of

the spinal cord. Myelin basic protein expression, as determined by the immunoperoxidase method, was found to be a more precise and sensitive method of demonstrating initial stages of myelination than conventional histological stains. However, this method offered no advantage over tinctorial methods for examination of the subsequent advancement of myelination towards maturity.

Galactocerebroside expression was seen on frozen sections of spinal cord in populations of subpial glial cells but it was not demonstrated in fixed, paraffin wax embedded tissues.

Carbonic anhydrase II was expressed in choroid plexus epithelium and in glial cells of white matter. Double immunoenzyme labelling showed that these latter cells also expressed glial fibrillary acidic protein and were thus identified as fibrous astrocytes.

Using monoclonal antibodies raised to BVD-MD virus the immunoperoxiase method demonstrated widespread neuronal, glial cell and ependymal cell infection of the spinal cord of fetal and newborn lambs. Cells within the peripheral nervous system were also infected. Double immunoenzyme labelling showed that glial fibrillary acidic protein expressing cells and myelin basic protein expressing cells were infected with virus. On morphological and biochemical characteristics the infected cells were identified as astrocytes, oligodendrocytes and transitional cells. Morphometric studies of glial fibrillary acidic protein expressing cells and myelin basic protein expressing cells show an astrocytosis and a deficiency of myelinating oligodendroglia in the 90 day fetus. These results suggest that myelin deficiency of BD is caused by defective differentiation of oligodendroglia or oligodendroglial precursor cells.

CHAPTER ONE. INTRODUCTION

Historical Perspective

Border Disease (BD) or 'B' disease was first described as a distinct clinical entity in lambs born in the border counties between England and Wales (Hughes et al., 1959). It is a congenital viral infection of sheep (Gardiner et al., 1972) and goats (Loken et al., 1982) characterised by embryonic and fetal death which may result in abortion, mummification, or still birth and by birth of small lambs of low viability with nervous symptoms and abnormally hairy, pigmented birth coats. Following its original descriptions the disease has been reported from many other European countries, (Alenius et al., 1986; Barlow & Dickinson, 1965; Hamilton & Donnelly, 1970; Leiss et al., 1982; Loken et al., 1982) and from New Zealand (Manktelow et al., 1969), Australia (Acland et al., 1972), the USA (Jackson et al., 1972), Canada (Physick-Sheard et al., 1980) and Syria (Willsmore & Roeder, 1984).

Two distinct clinical syndromes of cattle, bovine virus diarrhoea (Olafson et al., 1946) and Mucosal Disease (Ramsey & Chivers, 1953) were eventually shown to be caused by the pestivirus bovine virus diarrhoea-Mucosal disease virus (BVD-MD). Since the earliest descriptions of the disease abortion and neonatal death has been associated with infection of susceptible pregnant cattle. Experimental infections subsequently established that the virus is teratogenic (Ward et al., 1969).

Mucosal disease is invariably fatal and occurs in cattle which have a persistent BVD-MD viraemia acquired as fetuses (Roeder & Drew, 1984; Steck et al., 1980). The pathogenesis of Mucosal disease remains

controversial but most authors agree that post-natal exposure to another pestivirus ("super infection") is important for the onset of clinical signs (Littlejohns & Walker, 1985). Not all persistently infected calves, however, die shortly after birth and some may survive for a number of years as clinically healthy but persistently viraemic animals (Coria & McClurkin, 1978). Most post-natal infections of BVD-MD naive cattle are probably inapparent (Roeder & Drew, 1984).

Aetiology

BVD-MD and BD are caused by closely similar Pestiviruses (family Togaviridae). In sheep experimentally infected with BD virus it has been shown that recipricating, neutralising and complement fixing antibodies develop to BVD-MD (Huck et al., 1975) and sera from one natural outbreak of BD possessed higher neutralising antibody titres to the NADL strain of BVD-MD than to BD (Vantsis et al., 1979). The physical properties (hydrodynamic) of BD virus and BVD-MD virus are indistinguishable (Laude, 1979). With the exception of one report which describes viral like particles in turbinate epithelium of an experimentally infected cow (Chasey & Roeder, 1981) the morphology of the viruses has not been described as viruses are difficult to visualise by means of transmission electron microscopy (Scott et al., 1977). Recently, Gray and Nettleton (1987) described structures which appeared to be virions derived from modified endoplasmic reticulum in cell cultures infected with BD virus or BVD-MD virus. BD and BVD-MD are also distantly related to Hog Cholera (syn. Classical Swine Fever) virus.

Originally BD and BVD-MD were regarded as distinct species - specific entities but the antigenic heterogencity that exists between strains of BD virus and BVD-MD virus is probably no greater than the anti-

genic differences found among BVD-MD virus strains (Terpstra, 1985). Furthermore, infection of cattle with BD virus causes abortion and cerebral cavitation (Gibbons et al., 1974) and BD has been reproduced in sheep following infection with BVD-MD (Barlow et al., 1980b; Terlecki et al., 1980). Marked differences in pathogenicity for the ovine fetus have been observed with various BVD-MD virus strains (Snowdon et al., 1975). In this thesis, therefore, the designation BD virus or BVD-MD virus indicates that the isolate is respectively of ovine or bovine origins.

Transmission

Experimentally BD has been transmitted by several inoculation routes (sub-cutaneous, intramuscular, intraperitoneal, intravenous, intranasal) but it is likely that oral and intranasal routes are the most important in natural cases. Transmission of BD (Gardiner & Barlow, 1981) and BVD-MD (McClurkin et al., 1979; Meyling & Jensen, 1988) may occur by infected semen and, in persistently infected dams, there may be transovarian infection of the fetus (Barlow et al., 1980a; Westbury et al., 1979). Recently persistent viraemia has been detected in calves derived from embryo transfers (Liess et al., 1987).

Extensive outbreaks of disease have also been reported following the use of BVD-MD virus infected vaccines (Lohr et al., 1983). Interspecies infection occurs (French et al., 1974) and infected cattle may be a source of infection for sheep (Physick-Sheard et al., 1980).

Persistently infected lambs shed virus from all body secretions (Terpstra, 1978 & 1981). Such sheep may not show overt signs of disease (Terpstra, 1981; Westbury et al., 1979) and are a potent source of infection for in-contact animals.

Lateral transmission of infection after abortions in the first half of gestation may occur but spread is probably limited when sheep are kept at pasture (Plant et al., 1977). Experimental studies have shown that BD virus may persist in intra-abdominal cells of flies for up to 72 hours after blood sucking viraemic sheep and it is, therefore, theoretically possible that mechanical transmission of virus may occur under conditions of natural infection (Al-Muarrawi, 1986).

Pathogenesis

Fetal infection occurs following BD or BVD-MD viraemia and subsequent virus replication in the placentome where it multiplies to high titres (French et al., 1974; Snowdon et al., 1975). This maternal infection usually runs a subclinical course. Following BD virus infection of sheep vascular endothelial necrosis and epithelial degeneration of the maternal placentae (caruncle) has been observed (Barlow, 1972). Necrotic maternal tissue is subsequently phagocystosed by fetal trophoblast epithelium and it has been suggested that virus may pass to the fetus within cellular debris (Barlow, 1972). Other studies, however, indicate that virus may also pass the placental barrier without causing lesions (French et al., 1974; Parsonson et al., 1979). In view of the inconsistent nature of the lesions described by Barlow (1972), it is likely that placental necrosis is not of major importance for transmission of infection to the fetus.

Once the virus gains access to the fetus a generalised infection is established and virus and antigen may be demonstrated in most organs (Ohmann, 1982; Terpstra, 1978).

The ultimate outcome of infection is dependant upon a number of as yet incompletely defined factors which include the strain and dose of

virus, the genotype of the host and the developmental age of the fetus. Virus strain differences cause variable clinical signs and teratogenic defects of both cattle and sheep.

Plant et al. (1983b) described clinical variations of BD according to the source of the inoculum. Lambs born to ewes infected with inocula obtained from a lamb showing a hairy birth coat delivered lambs with a similar fleece but lambs born to ewes infected with tissue from a lamb with tremor and hairyness delivered lambs which also had both nervous disease and a hairy fleece. The BD virus strain BP-77 causes hypomyelinogenesis but strain H-77 produces hydranencephaly and arthrogryposis in the same breed of sheep (Barlow et al., 1979). In fetal calves older that 100 days gestation the NADL strain of BVD-MD did not cause significant lesions (Kendrick, 1971) but the Holmes strain caused cerebellar hypoplasia and occular lesions.

The dose of virus used may influence the outcome of infection (Richardson et al., 1976) and there is also some evidence to show that host genotype may affect the nature of disease. Following experimental infections with the same virus strain Barlow et al., (1979) described typical BD in lambs born of two breeds of sheep but no disease in lambs born to two other breeds. The numbers of animals used in this experiment were small and further corroboration of the effects of host genotype are required.

The gestational age of infection is also important in determining the outcome of infection. Early gestational infections prior to the onset of immune competence cause persistent infections of both cattle and sheep and result in persistent viraemia. Such persistently viraemic states may be induced by infections of cattle from approxi-

mately 40 to 125 days gestation (McClurkin et al., 1984; Orban et al., 1983). Persistent viraemic states have been caused in lambs by vertical transmission and from maternal infections occurring between 21 and 72 days gestation (Barlow et al., 1980a; Gard et al., 1976; Plant et al., 1976a&b; Plant et al., 1983a; Sweasey et al., 1979; Terpstra, 1981).

Teratogenic Effects

Reproductive Loss

Since the earliest descriptions of disease abortion and reproductive loss, of both cattle and sheep, have been one of the most consistent features of the disease. Reduced conception rates have been observed in seronegative cows exposed to BVD-MD virus at the time of breeding, either experimentally by intrauterine administration of virus suspension (Whitmore et al., 1981) or naturally by venereal contact with a virus shedding bull (McClurkin et al., 1979).

Cattle did not conceive to a persistently infected bull until after seroconversion occurred (McClurkin et al., 1979). Prolongation of the oestrus cycle was not observed and experimental intrauterine infections of cattle at oestrus showed reduced collection rates for fertilized ova (Grahn et al., 1984). This suggests that fertilization failure is the principal manifestation of reproductive loss following BVD-MD virus infection of seronegative cattle at oestrus.

Abortions, which are thought to be the result of fetal infections since maternal infection is usually asymptomatic, are probably most common in early gestational infections (Kendrick, 1971; Lewis et al., 1970; Manktelow et al., 1969).

Neuropathology and Neurochemistry

The morphologic teratogenic defects of pestivirus infection of the CNS are protean. The principle central nervous system (CNS) defect of classical BD is a deficiency of stainable myelin (Markson et al., 1959). Ultrastructural examination of such cases show that axons are invested with abnormally thin myelin sheaths composed of disproportionatly few myelin lamellae for a given axon diameter (Cancilla & Barlow, 1968). Splitting of myelin lamellae at the intraperiod line, persistence of abaxonal tongues of oligodendroglial cytoplasm and formation of lamellar lipid profiles within the myelin sheath are also reported (Barlow & Storey, 1977b; Cancilla & Barlow, 1968).

In surviving BD affected lambs myelination proceeds and clinical recovery is observed. Formed myelin sheaths thicken and, in one study resolution of hypomyelinogenesis was complete by six months (Cancilla & Barlow, 1971). Myelin deficits and neuroglial changes are found in lambs not showing clinical signs of tremor which suggests that the cause of neurological signs is unrelated to the observed histopathological changes (Barlow & Dickinson, 1965; Plant et al., 1976a).

Morphologically abnormal glial cell nuclei, lying in clusters or in dense chains, particularly around blood vessels, (sometimes referred as "hypergliosis of myelination": Osburn et al., 1972) are also frequently present. The nature of these cells is unclear. They have been variously categorised as being of microglial origin (Barlow & Dickinson, 1965); showing ultrastructural characteristics of astrocytes (Cancilla & Barlow, 1968) and resembling the "third neuroglial cell type" (Barlow & Storey, 1977a). These latter cells are considered to represent a multipotential glial cell. Although Barlow

and Storey (1977b) showed that a normal density of glial cell nuclei is present in spinal cord of BD fetuses they suggest that a diminished number of mature oligodendroglia are present, and it is suggested that the abundance of Type III glial cells represents a deviation in the normal differentiation pathways. Instead of small glioblasts differentiating to oligodendroglia, Type III glial cells are formed instead (Barlow & Storey, 1977b). However, the classification of Vaughn and Peters (1968) upon which the interpretation of this excellent morphologic study is based is no long widely accepted and the Type III glial cell is now considered to be a resting microglial cell (Peters, Palay & Webster, 1976).

Intrafascicular lipid is consistantly reported by workers at Moredun Research Institute (Barlow & Dickinson, 1965; Storey & Barlow, 1972) but is not reported by other authors.

Neurochemical analyses of spinal cord in BD affected lambs, have shown myelin dysgenesis. The neurochemical abnormality found, however, was shown to differ with clinical signs. Spinal cords of "Hairy" lambs are deficient in cerebroside and other lipids but spinal cords of "Shakers" were characterised by the presence of cholesterol esters. A deficiency of lipids was not an invariable feature of "shakers" (Patterson et al., 1971).

Detailed studies of "Hairy-Shakers" spinal cord show deficiencies in DNA, indicating a sub-normal total of cells, and the constituent long chain fatty acids of cerebrosides were deranged. Fewer than normal unsaturated long chain fatty acids were present. This form of myelin is probably unstable and may break down readily (Patterson et al., 1975). Myelination is normally a biphasic process. with early

myelin being richer in copper than mature myelin. The concentration of copper in myelin in BD affected lambs is abnormally high and it suggested that the myelin laid down in BD is immature and therefore unstable (Patterson et al., 1975).

Thus, neurochemical studies of "hairy-shaker" spinal cords showed that deficiences of stainable myelin are accompanied by a reduced total number of cells and myelin dysgenesis associated with a complex remodelling process of demyelination and remyelination. Myelin formed during fetal development in BD is probably inherently unstable.

Restricted evidence of hypomyelinogenesis with traces of interfascicular lipid have been reported in newborn lambs whose dams were inoculated at up to 120 days of gestation (Gardiner & Barlow, 1972). The response to infection at stages of gestation later than 90 days and even in newborn lambs may result in nodular periarteritis (Zakarian et al., 1975 & 1976). These lesions principally affect the arteries and arterioles of the meninges but are also present in other tissues. Lymphocyte and macrophage infiltration of adventitia and mild subendothelial and medial degeneration is present.

Although myelin dysgenesis occurs frequently with BD virus of sheep there are few reports of abnormalities of myelination following BVD-MD virus infection of cattle.

Done et al. (1980) described neurochemical evidence of dysmyelination in nine of ten calves experimentally infected at 100 days gestation and in two of these calves deficiencies of stainable myelin were demonstrated histochemically. Neurochemical abnormalities consisted of low total lipids, low cholesterol and low lipid hexose but the presence of cholesterol esters indicative of myelin degeneration was

not seen. Although not presented in this thesis the author has also seen deficiencies of stainable myelin in calves experimentally infected in-utero at 80 days gestation with a mixture of nine field stains of BVD-MD virus (Harkness et al., 1987). As with BD of sheep such lesions are associated with a persistent viraemia in affected calves. Deficiencies of myelin, abnormal glia and the presence of intrafascicular sudanophilic lipid have also been seen in five calves following naturally acquired infection (Binkhorst et al., 1983). BVD-MD antigen was demonstrated in tissues from four of five of these calves (Straver et al., 1983). Clinical neurological signs diminished with age but, unlike BD, deficiencies of stainable myelin persisted and moderate hypomyelination was found in a bull necropsied at 18 months of age (Binkhorst et al., 1983).

In recent years it has become increasingly recognised that bovine fetuses exposed to BVD-MD virus infection may show a complex of severe CNS and ocular lesions. The evaluation of the effects of BVD-MD has been inhibited by use of inappropriate terms to describe CNS defects. Nevertheless severe and moderate CNS lesions occur often with surprisingly little clinical effect. Hydranencephaly (defined as a lesions occurring as a consequence of a prenatal process which results in a reduction of the cerebral hemispheres to membranous sacs; Innes & Saunders, 1962) and porencephaly (defined originally as a defect extending from the cerebral hemispheres into the subjacent ventricle but now used to describe any excavation or cyst occurring in a young individual; Greenfield, 1959) are described following naturally occurring disease (Axthelm et al., 1981; Badman et al. 1981; Markson et al., 1976). Microencephaly and hydrocephalus are also reported infrequently (Done et al., 1980).

Cerebellar atrophy, dysplasia or hypoplasia may be induced following experimental (Brown et al., 1973 & 1974) or field infections (Axthelm et al., 1981; Markson et al., 1976). Experimental infections show granuloprival cerebellar hypoplasia which is characterised by severe granule cell loss and Purkinje cell heterotopia. More severe cortical destruction and cavitation of folial white matter was also present. Following experimental infection and surgical removal of fetuses Brown et al. (1974) described inflammation of meninges, folial oedema and necrosis of external granule cells. These observations led Brown et al. (1974) to conclude that the residual lesions should be classed as cerebellar atrophy as the process involves degeneration of preformed tissues. Not all strains of virus cause severe cerebellar atrophy. In at least some naturally occurring cases of BVD-MD infection lesions are mild and restricted to a reduction of molecular layer and internal granule cell layer and displacement of Purkinje cells (Ohmann, 1984).

Although cystic cerebral cavitation and cerebellar dysplasia are frequently recognised with BVD-MD infection similar lesions have not previously been recorded following naturally occurring BD virus infection of sheep. Several investigations, however, have described such lesions following experimental infections (Barlow, 1980; Plant et al., 1976a&b). The cerebral lesions are essentially the same as those described for BVD-MD infection with the exception that cysts of septum pellucidum were frequently encountered. Severe hydrocephalus was recorded in one case (Barlow, 1980). With the exception of one report (Plant et al., 1983b) myelin deficits have not been found in association with hydranencephaly but are seen along with porencephaly and cysts of the septum pellucidum. Lissencephaly (literally "smooth

brain"; a brain without convolutions) and white matter degeneration consisting of bilateral cerebral leucomalacia has also described following experimental infections from 19-72 days gestation (Plant et al., 1983b). These lesions were also associated with hypomyelinogenesis. Occasionally cystic cavitation of cerebellar white matter was also found in BD affected sheep. The principal cerebellar lesion is, however, cortical dysplasia consisting of rudimentary folia containing islands of germinal cells surrounded by rosettes of Purkinje cells and granule cells (Barlow, 1980).

Contradictory evidence of the pathogenesis and morphogenesis of these lesions is reported. Barlow (1980) suggests lesions are the result of necrosis in germinal cells and subtle developmental deviations but Clarke and Osburn, (1978) propose widespread inflammation and post inflammatory changes. The pathogenesis of these lesions has not yet been resolved.

Ocular lesions.

The developing eye is also susceptible to BVD-MD virus infection. In cattle ocular abnormalities of optic neuritis, cataract microphthalmia and retinal dysplasia have been described following experimental infections from 79-150 days of gestation (Bistner et al., 1970). The location of cataracts is variable and is recorded as equatorial, anterior and sub-capsular (Bistner et al., 1970; Ohmann, 1984). Milder lesions occurring with or without cataract or severe multifocal or total retinal atrophy have also been described (Barlow et al., 1986; Brown et al., 1975; Ohmann, 1984). Brown et al. (1975) studied the development of ocular lesions in fetuses infected at 90 and 150 days gestation. Acute retinal lesions were seen from 17 days post infection and were characterised by mononuclear cell infiltration,

retinitis, choroidits and proliferation of retinal pigment epithelium.

In one naturally occurring outbreak of BVD-MD infection in Germany hypochromasia of the iris was observed in association with hydranencephaly and cerebellar dysplasia in five calves (Brentrup et al., 1985).

Lesions of other tissues and organ systems.

Radiographically the skeletal system of newborn calve animals shows radio-opaque lines which are non-specific indications of fetal insult and growth retardation (so-called "growth arrest lines") (Terlecki et al., 1973; Done et al., 1980). Brachygnathia and arthrogryposis are rare sequels to both BD virus and BVD-MD infection (Ohmann, 1984; Plant et al., 1983 a & b; Vantsis et al., 1980).

Visceral lesions are not a consistent finding but in cattle myocarditis (Brown et al., 1974) and glomerulonephritis (Cutlip et al., 1980) have been reported. Persistently viraemic cattle may show enlargement of renal glomerular mesangium with IgM and C_3 deposits (Hewicker et al., 1987). Renal hypoplasia was present in a small number of experimentally produced BD lambs (Barlow & Gardiner, 1969). Axthelm et al. (1981) also recorded the presence of birefringent crystals in renal cortex of BVD-MD affected cattle. The nature of these crystals was not determined. Studies of congenitally deformed calves have shown that oxalate, which is also a birefringent crystal, is found in renal tubules of newborn calves following a wide range of teratogenic defects particularly those which involve much remodelling of skeletal elements (Gopal et al., 1978). It is likely that the crystals described by Axthelm et al. (1981) are of a similar composition.

Abnormalities of skin and hair occur in both cattle and sheep. In BD

hair follicles are enlarged and an increased frequency and degree of primary hair fibre medullation is present (Derbyshire & Barlow, 1976). This is the cause of the "hairyness" seen in classical BD. Skin and hair lesions of cattle are rare but alopecia and hypotrichosis (Casaro et al., 1971) and dermatitis have been described (Kendrick, 1971).

Nature of investigations and relevance of principal observations

Previous studies have shown that naturally acquired infections of pregnant cattle with BVD-MD may cause ocular and cerebral malformations or, more rarely, hypomyelinogenesis of the newborn calf. Naturally acquired infections of pregnant sheep with BD may cause hypomyelinogenesis. The morphogenesis of cerebral cavitation has been described in the fetal calf (and also for sheep following experimental infections with selected strain(s) of pestivirus) and the morphogenesis of spinal cord hypomyelination is described for the sheep. However, the pathogenesis of these lesions is little understood. This thesis presents observations of naturally acquired BVD-MD and BD virus infections and experimental BD virus infection of sheep which explore the pathogenesis of cerebral and ocular lesions of cattle and sheep and hypomyelination of sheep. A hypothesis to explain the protean manifestation of pestivirus infections of the CNS is suggested. The possibility of peripheral nervous system (PNS) hypomyelination is also investigated.

Chapters two and three describe the epidemiology, pathology and infection status of animals from naturally occurring outbreaks of BVD-MD of cattle and BD of sheep. A survey of the infection status (presence of virus or serum neutralising antibody) of animals with lesions of cerebral cavitation, cerebellar dysmorphogenesis, retino-

pathy or cataract selected from accession material presented to the diagnostic pathology unit at the Central Veterinary Laboratory is described in chapter four. These studies show that ocular and cerebral lesions may be seen following naturally acquired BD virus infection of sheep. Hence, naturally acquired infections with the viruses of BD and BVD-MD produce the same range of lesions in their respective hosts, albeit the frequency with which specific lesions are found differs between them. A novel photoreceptor cell atrophy of cattle is described. The studies also show that transmission of infection through a flock or herd may be rapid.

Few previous studies have detailed both the infection status and CNS pathology of individual animals. The studies reported here show that animals with gross cavitation of the cerebrum have usually responded immunologically and are invariably non-viraemic. At least some such animals show preservation of virus in CNS white matter at birth. Viraemic animals may show no significant changes, mild lesions of the cerebellum or eye or, in sheep, hypomyelinogenesis.

Immunohistochemical markers of glial cells of CNS are widely employed in neurobiology generally and some are commercially available. Their possible application for study of the developing CNS in pestivirus infections prompted a review of the literature to identify potentially suitable markers. Subsequently, an immunohistochemical investigation of the distribution and cellular localisation of four selected markers in sheep was performed. These studies showed glial fibrillary acidic protein is expressed in astrocytes and related cell types; myelin basic protein is expressed in myelinating oligodendroglia and myelin; carbonic anhydrase II is expressed in fibrous astrocytes and choroid plexus epithelium; galactocerebroside is expressed in some sub-pial

glial cells of the fetal lamb spinal cord.

Two of these markers, GFAP and MBP, were used to study glial cells of fetal and neonatal lamb brains whose dams were experimentally infected with BD virus. Using a double immunolabelling technique BD virus was shown to infect astrocytes and oligodendrocytes of persistently viraemic animals and of a porencephalic, seropositive, non-viraemic, newborn lamb. Transitional cells were infected in fetuses. Quantitative analysis of GFAP expressing and MBP expressing cells of viraemic sheep show an astrocytosis and a deficiency of myelinating oligodendroglia. These results conflict with those of a previous morphometric study.

There are only two reports in the literature of the effect of BD virus on the PNS and both of these were performed on formalin fixed material. One of these studies suggested that myelin defects might also occur in the PNS.

An ultrastructural study of the PNS of a BD virus infected fetus and newborn lambs is presented. The results obtained are ambiguous but some evidence to suggest that there may be deficiencies of PNS myelin was found.

General materials and methods

Animal accommodation and disease security.

Persistently infected sheep and calves were housed in enclosed disease secure accommodation at the Central Veterinary Laboratory or Worcester Veterinary Investigation Centre. At the Central Veterinary Laboratory staff attending infected animals did not attend uninfected groups but at Worcester, where only one animal attendant was available, care was taken to prevent transmission of infection by

examining and sampling infected sheep last.

Sampling methods.

Five millilitres clotted and EDTA treated blood samples were obtained from jugular or cephalic veins using evacuated tubes. Fetal blood samples were taken following euthanasia of the dam and hysterotomy. For each fetus the umbilical vein was exposed and incised and blood collected into a sterile universal container.

Samples of nasopharyngeal mucosal cells were obtained by using Belmont brush swabs in yearling and adult cattle and laryngeal swabs were used for young calves. For transport, harvested cells were transferred to five ml of Eagle's minimal essential medium supplemented with 10 per cent fetal calf serum (BVD-MD virus and antibody free) and antibiotics.

Virus antigen detection.

Nasopharyngeal cells were washed in 0.01M phosphate buffered saline pH 7.6 and resuspended in 5µl of buffered saline. Fifty µl aliquots of the cell suspensions were dried on to the wells of Teflon coated slides. The cell preparations and, where sampled, 6 µm thick sections of frozen tissues were fixed in cold acetone, exposed to fluorescine isothiocyanate-conjugated bovine antiserum to BVD-MD virus and counterstained with Evans's blue stain.

Virus isolation.

Tissues and blood clots were ground with sterile sand, suspended in tryptose phosphate broth with antibiotics and clarified by centrifugation. Buffy coat cells, harvested from EDTA treated blood samples were suspended in sterile Dulbecco phosphate buffered saline containing antibiotics.

Duplicate Leighton tube cultures of calf kidney cells were established by seeding simultaneously 1.2 ml aliquots of cell suspension with 200 µl aliquots in inoculum. After four or five days in culture the coverslips were removed, washed briefly in phosphate buffered saline, fixed in acetone and stained with conjugated antiserum.

Serology.

Sera were tested in a microtitre serum neutralisation test against 100 median cell culture infectious doses of NADL strain BVD-MD virus. Sera were stored at -20°C prior to analysis and were inactivated by heating for 30 minutes at 56°C in a water bath before being tested.

Fixation and tissue processing.

At necropsy tissue samples were taken from a full range of tissues and in each case the brain, spinal cord and eyes were fixed *in toto*. Eyes were fixed in Zenker's solution for two hours at full strength, two hours at half strength, washed in running tap water for 12 to 14 hours and placed in 70 per cent ethanol until processed further. All other tissues were fixed in neutral phosphate buffered ten per cent formalin. Following fixation CNS tissue was sampled in the standardised fashion used routinely at the Central Veterinary Laboratory. Half coronal sections of the left side of cerebrum were taken from the frontal cortex at the level of the head of the caudate nucleus, parietal cortex at the optic tract; occipital cortex at the hippocampus; midbrain at the rostral colliculi; medulla at the cerebellar peduncles and obex and spinal cord was sampled at segments C_3, T_1 and L_{5-6}. A saggital slice of cerebellum was also removed In addition to these samples, sections were also taken from lesion sites. Tissues were processed to paraffin wax sectioned at 5 µm and routinely stained with haematoxylin and eosin. Luxol fast blue

cresyl violet (LFBCV) was used for histochemical staining of myelin. Details of other specialised fixation, tissue processing and staining processes are given in their relevant chapters.

CHAPTER TWO

AN INVESTIGATION OF NATURALLY OCCURRING PESTIVIRUS FETOPATHOGENICITY IN CATTLE : CHANGING SEQUELAE WITH FETAL MATURATION

INTRODUCTION

Of the viruses known to cause reproductive failure in cattle the pestivirus, BVD-MD virus, is now widely considered to be the most important viral pathogen of the bovine fetus in Britain (Done et al., 1980; Roeder & Drew, 1984). Studies of natural and experimentally induced disease have reported a wide range of teratogenic defects. Attention has usually been drawn to naturally occurring outbreaks by the arresting nature of the pathological lesions. Classically some combination of ocular defects (Bistner at al., 1970; Brown at al., 1975), cerebellar dysplasia (Brown et al., 1973, and 1974) and porencephaly or hydranencephaly (Axthelm et al., 1981; Badman et al., 1981; Horvath, 1976; Markson et al., 1976) is described. The factors influencing the pathogenesis of the widely differing clinical and pathological abnormalities are, however, not understood clearly.

In naturally occurring outbreaks of BVD-MD virus infection information relating to the source of infection, rate of spread and gestational age of infection have not usually been available. Presented here are the results of an investigation of one incident of a reproductive disorder in which the relationship between the gestational age of fetuses at the time of introduction of infection and the characteristics of the ensuing lesions indicated that increasing fetal maturity modulated the fetopathogenicity of BVD-MD virus.

MATERIALS AND METHODS

The cattle and their history

The herd comprised 60 Ayrshire milking cows calving throughout the year after artificial insemination or service by a bull for persistent repeat breeders. The only addition to the herd in 1982 was a

down-calved heifer which was introduced to the milking herd on October 10th. The heifer did not milk well and in March 1983 developed diarrhoea progressing to dysentery and death ten days later. Abomasal ulceration was seen at necropsy by the farmer's veterinarian. Although no examination of this animal was possible it is suspected that she was persistently infected with BVD-MD virus and was the source of infection for the herd. All gestational ages quoted in this chapter were calculated from the presumptive date of the introduction of infection.

A reproductive problem was first indicated by five abortions in December 1982; a further three abortions occurred between February and July 1983. Cows inseminated between January and March 1983 returned to service several times and calves conceived between April and July 1982 were noticed by the farmer to be small and lacking in vigour when born between February and 1 April 1983.

Investigations Performed

In June 1983 a detailed examination of herd records was initiated to determine when infection might have occurred; subsequently the serological and virological status of calves which had been in utero at the time of arrival of the suspect heifer was examined. In July 1983 blood samples and nasal swabbings were collected from all calves born between October 1982 and June 1983 (and, thus conceived between January and October 1982) to explore their infection status by examining for neutralising antibody and the presence of infectious virus. Four calves were selected for necropsy examination on 16th August on the basis of putative gestation age at infection and infection status. The farm was revisited in November 1983 to gather

information on events occurring in the herd and to sample all additional calves born after July 1982 so that their infection status could be examined.

Examination of purchased calves

On arrival at the laboratory the four calves, identified as JC1, 103, 93 and 94, were examined clinically, blood sampled and killed by barbiturate anaesthesia. At necropsy samples were taken from a full range of tissues. Tissue sampling and virological techniques were as outlined in general materials and methods. Additional special staining techniques of von Kossa, phosphotungstic acid-haematoxylin for astrocytes and a trichrome stain were used as required. Tissue samples from the right globe of calf 93 were also embedded in araldite (Ciba-Geigy), sectioned at 1 µm and stained with toluidine blue.

RESULTS

Overt sequelae to infection

Abortions occurred only in cows which were at less than 100 days of gestation, or, conceived soon after the introduction of infection to the herd (Table 1. and Fig. 1a & b). The group of calves which were reported stunted and lacking in vigour were between 81 and 168 days of gestation when infection of their dams is likely to have been initiated (Fig 1a-1c).

Infection status of the calves

The fate of each fetus conceived between January and November 1982 and the infection status of each surviving calf are recorded in Table 1 together with the gestational age at the putative time of introduction of infection. Figure 1 represents graphically the relationship between introduction of the suspect heifer and fate of calves

born subsequently.

Viraemia and excretion of virus was detected in five calves which were all presumed to have been infected before 100 days of gestation. No serological or virological evidence of infection was found in seven calves conceived between January and February 1982, and two calves conceived in October 1982. All the remaining 19 calves were seropositive with titres ranging from 1/10 to 1/480. Examination of the progeny of another 12 cows which conceived between November 1982 and January 1983 (and therefore after the introduction of infection) revealed no evidence of abnormality.

Two of the calves selected for necropsy were persistently infected (JC1 and 103) having been at 25 and 31 days of gestation respectively at the putative time of introduction of infection. BVD-MD virus was isolated from leucocytes and antigen was visualised in the kidney and thyroid cells of both these seronegative calves in confirmation of their earlier identification as persistently infected. In contrast all virus isolation and antigen detection attempts were negative in seropositive calves 93 and 94 which had been at 153 and 146 days of gestation respectively at the time of introduction of infection.

Clinical Signs and Gross Examination of Purchased Calves

Persistently infected calves JC1 and 103 were both small and displayed muscular tremor which was most clearly visible around the head while calves 93 and 94 only exhibited stunting.

Gross examination of viscera was unremarkable in all calves with the exception of congestion and collapse of the antero-ventral lobes of the lungs in calf JC1 as is commonly seen in calves necropsied rout-

inely at this laboratory.

Abnormalities of the globes were seen only in calf 93 in which multifocal areas of retinal thinning (0.1 to 0.8 cm diameter) were present throughout the tapetal and non-tapetal retinas of both globes (Fig. 2).

The cerebellar whole brain weight percentage of calves 93 and 94 were low at 8.4 and 7.8 per cent respectively compared to more than 10 per cent for each of the other two calves. The only other grossly visible abnormality was flattening and distortion of the dorsal cerebellar vermis in calf 94.

Histopathology

Histological examination revealed mild cerebellar dysplasia in calves 93 and 94 with multifocal areas of rarefaction and narrowing of the internal granule cell layer. Small cysts centred in the white matter of the peripheral folia of cerebellar lobules VII (Fig. 3) and VIII of calf 94 corresponded to the areas of distortion seen at gross examination and were lined by astrocytes. Focal glial scars were also present in folia unaffected by cysts.

In calf 93 much of the retina was histologically unremarkable with a photoreceptor nuclear layer 7 to 10 cells deep. However multifocal areas of photoreceptor cell atrophy sometimes accompanied by thinning and rarefaction of the inner nuclear cell layer were present. In the most severe lesions the photoreceptor layer was replaced by glial and connective tissue plaques (Fig. 4). Invasion of the sensory retina by retinal pigmented epithelial cells, foci of retinal disorganisation (rosette) (Fig. 5), minute foci of mineralisation and multifocal depigmentation of the retinal pigment epithelium in tapetal

zones were also present.

No significant histological lesions were detected in calves JC1 and 103. The correlation between pathological and virological findings is summarised in Table II.

DISCUSSION

The source of infection in this outbreak cannot be established with certainty but the history of poor productivity and death from dysentery after protracted illness of the introduced down-calved heifer suggests strongly that this animal was persistently infected with BVD-MD virus. The proposition that she was the source of the infection for other cattle is further supported by the temporal relationship between her introduction, the abortions and the subsequently demonstrated persistent infections in calves which had been *in utero* at the time. This heifer also represented the only introduction of new stock to the farm in the preceeding year. Previous studies have shown that persistent infections are generated only by infection of the fetus at up to 125 days of gestation (Liess *et al.*, 1984; McClurkin *et al.*, 1984) and in the present study only the cows which had been pregnant for 81 days or less at the time of introduction of the suspect heifer subsequently produced viable persistently infected progeny. Abortions only occurred in fetuses which were less than 99 days old at the time of introduction of infection. This is in agreement with previous studies which suggest that abortions following pestivirus infections of both cattle and sheep are most frequent in the first trimester of pregnancy (Barlow & Gardiner, 1969; Kendrick, 1971).

The precise time of infection of the fetus cannot be determined exactly because transmission to individual cows and transmission within them to the fetuses would have involved an unpredictable delay. However, the indications are that transmission occurred rapidly and fetuses became infected within a very short time of introduction of the heifer; other studies have shown that approximately seven days are required for transplacental transmission to occur in sheep (Hadjisavvas et al., 1975; Parsonson et al., 1979). Assessments of the gestational ages of the fetuses at exposure are therefore approximate but are none-the-less of value in interpreting the events which occurred in this herd. The apparent rapid spread of infection through the herd in this case is in contrast to the much slower rate of spread (two and a half years) of infection reported by Barber et al., (1985).

These findings illustrate the serious consequences of introducing infection to pregnant cows in the first 100 days of gestation. Of 18 cows in this category seven aborted, five produced persistently infected progeny, and six appeared to have escaped obvious damage. In all, 39 cows were pregnant at the time of introduction and the progeny of 18 were clearly damaged. Apart from one abortion affecting a cow which conceived 23 days after the introduction of infection all the progeny of cows which conceived later appeared to have escaped damage and probably infection.

It is of considerable diagnostic significance that the persistently infected calves in this study lacked macroscopic and microscopic lesions as did those reported by other workers (Binkhorst et al., 1983; Done et al., 1980). The lesions reported by Binkhorst et al., (1983) to accompany persistent infection in calves, namely hypo-

myelinogenesis and the presence of abnormal glia, closely resemble those occurring in BD of sheep. Myelin defects detectable at birth by relatively insensitive histological techniques resolve rapidly and quickly become undetectable. By 20 weeks of age there is almost complete neurochemical recovery (Sweasy & Patterson, 1979). Thus, the failure to detect deficiencies of stainable myelin in the two trembling calves necropsied at three months of age was not unexpected and did not imply that such lesions were not present, at least at birth. Indeed the clinical sign of tremor indicated that a neuromuscular defect was present. Evaluation of cholesterol esters which correlates with the clinical sign of shaking in lambs with BD (Patterson, 1982) may have been more rewarding.

In contrast, in the calves which were able to eliminate infection after infection in the second trimester, presumably by an active immune response, the lesions were more severe and were characterised by atrophy and degeneration in neural tissue. It is well established that calves infected *in utero* via their dams from as early as 94 but more commonly 100 days of gestation can respond immunologically and clear themselves of infection (Braun *et al.*, 1973; Done *et al.*, 1980; Duffell *et al.*, 1984; Kendrick, 1971; Liess *et al.*, 1984) Orban *et al.*, 1983; Ward *et al.*, 1969). The pattern is seen in many other studies of BVD-MD virus fetopathogenicity (Braun *et al.*, 1973; Brown *et al.*, 1973, 1974 & 1975; Scott *et al.*, 1973) from which it may be concluded that dysplastic central nervous system lesions are a common sequel to maternal infections occurring at between 94 and 150 days of gestation, whereas persistent infections accompanied occasionally by lesions of dysmyelinogenesis (Done *et al.*, 1980) are generated by infection in early gestation at up to

125 days. Thus, it would appear that the degenerative dysplastic or atrophic lesions encountered after infection are associated temporally with, and conceivably induced by, even if only in part, the early immune response of the fetus to the virus infection.

Previous studies of ocular lesions caused by BVD-MD show two types of lesions. The first ocular changes attributed to BVD-MD infection were severe florid lesions which resulted in phthysical eyes characterised by cataract and retinal dysplasia (Bistner et al., 1970; Scott et al., 1973). The second type of lesion is more subtle and consists of chorioretinopathy and may also sometimes by accompanied by cataracts (Brown et al., 1975; Ohmann, 1984). The lesions described here are much less marked than those of previous studies and is the first report to describe multifocal selective photoreceptor cell atrophy.

The cerebellar lesions are likewise mild and in this instance had no clinical counterpart. Granule cell loss was mild and cystic cavities of cerebellar white matter small. These lesions did not result in the extensive destruction of folia previously described but nevertheless such lesions are typical of midgestational BVD-MD infection (Brown et al., 1973).

In conclusion the results of this study show that the introduction of cattle persistently infected with BVD-MD to susceptible herds probably results in rapid spread of infection through the herd and may cause considerable economic loss to individual farmers. The study further describes a novel pathological feature of BVD-MD infection, namely selective photoreceptor cell atrophy and indicates the subtlety of pathological lesions which may occur following infection

with some strains of BVD-MD virus. The relationship between gestational age and outcome of infection also shows that infections occurring before approximately 100 days may cause abortions and persistent viraemia. Infections between approximately 100-150 days gestation result in elimination of infection, a serological response and ocular and or cerebral dysmorphogenesis.

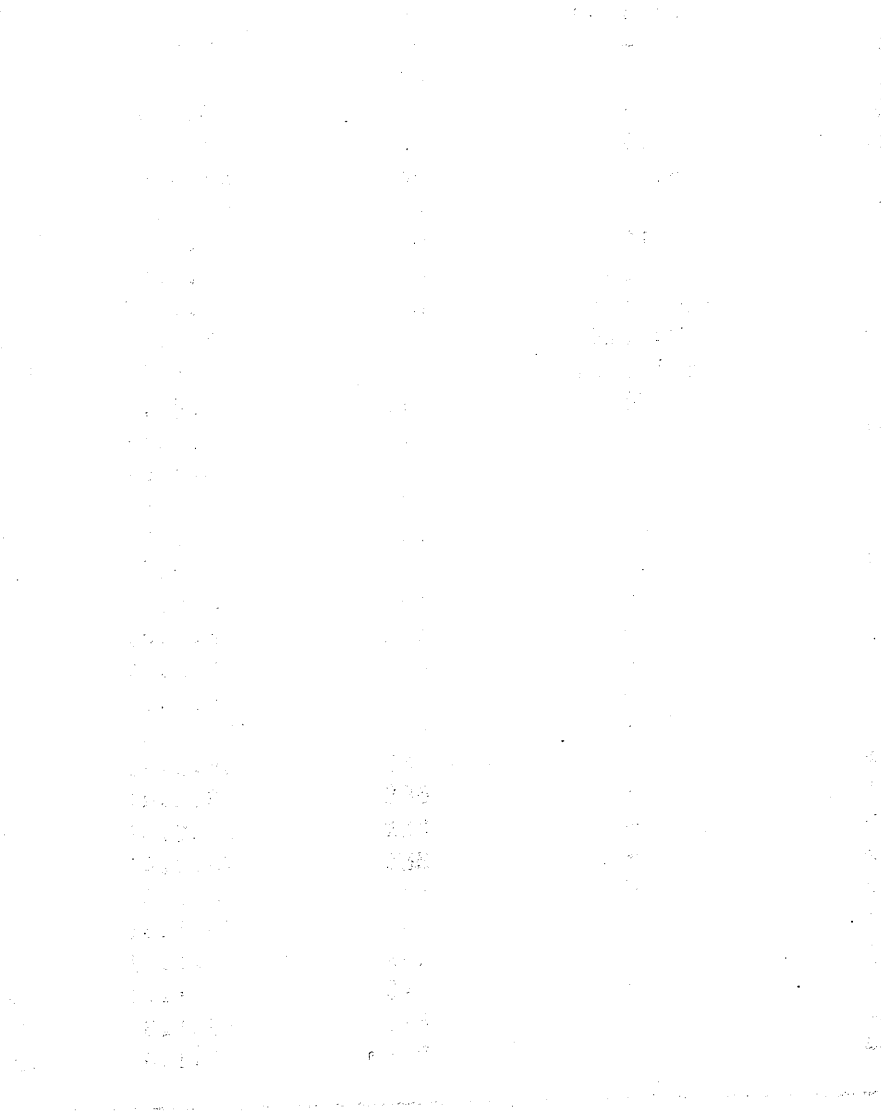


TABLE 1 Gestational outcome and status of the progeny of pregnant cows exposed to infection at differing stages of gestation.

DAM/CALF	CONCEPTION	GESTATION DAY on 10.10.82	OUTCOME	ANTIBODY TITRE $^{-1}$
cow A	2.11.82	CL	abortion	
calf 106	13.10.82	CL	-	<10
calf 105	4.10.82	6	-	<10
cow B	15.9.82	25	abortion	
*calf JC1	15.9.82	25	PI	<10
calf 104	11.9.82	29	-	80
*calf 103	9.9.82	31	PI	<10
cow 12	6.9.82	34	abortion	
cow 103	6.9.82	34	abortion	
calf 102	5.9.82	35	-	15
calf 101	26.8.82	45	-	60
calf 100	20.8.82	51	-	80
calf 99	17.8.82	54	PI	<10
cow 89	12.8.82	59	abortion	
calf 98	7.8.82	64	-	60
calf 97	1.8.82	70	PI	15
cow 76	27.7.82	75	abortion	
cow 104	21.7.82	78	abortion	
calf 96	21.7.82	81	PI, ST	<10
heifer 10	3.7.82	99	abortion	
calf 95	21.6.82	111	ST	30
*calf 94	17.5.82	146	ST	160
*calf 93	10.5.82	153	ST	240
calf 92	8.5.82	155	ST	10
calf 91	2.5.82	161	ST	10
calf 90	25.4.82	168	ST	160
calf 88	8.4.82	185	-	10
calf 87	16.3.82	208	-	40
calf 84	28.2.82	224	-	<10
calf 83	25.2.82	227	-	240
calf 80	18.2.82	234	-	<10
calf HC3	18.2.82	234	-	<10
calf 78	16.2.82	236	-	120
calf 77	14.2.82	238	-	<10
calf 76	14.2.82	238	-	<10
calf 74	30.1.82	253	-	<10
calf 70	18.1.82	265	-	<10
calf 69	16.1.82	267	-	240
calf 68	11.1.82	272	-	60
calf 67	8.1.82	275	-	480
calf HC2	4.1.82	279	-	480

CL conceived later ST stunted PI persistently infected
- not viraemic * selected for necropsy

TABLE II Summary of findings in the four calves necropsied.

CALF	GESTATION DAY at 10.10.82	CEREBELLUM WHOLE BRAIN WEIGHT Percentage	HISTOLOGICAL EXAMINATIONS cerebellar lesions	HISTOLOGICAL EXAMINATIONS ocular lesions	VIRUS INFECTION	ANTIBODY TITRE $^{-1}$
JC1	25	10.4	-	-	+	<10
103	31	10.0	-	-	+	<10
93	153	8.4	+	+	-	240
94	146	7.8	+	-	-	160

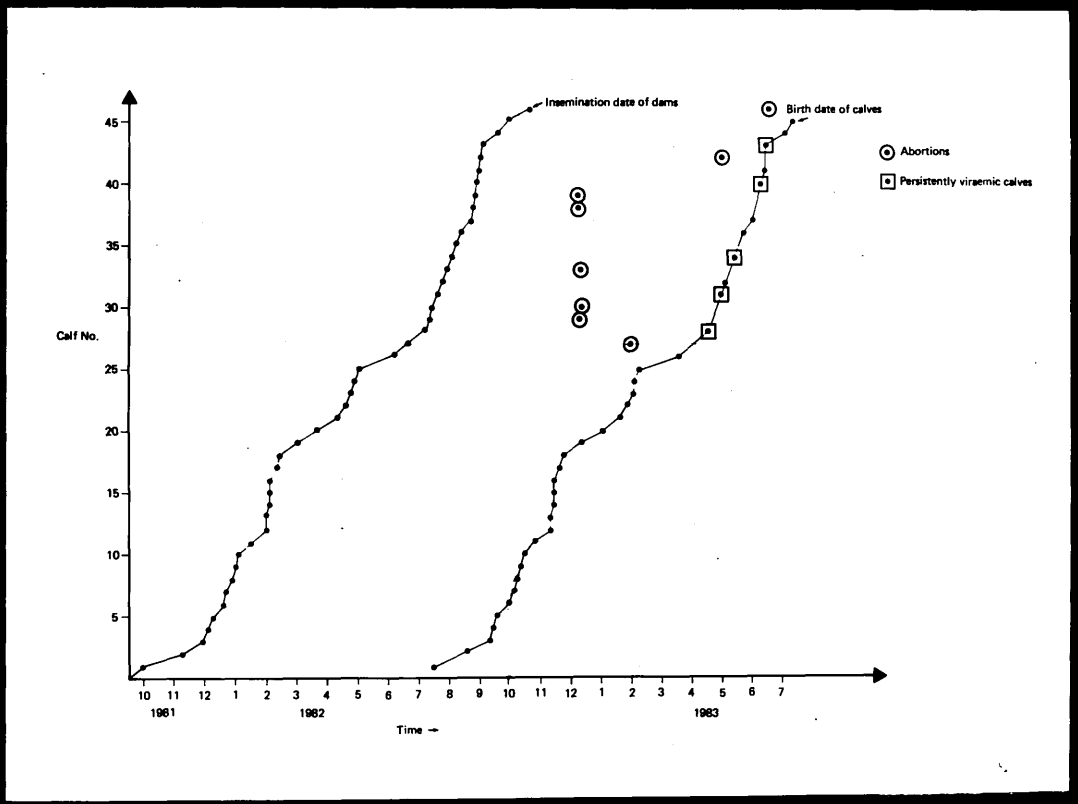
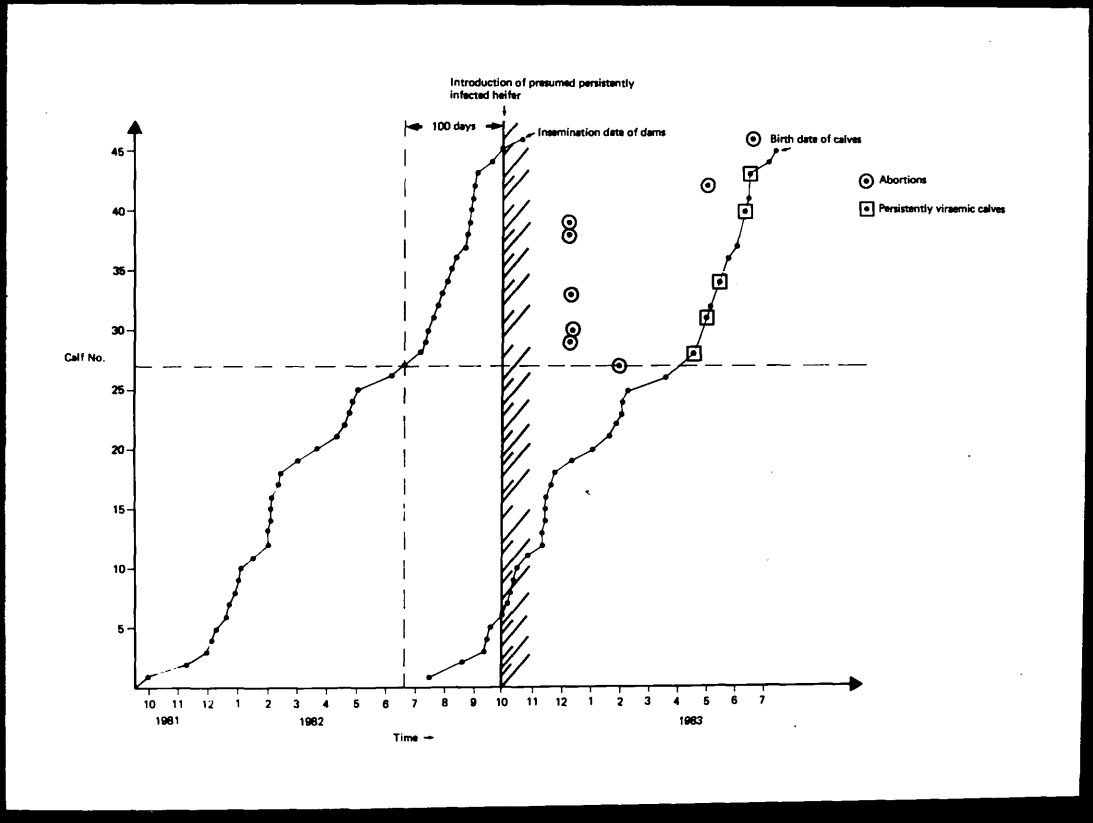

Insemination date of dams
Birth date of calves
Abortions
Persistently viraemic calves
Calf No.
45
40
35
30
25
20
15
10
5
10 11 12 1 2 3 4 5 6 7 8 9 10 11 12 1 2 3 4 5 6 7
1981
1982
1983
Time →

FIGURE 1a Figure showing insemination dates of dams and date of birth of calves. Dates of abortions and birth of persistently infected calves is also shown.

FIGURE 1b Figure shows relationship of introduction of heifer to birth of persistently infected calves and abortions. Cows in the first 100 days of pregnancy lie between the vertical hatched line and the vertical solid line which represents the introduction of the heifer. Calves born to cows in the first 100 days of gestation at the time of introduction of the heifer lie above the horizontal hatched line.


Introduction of presumed persistently infected heifer
100 days
Insemination date of dams
Birth date of calves
Abortions
Persistently viraemic calves
Calf No.
45
40
35
30
25
20
15
10
5
10 11 12 1 2 3 4 5 6 7 8 9 10 11 12 1 2 3 4 5 6 7
1981
1982
1983
Time

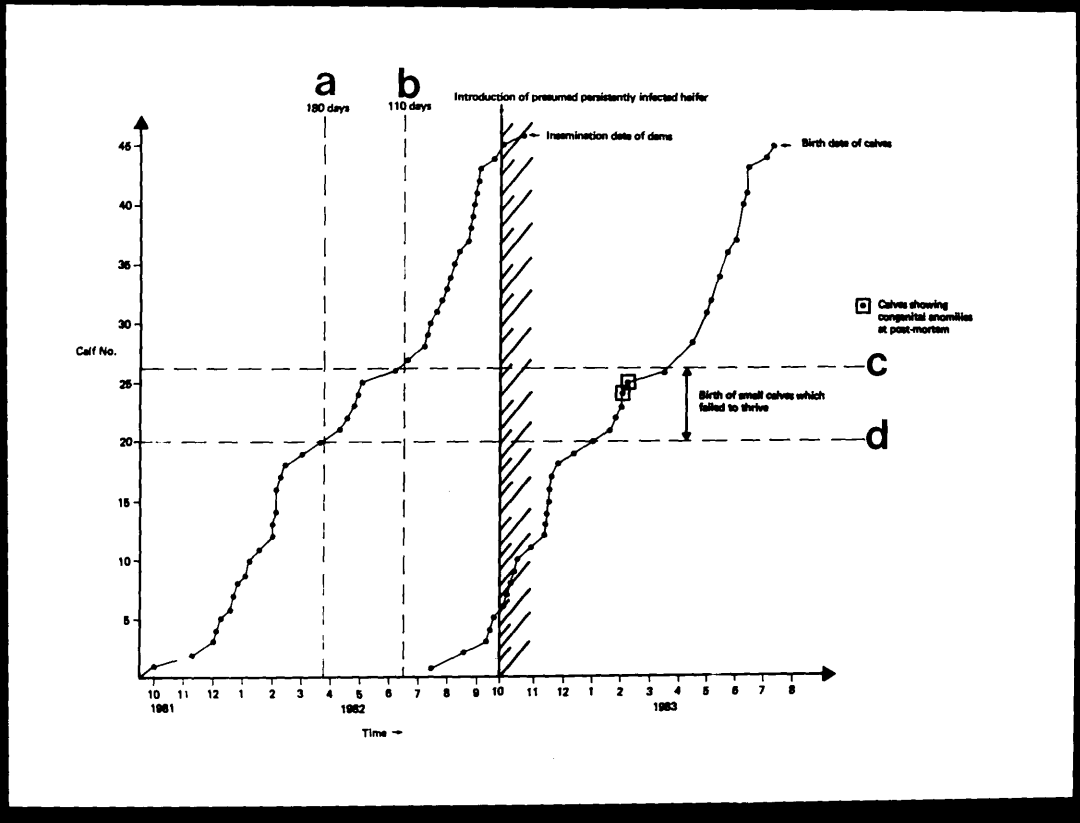

a
180 days
b
110 days
Introduction of presumed persistently infected heifer
Insemination date of dams
Birth date of calves
Calves showing congenital anomilies at post-mortem
Birth of small calves which failed to thrive
c
d
Calf No.
45
40
35
30
25
20
15
10
5
10 11 12 1 2 3 4 5 6 7 8 9 10 11 12 1 2 3 4 5 6 7 8
1981
1982
1983
Time

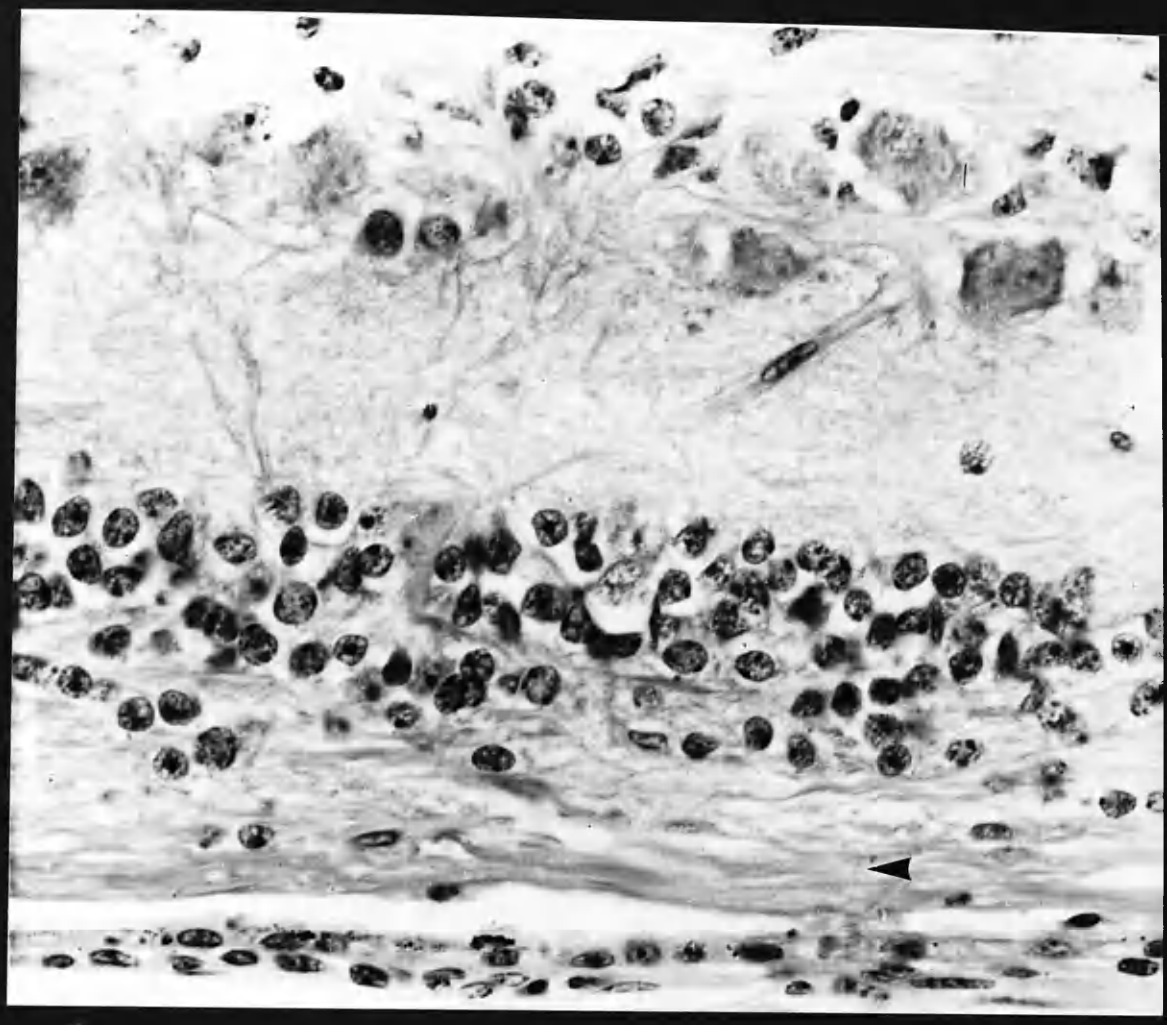
FIGURE 1c Figure showing relationship of introduction of heifer to birth of stunted calves and calves 93 and 94. Cows at gestational ages 110 to 168 days are represented between lines A and B. Calves born to these cows are represented between lines C-D.

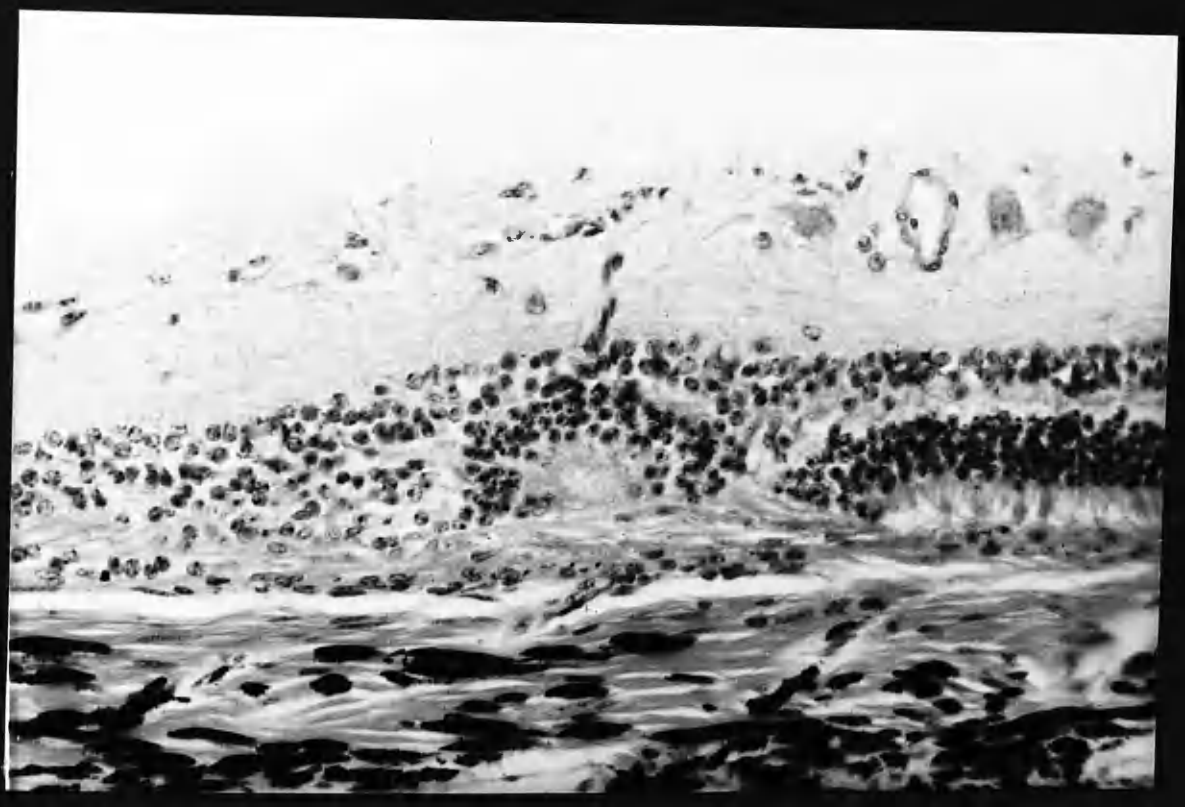
FIGURE 2 Focal areas of retinal thinning (arrow) in the left globe of calf 93 x 3.5

FIGURE 3 Cerebellum of calf 94. There is cystic cavitation of the white matter of cerebellar vermis lobule VII with absence of normal overlying cerebellar cortex. HE x 6.3

FIGURE 4 Retina of calf 93. There is rarefaction, narrowing and fusion of the inner and outer nuclear layers with complete atrophy of rods and cones. The rod and cone layer is replaced by a fibrous plaque (arrow). HE x 820

FIGURE 5 Retina of calf 93. There is an abrupt transition between unremarkable and disorganised sensory retina in which is seen a focal adhesion of the sensory retina to the retinal pigment epthhelium. HE x 425





CHAPTER THREE

AN INVESTIGATION OF NATURALLY OCCURRING OVINE PESTIVIRUS FETOPATHOGENICITY: CLINICO-PATHOLOGICAL VARIATIONS ON A SINGLE FARM.

INTRODUCTION

Border disease (BD) infection of ewes at susceptible stages of pregnancy, is characterised by a range of clinico-pathological conditions. Since the first description by Hughes et al. in 1959 an abnormal birthcoat and tremor with hypomyelinogenesis has been recognised as one of the most consistent features of the disease. However, in experiments concerned with fetal infection of BD virus other neuropathological abnormalities have been noted. Zakarian et al. (1976) described periarteritis in fetuses infected late in gestation (90 days after conception) and there have been sporadic reports of hydranencephaly and cerebellar dysplasia from several sources (Barlow, 1980; Barlow & Gardiner, 1969; Clarke & Osburn, 1978; Plant et al., 1983a; Vantsis et al., 1980). Cases of hydranencephaly and cerebellar dysplasia have also occasionally been seen in BD experiments carried out at the Central Veterinary Laboratory (unpublished).

This chapter describes the clinical and pathological findings in cases of BD seen on one farm. Details of virological studies and their correlation with pathological lesions is also presented.

MATERIALS AND METHODS

Flock history and source of animals

The flock of origin was a 500 ewe flock of Beulah and Beulah cross Suffolk ewes with Suffolk rams situated in Buckinghamshire. Studies commenced in the early lambing period when a clinical diagnosis of BD was confirmed in the laboratory. The farmer had purchased 100 Beulah ewe lambs from mid Wales in the previous autumn. When BD appeared in the progeny of the established flock the replacements were suspected

of having introduced the infection although they had been isolated from the main flock until lambing and the only apparent possibility of transmission was by the movement of rams. Subsequent investigations revealed that none of the replacement stock were seropositive to BVD-MD virus and only a small proportion (17 per cent) of the established flock were seropositive; this number included the dams of affected lambs. The pregnant sheep which produced affected lambs had previously had some contact with a group of fattening steers, the survivors of which all had high BVD-MD virus neutralising antibody titres. Perhaps significantly, two unthrifty steers, purchased when the ewes bearing affected lambs were 35 to 70 days pregnant, had been culled. Thus, although the origin of infection could not be established with certainty it is possible that infection was introduced by persistently infected cattle.

Eight frank abortions were observed and in total 17 clinically typical BD lambs were born. Subsequently it was recognised that there were at least an additional four lambs showing deviant behaviour and other lambs that failed to thrive.

Initially one severely affected BD lamb (1931) and three other abnormal lambs (1929, 1930 and 1932; see Table III) each approximately three months old were transported to the Central Veterinary Laboratory. After weaning all but three of the remaining BD lambs originally identified had died. At approximately five months of age these three and seven additional clinically abnormal lambs were combined with eight contemporary control lambs, from an uninfected sub-flock maintained on neighbouring premises, and transported to the laboratory. On arrival all the sheep were housed in boxes within one disease secure premises where they remained until slaughtered at up

to 18 months later. Ewe lamb 2093 mated to a BD ram gave birth at one year of age to a typical BD lamb. The lamb was necropsied at one week of age. Thus, in all, 23 sheep were available for study of which 15 had been selected as clinically abnormal. No significant clinical or pathological abnormalities were present in eight control sheep and they will not be discussed further.

Assessment of the clinical, pathological and virological findings of the remaining 15 sheep indicated three distinct groupings (summarised in Table III) and for convenience of presentation the results will be presented as such. In brief, Group A consisted of lambs persistently infected with BD virus, Group B were seropositive lambs with gross morphologic changes in the CNS and Group C were similarly seropositive but lacked gross CNS abnormalities.

Sampling Procedures

These were as described in the general materials and methods except that suspensions prepared from blood and secretions or tissues were inoculated into preformed cultures of calf kidney cells for isolation attempts.

Histology

With the exception of lamb 2088 which died, the sheep were killed with intravenous pentobarbitone sodium. The following special stains were used in addition to those listed in General Materials and Methods: Perls' Prussian blue, Romanes' (for axons), von Kossa's (for calcium salts) and a trichrome.

RESULTS

Clinical Signs

Group A.

The variable degree of continuous muscle tremor which these lambs suffered from birth was enhanced by stimulation and gradually ameliorated as the sheep matured but was still elicited by stress even at 15 months of age. The tremor of lamb 1931 had become unnoticeable within weeks of birth but returned at 12 weeks of age and increased in intensity for a week until the lamb was unable to stand. It was very weak on arrival at the laboratory and died within 24 hours.

At 33 weeks of age lamb 2088 declined in condition and died a week later. It showed terminal dysentry, ocular and nasal discharges.

The growth of several lambs was abnormal. Ewe lamb 2093 was stunted whereas twin ram lambs 2091 and 2092 were as large as control rams of the same age but differed in conformation being "blocky" in appearance. Ewe lamb 2089 was intermediate in size.

All lambs had hairy birth coats and the mature fleece of the twin rams remained abnormally fine and kempy.

Lamb 2975, born of PI parents was vigorous and sucked well despite marked tremor; it had a domed head and possessed a patch of black neck wool as did its dam.

Group B.

Four of the sheep (1929, 1930, 1932 and 2086) showed behavioural abnormalities inconsistent with normal herding. They had visual deficits and as a result would not pass through gateways. They easily became disorientated and when startled would run in a straight

line until physically stopped by an obstruction. If unstimulated they walked in circles for longer periods of time, the direction being constant for an individual and the head was inclined towards the centre. In addition sheep 1929 spent much of its time pressing its head firmly into the corner of the box and had a horizontal nystagmus. The four sheep had long, relatively straight, stapled fleeces, were lightly built with fine limb bones and had normally shaped heads. All four had a gait abnormality which consisted of medial circumduction of the foot to give abduction of the hock (talus varum). Sheep 1932 was the only one of the four with tremor visible as a fine trembling of the head.

Behavioural abnormalities of ram 2095 were less marked but it became progressivley restless, responded aggressively to handling and even attacked its pen mates.

Group C.

Clinical abnormalities were not marked in this group. Sheep 2087 was slightly built and suffered a marked abduction of the hock joints, particularly the right, and sheep 2090 had a fine but definite head tremor throughout life. Ewe 2094, the twin of sheep 2086 (Group B), was stunted with a patchilly pigmented fleece and a pronounced patch of dark wool on the neck dorsum.

Infection Status of Sheep.

Group A

All sheep in this group yielded BD virus from a large proportion of samples tested and antigen visualisation was observed in the majority of tissues examined. With the exception of two animals the remaining animals all showed no evidence of virus neutralising antibody at a

dilution of 1/10. Animal 2091 occasionally had detectable virus neutralising antibody but the titre on no occasion exceeded1/12. Although initially seronegative,ewe 2093 seroconverted at 6 months of age and remained seropositive thereafter. The maximum antibody titre recorded was 1/80.

Group B

In marked contrast to classical BD lambs, BD virus was not isolated from any of nine sampling occasions, including tissue samples taken at necropsy. BD virus antigen in tissues was also not visualised following direct immunofluorescence. Consistently high virus neutralising antibody titres were recorded in sera of all these sheep and these levels were maintained up until necropsy at approximately one and a half years of age.

Group C

The virology and serology results of this group are similar to those of group B. All virus isolation attempts were negative and three sheep showed elevated antibody titres ranging up to 1:17,000. One animal (2094), the twin of hydranencephalic lamb 2086 from group B, was seronegative until 34 weeks of age after which time it seroconverted and was consistently seropositive.

Gross pathology

Group A

There were no gross abnormalities of the CNS in this group but visceral abnormalities were seen in the four animals examined when adult (2089, 2091, 2092 and 2093). Caseous 0.5cm diameter circumscribed nodules were present in the lungs of three sheep and small 1 to 2mm diameter pale foci were present throughout the kidney cortices in all

four. In two sheep (2091, 2092) the hearts were enlarged and flabby and there were numerous small (1mm diameter) pale foci in the ventricular myocardia. Fibrinous pericarditis was present in two sheep. Cysticercus tenuicollis cysts were found in one animal and lungworms were present in the bronchi of two. Numerous intestinal polyps, each approximately 5mm in diameter, were present in the ileum of lamb 1931.

The lungs of lamb 2088 which died had congestion, oedema and red hepatisation of 60 per cent of the lung lobes. The thymus was very small and there were widespread intestinal mucosal haemorrhages with blood present in the lumen. Marked ulceration of the dorsum of the tongue and soft palate extended into the pharynx.

Group B

The most striking feature of this group was hydranencephaly. At its most severe cavitation of the cerebral hemispheres involved the entire neopallium (Fig. 6) and the hemispheres were replaced by a fluctuant, fluid-filled sac covered by a translucent membrane which collapsed as the calvarium was removed. The frontal lobes were generally spared. The basal ganglia, thalamus, amygdaloid bodies and hippocampus could be identified through the membrane and appeared normal. Cavitation ranged from hydranencephaly in sheep 1929 (Fig. 7a), 1930 and 2086, to focal cystic cavitation of cerebrum in sheep 1932 and 2095 (Fig. 7b). Two animals (2086 and 2095) had hypoplastic cerebella which displayed abnormal lobulation. Coronal sections showed poor differentiation of lobulus (L.) flocculus and L. paraflocculus. In one sheep (2095) L. ansiformis, L. flocculus and L. paraflocculus could not be individually identified.

The eyes from two animals (1929 and 2095) showed mild diffuse thinning of the midzonal non-tapetal retina and one of the eyes had a fold which extended through the tapetal and non-tapetal retinas.

Group C

With the exception of sheep 2087, which still retained a large thymus extending well up the neck, gross necropsy was unremarkable.

Histopathology

Group A

Abnormalities of the CNS were present in two animals. In lamb 2975 there was hypomyelinogenesis of the white matter in the cerebellum and spinal cord. Abnormal glial cells lay in clusters or short chains in the white matter. These cells contained irregular or ovoid nuclei with no nucleoli and little chromatin.

Similar lesions were found in lamb 1931 and in addition there was moderate multifocal gliosis mainly affecting the brain stem. There was extensive parasitic infestation of several organs. Polyps with associated coccidia and cryptosporidia were found in the small intestine and in the liver there was cholangitis, biliary hyperplasia, focal haemorrhage and necrosis suggestive of parasitic migration.

Non-suppurative myocarditis and myositis with focal eosinophil infiltration was a consistent feature in all the remaining animals. Lamb 2088, which died from acute exudative pneumonia, showed pulmonary lesions characteristic of pasteurellosis. There were large numbers of immature protozoan bodies in the subcapsular sinuses of the prescapular and mesenteric lymph nodes. Mature sarcocysts were present in moderate to large numbers in the tissues of all five animals. Multifocal mononuclear cell infiltration was present in pancreas,

salivary glands, liver, spleen, kidney, heart and lung. The larger foci present in the kidney, myocardium and lung contained central lymphoblastic clusters resembling germinal centres. In non-parasitised regions of the lung prominent diffuse peribronchiolar and peribronchial lympho-reticular hyperplasia was present.

Group B

Significant lesions were restricted to the CNS; hydranencephaly or cystic cavitation of the white matter affected all sheep. The cavities were lined by compressed myelin sheaths or by a thin border of astrocytes.

Cavities and cysts were centred on the white matter and occasionally small cysts were seen within the white matter cores of gyri (Fig. 8). Rarefaction of myelin and glial scars were also present in gyri. Numerous small densely cellular glial cell rests were scattered throughout the cerebral cortex but were found mainly in the basal ganglia and para-ependymal areas. The cortex was generally well organised but pyramidal neurons were misaligned, absent or scattered randomly throughout the very thin cortical remnants that formed the dorsal boundaries of hydranencephalic cavities. In one animal (1930) haemosiderin pigment was present in the choroid plexus. The cortex overlying cavities varied in thickness. In some animals it was preserved to the full normal width. In some parts only residual cortical elements persisted often with fine diffuse particulate mineralisation of the neuropil.

The cerebella were variably affected. In general lesions were multifocal. The centro-lateral lobes, where foliation was rudimentary, (particularly l. flocculus and l. paraflocculus), were often the most

severely affected. However, in two cases lesions were mild, focal and restricted to one folium. In areas where disturbance of the cortical architecture was greatest there were nodules of granule cells surrounded by Purkinje cells and foci of mineralisation primarily associated with the blood vessels. In some regions granule cell heterotopia was a prominent feature and was sufficiently extensive to form an intermediate granule cell layer (Fig. 9). There was multifocal rarefaction of the internal granule cell layer with narrowing of both the molecular layer and the internal granule cell layer. In these regions Purkinje cell heterotopia with bizarre dendritic arborisation was a feature (Fig. 10). There was occasional chromatolysis of Purkinje cells and eosinophilic spheroids. The latter, interpreted as swollen cell processes, were present in the granule cell and molecular layers. Cysts and small cavities were occasionally seen in white matter.

The globes of two sheep (1929 and 2095) showed multifocal photoreceptor cell atrophy accompanied by rarefaction of the outer nuclear layer. In some foci rod and cone receptors and the inner nuclear layer were also rarefied (Fig. 11). Multifocal depigmentation of the retinal pigment epithelium overlying non-tapetal retina and pigment cell invasion of the sensory retina were also features. A retinal fold (Fig. 12) was present in the globe of one sheep.

Group C

With the exception of the small numbers of plasma cells present in the adventitia of sulcal meningeal vessels, no significant abnormalities were detected.

DISCUSSION

This investigation describes a range of clinico-pathological conditions caused by infection of fetuses with BD virus and is the first to describe hydranencephaly, cerebellar dysplasia and multifocal retinal atrophy in surviving field cases. The alterations in mental state, the locomotor and postural deficits and nystagmus of sheep in group B are consistent with cystic cavitation of the cerebrum or central vestibular syndrome (Palmer, 1976). These clinical signs correlate with observed pathological changes and are similar to the intracranial malformations described as "alternative pathology" by Barlow (1980).

Persistently infected ewes have been shown to give birth to affected lambs (Westbury et al., 1979) and such a case with marked hypomyelinogenesis was seen in this study. Another lamb was similarly affected but also had multifocal gliosis of the brain stem; the cause of the encephalitis was not determined but infection by another agent is suspected. Similar changes have been observed at this laboratory after experimental infection of newborn lambs with BD virus contaminated with adenovirus (S. Terlecki personal communication).

Older animals in Group A did not show hypomyelinogenesis. This is consistent with experimental work of Sweasey and Patterson (1979) and Cancilla and Barlow (1971) who showed that there was complete neurochemical and histological recovery of the CNS in persistently infected lambs by 20 weeks and six months respectively. These sheep had generalised lymphocytic infiltrations in many organs resembling those described by Barlow et al., (1983) which were attributed to the response of PI lambs to superinfection with "homologous" BD virus. However, the distribution of the lesions differs and in particular

evidence of choroiditis, thyroiditis and intestinal lymphoproliferation with germinal centre formation was absent in the present cases.

The mechanisms that result in destructive cerebral lesions in pestivirus infections are poorly understood. Barlow (1980) described necrosis of the germinal layers of the cerebellum in lamb fetuses infected with BD virus at between 75 and 85 days of gestation. Brown et al. (1974) described similar leisons in bovine fetuses infected with BVD virus at approximately 150 days of gestation. Morphologically similar cerebellar cortical dysplasia resultant upon necrosis of germinal cells has also been produced experimentally in rats by x-irradiation or cytotoxic drugs (Hopewell, 1974; Yamono et al., 1978). Thus, the virus may find a favourable environment for replication in metabolically active fetal cells undergoing mitosis and differentiation. The lesions seen in the sheep of Group B would be consistent with the destruction of primitive cells, neuroblasts and glioblasts, in the matrix and intermediate zones of the developing neocortex and in the external granule cell layer of the cerebellum. However, necrosis of stem cells alone is unlikely to result in the extensive tissue destruction seen in the cerebral leisons. While the multipotentiality of the mantle layer remains, rapid proliferation of these cells would be expected to partially repair the defect. Ependymal destructions or capillary thrombosis such as recorded by Barlow (1980) and cerebellar folial oedema described in BVD-MD virus infec-

ted bovine fetuses by Brown et al. (1974) probably also contribute to the severity of the tissue destruction. Variation in the extent of cavitation may in part be related to the changing availability or susceptibility of target cells.

A wide disparity was evident in the nature of the lesions present in one of the sets of twins. One was hydranencephalic whereas the other had mild diffuse meningeal perivascular lymphocytic infiltrations. This suggested that the less affected lamb was either more mature than the hydranencephalic lamb at a simultaneous time of infection or infection was asynchronous. This former situation could have arisen as a result of idiosyncratic differences in their rates of development.

Ocular lesions have been described in cattle infected with BVD-MD virus (Bistner et al., 1970; Brown et al., 1975), but not in lambs exposed to BD virus infection in utero. Two types of lesion are reported in cattle; mild lesions restricted to the retina and more florid lesions with phthisis bulbi, microphthalmia, cataracts and retinal disorganisation. The ocular lesions present in sheep 1929 and 2095 in the present study were mild, limited to the retina and are similar to those described in BVD infected cattle by Ohmann (1984) and those described in Chapter two.

Various pathogenesis factors have been invoked to explain the differing forms of CNS malformations occurring in experimentally produced BD including virus strain (Barlow et al., 1979), dose of virus (Richardson et al., 1976), breed and immune status of dams (Barlow, 1980). Whilst these factors are undoubtably of importance the results presented here, and also in the preceeding chapter, suggest

that fetal age is probably of paramount importance in modulating the fetopathogenic effects of pestivirus infection. In this study breed of sheep, and presumably also immune status of the dam and virus strain were constant.

Previous studies have shown that persistent infections result from maternal infections occurring between 21 and 72 days gestation (Barlow et al., 1980a; Gard et al., 1976; Plant et al., 1983a; Plant et al., 1976a&b; Sweasy et al., 1979; Terpstra, 1981. Following inoculation of the dam approximately seven days are required to establish fetal infections (Hadjisavvas et al., 1975; Parsonson et al., 1979). Thus it appears that the ovine fetus acquires the ability to produce virus neutralising antibodies to BD virus between approximately 61-79 days of gestation.

Reports in the literature show that severe cystic cerebral cavitation and cerebellar dysplasia (so-called "alternative pathology," Barlow, 1980) have all been initiated following maternal infection between 54 and 71 days gestation (Barlow, 1980; Barlow & Gardner, 1969; Plant et al., 1983a) and therefore fetal infections between 61-78 days of gestation. The correlation of the timing of the acquisition of immune responsiveness and generation of "alternative pathology" lesions is so similar that the possibility of a causal relationship may be suggested. Such developmentally related host immune mediated CNS pathology is not unique and has been described for example in Borna disease of rats (Narayan et al., 1983).

The findings presented here are in accord with the above suggestion. All animals in group B (cerebral cavitation and cerebellar dysplasia) demonstrated a virus neutralising antibody titres to BD and thus were

infected at gestational ages greater than 61-79 days. The hypothesis is also compatible with findings of Clarke and Osburn (1978). These authors describe an acute lymphoid response following fetal infection between 66-91 days gestation and gross cavitation of the CNS occurred in two fetuses infected at 66-91 days gestation.

The above findings are controversial and Barlow (1980) considers his results are in conflict with those of Clarke and Osburn (1978). Barlow (1980) suggests that cerebral cavitation and cerebellar dysplasia result from necrosis and inflammation in germinal cells of the respective tissues.

The possibility that acquisition of immune responsiveness may only bear a fortuitious temporal relationship to the development of "alternative pathology" lesions, should be considered. The ability of the fetus to eliminate infection may simply correspond to the availability of susceptible target cells in the CNS which permit evolution of "alternative pathology" lesions. These arguments are further considered in Chapter four.

In summary this study is the first to describe cerebral cavitation, cerebellar dysplasia and photoreceptor cell atrophy in naturally occurring BD of sheep. The range of lesions seen on this single farm and the infection status of sheep suggests that time of fetal infection is the most important factor which determines the outcome of infection. Cerebral cavitation, cerebellar dysplasia and retinal atrophy occur following midgestational infections.

TABLE III Composition of groups and criteria for their selection

GROUP	ANIMAL NUMBER	AGE AT NECROPSY (WEEKS)	SELECTION CRITERIA
A	1931	14	Typical BD, persisent infection
	+2088	34	
	2089	73	
	*2091	73	
	*2092	73	
	2093	73	
	2975	1	
B	1929	53	Behavioural, locomoter and postural abnormalities, not persistently infected
	1930	58	
	1932	59	
	**2086	59	
	2095	61	
C	2087	62	Not persistently infected, failure to thrive, limb deviation
	2090	60	
	**2094	62	

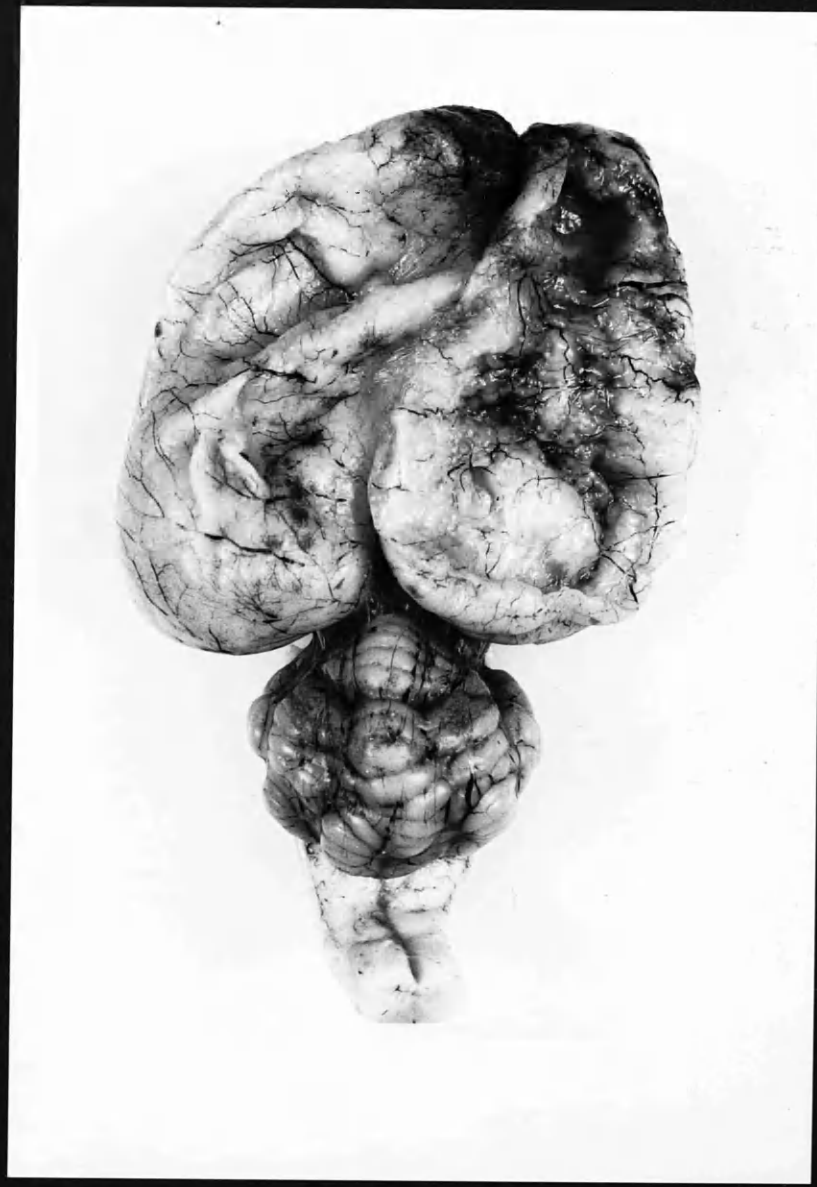
* and ** indicate twins

+ animal died all other euthanased

BD = Border Disease

FIGURE 6

Hydranencephalic brain of sheep 2086.



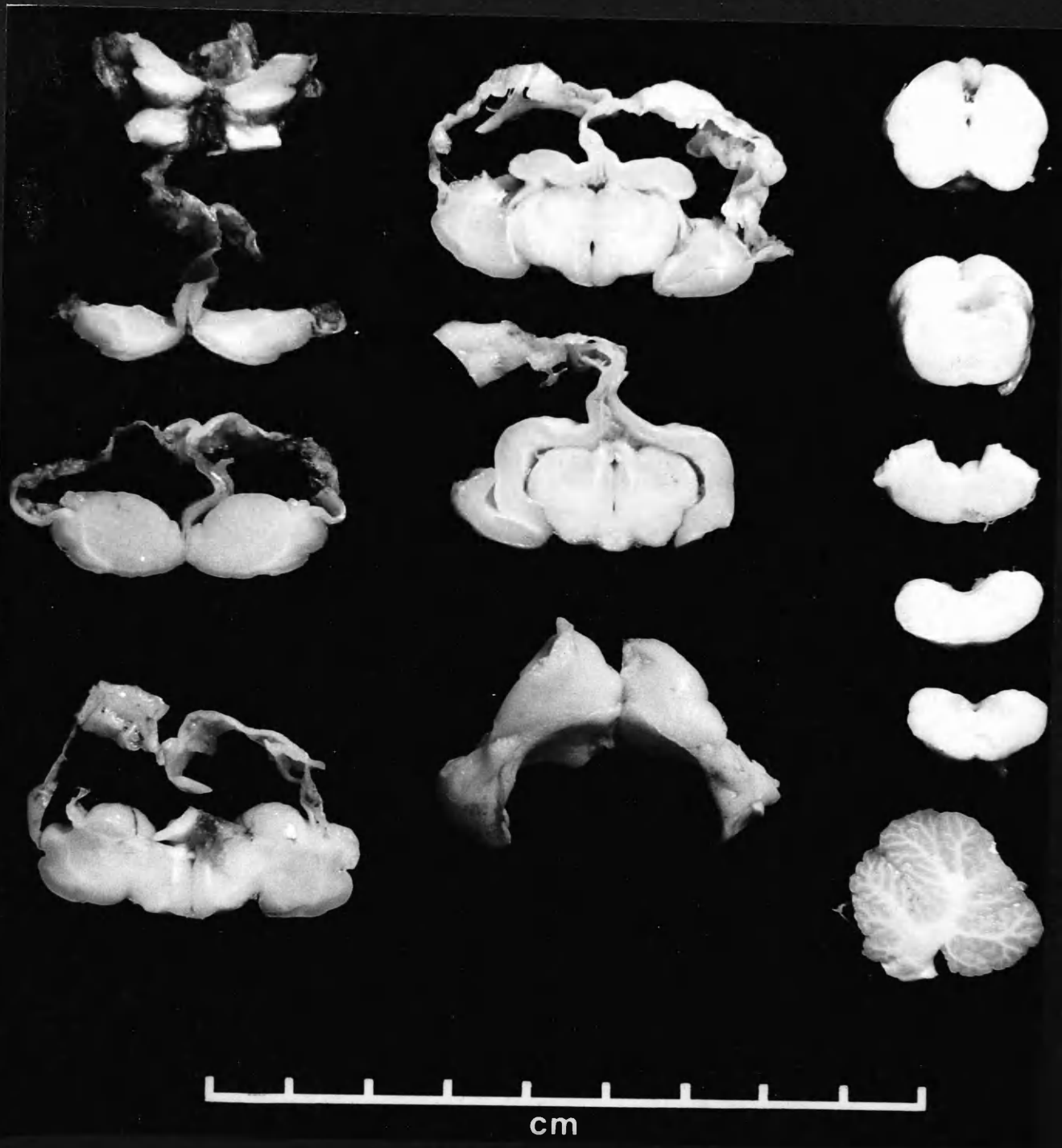
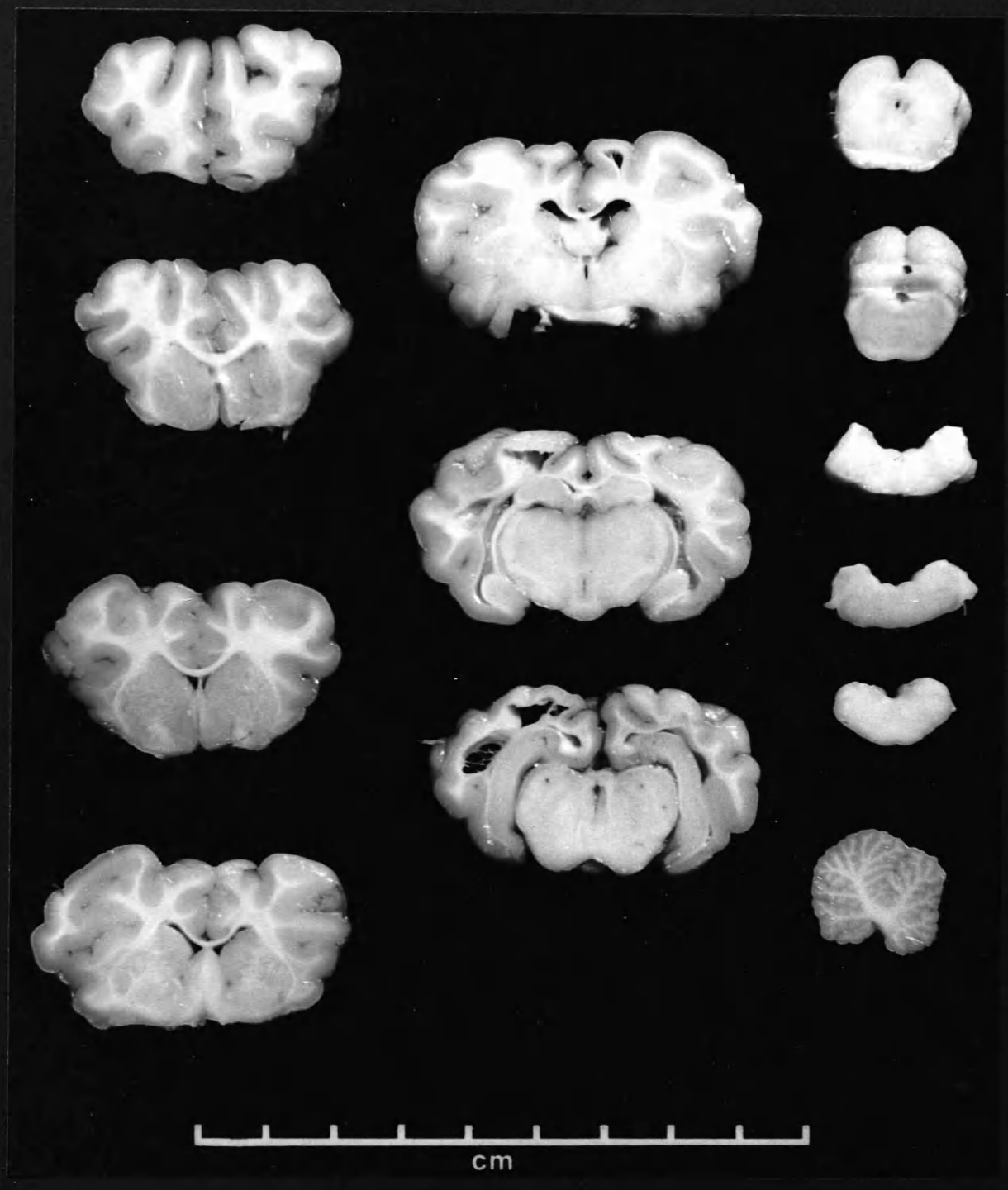

cm

FIGURE 7

Coronally sliced brains of two sheep showing varying degrees of cerebral cavitation:

(a) sheep 1929 - hydranencephaly

(b) sheep 2095 - cystic cavitation of parietal and occipital cortex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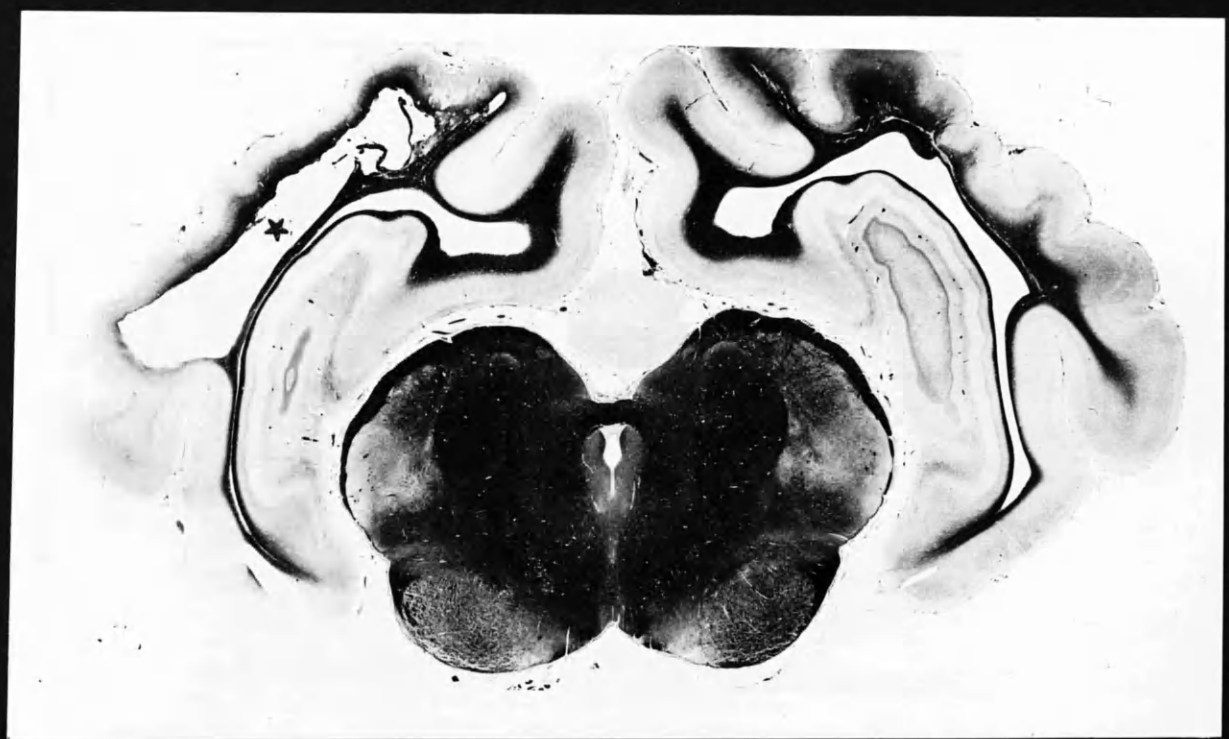


FIGURE 8

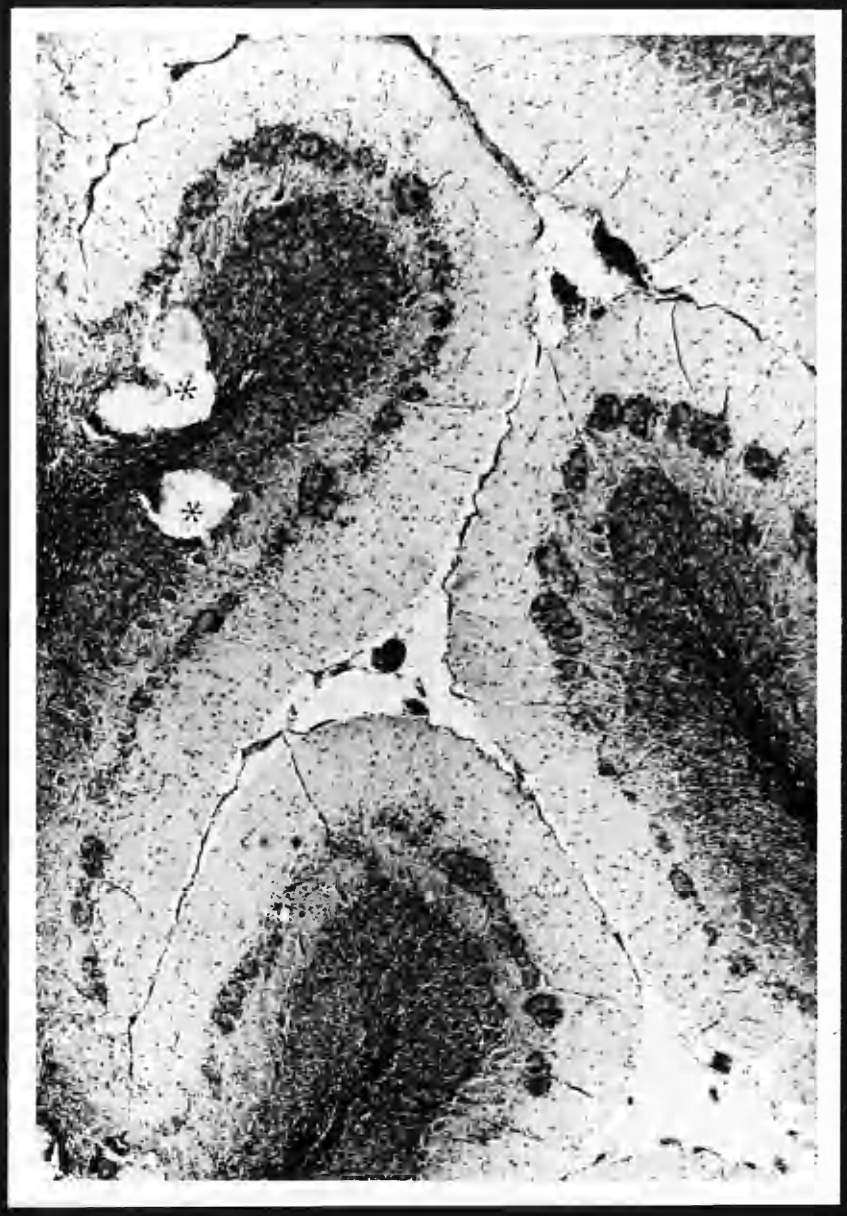
Cerebrum of sheep 2095 - focal cavity (asterisk) centred on the white matter and extending into the grey matter of the occipital cortex. LFBCV x 2

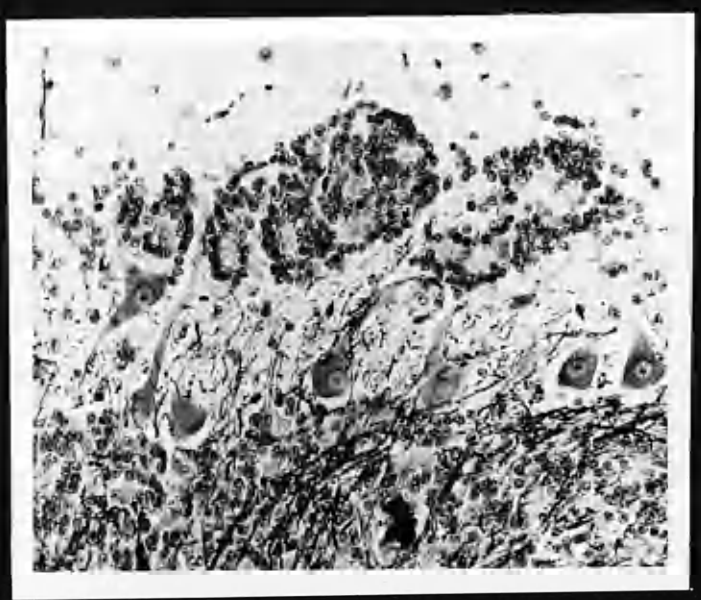
FIGURE 9

Cerebellum of sheep 2095 - marked heterotopia of granule cells. The unstained areas (asterisks) correspond for foci of mineralisation displaced on sectioning. LFBCV x 50

FIGURE 10

Cerebellum of sheep 2095 - Detail of cerebellar cortex form Figure 9 showing heterotopia of granule cells and Purkinje cells with abnormalities of purkinje cell arborisation. LFBCV x 350





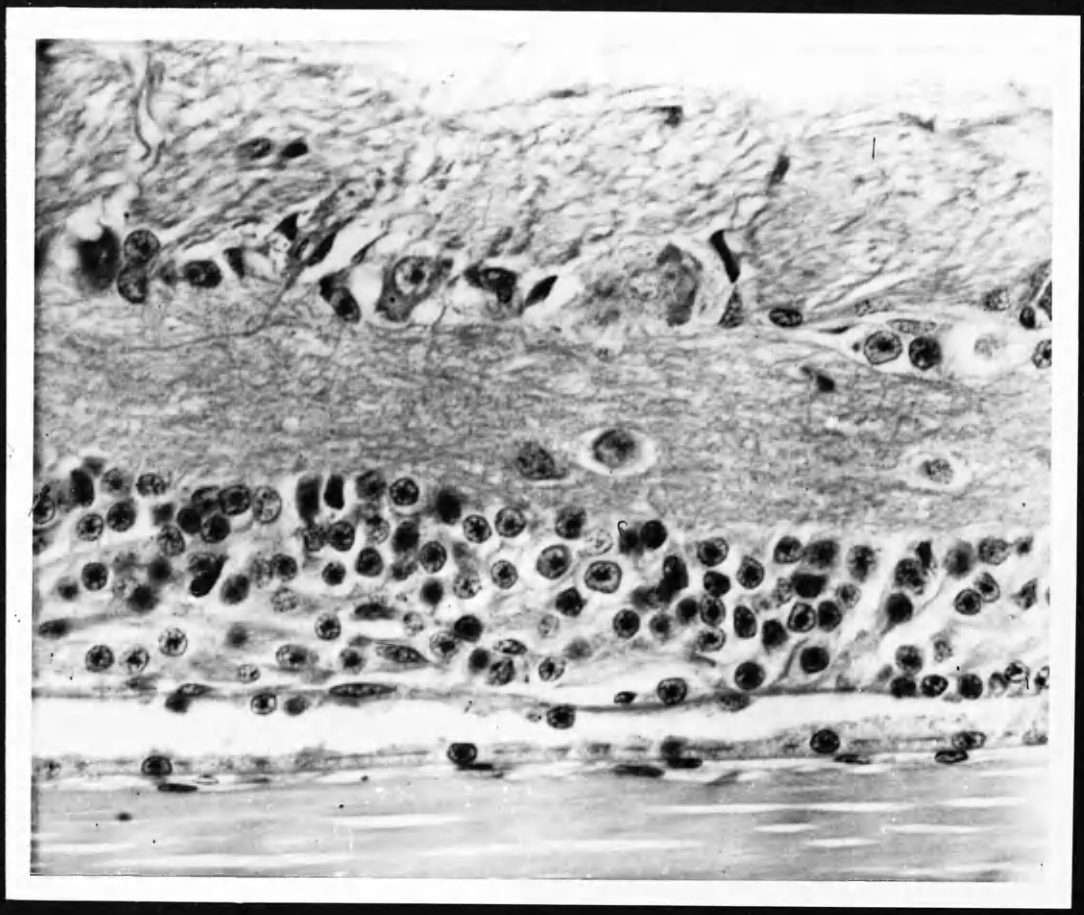
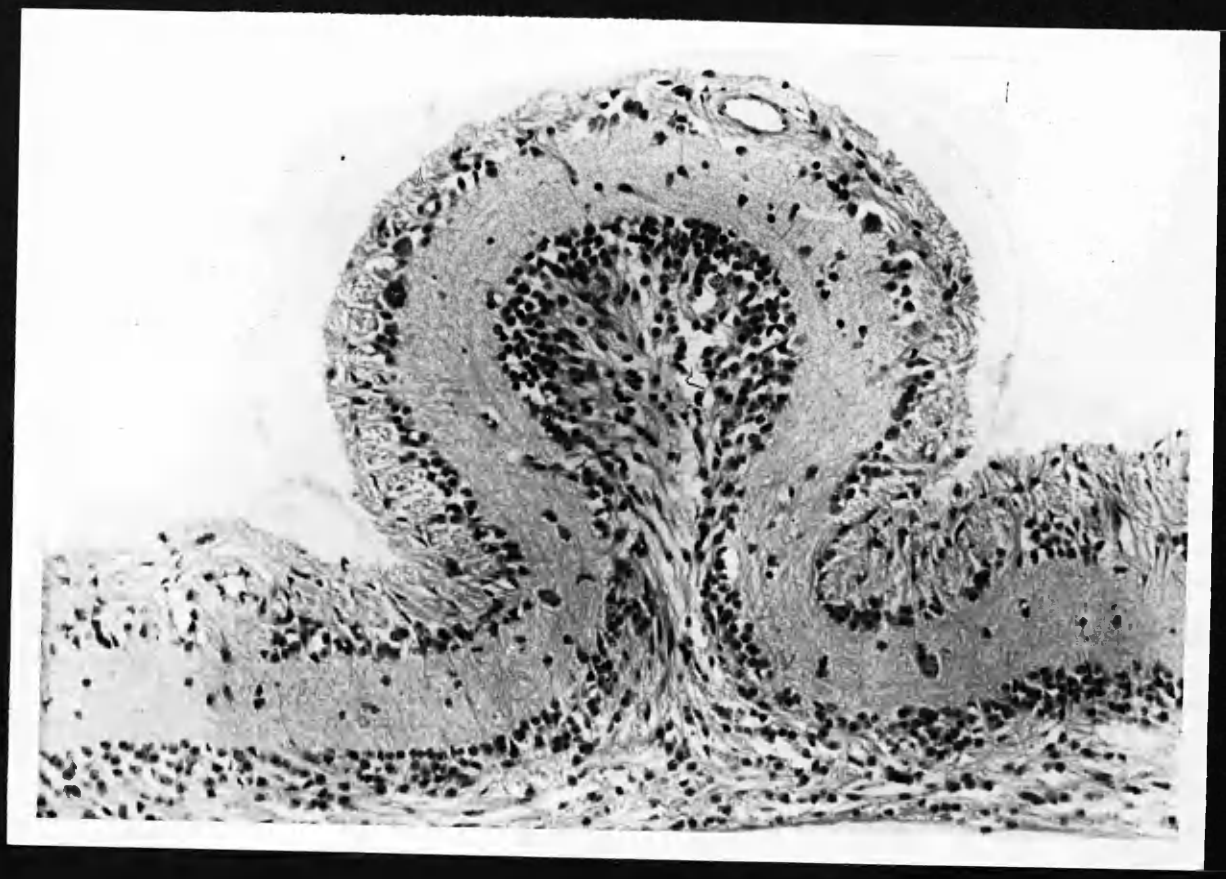


FIGURE 11

Retina of sheep 1929 - focal rarefaction of the inner nuclear layer and absence of rod and cone receptors. HE x 400

FIGURE 12

Retina of sheep 1929 - retinal fold with rarefaction of nuclear layers. HE x 140.



CHAPTER FOUR

THE INFECTION STATUS OF CATTLE AND SHEEP CONGENITALLY AFFECTED WITH CENTRAL NERVOUS SYSTEM OR OCULAR LESIONS CONSISTENT WITH NATURALLY ACQUIRED PESTIVIRUS INFECTION.

INTRODUCTION

Factors affecting the nature of pathological lesions found in cattle and sheep congenitally infected with pestivirus include the genotype of the host, virus strain, dose of virus and prior exposure of the dam to pestivirus. While these factors undoubtably influence the outcome of infection probably the most important factor is the gestational age at which the fetus becomes infected.

Hypomyelinogenesis of sheep is reported following infections between 15-72 days gestation (Barlow, 1983; Plant et al., 1983b; Shaw et al., 1967). Such lesions are, with some apparently rare exceptions (Plant et al., 1983b), associated with viraemia. This is consistent with experimental production of persistent viraemia which occurs as a result of vertical transmission from persistently infected ewes and from maternal infections between 21-72 days gestation (Barlow et al., 1980b; Gard et al., 1976; Plant et al., 1983a; Plant et al., 1976a&b; Sweasey et al., 1979; Terpstra, 1981). Restricted lesions of hypomyelination is reported following infection of dams at 120 days gestation but the virus status of these sheep was not stated (Gardiner & Barlow, 1972). Though rare, dysmyelinogenesis of cattle associated with BVD-MD viraemia following possible exposure of the dam to BVD-MD virus between three and five months gestation has also been reported (Binkhorst et al., 1983; Straver et al., 1983). The isolation of virus in calves infected in-utero at this time is consistent with experimental production of viraemia in cattle following direct inoculation of the fetuses at 49-125 days gestation (McClurkin et al., 1984).

The relationship between lesions of cystic cerebral cavitation, cere-

bellar dysplasia or atrophy and retinopathy or cataract and the infection status of the host is less clear than is the case of animals with deficiencies of myelin. Calves with lesions of cerebellar dysplasia or retinopathy described in Chapter Two of this thesis showed a serological response and were probably exposed to infection at approximately 100-168 days of gestation. Consideration of the literature shows that lesions of cystic cerebral cavitation, cerebellar dysplasia or ocular lesions of calves are reported following infections from 79 to 150 days gestation (Bistner et al., 1970; Brown et al., 1974; Trautwein et al., 1986). However, the presence of viraemia or the serological response of such animals is rarely stated but several reports indicate at least some affected calves show a serological response to BVD-MD virus (Badman et al., 1981; Markson et al., 1976; Scott et al., 1973). Ohmann (1984) describes retinopathy, cataract and mild cerebellar dysplasia in calves which were probably exposed as fetuses between 14-75 days gestation. Several affected calves yielded virus on culture. Retinopathy has also been described in a persistently viraemic bull (Barlow et al., 1986).

The gestational age at which sheep with lesions of cystic cerebral cavitation described in Chapter Three were infected could not be established but all affected animals showed a serological response to BD virus. The ability of the ovine fetus to mount an immune response to BD virus as assessed by detection of serum neutralising antibody in fetuses and newborn lambs has been demonstrated as early as 52 days gestation (Terpstra, 1981). Thus the dams of the aforementioned sheep were presumably infected at 52 days of gestation or later. This is in accord with the experimental production of hydranencephaly,

porencephaly or cerebellar dysplasia reported following infection of fetuses from 54-71 days gestation (Barlow, 1980; Plant et al., 1983a & b).

This Chapter presents a retrospective study of the serological and virological findings of cattle and sheep congenitally affected with CNS or ocular lesions suggestive of pestivirus infection. The aim of this study is to correlate the infection status of animals with the types of malformations observed.

MATERIALS AND METHODS

Cases of sheep and cattle with CNS or ocular lesions suggestive of BVD-MD or BD virus infection were selected from material submitted over a five year period (1982-1987) to the Central Veterinary Laboratory. A questionnaire soliciting information regarding the pestivirus infection status of these animals was circulated to veterinary investigation centres in England and Wales. Replies to the questionnaire were received from 29 of 37 cases. Sufficient information was obtained for 20 cases to warrent their inclusion in this study. Cases were selected where: i) evidence of persistent viraemia was obtained, or, ii) where the morphologic diagnosis was strongly suggestive of BVD-MD or BD virus infection and both serology and virology had been performed. Further evidence of BD virus infection of contemporary lambs (virus isolation) or ewes (rising titres) in the flock of origin was also obtained for sheep showing lesions suggestive of BD virus induced encephalopathy or retinopathy.

The morphologic criteria used for selection of cases was: for cerebral lesions hydranencephaly or porencephaly; for cerebellar lesions granuloprival type cerebellar dysplasia or cystic cavitation and

atrophy and for ocular lesions cataract or retinopathy.

Data extracted from files showed that, consistent with previous studies, lambs (n=22) with pathological lesion of classical Border disease ie hairy-shakers with hypomyelination, were invariably viraemic. These lambs are not considered further in this study. Excluded from the study are sheep showing lesions consistent with swayback. Virological and serological investigations were performed at the Central Veterinary Laboratory or at the Veterinary Investigation Centres concerned. Virus antigen detection was determined by direct immunofluorescence of tissues using bovine anti-BVD-MD antiserum and virus isolation was determined by immunofluorescence or immunoperoxidase techniques following cell culture. Virus isolation or antigen detection results were only accepted as valid where corroboration of results was obtained by two laboratories (ie at a veterinary investigation centre and at the Central Veterinary Laboratory or where demonstration of virus was obtained by both virus isolation and virus antigen detection methods.

Tissue fixation and processing is as described in Chapter One but some globes were presented fixed in formalin. Selected sections of kidney were stained by Yasue's method for calcium oxalate (Yasue, 1969) and selected section of brain and kidney were stained by the following methods; von Kossa's, Perls' prussian blue reaction; sudan black; masson fontana; Schmorl's stain for lipofuscin; long Ziehl-Neelson method for lipofuscin; a trichrome stain or, following treatment with α-amylase for 14 hours (Stam & Roukema, 1973), with periodic acid-Schiff (PAS). Unstained sections mounted in entolan (a non fluorescent mounting medium) were used for fluorescence studies.

RESULTS

The results of this survey are presented in four sections,

i) Lesions present in viraemic bovines

ii) Lesions present in bovines seropositive to BVD-MD

iii) Lesions present in non-viraemic and seronegative bovines.

iv) Lesions present in sheep.

Lesions present in viraemic bovines.

Virus was isolated or antigen detected in three calves (Table IV). These calves did not possess antibody to BVD-MD virus. Lesions of cystic cerebral cavitation or cerebellar atrophy were not present in any calf. However, cerebellar lesions were present in one calf and consisted of mild focal localised rarefaction of the internal granule cell layer, disorganisaton of cortex and multifocal mineralisation of the intima of blood vessels. Hypoplasia of the cerebellum, as shown by the cerebellar whole brain weight percentage (10.55%), was not present.

In two calves ocular lesions were present. In one calf lesions were restricted to lenticular tissue and consisted of bilateral nuclear and posterior polar cataract. In the other calf unilateral posterior polar cataract and bilateral chronic retinal detachment (Fig. 13) was present.

Renal lesions were present in two calves and consisted of moderate to severe multifocal interstitial fibrosis, with multifocal tubule and glomerular atrophy (Fig. 14). In one calf colourless plates, rosettes, spicules and concentric clusters of crystal were present within cytoplasm of tubule epithelium and were extracellular in tubule lumens. The crystal was birefringent under polarised light, and stained black

with Yasue's method. A yellow brown granular pigment was also multifocally distributed in tubule epithelium. This pigment was dark brown stained with Masson-Fontana but did not react for calcium salts by von Kossa's method nor did it react for ferric iron with Perls' prussian blue reaction. Multifocal cortical tubule mineralisation was also a feature. Pasteurellosis, and Pasteurella haemolytica biotype A was isolated from both.

Lesions present in bovines seropositive to BVD-MD

Eight calves and two fetuses had antibody to BVD-MD virus and in all of these animals attempts at virus isolation or virus antigen detection were unsuccessful (Table V). In six of these animals hydranencephaly (Fig. 15) or porencephaly was present and in all but one of these severe cerebellar lesions were also present. One calf showed unilateral porencephaly with hydranencephaly of the contralateral hemisphere. On gross examination a brown pigment lined cystic cavities of two cases. Histological examination showed pigment in phagocytes within and at the margin of cavities. This pigment was brown-black with Masson-Fontana, and did not stain for neutral lipid, calcium or ferric iron. The pigment was autofluorescent and stained for lipofuscin with Schmorl's stain.

Additional cerebral lesions included cavum septum pellucidum and mild multifocal non-suppurative encephalitis. In two calves clusters of irregularly oval, laminated basophilic structures frequently with an eosinophilic core were present. They were non fluorescent, non birefringent, PAS negative, von Kossa negative, and ferric iron positive. They were also incompletely degraded by α-amylase and thus were identified as corpora amylacea (Stam & Roukema, 1973). They were mainly located in basal ganglia and thalamus. Gross examination

of the brain of calf nine showed complete absence of corpus callosum (Fig. 16), including the hippocampal commisure, and cerebellar hypoplasia. Rostral and posterior commisural fibres were present. Heterotopic bundles of myelinated fibres, the so-called bundles of Probst were present and a thin translucent membrane traversed obliquely from the dorso-medial border of the lateral ventricles to the fornices. Histological examination showed mild non-suppurative meningitis in this case also.

Cerebellar atrophy or hypoplasia and dysplasia was present in nine cases. Lesions classified as hypoplasia showed mild, moderate or severe diffuse granule cell loss (hypoplasia) often with localised granule cell and Purkinje cell heterotopia (so-called granuloprival cerebellar dysplasia) (Fig. 17). In such cases the cerebellar whole brain weight ratio was low (5% case nine, 4% case six). Where lesions were classified as atrophic (see Fig. 15), granule cell loss and Purkinje cell heterotopia were present but were minor components of the lesion. The predominant finding was severe cystic cavitation of white matter and remaining cortical tissue showed severe disorganisation. Little cerebellar tissue remained and whole brain weight ratios were not evaluated.

In only two cases in this group were eyes presented for examination. One case showed severe bilateral diffuse photoreceptor cell atrophy (Fig. 18) with gliosis of optic nerve and the other showed bilateral pseudorosettes and rosettes of photoreceptor cells. Lesions of other organs or tissues included brachygnathia and those consistent with pneumonic pasteurellosis. In case nine, a seven month-old fetus, systemic inflammation consisting of organising fibrinopurulent exudate was present on serous membranes of heart, kidney and lung.

Multifocal suppurative alveolitis and bronchiolitis was associated with argyrophilic organisms, morphologically similar to Campylobacter. sp. Culture of fetal stomach contents resulted in isolation of a bacterium identified as Campylobacter. sp. and characterised by biochemical tests as C. fetus subs. fetus.

Lesions present in non-viraemic, seronegative bovines.

Two calves showed morphological lesions typical of BVD-MD infection but lacked antibody to BVD-MD virus (titre less than 1:10) yet virus isolation/antigen detection procedures were unsuccessful (Table VI). One calf showed cerebral lesions of hydranencephaly and cavum septum pellucidum. Lesions of cerebellum were severe and atrophic with marked cystic cavitation. The only recognisable residual elements of the cerebellum were small nodules of tissue at the cerebellar peduncles. Ocular lesions of multifocal photoreceptor cell atrophy were also present. No cerebral lesions were present in the second calf and cerebellar changes were mild. The cerebellum was grossly normal and the cerebellar whole brain percentage was 8.94%. Histologically lesions were of the granuloprival type. Ocular lesions of chronic retinal detachment were also present.

Lesions present in sheep.

Five sheep showed lesions suggestive of pestivirus "alternative pathology" (Barlow, 1980) (Table VII). In each case additional evidence of BD virus infection was obtained in contemporary lambs or in ewes in the flocks of origin. Lambs four and five originated from the same flock. Two seropositive sheep showed mild to moderate porencephaly and cerebellar dysplasia. Cerebellar lesions consisted of multifocal rarefaction of internal granule cells with generalised multifocal granule cell and Purkinje cell heterotopia. Foci of min-

eralisation were present and sheep one showed a cavity of the white matter (Fig. 19). One lamb showed no CNS changes but unilateral ocular changes of retinal-vitreous adhesions, hyperplasia of the retinal pigment epithelium, focal loss of photoreceptor cells, rosette formation, focal retinal detachment, focal gliosis, and invasion of sensory retina by retinal pigment epithelium (Fig. 20).

Two further sheep showed severe bilateral porencephaly (Fig. 21) and cerebellar dysplasia. The cerebellar lesions were similar to those described above. As with calves shown in table VI showing similar lesions, these sheep were seronegative and non-viraemic.

DISCUSSION

In this survey lesions of hydranencephaly or porencephaly, cerebellar atrophy or severe granuloprival cerebellar dysplasia of both cattle and sheep were generally (13/17) associated with a serum neutralising antibody response and such animals were invariably non viraemic. Lesions in viraemic calves were mild and grossly visible CNS or ocular changes were not evident. The cerebellar whole brain weight percentage was within the normal range. (normal range = 8.5-11.1% personal observations and Scott et al., 1973).

Calves with severe cerebral and cerebellar lesions had antibody to BVD-MD virus but it was not determined whether this was colostral in origin, or, whether seroconversion had taken place. However, the similar nature of these lesions to those previously described for BVD-MD infection (Brown et al., 1973; Done et al., 1980; Markson et al., 1976; Scott et al., 1973; Trautwein et al., 1986) suggests that these lesions were caused by BVD-MD virus infection. Furthermore, two fetuses (Table V, calves two and nine) showed a serological res-

ponse which is indicative of a transplacental (in-utero) infection by BVD-MD virus. The nature of the cerebral and cerebellar lesions present in seropositive sheep is similar to those described in Chapter Three and also to those described as "alternative pathology" by Barlow (1980). BD virus infection is the only known cause of such lesions of British sheep. Lesions suggestive of copper deficiency were not present.

All cases of cystic cerebral cavitation and severe cerebellar atrophy or dysplasia presented in this study were not viraemic. One case excluded from this study did show severe cerebellar atrophy in association with virus antigen detection. This case was excluded from the study as only antigen detection procedures were employed and because during virus isolation attempts bacterial contamination occured which may have compromised interpretation of the fluorescent antibody test. The possibility that some cases of severe cerebellar dysplasia may be associated with viraemia cannot be entirely excluded. In a recent study (Trautwein et al., 1987) demonstrated virus in cerebrospinal fluid in a calf with hydranencephaly and cerebellar hypoplasia. Nevertheless, the results of this study combined with results in previous Chapters demonstrate that most animals with lesions of severe cystic cavitation and cerebellar dysplasia are seropositive and virus is not present in tissues.

In two calves and two sheep with cerebral and/or cerebellar and ocular lesions neither virus isolation, virus antigen detection nor a serological response was found. Several viruses are known to cause cerebral cavitation of cattle and sheep including Wesselsbrons disease virus (Coetzer et al., 1979), Akabane virus (Inaba, 1979), bluetongue virus (Osburn et al., 1971) and BVD-MD (Markson et al.,

1976; Scott et al., 1973). The only viruses currently known to occur in Britain capable of causing such lesions are BVD-MD and BD. Mid-gestational maternal pyrexia may also cause cystic cerebral cavitation (Hartley et al., 1974) of the fetus. However, ocular and cerebellar lesions such as were present in the calves and lambs described in this study are not reported following maternal pyrexia. In view of the close similarity of the cerebral, cerebellar and ocular lesions in these cases to those of the serological responder group and to those of previous reports of experimental disease of both cattle (Bistner et al., 1971, Brown et al., 1974) and sheep (Barlow, 1980) it is likely that the lesions in these cases are caused by BVD-MD or BD virus infection. The absence of a serologic response (that is, one with a titre of less than 1:10) or virus in tissues in these cases is puzzling. BD and BVD-MD virus are notoriously capricious viruses in the laboratory and the possibility that the laboratory virus isolation results are erroneous should be borne in mind. Ontogenically, cellular immune surveillance functions preceed the ability of the host to mount a humoral response. In sheep a cellular inflammatory response mediated by large mononuclear cells may represent the earliest and most primitive fetal defence mechanism (Osburn, 1981). Experimental studies show that the bovine fetus becomes immunologically competent to BVD-MD at 90-100 days gestation with specific neutralising antibody produced some 30 days later (Schultz, 1973). Previous studies have also shown that BD virus infected sheep may show weak and transient serological responses and others may seroconvert after long periods of seronegativity (Terpstra, 1981; this thesis Chap 3 p.48). Thus some nonviraemic, seronegative animals with severe CNS or ocular lesions may have eliminated the viral infection without responding serologically.

These results also suggest the sequential pattern of development of lesions and their relationship to gestational age of infection. Ocular and cerebellar lesions resultant upon necrosis of primary or secondary lens fibres, neuroblasts of the retina or fetal external granule cells of the cerebellum occurred in viraemic calves. Degeneration of differentiating or mitotically active cells is probably directly related to viral infection of such cells. Persistent viraemia may occur in calves infected at up to 125 days gestation (McClurkin et al., 1984). However, hydranencephaly, porencephaly and cystic cavitation of the cerebellum occurred only in non viraemic calves and sheep. Lesions of cystic cavitation probably have a different causation from those seen in viraemic calves and were only found in seropositive or non-viraemic seronegative animals. These observations provide some evidence to suggest that such lesions are wholly or partly mediated by the fetal immune response. Thus the variation in pathological lesions following in-utero infection is dependent upon the availability of susceptible target cells particularly neuroblasts, and possibly also on the development of immunologic responsiveness. Lesions of cataract, retinopathy and mild cerebellar dysplasia must preceed those of cystic cavitation as the former lesions are found in persistently viraemic calves; Lesions of cystic cerebral cavitation occur in animals able to eliminate virus and generally in animals able to respond serologically and hence occur after 90 days gestation (Schultz, 1973).

Several pathological features novel to BVD-MD or BD virus infection were seen. Cavum septum pellucidum, seen frequently in "alternative-pathology" of sheep (Barlow, 1980) but not previously described in BVD-MD infected cattle, was present in two calves.

Renal tubule and glomerular atrophy has not previously been reported in BVD-MD infected calves. Axthelm et al., (1981) described birefringent crystals in kidneys but did not further characterise the nature of the crystal. The crystal present in the kidneys of one calf had optical (Peterson & Kuhn, 1965) and histochemical (Yasue, 1969) properties of oxalate. Oxalate crystal has previously been described in calves showing various serious congenital defects, usually of a skeletal nature. It is suggested that crystal formation is due to accumulation of glycoxylates following breakdown of connective tissues (Gopal et al., 1978).

The significance of corpora amylacea formation in two calves is not known. These structures are present in senescent human brain (Ramsey, 1965) but they have not previously been described in developmental CNS disorders. Corpora amylacea have been found in brains of newborn lambs affected with a lethal congenital renal hypoplasia (personal observation).

Previous descriptions of ocular disease in BVD-MD infected cattle and also in sheep (Chapter Two) have invariably been in associated with cerebral or cerebellar pathology (Bistner et al., 1970; Brown et al., 1973). However, this study shows that ocular lesions may occur in the absence of CNS pathology. The lesions present in viraemic calf three are, though mild and less florid, similar to those described by Bistner et al. (1970) in a calf infected at 78 days gestation. Although virus isolation results are not reported by Bistner, in view of the gestational age of infection it is likely that this animal would also have been viraemic.

Finally agenesis of the corpus callosum has not been described

following pestivirus infection of ruminants. The corpus callosum is the largest horizontal commissure of the brain. It connects the cerebral hemispheres and forms the roof of the lateral vetricles. Partial or complete agenesis of the corpus callosum is a rare and sporadically occurring defect (Innes & Saunders, 1962). The normal development of the corpus callosum in cattle has not been well studied. In man the first callosal fibres are seen anterior to the foramina of Monro at 12 weeks gestation and by 17 weeks gestation the adult form has been reached (Kendall, 1983). A causal relationship between BVD-MD virus infection and callosal agenesis has not been established in this case. However, assuming callosal development in the bovine occurs at a correspondingly similar period of gestation to that in man this period of gestation coincides with the putative gestational age at which BVD-MD infection occurred in this fetus (90-150 days) (Brown et al., 1973). Also callosal agenesis has been reported following maternal (human) infection with a related virus, rubella, which is also a member of the family Togaviridae (Friedman & Cohen, 1947).

In summary these studies show that animals with grossly visible CNS lesions suggestive of BVD-MD or BD virus infection are likely to be seropositive to BVD-MD or BD virus and attempts to isolate virus from viscera and blood will probably be unrewarding. The results also show that in at least some cases ocular lesions occur in the absence of grossly visible CNS lesions and in such cases virus may be isolated. Some animals with severe BD or BVD-MD induced cerebral or cerebellar changes may show neither a serological response nor persistent viraemia.

TABLE IV. Table showing distribution and severity of lesions in viraemic calves.

Case No	Age of Calf	Ingestion of Colostrum	Serology of Calf	Serology of Dam	Virus Isolation or Antigen Detection	Severity of pathological lesions.			Other Lesions Present
						Cerebrum	Cerebellum	Ocular	
86/066	60 days	Yes	<1:20	1:480	Positive	-	+	-	Nephropathy with tubule atrophy and pigmentation. Pneumonic Pasteurellosis.
85/1276	12 days	Yes	<1:10	N.E.	Positive	-	-	+	Nephropathy: multifocal tubule atrophy.
86/1607	42 days	Yes	<1:20	N.E.	Positive	-	-	+	Pneumonic pasteurellosis.

Legend N.E. Not Examined
\+ Mild
++ Moderate
+++ Severe
\- Absent.

TABLE V. Table showing distribution and severity of lesions in seropositive bovines

Case No	Age of Calf	Ingestion of Colostrum	Serology of Calf	Serology of Dam	Virus Isolation or Antigen Detection	Severity of Pathological Lesions			Other lesions present
						Cerebrum	Cerebellum	Ocular	
85/0772 1	28 days	Yes	1:160	>1:960	Negative	+++	+++	N.E	Pneumonic pasteurel-losis.
86/1004 2	Full term fetus	No	1:60	>1:10	Negative	++	-	N.E.	Brachynathia.
86/1275 3	3 days	Yes	1:60	N.E.	Negative	+++	+++	N.E.	None Identified.
84/1827 4	14-21 days	Yes	1:60	1:80	Negative	+++	+++	N.E.	None Identified.
84/1714 5	56 days	Yes	1:120	N.E.	Negative	-	++	N.E.	None identified.
83/1129 6	63 days	Yes	1:120	N.E.	Negative	-[2]	++	+++	None identified
84/240 7	4 days	Yes	1:40	N.E.	Negative	+++[1]	+++	N.E.	None idenitifed.
86/1243 8	7 days	Yes	1:120	N.E.	Negative	+++[3]	+++	N.E.	None identified.
86/1481 9	210 days gestation	No	1:640	N.E.	Negative	-[4]	++	N.E.	Systemic inflamation comprising chronic peritonitis, pleurisy and pericarditis. Campylobacter fetus isolated.
E781/87 10	10 days	Yes	1:120	N.E.	Negative	+++	+++	+	None identified

Legend N.E. - Not Examined
- - No Lesions Present
+ - Mild Lesions
++ - Moderate Lesions
+++ - Severe Lesions

1. Corpora amylacea present in basal ganglia
2. Mild non-suppurative encephalomyelitis
3. Cavum Septum Pellucidum
4. Mild non-suppurative meningitis and agenesis of corpus callosum

TABLE VI Table showing distribution and severity of lesions in non-viraemic, seronegative bovines.

Case No	Age of Calf	Ingestion of Colostrum	Serology of Calf	Serology of Dam	Virus Isolation or Antigen Detection	Severity of Pathological Lesions			Other Lesions Present
						Cerebrum	Cerebellum	Ocular	
1 86/1201	12 days	No	<1:10	N.E.	Negative	+++[+]	+++	++	
2 84/1753	42 days	Yes	<1:10	N.E.	Negative	-	++	+++	Calf diphtheria (C. pyogenes and fusiformis necrophorus isolated from pharynx.

Legend - absent
+ mild
++ moderate
+++ severe
N.E. Not Examined

1. Cavum septum pellucidum.

TABLE VII

Table showing severity and distribution of lesions in sheep.

Case No.	Age of Lamb	Ingestion of Colostrum	Serology of Lamb	Virus Isolation or Antigen Detection	Severity of Pathological Lesions			Other History of BVD infection in Flock
					Cerebrum	Cerebellum	Ocular	
1 284/84	100 days	Yes	1:120	Negative	+[+]	++	N.E.	)Evidence of rising titres to)BVD-MD virus in flock))))
2 115/84	14 days	Yes	1:160	Negative	++	++	N.E.	
3 0322/82	120 days	Yes	>1:10*	Negative	-	-	++	One of four scouring lambs, the other three were persistently infected with BD virus.
4 617/83	120 days	Yes	<1:10	Negative	+++	++	-	)BD virus isolated from several)contemporary lambs.))))
5 616/83	120 days	Yes	<1:10	Negative	+++	++	-	

Legend - no lesions present
+ mild lesions
++ moderate lesions
+++ severe lesions

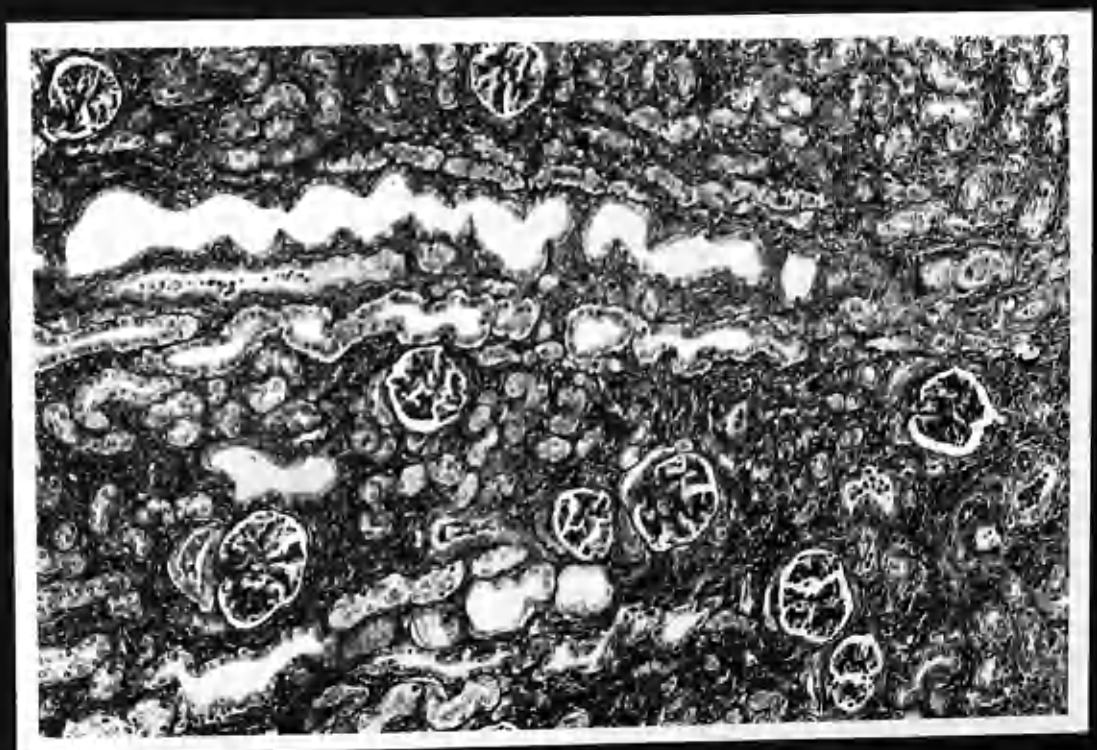
+ Lissencephaly severe bilateral hydrocephalus

* Final antibody titre not determined. Titre established as >1:10 on routine screening.

Legends for Figures

Figure 13. Retinal lesions of a viraemic calf 3 showing fibrovascular plaques of the choriocapillaris, focal depigmentation of the retinal pigment epithelium and retinal pigment epithelial proliferation and reduplication consistent with chronic retinal detachment. MSB x 100

Figure 14. Renal lesions of a viraemic calf 2 showing multifocal tubule and glomerular atrophy and interstitial fibrosis. HE x 250



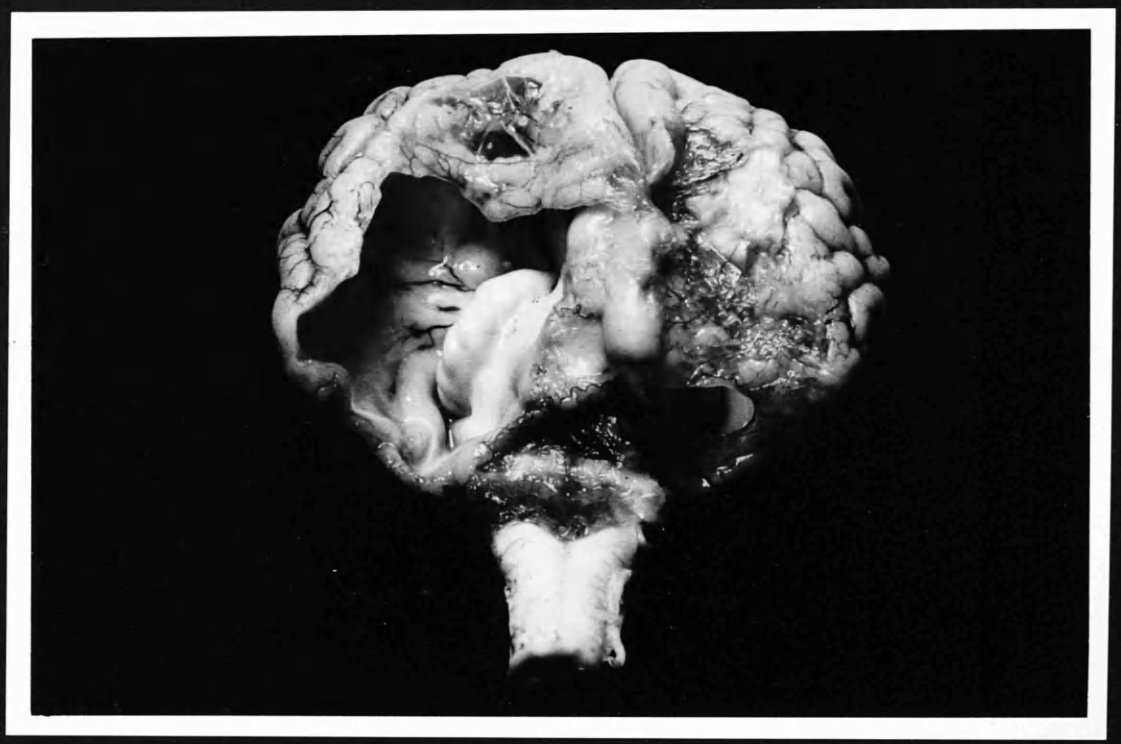


Fig 15

Figure 15. Cerebral lesion of non-viraemic calf 4 showing bilateral hydranencephaly, and severe cerebellar dysgenesis. Membraneous cerebrocortical remnants are preserved at the right cerebral hemisphere but are artefactually removed at the left hemisphere. Note the preservation of normal hippocampus (x 1).

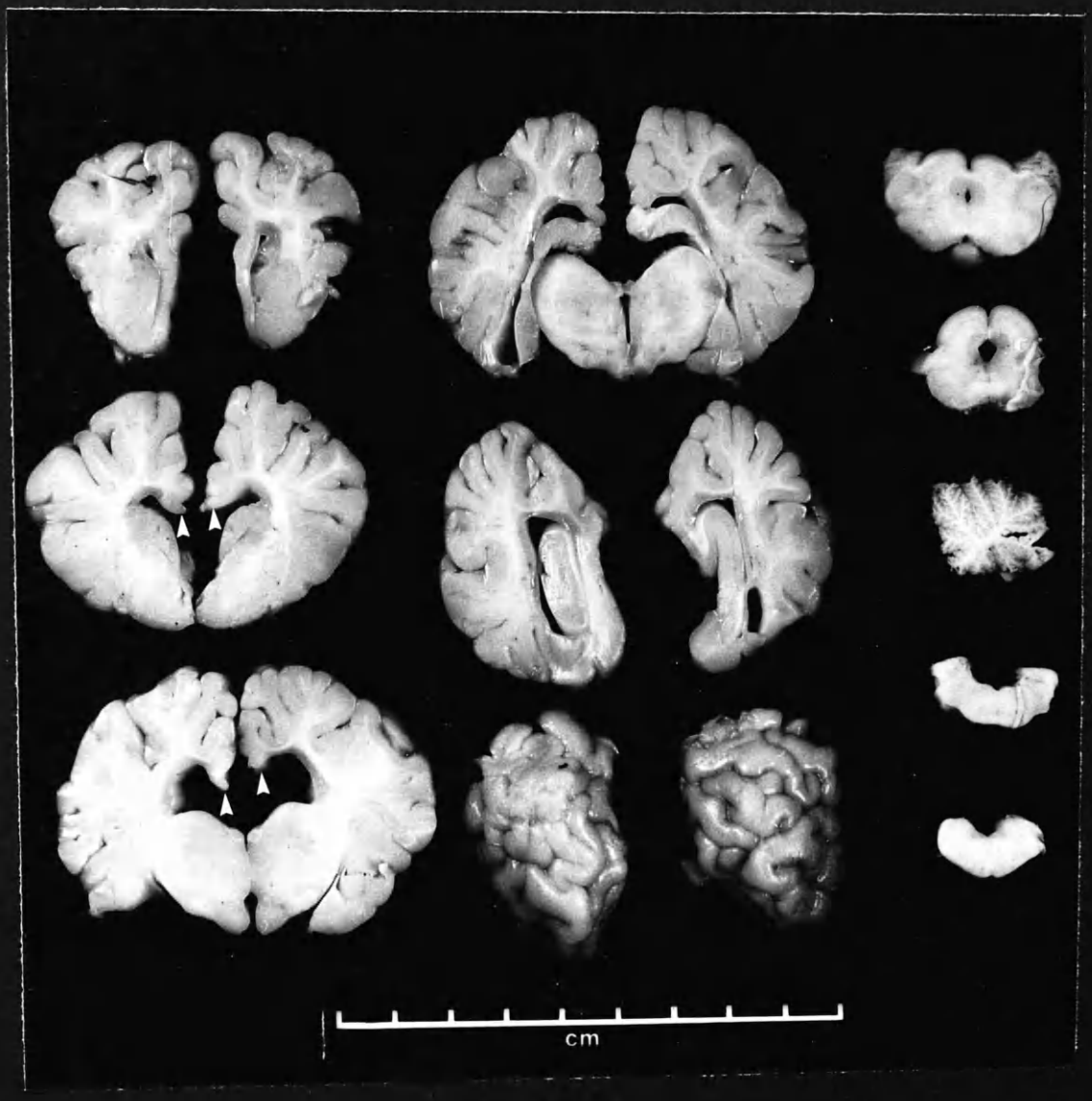

cm

Figure 16. Cerebral lesion of a non-viraemic calf 9 showing agenesis of corpus callosum with so-called bundles of Probst (arrow) and cerebellar hypoplasia (x ½)

Figure 17. Cerebellar lesions of non-viraemic calf 9 showing a cerebellar lobule with rarefaction of internal granule cell layer. HE x 70

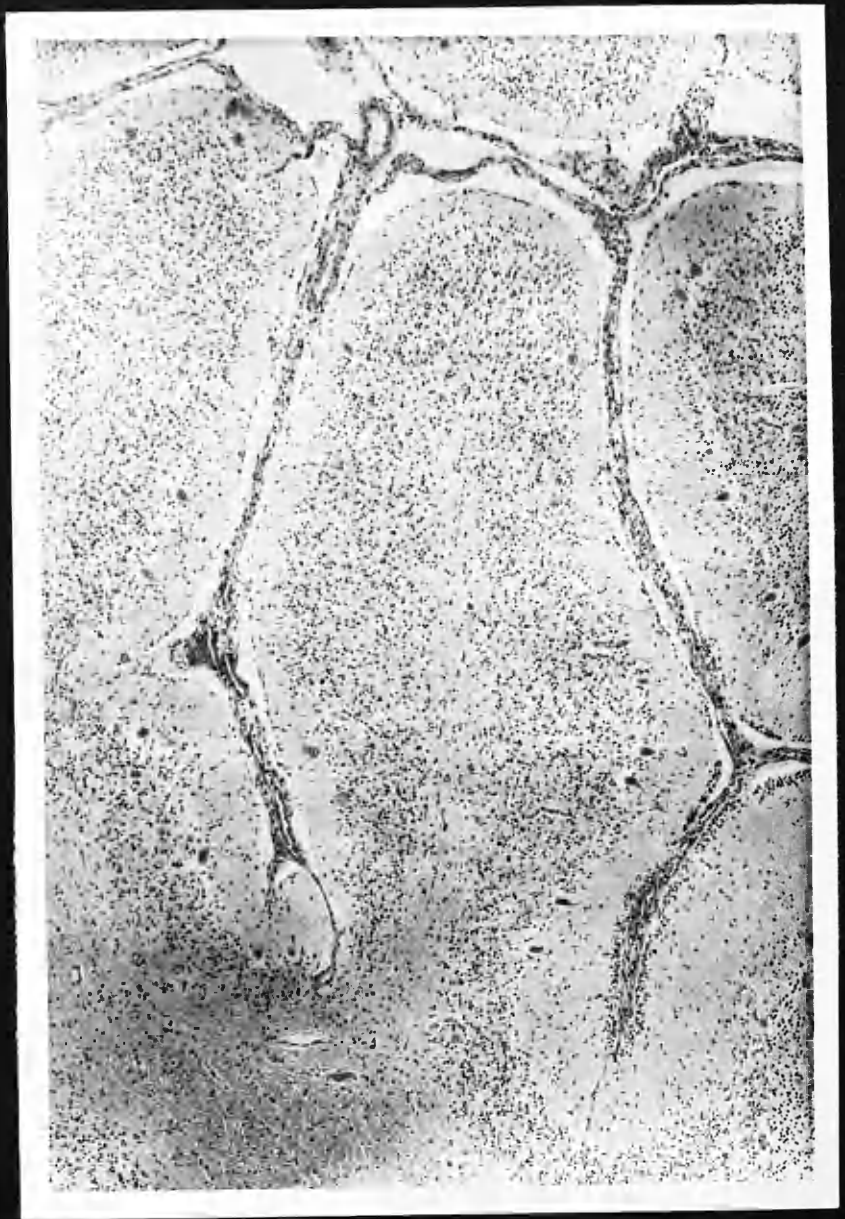
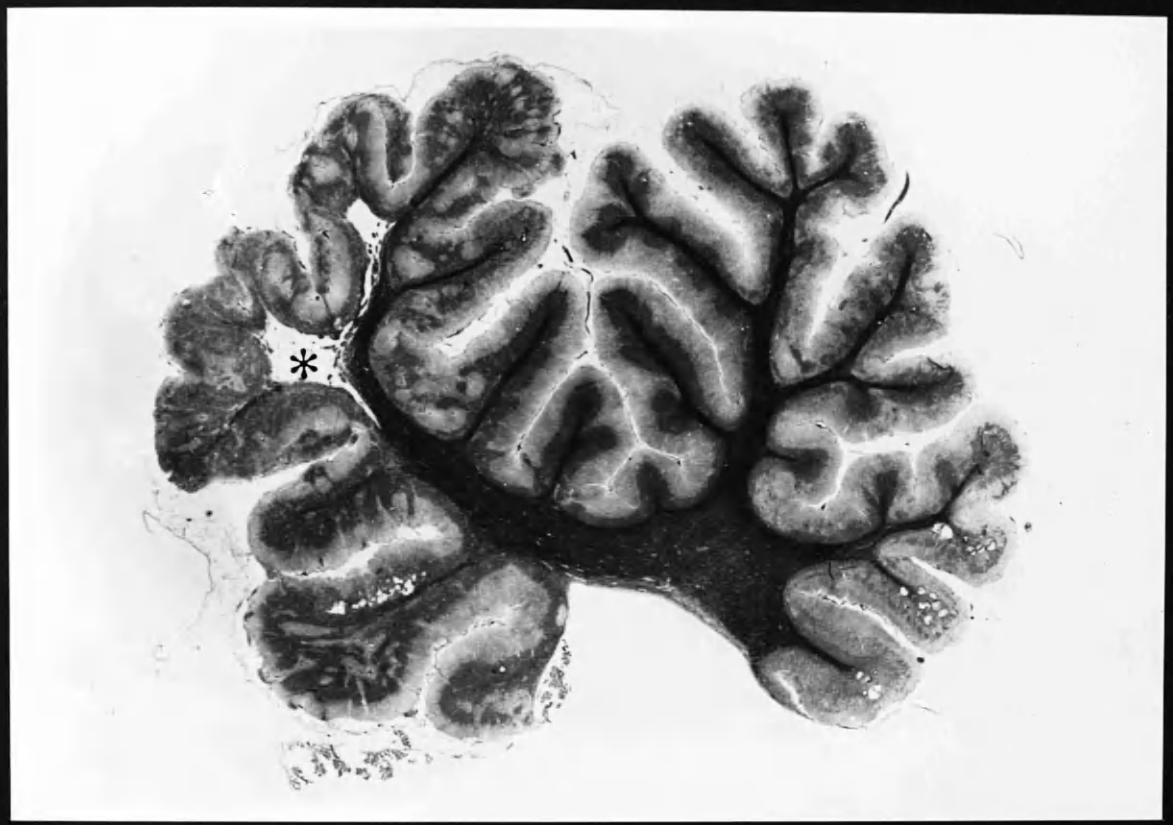


Figure 18. Retinal lesions of non-viraemic calf 6. Showing complete loss of outer and inner segments of rods and cones and loss of the outer nuclear layer. HE x 400

Figure 19. Cerebellar lesion of non-viraemic sheep 1 showing incomplete lobulation and marked disorganisation of cortex.

Focal cavitation of white matter (asterisk) is also present. Foci of mineralisation present in lobules 1, 2 and 9 have been artefactually displaced. LFBCV (x5)



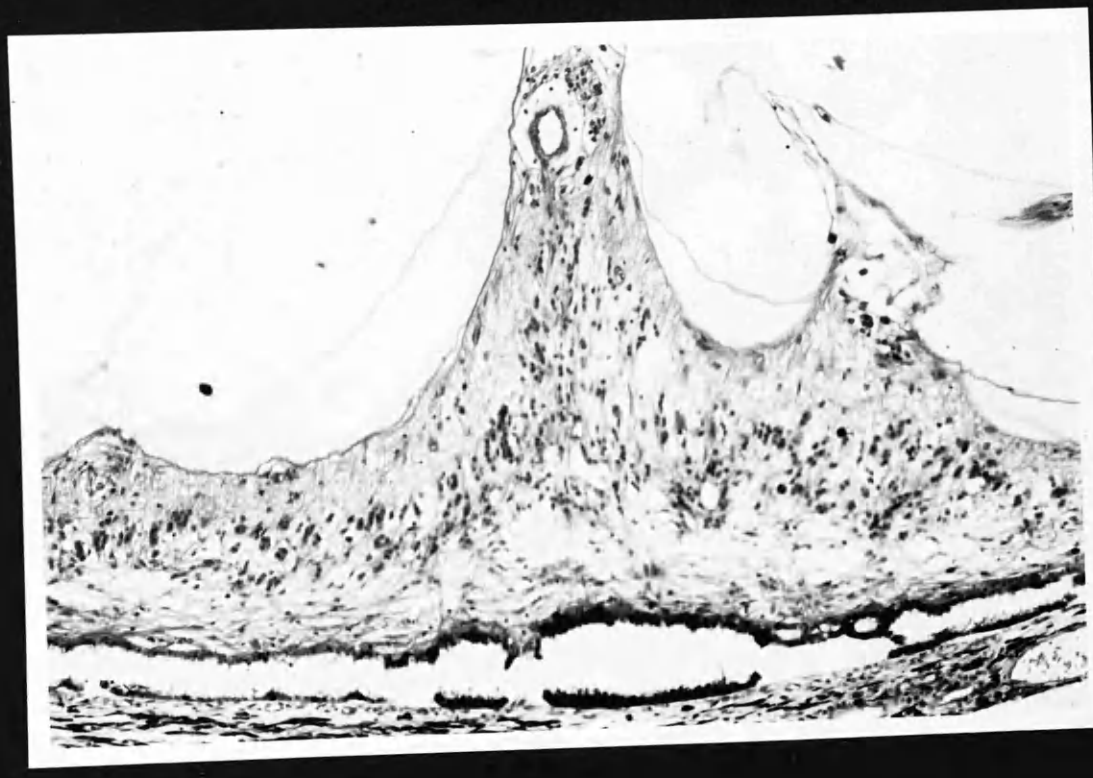
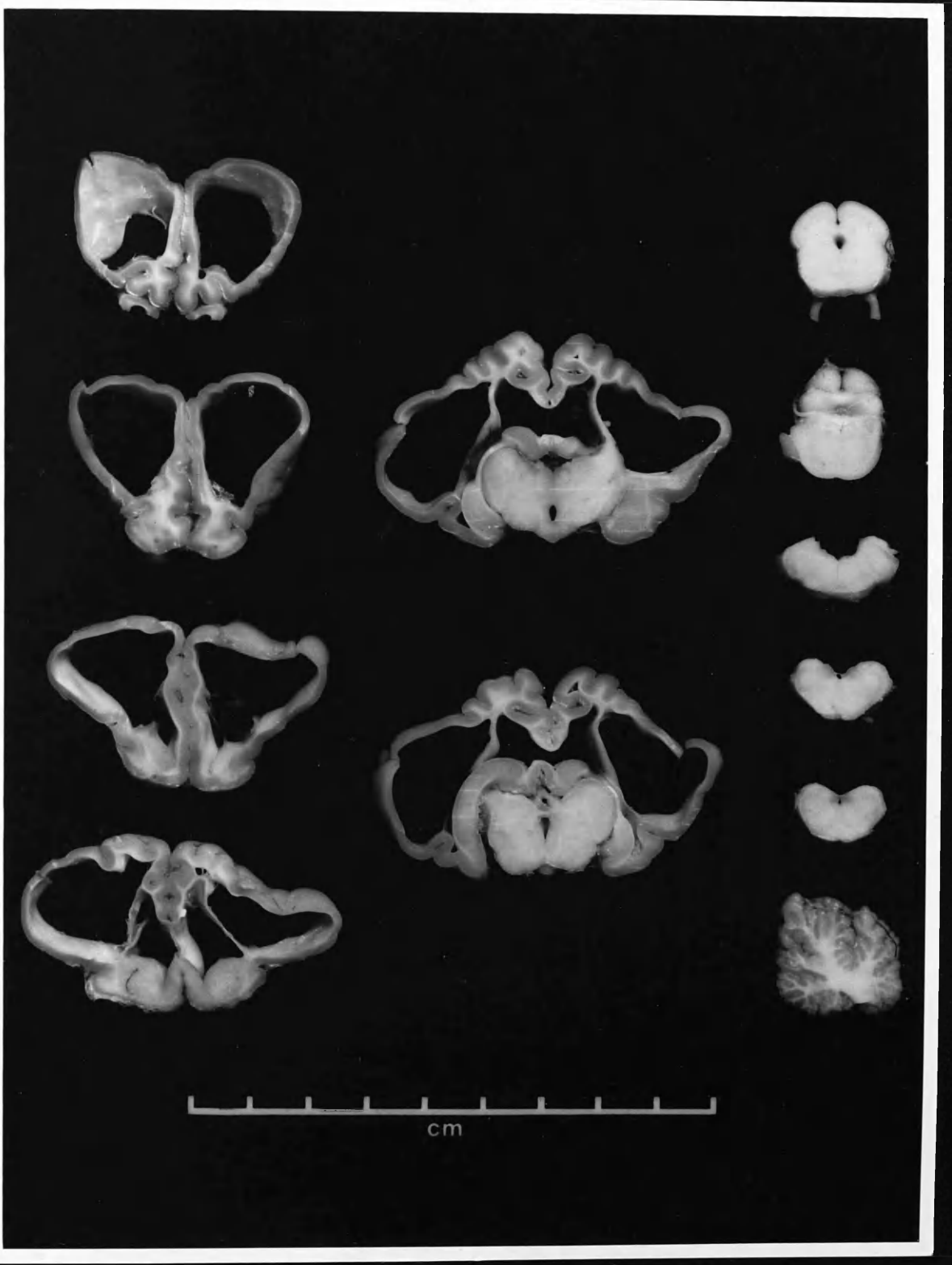


Figure 20. Retinal lesions of non-viraemic sheep showing separation of sensory retina from retinal pigment epithelium, atrophy and replacement gliosis of photoreceptor cell layer, disorganisation of hypoplastic outernuclear layer and tenting (retinal-vitreous adhesion) of sensory retina. HE x 100

Figure 21. Coronal sections of brain of a non-viraemic sheep showing marked bilateral porencephaly and moderate compensatory hydrocephalus. The cerebellum is also hypoplastic. (x 1)


cm

CHAPTER FIVE

GLIAL CELL MARKERS IN THE CENTRAL NERVOUS SYSTEM

INTRODUCTION

1.1 Historical perspective and definitions

Immunocytochemistry or immunohistochemistry is a method of identifying constituents in tissue sections by means of a specific antigen-antibody reaction and extends considerably the range of structures visualised by conventional staining methods. The technique was initially considered to be unreliable when first described by Coons (1950) but it is now well established as a highly specific research tool in the biological sciences. Visualisation of the reaction products was first achieved by fluorescence microscopy of conjugated antibody - fluorescein isocyanate reacted with tissue antigen in frozen sections. A wide range of antibody labels; including principally fluorescein, peroxidase, biotin-avidin, ferritin, haemacyanin and gold; are now available. Visualisation, depending upon the label used, may now be achieved by light microscopy, by transmission electron microscopy or by fluorescence microscopy (Polak & Van Noorden, 1983). Because of its suitability for formalin-fixed paraffin wax-embedded material and light microscopic visualisation the peroxidase technique is currently the most widely used of these labels.

Immunocytochemical techniques were used initially to localise antigens already known to exist in tissues. With refinement the techniques are now more specific and so reliable that it is possible to localise and identify molecules not previously demonstrated biochemically. To begin with also, the technique was employed to find and identify pathogenic organisms and immunoglobulins in tissues. Indeed, in veterinary pathology, the former use in particular is still the major application of the method.

A wider application has emerged in which antigen-antibody reactions or other binding mechanisms are used to visualise cells by specific molecular configurations. Such substances have become termed 'markers'.

Varon (1978) defined a cell-specific marker as a property that is expressed uniquely or prevalently in a given cell class. For the purpose of this review 'markers', with the exception of lectins, included here because of their suitability as markers for microglia are molecular configurations to which antibodies can be raised and combined with a visualisation system to identify discrete cell populations.

1.2 Aims of the review

Immunocytochemical neural cell markers are now widely applied in medical pathology and although employed extensively in laboratory animals for neurobiological research, little has been published on their uses in veterinary neuropathology. In veterinary research the priority given to infectious diseases and limiting economic factors, has largely prevented the prolific production of specific antibodies to host tissues that is occurring in research into human diseases. In medical research much impetus in this direction has come from the search for new features of intracranial tumours that might prove of diagnostic or prognostic value.

In veterinary pathology, therefore, the use of markers must of necessity evolve mainly from opportunities provided by materials developed in medical research programmes. There are indications that this is proving possible and offers considerable scope for the veterinary histopathologist to utilise markers to replace some conventional tinctorial methods.

However there is a paucity of published information on application of neural cell markers in animal disease study. The main purpose of this review is to examine the literature on glial cell specific antigens as markers of major glial cell types of the CNS with reference to their their potential or realised value in morphologic studies of veterinary neuropathology in general and in particular, in neuropathogenesis studies of BD. Details of the morphogenesis of CNS hypomyelination and cerebral cavitation following pestivirus infections have been described but the pathogenesis of these changes is little understood. Although BD virus or BVD-MD virus has been demonstrated in the CNS of lambs and calves following maternal infection (Ohmann, 1984; Terpstra, 1985), the role of the virus in the formation of CNS lesions is not established. Immunocytochemical markers are potentially valuable for studies of presumed glial cell defects in the hypomyelinated brains of persistently viraemic BD lambs.

The methodology and techniques used to visualise CNS markers are those employed in immunocytochemistry generally (Polak & Van Noordon, 1983) and will not be reviewed here.

1.3 The uses of markers

Nervous system markers can be employed in the study of structure, function and development of the nervous system. Morphological studies, which have provided the conventional classification of cells of the nervous system, define essentially four types of cells - oligodendrocytes, astrocytes, microglia and neurons. While this classification is currently used by anatomists and pathologists as a basis for "cell type" new knowledge may show its imperfections and provide alternative taxonomies. Indeed one of the most important uses of markers is to

identify aspects of organisation or function of cells not readily apparent by tinctorial or other methods.

This is borne out by a rapidly expanding literature which identifies nervous system proteins defining sub-sets of neurons and, to a lesser extent, sub-sets of glia (discussed in detail below). In addition to applications for elucidating tissue differentiation, microanatomy and cellular physiology neural cell markers can also be used to identify "cell types" in situations where characteristic morphologic stigmata are lacking. In developmental neurobiology, for example, markers have been used to study the histogenesis of neural cells (Choi, 1981). They have also been used to show pathophysiological changes occurring in cells prior to morphologic abnormalities and therefore inform on pathogenesis (Itoyama et al., 1982). In man a major pathological application for neural cell markers has been the histological investigation of intracranial tumours but there is presently little application of such markers in disease diagnosis of domestic animals. Markers have also been used as an adjunct to morphometric analysis to delineate specific cell populations.

1.4 Cell specialisation and organisation

Although the range of physiological properties possessed by stem cells is retained, at least potentially, by all but the most specialised of cells during ontogeny each specialised tissue becomes structurally adapted to express a restricted range of these physiological properties. The nervous system is uniquely specialised with respect to irritability and conductivity.

Cell constituents that can be utilised as cell markers are components of the cell membrane or intracellular substances. They are mostly

macromolecular proteins. The specificity of the cell property or constituent to a cell population determines its informational value as a marker. Such information may be related by light microscopic visualisation to cell distribution and special organisation or with increased marker specificity and ultrastructural localisation to subcellular structure and function. While markers frequently give information which correlates with structural organisation most applications of markers, at least initially, are empirical but may later give knowledge of functional significance.

1.5 Variabiliy of Marker expression

Many terms are used to indicate the immunocytochemical antigen/antibody reaction, but in this review tissue antigens are said to be expressed by cells and to react with applied antibody.

It is important to distinguish between the intrinsic capabilities of a cell and the extent to which neural cell markers become expressed in a given cellular or humoral environment. In some circumstances the cell constituent which the cell marker antibody recognises may be "switched off" but under different circumstances expression is "switched on " and may even be enhanced . For example, human glioma cells show enhanced glial fibrillary acidic protein expression on contact with collagen (Herpers et al., 1984). Raff et al. (1983a) also described a cell from the optic nerve of the 7 day old rat which in culture with added fetal calf serum showed astrocytic differentiation and without the serum oligodendrocytic differentiation . It is, therefore, important to employ an appropriate range of control tissues when using cell markers in novel situations or in pathological tissues since their specificity is dependent not only upon antigen/antibody relationships but also upon expression or availability of

that cell marker protein.

Much of the work done on neural cell markers has been carried out on human or rodent tissues and the value of such markers in domestic animal microanatomy and pathology has still to be evaluated. Nevertheless, many proteins are well conserved (i.e. preserved in phylogenetically diverse groups of animals) and although they may be present in differing quantities or at differing stages of development and may be variably expressed they may prove valuable interspecies cell markers. Table VIII shows some examples of the interspecies preservation of markers.

2. Non specific glial cell markers

2.1 *S100*

S100 is a highly soluble acidic protein which was first isolated from brain by Moore and McGregor (1965). In the rat it is present in many tissues but it is at least 10,000 fold more concentrated in brain than elsewhere in the body (Moore, 1972) and is generally found in higher concentrations in white matter than in grey matter.

S100 has been demonstrated in a wide variety of vertebrates and antiserum to bovine brain S100 cross reacts with brain extracts from other mammals and from birds, fishes and reptiles (Moore, 1982). S100 was one of the first glial specific proteins identified in tissue sections and was originally demonstrated by immunofluorescence (Hyden & McEwan, 1966). More precise localisation of S100 in adult rat brain using the peroxidase labelled antibody technique has demonstrated it in the nucleus and cytoplasm of astrocytes and in a proportion of oligodendrocytes but not in neurons (Ludwin et al., 1976) . Weak expression of S100 in Golgi epithelial cells of the rat cerebellum

is also described. S100 is found in tissues originating from the neural crest such as Schwann cells (Stefansson et al., 1982) and melanocytic tumours (Kindblom et al., 1984) and it is also found in tissues other than those of neuroectodermal origin (Kahn et al., 1983).

Although the glial localisation is well established its neuronal localisation of S100 is controversial. Hyden and McEwen (1966) found S100 in neuronal nuclei as well as in glia. In the developing nervous system of the rat Rapport et al. (1974) noted S100 in Purkinje cells. Michetti et al. (1974) also considered that low levels of S100 could be found in some neurons of adult rat brain. Ultrastructural immunocytochemistry of rat cerebrum and cerebellum further localised the protein to synaptosomes and particularly to the postsynaptic membrane (Haglid et al., 1974 & 1975) but Matus and Mughal (1975) were unable to confirm synaptic localisation of S100 in rat brain. There may be considerable differences in localisation of S100 with species and stage of development.

In mouse brain low levels of S100 are present at post-natal day 1, rise sharply at day 7 and then again increase rapidly between days 14-28 (Cicero et al., 1972). These increases in S100 in the mouse correspond to the initial period of myelination. In embryonic rat brain S100 is synthesized by primitive cells adjacent to the ventricle (Cimino cited by Moore, 1982). The migration of S100 expressing cells from these subependymal sites and their subsequent differentiation into astrocytes and oligodendrocytes has also been shown (Haglid et al., 1977). In the chick only well-differentiated oligodendrocytes express S100 (Hartman et al., 1977).

3. Oligodendrocyte markers

Oligodendrocyte markers may be subdivided into three groups based on their biochemistry and sub-cellular distribution. Some protein, glycolipid and lipid markers of fully differentiated oligodendrocytes are associated with the myelin sheath and mesaxon (3.1-3.5). Other markers are enzymes located in the cytosol (3.1, 3.6, 3.7). The remaining oligodendrocyte markers (3.8 and 6.0) are located on the cell surface and their nature is as yet unknown.

3.1 *Myelin Basic Protein (MBP)*

MBP is specifically localised in myelin and comprises 30 per cent or more of total myelin protein. MBP was originally located in myelin using fluorescein labelled antibodies (Rauch & Roffel, 1964). The amino-acid sequence of MBP is known for the rabbit (Martenson et al., 1981) and for a few other species and is structurally similar in most mammals but monoclonal antibody studies have shown interspecies differences. Sires et al. (1981) reported a monoclonal antibody raised to monkey (Macaca nemistrina) MBP which reacted with human, monkey and bovine tissue MBP. It also reacted with rat L-BP which is one of two forms of MBP in this species, the other being designated S-BP (Hartman et al., 1979) but does not react with pig, guinea pig, and chicken MBP.

Ultrastructural immunocytochemical results of localisation of MBP are conflicting. Some studies showed MBP in the intra period line (Sternberger et al., 1979). Omlin et al. (1982) overcame technical problems, largely difficulties in penetration of antiserum, by studying teased optic nerve fibres of 7-11 day old rats and showed localisation of MBP to the period line. Using the peroxidase-anti-

peroxidase method Sternberger et al (1978a) found that cell bodies and processes of oligodendrocytes in the CNS of the newborn rat react intensely for MBP. Ultrastructural immunocytochemical localisation of MBP in developing rat brain showed diffuse distribution within the oligodendrocyte cytosol or random association with the surfaces of subcellular structures (Schwob et al., 1985). MBP is not restricted to the perikarya; it extends throughout cell processes enveloping newly myelinating axons. MBP staining, observed within oligodendrocyte cytoplasm immediately before myelination, gradually diminished as the CNS matured, until in the adult it was only visualised in myelin (Hartman et al., 1979; Sternberger et al., 1979).

Neonatal rat oligodendrocytes also elaborate myelin and MBP in vitro (Mirsky et al., 1980). In cultures of neonatal (1 day old) mouse brain MBP was expressed only after 14 days (Bologa-Sandru et al., 1981a). Barbarese and Pfeiffer (1981) showed that oligodendrocytes, unlike Schwann cells, produce MBP and membranous structures resembling "unfurled" myelin in vitro in the absence of axonal contacts suggesting that MBP production is an intrinsic property of the oligodendrocyte. In vitro also, myelination parallels the situation seen in vivo in that MBP gradually disappears from the perikara of the oligodendrocyte and accumulates in the membrane myelin. Zurbriggen et al. (1984) demonstrated MBP and Myelin-Associated Glycoprotein (MAG) expressing oligodendrocytes in dissociated cell cultures of neonatal canine brain using monoclonal antibodies to human derived antigens.

Although it has long been understood that oligodendrocytes do not divide in mature brain, tritiated thymidine can be incorporated into oligodendrocyte nuclei following trauma (Ludwin, 1984). Ludwin and Sternberger (1984) showed a population of cells in adult mouse brain

capable of reverting to developmental status, at least at a functional level. Following demyelination induced by cuprizone intoxication oligodendrocytes expressed cytoplasmic MBP during the period immediately prior to and during remyelination. Remyelinated sheaths resembled normal myelin sheaths in their distribution of MBP.

Oligodendrocytes of the adult mouse brain do not invariably respond to insult by the production of specific myelin proteins. Ludwin (1985) showed that although oligodendrocytes in adult mouse brain proliferate at the margins of excision wounds they do not express cytoplasmic MBP. Ludwin (1985) suggests that the production of myelin proteins depends on a specific stimulus, perhaps from axonal contacts (such as has been shown in the peripheral nervous system [PNS]), which is not necessarily the same as the stimulus for mitosis.

3.2 *Proteolipid Protein (PLP)*

PLP is the major structural protein of the central nervous system and comprises 50% of myelin in human brain (Eng et al., 1968). Agrawal et al. (1977) showed that myelin proteolipid is confined to the myelin sheath and is not represented in oligodendrocyte perikarya in the adult rat. However, in the 10 day old rat PLP is expressed as coarse granular staining in the cytoplasm of actively myelinating oligodendrocytes in corpus callosum but is not present in astrocytes or neurons (Agrawal et al., 1977). Studies of myelination in both the developing rat and chick brains (Hartman et al., 1979) showed that myelin protein production began after neurite pathways had been established and oligodendrocytes had migrated into these preformed pathways. Both MBP and PLP were produced only after the process of ensheathment had started (Hartman et al., 1982).

Serial sections showed also that individual cells did not simultaneously express both PLP and MBP. PLP expression was seen only in those cells closely associated with neurites. As myelination progressed MBP expression decreased and PLP became apparent in oligodendrocyte populations. These authors concluded that in developing rat brain MBP production is "switched-off" prior to the production of PLP. The proportions of PLP and MBP in myelin sheaths are not constant in mature tissue (Hartman et al., 1982). In the rat and bovine brains PLP is the predominant protein in small diameter sheaths and MBP is the major protein in large diameter sheaths. Ultrastructural immunocytochemical studies of developing (8 day old) rat brain during an active period of myelination showed that staining of PLP in oligodendrocytes is restricted to membranes of rough endoplasmic reticulum and Golgi apparatus. PLP is absent from cell processes that initially envelop the axon (Schwob et al., 1985). The latter study was unable to resolve localisation of PLP to the intra period line or period line but PLP expression was constantly associated with myelin sheaths undergoing compaction. The authors concluded that the role of PLP in the formation of myelin occurs at a later stage of myelination than MBP and may relate to the compaction of myelin lamellae.

Recent studies of the myelin deficient rat shows changes of myelin periodicity which are associated with abnormally fused intraperiod lines. These changes are associated with marked reduction of mRNA for PLP and absence of immunocytochemically detectable PLP. These results further suggest that PLP has a role in compaction of myelin sheaths and is particularly important in stabilising the 1.5 nm gap between extracellular leaflets of compact myelin (Duncan et al.,

1987). In the mouse "jimpy" and "quaking", which also have myelin abnormalities, proteolipid proteins were decreased in whole brain extracts (Lerner et al., 1974). As with the myelin deficient rat the "jimpy" mutant also has a deletion of the gene coding for myelin PLP (Nave et al., 1986).

3.3 *Wolfgram proteins*

In addition to the main myelin proteins, MBP and PLP, a third class of myelin proteins, called Wolfgram proteins, has also been described (Wolfgram, 1966). These are high molecular weight proteins and two major fractions, designated W_1 and W_2, have been isolated from rat brain (Mandel et al., 1978). These proteins show structural similarities in various species. Immunodiffusion experiments, using antisera raised to rat W_1 and W_2, show complete identity between rat, chicken, mouse and human Wolfgram proteins (Mandel et al., 1978). Immunocytochemical localisation of Wolfgram proteins in the rat show expression of the protein at the "periphery" of oligodendrocytes (Roussel et al., 1977). Ultrastructural studies, in the actively myelinating 18 day old rat, have further shown expression of Wolfgram proteins at the major dense line, predominantly at the innermost and outermost lamellae of the myelin sheath (Roussel et al., 1978). In the mutant mice, "jimpy" and "myelin synthesis deficiency", studies showed that Wolfgram protein expression was infrequent, and therefore consistent with the marked decrease in oligodendrocytes reported in these mutants. Myelinated fibres showing W_1 expression were also scarce (Delannoy et al., 1977).

3.4 *Myelin-associated glycoprotein (MAG)*

Although it is not a major structural component of the CNS myelin

sheath MAG is an important high molecular weight glycoprotein of myelin (Quarles et al., 1973). In mature rat CNS myelin MAG is confined to a "peri-axonal ring" (Itoyoma et al., 1980a; Sternberger et al., 1979). In the PNS MAG similarly localised between the Schwann cell plasmalemma and the axolemma (periaxonal space) (Sternberger et al., 1979) and is present also in Schmidt-Lanterman incisures (Trapp et al., 1984). Based on the biochemical features of MAG, Trapp and Quarles (1982) proposed that the MAG molecule projected into the periaxonal space and was instrumental in maintaining the integrity of the space.

Winchel et al. (1982) induced intramyelinic vacuoles and separation of compact myelin lamellae from oligodendrocyte processes surrounding axons by exposing 18 day old rats to hexachlorophene. MAG staining was limited to periaxonal regions and no MAG expression was present in compact myelin at abaxonal margins of vacuoles. No additional sites of MAG expression were revealed by the splitting of myelin lamellae. Further evidence that MAG plays a role in maintaining the periaxonal space comes from experimental B, B^{1}-Iminodipropionitrile neuropathy of rats (Trapp et al., 1984). In chronic intoxication multiple Schwann cell processes invaginate into giant axonal swellings and separate the internodal axoplasm into two concentric compartments: a central zone of normally orientated axoplasm and an outer zone containing malorientated neurofilaments. The periaxonal space adjacent to the Schwann cell mesaxon nevertheless remains constant at a width of 12-14nm. The expression of MAG in such membranes, which are clearly separated from compact myelin, shows that MAG does not have an obligatory localisation near compact myelin and is consistent with a functional role for MAG in maintaining the periaxonal space of

myelinated fibres. Webster et al. (1983) reported ultrastructural immunoexpression of MAG in compact myelin, a finding that Quarles and Trapp (1984) consider controversial.

The labelled peroxidase anti-peroxidase method has been used to study MAG in the developing rat nervous system (Sternberger et al., 1979). Like MBP, MAG was found in oligodendrocyte cytoplasm prior to myelination and the intensity of expression increased during early development and declined during the period of rapid myelination. The distribution of MAG was also similar to that of MBP but the former peaked later and persisted longer. MAG expression could still be detected at 52 days of age whereas no cytoplasmic MBP expression was evident at 25 days of age.

Zurbriggen et al. (1984) showed expression of MAG in cultures of oligodendrocytes from neonatal dog brain.

In Progessive Multifocal Leucoencephalopathy (PML) a CNS papovavirus infection of man, Itoyama et al. (1982) studied immunostaining distribution of JC virus, MAG and MBP in serial sections of demyelinating foci. At the centre of foci, severe demyelination was accompanied by decreased or absent MAG and MBP staining but in a zone borderings active demyelination MAG staining of periaxonal regions was decreased while MBP staining remained normal. This zone also contained the highest density of virus infected oligodendrocytes and it is suggested from this model that MAG decrease, apparently preceeding active demyelination, may have a predictive role in indicating oligodendrocyte changes leading to myelin breakdown.

In Multiple Sclerosis also, morphologically normal white matter adjacent to demyelinated areas has decreased MAG expression without changes

in MBP expression (Itoyama et al., 1980b).

By contrast in canine distemper, a paramyxovirus infection also characterised by a demyelinating lesion, there is little or no evidence for primary oligodendrocyte infection and Vandevelde et al. (1983) were unable to demonstrate MAG decrease preceeding disappearance of MBP in the early demyelinating lesion. Losses of both MAG and MBP remained matched throughout the progress of the lesion.

Demyelination in Experimental Allergic Encephalitis is also accompanied by simultaneous decrease in MAG and MBP expression (Webster et al., 1985).

3.5. *Galactocerebroside (GC)*

GC is the major glycolipid of myelin and studies of GC expression have largely been performed in cultured cells. GC was initially shown to be a marker for rat oligodendrocytes by double labelling experiments in culture (Raff et al., 1978). In cultures of rat optic nerve Kennedy et al. (1980) found that antibodies to GFAP, GC and tetanus toxin each bound to separate populations of cells. GC is also expressed on the surface of oligodendrocytes from bovine brain in suspension culture (Lisak et al., 1979). Oligodendrocytes isolated from neonatal rat optic nerves 6-7 days before the initiation of myelination expressed GC and MBP in culture (Mirsky et al., 1980). Isolated human fetal oligodendrocytes gave similar results (Kennedy et al., 1980).

Some cultured oligodendrocytes from dissociated mouse brain express GC initially but later express GC and MBP (Bologa-Sandru et al., 1981a). In dissociated brain cultures from day old mice the population of GC expressing cells increased from 7-14 days and MBP was

expressed only after 14 days. These results indicate that GC is expressed on the cell membrane of well differentiated oligodendrocytes before the appearance of MBP (BologaSandru et al., 1981a). Following destruction of GC expressing cells in culture of newborn mouse brain by complement dependent cell mediated cytotoxicity GC expressing cells re-emerged in culture 2 days later (Bologa et al., 1982). These results show that a population of cells which do not express GC are precursors of GC expressing cells.

In an immunohistochemical study of neonatal mouse brain (Zalc et al., 1983) the start of GC expression in the CNS coincided with myelination.

In the "jimpy" mouse mutant there is failure of myelination and some glial cells show ultrastructural characteristics of immature oligodendrocytes (Meier & Bischoff, 1975). Dissociated brain cell cultures from the "jimpy" produced cells which expressed GC but not MBP (Bologa-Sandru et al.,1981b), suggesting that in the mutant early myelination proceeds normally but that later stages of myelination, corresponding to the period of MBP expression, is abnormal.

3.6 *Carbonic Anhydrase (CA)*

This enzyme largely controls the exchange of CO_2 between cells and extra cellular fluids by catalysing the hydration of CO_2 and is important in the control of cell volume. CA has a possible role in the regulation of brain volume (Bourke et al., 1975). It exists as two main isoenzymes and is present in all basic tissue types. Regional differences of enzyme activity exist within the CNS (Nishimura et al., 1963). Giacobini (1961) showed by microdissection and biochemical techniques that the enzyme is present to a greater extent in glial

cells than in neurons. Later immunocytochemical studies confirmed the principal glial localisation of this enzyme (Mandel et al., 1978), and Ghandour et al. (1980) showed that the isoenzyme CAII (syn. CA C) is especially rich in oligodendrocytes.

Light and electron immunocytochemical studies of CAII employing double labelling techniques with the astrocyte marker GFAP have shown that CAII is exclusively located in cells with oligodendrocyte characteristics in rat cerebellum (Ghandour et al., 1980). Kumpulainen and Korhonen (1982) found expression of CAII in choroid plexus cells and in the glial networks around neurons of the retina in mouse. Only the outer lamellae of myelin sheaths contain CAII in the CNS of the rat (Ghandour et al., 1980) and no expression of CAII was found in mouse PNS myelin or Schwann cells (Kumpulainen & Korhonen, 1982). CAII is, therefore, considered a useful marker for mature oligodendrocytes.

In the day old mouse no CAII activity could be demonstrated in brain but in 8 day old mice CAII was expressed in the caudal brain stem and by 22 days of age the distribution was very similar to that in mature brain (Kumpulainen & Korhonen, 1982). This initiation and extension of CAII expression corresponds with the topographic and temporal development of myelination in the mouse.

3.7 *L-Glycerol 3-phosphate dehydrogenase (GPDH)*

GPDH is a soluble NAD-dependent cytoplasmic enzyme catalyzing the conversion of dihydroxyacetone phosphate to glycerol 3-phosphate (α-glycerolphosphate). It is thought to be important in the NAD dependent glycolytic reaction, in the glycerol phosphate cycle and in phospholipid synthesis. Immunoperoxidase staining of adult rat brain

has shown expression of the enzyme exclusively in oligodendrocytes (de Vellis et al., 1977). Other brain enzymes have not yet been localised immunocytochemically but levels of 2,, 3,-cyclic nucleotide 3,-phosphohydrolase, for example, are high in myelin (Kurihara et al., 1971); low in myelin deficient mutants (Sarlieve et al., 1976) and are present in bulk fractions of bovine oligodendrocytes (Poduslo, 1975). Thus, this enzyme is a potential oligodendrocyte marker.

3.8 *Nervous System Antigen.1 (NS-1)*

A series of brain specific proteins have been identified using antisera raised against intracranial neoplasms. One, derived from a putative oligodendroglioma, is designated NS-1. Levels of NS-1 in normal mouse brain are greater in white matter than in grey matter (Schachner, 1974). The protein is expressed on normal oligodendrocytes but not on astrocytes, neurons or non-neural cells (Schachner & Willinger, 1979).

4. Astrocyte markers

4.1 *Glial Fibrillary Acidic Protein (GFAP)*

GFAP was first demonstrated in the brains of patients with multiple sclerosis where it was located in the characteristic plaques (Eng et al., 1971). It has since been demonstrated immunochemically in a wide variety of vertebrates including mammals, birds and fish (Dahl & Bignami, 1973). GFAP is a sub-unit of intermediate filaments of astrocytes (Yen et al., 1976) and is therefore a specific marker for astrocyte cytoplasm (Dahl & Bignami, 1973). Bignami and Dahl (1974a) found selective staining of astrocytes located in the white matter (type II astrocytes) but Ludwin et al. (1976) noted staining, albeit

weaker and somewhat variable, in astrocytes of grey matter (type I astrocytes). During some periods of development and during inflammatory processes GFAP is also expressed in ependymal cells (Eng & Rubenstein, 1978; Roessmann et al., 1980). It is also expressed in astrocyte related cells, principally Müller cells of the retina (Bignami et al., 1980), and Bergmann glial cells (syn. Golgi epithelial cells) and their processes (Bergmann glial fibres) in the cerebellum (Ludwin et al., 1976). In the cerebral cortex of the adult rat GFAP is present in cell processes mainly around small arterioles and venules and in the external glial limitans membrane (Ludwin et al., 1976).

GFAP is expressed early during development although the onset of expression varies with species. Antanitus et al. (1976) described GFAP expressing radial processes in the human fetal cerebrum at 10-11 weeks of gestation. GFAP expressing fibres have also been described in the medial wall of the lateral ventricles at the 18th embryonic day in the rat (Bignami & Dahl, 1974b) but these show no radial orientation. However, it is only first expressed around the time of birth in the mouse (Bignami & Dahl 1974a). Raff et al. (1979) showed that mature astrocytes expressed GFAP in vitro.

Following injury astrocytes enlarge and show increased fibrillary content. Experimentally, stab wound trauma in both adult and newborn rat brain increases the expression of GFAP in the normally weakly-reactive protoplasmic astrocytes (Bignami & Dahl, 1976). Radial glia of newborn rats also express GFAP following such injury and it is suggested that radial glia are in fact early forms of astrocytes (Bignami & Dahl, 1974b).

A recent study utilising intracytoplasmic glycogen to identify radial glia and astrocytes supports this hypothesis (Kadim et al., 1988). In pathological conditions involving the proliferation of astrocytes such as experimental scrapie of mice and tuberous sclerosis in man there is increased GFAP in the enlarged reactive astrocytes (McKenzie, 1983; Stefannson & Wollmann, 1980). Abnormal astrocyte distribution and abnormal Bergmann glial fibres have been shown in the "reeler" mouse cerebellar cortex using GFAP (Ghandour et al., 1981a).

4.2. *Glutamine Synthetase*

Glutamine synthetase is an enzyme of astrocytes which has been tentatively ascribed the function of uptake and metabolism of the neurotransmitters gamma-aminobutyric acid (GABA) and glutamate (McGeer et al., cited by Lagenaur, 1984). This suggests that the immunoexpression of this enzyme provides a valuable marker for these specific astrocyte functions. The enzyme has been demonstrated immunocytochemically in rat (Norenburg, 1979) and biochemically in mouse (Schousboe et al., 1977) and cat brain (Berl, 1966). There is particularly strong expression of glutamine synthetase in the hippocampus and cerebellum where high concentrations of the neurotransmitters GABA and glutamate are found. The unlabelled peroxidase method was used initially to demonstrate the localisation of this enzyme within glia of rat cerebral cortex, basal ganglia and cerebellum (MartinezHernandez et al., 1977). In the white matter a perivascular distribution was evident but the methods used did not resolve whether expression was in oligodendrocytes or astrocytes. Norenburg and Martinez-Hernandez (1979) showed by ultrastructural immunocytochemistry that the enzyme was located specifically in astrocyte cytoplasm of the adult rat brain. This is consistent with the high activity of the enzyme in

cultured mouse brain astrocytes (Schousboe et al., 1977). Glutamine synthetase has also been demonstrated in Müller cells of rat retina (Reipe & Norenburg, 1977).

4.3 *Fructose-1, 6-diphosphate aldolase (aldolase)*

Aldolase is present in mammalian tissues as the isoenzymes aldolase A, B and C. Aldolase A is present in most tissues, aldolase B is usually confined to liver and kidney and aldolase C is found chiefly in brain. Although the aldolase A isoenzyme is found in the brains of various mammals (Lebherz & Rutter, 1969) little is known of its immunocytological localisation. In man astrocytes throughout the brain express aldolase C. Marked expression of aldolase C is also seen in Purkinje cells including their processes. Weak expression of the enzyme is also seen in some cerebrocortical neurons (Kumanishi et al., 1985; Thompson et al., 1982).

4.4. *Creatine Kinase (CK)*

CK is a soluble enzyme with 3 isoenzyme forms. It catalyses the reaction of creatine and adenosine triphosphate to creatine phosphate and adenosine diphosphate. Each isoenzyme is formed from two distinct subunits M and B. In brain tissue only CK-BB is found and immunohistochemical localisation suggests astrocyte specificity (Thompson et al., 1980).

4.5. *Vimentin*

Vimentin is a protein sub-unit of an intermediate filament and was initially demonstrated in fibroblasts. It is also found in many other tissues but especially in mesenchymal cells. It is expressed by cells in the walls of larger blood vessels, astrocytes and ependymal

cells in adult mouse and rat brain (Fields & Yen, 1981; Schnitzner et al., 1981). It is also expressed by cultured human endothelial cells (Franke et al., 1979).

In embryonic mouse brain vimentin is detectable as early as embryonic day 11 and is located in radial fibres of the neural tube and cells lining the lumen (Schnitzner et al., 1981). Similarly, in cultures of early postnatal mouse brain, astrocytes express vimentin whilst also expressing GFAP (Schnitzner et al., 1981). These studies show that vimentin is a marker in the primitive neuroectoderm for astrocyte and ependymal cell precursor cells.

5.0 Monoclonal Antibodies recognising sub-populations of glia

Several workers have raised monoclonal antibodies to neural antigens using whole brain or selected neuroanatomic regions such as corpus callosum and cerebellum, as sources of immunogen complexes. Four such antibodies (01, 02, 03 and 04) have been produced by immunizing mice with homogenised white matter from bovine corpus callosum (Sommer & Schachner, 1981). Antibodies to all four "O" antigens are cytotoxic to a population of cultured mouse glia when mixed with complement, indicating that the antigen is expressed on the cell surface. All four "O" antigens are expressed on cultured glia which also express GC but not GFAP (Sommer & Schachner, 1981). In addition 03 and 04 are expressed on another population of cells which, although negative for GC, GFAP and tetanus toxin, show ultrastructural characteristics of oligodendrocytes (Schachner et al., 1981a). Initially, in dissociated neonatal mouse cerebellum cultures only 03 and 04 antigens are expressed, whereas from postnatal day 7 the cultures expressed 01, 02, 03 and 04, (Schachner et al., 1981b). 01 and 02 expressing

cells were removed from suspension cultures of 7 day old mouse cerebellum by reacting cells in the presence of antibody and complement. In the remaining culture, comprised supposedly of only 03 and 04 cells, 01 and 02 expressing cells reappeared after 1-2 days. The results of these experiments show that 01, 02, 03 and 04 antigens are expressed on the surface of oligodendrocytes and furthermore suggest that 03 and 04 expressing cells are precursors of 01, 02 and GC expressing oligodendrocytes.

There are several monoclonal antibodies which define astrocyte subsets. Monoclonal antibodies designated C_1 and M_1 were prepared in a similar manner to the '0' antigens of oligodendrocytes. C_1 is localised in radial fibres of the embryonic mouse CNS, and in ependymal cells and Bergmann glia of the adult mouse. It is also present in Müller cells of the mouse retina. M_1 is localised in astrocytes of mouse brain white matter. M1 and C1 therefore appear to be specific for different sub-populations of astrocytes but they have not as yet been identified in species other than mouse (Schachner et al., 1981a).

Other monoclonal antibodies: Thy-1, H-2, Stage Specific Embryonic Antigen 1 (SSEA-1), A2B5, M3 and M4 define subsets of astrocytes in the cerebellum of the mouse (for a brief review see Lagenaur, 1984). Many of these are potentially useful markers for studying astrocyte differentiation and maturation. For example,polysialogangliosides, to which A2B5 bind, are expressed on the surfaces of fibrous astrocytes but not on the surfaces of protoplasmic astrocytes (Raff et al., 1983b). A2B5 also reacts in rat brain with neurons, immature oligodendrocytes and oligodendrocyte precursors (Abney et al., 1983), a finding which led these authors to suggest that fibrous astrocytes may be more closely related to oligodendrocytes and neurons

than to protoplasmic astrocytes. SSEA-1 possibly defines a population of less mature astrocytes (Lagenaur et al., 1982).

6. The ontogenesis of glial cell specific proteins

The histogenesis of neuroglia remains unclear, partly because morphologic criteria for the recognition of the earliest forms of neuroglial cells is lacking and partly because of the long period of differentiation between the initial recognition of glial elements and their final differentiation (Jacobson, 1978). Current research on the expression of nervous system proteins suggests that oligodendrocytes and astrocytes derive from a common precursor cell.

Raff et al. (1983a), using 7 day old rat optic nerve, was the first to show that there were cells that expressed both GFAP and GC. These cells could be induced to develop into fibrous astrocytes (distinguished from protoplasmic astrocytes on the basis of morphology and growth characteristics) or oligodendrocytes depending on the culture medium. A summary of the putative ontogenesis of cultured rat glia is shown in Figure 22.

Choi et al. (1983) also showed that glial precursor cells may show characteristics of astrocytes and oligodendrocytes. In radial glial cells of human fetal spinal cord GFAP is expressed early in development (by 6-8 weeks) (Choi, 1981) but "transitional" cells (Choi et al., 1983), with ultrastructural and immunohistochemical characteristics intermediate between astrocytes and oligodendrocytes at 15-16 weeks, suggest that radial glia may give rise to both astrocytes and oligodendrocytes. A further study showed that the processes of GFAP expressing "transitional" cells extended into and contributed to myelin sheaths (Choi & Kim, 1984; Choi, 1986a&b).

By 17-18 weeks the GFAP expression of these cells was no longer evident and it was suggested that GFAP may be expressed transiently by myelin forming glia. A sequential study of developing glial cells within the sub-pial region of mouse spinal cord confirmed that "transitional" cells with ultrastructural features of both astrocytes and oligodendrocytes express GFAP. A summary of the ontogenic expression of oligodendrocyte proteins is shown in Table IX.

Supportive evidence for a common progenitor of oligodendrocytes and astrocytes comes also from in-vitro work (Choi & del Cerro, 1984). In cultures of astrocytes from an 11 week old human fetus 99 per cent of cells expressed GFAP. However, long term culture of these cells for two and a half years allowed the development of MBP expressing cells. These cells resembled oligodendrocytes on ultrastructural examination and produced multilayered compact myelin.

7. Microglia

Problems in nominating suitable markers for microglia arise because of uncertainty as to their origins. Current evidence suggests that, under differing circumstances, cells previously all classified as microglia have heterogenous origins. Some cells infiltrating the brain during inflammation express α-1-antichymotrypsin and lysozyme and have been shown to derive from circulating blood monocytes and are therefore mesodermal in origin (Esiri & Booss, 1984; Oehmichen, 1982). It is suggested that because so-called "resting" microglia do not express monocyte antigens they are not derived from monocytes. As yet there is no suitable immunocytochemical marker available for the identification of "resting" microglia and the best means of demonstrating these cells remains del Rio Hortega's weak silver

carbonate method.

Recent investigations have employed lectins for identification of microglia. Lectins are sugar binding proteins of non-immune origin. Ricinus communis agglutinin -1 (RCA-1) has been shown to be a marker for human microglia (Mannoji et al., 1986) but attempts at demonstrating rat microglia with lectins have met with mixed results. Schelper et al. (1985) claimed that the B4 isolectin of Griffonia simplicifolia seeds (GSA-B4) bound to monocyte derived brain macrophages and could be used to distinguish these cells from microglia. Streit and Kreutzberg (1987) however, found (in tissue fixed in formal sublimate and glutaraldehyde) that GSA-B4 bound to microglia. This latter study did not show RCA-1 binding of microglia. Species specific binding of lectins has also been demonstrated for vascular endothelium and therefore more extensive testing is required to establish the usefulness of lectins as markers of microglial cells in domestic animals.

Conclusions

Immunocytochemical nervous system markers, their importance already established in contemporary neurobiological research, seem likely, much as did the staining methods of classical histology, to become routine methods of the future. Some nervous system antigens are now proven interspecies markers of major cell types and provide a more reliable method of staining cell bodies and cell processes than previous capricious silver and gold impregnation techniques. GFAP, in particular, emerges as a universal marker for astrocytes and is present during early stages of astrocyte differentiation. CAII is the most appropriate marker for mature oligodendrocytes, MBP marks myelin sheaths and the cytoplasm of myelinating oligodendroglia and GC is a marker for premyelinating oligodendrogia, at least in cultured

cells. Recently RCA-1 and GSA-B4 have been shown to identify respectively human and rat microglia but an interspecies specific lectin has yet to be identified. Thus the markers GFAP, CAII, MBP and GC are potentially valuable tools for studying glial cell differentiation and maturation in the brains of fetuses malformed following pestivirus infection. These markers may be of particular value in studies of dysmyelinogenesis.

TABLE VIII

TABLE SHOWING PHYLOGENETIC CONSERVATION OF MARKERS

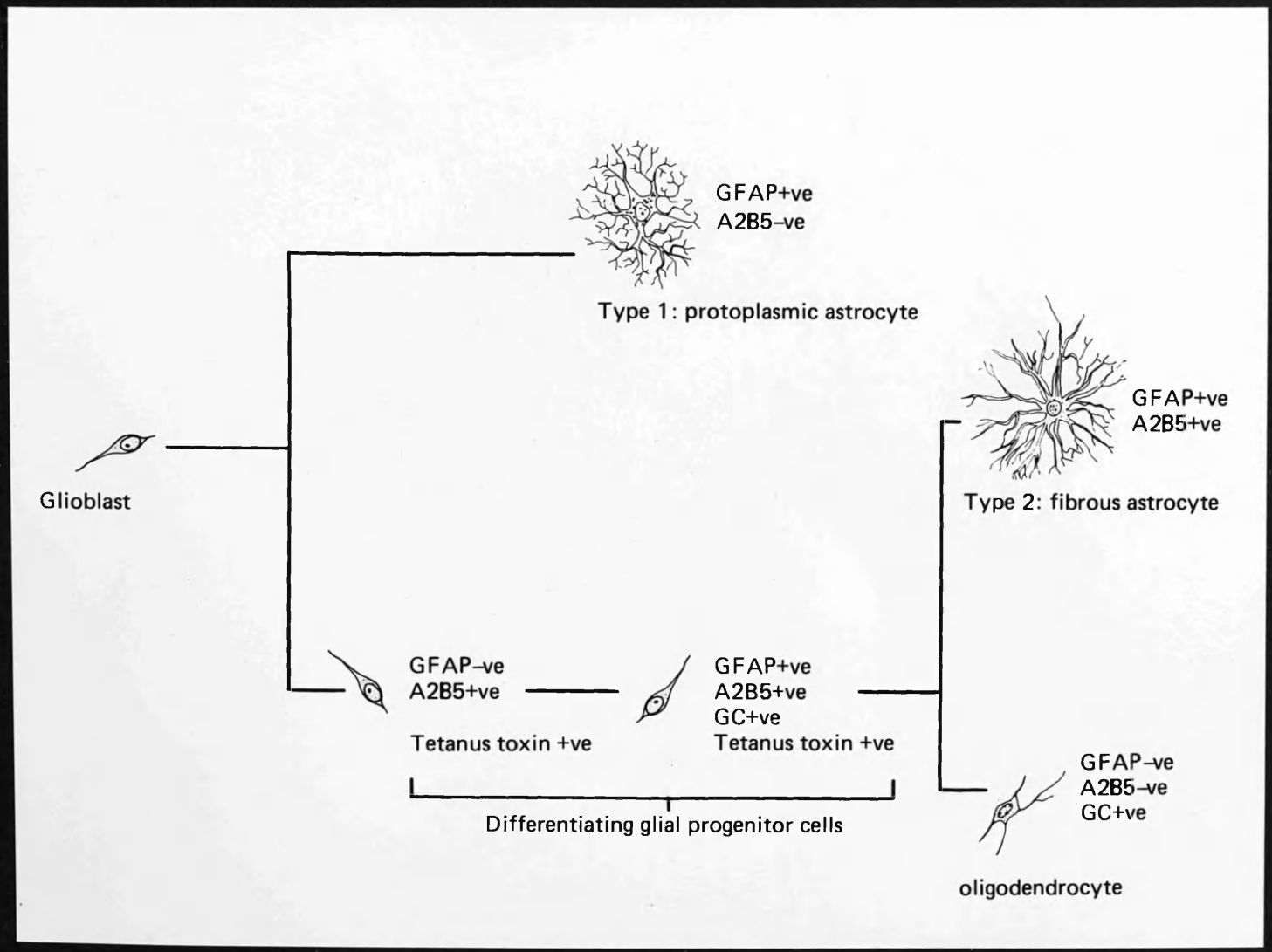
Marker	Source of antigen	Examples of reacting species	Reference
S100	Bovine	Mammals) Birds) Fish) Reptiles)	Moore, 1982
MBP	Monkey (Macaca nemistrina)	Human) Monkey) Bovine) Rat L-BP)	Sires et al., 1981
MBP	Rat	Chicken) Rat)	Hartman et al., 1979
MBP	Human	Dog	Zurbriggen et al., 1984
PLP	Rat	Rat) Bovine)	Hartman et al., 1982
	Rat	Chicken	Hartman et al., 1979
	Rat	Human	Agrawal et al., 1977
CAII	Human	Mouse	Kumpulainen & Korhonen, (1982)
	Human	Human	Kumpulainen & Nyström, (1981)
	Rat	Rat	Ghandour et al., 1980
MAG	Human	Man) Monkey) (Species not stated))	
		Calf) Dog) Rabbit) Guinea pig)	Steck et al., 1983
GC	Mouse	Mouse	Raff et al., 1978
	Rat	Rat	Zalc et al., 1983
NS-1	Mouse	Rat) Cat)	
		Rabbit) Bovine) Human)	Schachner, 1974
GFAP	Rat	Mammals Fish	Dahl & Bignami, 1973
Glutamine Synthetase	Rat Mouse	Rat Mouse	Norenburg, 1979 Martinez-Hernandez et al., 1977
01-04	Bovine	Rat) Mouse)	Schachner et al., 1981a, 1981b
		Human Chicken	

TABLE IX

THE ONTOGENIC EXPRESSION OF NERVOUS SYSTEM MARKERS OF OLIGODENDROCYTES IN DEVELOPING RODENT BRAIN

Stage of maturation	Oligodendrocyte marker	Species	Reference
Premyelinating	03-04	Mouse	Schachner et al (1981a)
	GFAP	Mouse	Choi (1984)
	GFAP/GC	Rat)	
	GC	Rat and Mouse)	Raff et al (1983a)
Myelinating	GC/MBP	Rat)	Bologa-Sandru et al
	MBP	Rat)	(1981a, 1981b)
	MBP/PLP	Rat)	Hartman et al (1979)
	PLP	Rat)	
Mature	CAII	Rat and Mouse	Ghandour et al (1980)

Figure 22. Schema of ontogenesis of rat optic nerve glial proteins in cultured cells (Raff et al., 1978 & 1983a&b)


GFAP+ve
A2B5–ve
Type 1: protoplasmic astrocyte
GFAP+ve
A2B5+ve
Type 2: fibrous astrocyte
Glioblast
GFAP–ve
A2B5+ve
Tetanus toxin +ve
GFAP+ve
A2B5+ve
GC+ve
Tetanus toxin +ve
Differentiating glial progenitor cells
GFAP–ve
A2B5–ve
GC+ve
oligodendrocyte

CHAPTER SIX

AN IMMUNOHISTOCHEMICAL STUDY OF THE TOPOGRAPHY AND CELLULAR LOCALISATION OF FOUR NERVOUS SYSTEM PROTEINS IN OVINE PERIPHERAL AND CENTRAL NERVOUS SYSTEMS

INTRODUCTION

Immunocytochemical staining offers major advantages over transmission electron microscopy and capricious silver and gold impregnation techniques for morphologic and quantitative studies of glial cell abnormalities of BD. The presence of some glial cell specific proteins early in the development and differentiation of neural cells may prove particularly valuable in pathogenesis studies relating to the ontogeny of glia in BD.

The use of such proteins has, however, been relatively neglected in veterinary pathology. The paucity of published information and species variation in glial protein expression requires, however, that the distribution and cellular localisation of such proteins first be investigated in normal sheep. This chapter describes the cytological and topographical distribution of GFAP, an astrocyte marker which is present from early periods of differentiation; GC, a marker for pre-myelinating oligodendroglia; MBP, a marker for myelinating oligodendroglia and CAII, a marker for mature oligodendroglia. The preservation of these tissues in a limited range of fixatives is also compared.

MATERIALS AND METHODS

Source of animals

Newborn lambs of two breeds, two breed crosses, a 29 day old lamb and an adult sheep were killed by intravenous injection of pentobarbitone sodium. A further sheep, 80 days pregnant, was also killed by pentobarbitone sodium and its fetus removed by hysterotomy (see table X).

Fixation and processing of tissues

Brains and spinal cords including dorsal and ventral spinal nerve

roots with dorsal root ganglia were placed in one of the following fixatives: 10% neutral phosphate buffered formalin (BF); Carnoy's fixative; a modified Karnovsky's fixative consisting of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1M phosphate buffered saline pH 7.4 or formol sublimate. Tissues placed in formalin were fixed 1 week or 18 months prior to paraffin wax embedding (Table X). Tissues fixed in modified Karnovsky's solution and Carnoy's fixative were processed in the following way. After 1 hour in fixative the cerebellum was removed and para-sagitally sliced. The remaining brain and selected spinal cord segments were coronally sliced at 0.5cm intervals. After a further two hours in fixative selected tissues were trimmed and placed in fresh fixative overnight prior to paraffin wax embedding. Selected samples of spinal cord were taken from the 80 day old fetus and a newborn lamb and quenched in liquid nitrogen as described in Chapter Seven. Brain tissue from a four week old mouse fixed in Carnoy's solution was used to control CAII immunostaining. After 30 minutes fixation the mouse brain was coronally sliced at 0.5cm intervals and retained in fixative for a further 4 hours prior to processing to paraffin wax. Brains obtained from newborn and 10 day old mice, fixed in formalin or formal sublimate for up to 26 hours were employed to control MBP immunostaining.

Source of antisera

Commercially available polyclonal MBP and GFAP antisera (Dako UK Ltd) was used. For double staining a GFAP monoclonal antibody was used (ICN Ltd). CAII antisera was raised by inoculation of rabbits with bovine CAII. Details of antisera production and testing have previously been published (Steart, 1983). Antisera to GC was raised according to the method of Raff et al. (1983a) as follows.

Preparation of Glactocerebroside Vaccine

18ml of liquid paraffin was emulsified (by agitation with a Silverson homogeniser) with 2ml of Arlacel (an emulsifier). 25mg of purified cerebroside (Sigma Chemicals) consisting of 60% Type 1 (hydroxy fatty acids) and 40-50% Type 2 (non-hydroxy fatty acids) was suspended in 5ml of neutral buffered saline. This was then added dropwise to the emulsified paraffin oil during continued agitation. Twenty five one ml doses (1mg/ml) of a vaccine were thus prepared with a 1:4 antigen/oil ratio.

Inoculation of Rabbits

1ml of the antigen/oil vaccine was inoculated intramuscularly (hind limb) or subcutaneously (under the skin of the back) into each of three rabbits over a period of two months (see Table XI).

Testing of antisera

Sera prepared from blood samples taken from the marginal ear vein of all three rabbits were reacted by the peroxidase-anti-peroxidase method on acetone fixed cryostat sections of ovine fetal spinal cord segment T1. The gestational age of the fetus used as control was 80 days. The results of the test bleeds is shown in Table XII. Intra-cytoplasmic expression of cerebroside in white matter glia was taken as a positive result. Serum taken from rabbit 1 was used for all further tests detailed in this study.

Immunohistochemistry

Dewaxed sections were taken to absolute alcohol and treated for 10 minutes with freshly prepared 2% hydrogen peroxide (100 vols) in methanol to inhibit endogenous peroxidases. Prior to primary antisera incubation sections to be stained for GFAP were pretreated for 10

minutes with freshly prepared 0.1% trypsin in 0.1% calcium chloride, pH 7.8 at 37°C After a rinse in tap water and followed by Tris-buffered saline, pH 7.6 (TBS), all sections were treated with normal ovine serum and 2% bovine serum albumen to block non-specific binding sites. Primary antiserum (anti-GFAP, anti-MBP, anti-cerebroside or anti-CAII) was then applied for 30 minutes at room temperature or for 24 hours at 4°C and washed three times with TBS. The primary antisera dilutions used are shown in Table XIII. Sections were then treated with appropriate bridging antiserum in excess (swine anti-rabbit, rabbit anti-mouse at 1:50) for 30 minutes and subsequently with a rabbit peroxidase-anti-peroxidase (PAP) complex. A TBS wash for 30 minutes was then followed by 10 minutes in 3,3'-diaminobenzidine tetrachloride (DAB: Sigma). Sections were washed in tap water, counterstained with Meyer's haemalum for 20 seconds, blued in running tap water for 15 minutes, dehydrated, cleared and mounted in DPX. Sections which were not treated with primary antiserum but which were otherwise identically handled accompanied each batch of tissue sections as negative controls. Double staining for GFAP and CAII was performed on selected section of Carnoy's fixed tissues. In this technique staining for CAII and GFAP was essentially as described above but the GFAP reaction, developed simultaneously, was modified in the following ways: trypsinisation was not performed, endogenous phosphatase activity was inhibited by levamisol, a GFAP monoclonal antibody was used, bridge antiserum was goat anti-mouse IgG, mouse alkaline phosphatase anti-alkaline phosphatase antibody complex was used in place of PAP complex and the colour reaction product was developed using fast blue. Sections were coverslipped with aquamount.

Paraffin wax sections were also cut at 5μm and 10μm and stained with

HE and or Luxol fast blue cresyl violet (LFBCV) respectively.

Samples of spinal cord quenched in liquid Nitrogen were cut on a cryostat at 15μm fixed in acetone and reacted with applied antibody according to the methods described and coverslipped with glycerol.

RESULTS

Distribution of GFAP

GFAP expression was present in perikarya and processes but not nuclei of a proportion of glial cells diffusely throughout the CNS and also in the peripheral nervous system (PNS). Cell processes within the sub-ependymal network, glial limitans, pericapillary membrane and dorsal and ventral spinal nerve roots expressed GFAP. Excellent preservation of GFAP was seen irrespective of the fixative used but tissues fixed in formalin for 18 months prior to embedding showed only weak expression of GFAP.

GFAP immunoreactive glial cells of white matter showed numerous tortuous, and branched processes (Fig. 23) with those of grey matter having fewer and finer processes (Fig. 24). The end feet processes of some grey and white matter GFAP expressing cells contributed to perivascular membranes (Fig. 24) and to the glial limitans membrane. In spinal cord GFAP expressing processes extended for a few millimeters into the dorsal and ventral spinal nerve roots creating a dome shaped evagination which defined the junction of the CNS and PNS. Immunoexpression of GFAP was also seen in a population of ependymal cells and in processes which formed a sub-ependymal network. These were most frequent in dorsal and dorso-lateral ependyma of the central canal of the spinal cord and of the lateral ventricles. Such cells were columnar with long processes which radiated into cerebral cortex

through the sub-ependymal glial network and the corpus callosum (Fig. 25a). Within the sub-ependymal glial network of the lateral ventricle similar unipolar (Fig. 25b) or, more rarely, bipolar cells were located immediately subjacent to the ependyma.

In the cerebellum parallel GFAP stained cell processes traversed the molecular layer (Fig. 26), extended through the external granule cell layer and divided at the pia into a spray of 3 to 5 fine processes which terminated with distinctive button-like end feet on the pial membrane (Fig. 27). The nuclei of these cells were located at the level of Purkinje cells. Perinuclear cytoplasm was scant and each cell gave rise to only one major process.

Some fusiform cells situated between and with their long axis parallel to myelinated fibres in the PNS showed weak to moderate GFAP expression. GFAP expression was also seen in amphicytes bordering neuronal perikarya of dorsal root ganglia (Fig. 28).

Distribution of GC

GC expression was seen only in liquid nitrogen frozen fetal tissue and was present at all levels of the spinal cord examined (cervical, thoracic and lumbar). In fetal spinal cord GC expression was present in cytoplasmic processes of a proportion of glial cells of white matter. Weak expression was also seen in the perikarya of ventral horn neurons. Expression of GC in glia was seen most frequently in sub-pial regions (Fig. 29a) and also in glia adjacent to the ventral longitudinal median fissure. In this location reactive cells were usually unipolar with cell nuclei located on the pia mater but bipolar cells in subjacent white matter were also present (Fig. 29a&b).

Distribution of MBP

MBP was demonstrated in myelin sheaths of CNS and PNS and, in one newborn lamb, in the cytoplasm of glial cells of the corpus callosum and cerebrocortical white matter. The distribution of myelin sheath MBP expression was not altered by different fixatives but tissues preserved in formalin for eighteen months prior to embedding showed reduced MBP expression. Tissues fixed in Carnoy's fixative showed artefactual diffusion of reaction product.

The distribution of LFBCV stained myelin was similar in all lambs irrespective of fixative used and corresponded to the distribution of MBP expression seen for lamb tissues fixed in modified Karnovsky's solution and BF. For newborn lamb tissues fixed by modified Karnovsky's solution and BF, MBP expression was seen in white matter of all funiculi of the spinal cord, the cerebellar vermis, medulla, mid-brain and thalamus (including the optic tract and cranial nerve roots) and in the internal capsule. MBP expression was weak to moderate in sub-cortical cerebral white matter and was absent in gyral white matter immediately subjacent to cortical grey matter. Expression was also absent in the corpus callosum. Tissues fixed in formol sublimate showed a more extensive distribution and an enhanced intensity of MBP expression than tissues fixed in modified Karnovsky's solution or BF. For formol sublimate fixed tissues MBP expression was seen in the anatomic locations described above and also in sub-cortical gyral white matter of the cerebrum and corpus callosum. Also present were occasional cells with granular or evenly distributed intracytoplasmic expression of MBP (Fig. 30).

In the 29 day old lamb and in the adult sheep both LFBCV staining and immunohistochemical staining showed a similar distribution of myelin-

ated fibre tracts. MBP expression extended throughout all myelinated tracts of the brain and spinal cord including sub-cortical white matter and corpus callosum. Intra-cytoplasmic expression of MBP was not present.

MBP expression within myelin sheaths of the PNS, though present, was weaker than in CNS myelin. Expression was seen in tissues fixed in modified Karnovsky's solution, Carnoy's and BF but not in tissues fixed in formol sublimate.

Distribution of CAII

CAII expression was not present in neuropil or myelin of neonatal lambs but, in 29-day-old and adult brains, expression was seen in a population of glial cells. Expression of CAII was not seen in tissues fixed in formalin and was weak in tissues fixed in modified Karnovsky's solution, but tissues fixed in Carnoy's fixative showed weak to strong expression of CAII depending on the age of the sheep and the neuroanatomic site sampled.

In all ages of animals examined CAII expression was found in cytoplasm (especially the microvillous border) of epithelial cells throughout the choroid plexus (Fig. 31). In the 29-day-old lamb weak expression of CAII was seen in the neuropil of ventral horn and in processes and perinuclear cytoplasm of some glial cells in the ventral and lateral funiculi of the spinal cord. These cells possessed from one to four short, sharply demarcated, unbranched tapering processes. The nuclei of these cells were large, and contained relatively little chromatin. Occasionally one or two nucleoli were visible. Unstained glial cells with variable nuclear morphology were also present in white matter. In adult sheep CNS expression of CAII

extended to involve glial cells of white matter throughout the brain and spinal cord and such cells contributed process continuous with CAII positive pericapillary membranes. CAII expression was also seen in glial cells and their processes within ventral horn but neurons were unstained.

CAII expression of sheep brain contrasted markedly with CAII expression of mouse brain. In mouse brain, CAII expression was seen in the perinuclear cytoplasm of glial cells (rarely extending to involve short cell processes) and in myelin sheaths. In double stained preparations of mouse brain, glial cells expressed CAII or GFAP (Fig. 32a). In the 29-day-old and adult sheep brains white matter CAII expressing glia also expressed GFAP (Fig. 32b), and in grey matter GFAP positive glia were present that were CAII negative. CAII positive-GFAP negative processes and CAII positive-GFAP positive glia were also present in ventral horn grey matter of spinal cord.

DISCUSSION

These results demonstate that the CNS proteins GFAP, MBP, GC, and CAII are conserved in ovine CNS and, when compared with information on other species, indicate inter-species differences in the cellular localisation of CAII and GFAP.

Previous studies in several different species have shown that GFAP is a stable protein and is well conserved following a variety of fixation methods including formalin. MBP expression is optimally demonstrated following fixation in formol-sublimate (Sternberger et al., 1978a) and CAII is best demonstrated following fixation with Carnoy's fixative or a modified Karnovsky's fixative (Kumpulainen and Korhonen, 1982; Langley et al., 1980). The results of this study suggest that

previously selected fixation methods are also suitable for sheep tissue but with one exception. Kumpulainen and Korhonen (1982) were able to demonstrate CAII expression in mouse brain following perfusion fixation with modified Karnovsky's solution. Poor demonstration of CAII in ovine brain fixed in modified Karnovsky's solution may be due to species differences in antigen perservation or expression or due to delayed penetration of fixative in immersion fixed ovine brain.

GFAP

These results show that the distribution of GFAP in sheep is similar to that found in rodents and man (Bignami & Dahl, 1974a; Ludwin et al., 1976; Pelc et al., 1986). Immunocytochemical techniques show that GFAP is expressed in fibrous astrocytes, protoplasmic astrocytes and Bergmann glial cells of rat, mouse and human brain (Fields & Yen, 1981; Ludwin et al., 1976; Pelc et al., 1986). Previous studies have shown selective staining of fibrous astrocytes (but not protoplasmic astrocytes) in the rat (Bignami & Dahl, 1974a) and in another study GFAP was not expressed in Bergmann glial fibres of adult sheep brain (MacKenzie, 1983). By omitting pre-trypsinisation of sections prior to incubation with antibody, diminished distribution of GFAP expression was found including absence of GFAP expression in protoplasmic astrocytes and Bergmann glial fibres. Thus, some of the reported differences in distribution of GFAP between species may be related to technique. However, species differences in expression may also occur. GFAP expression is not reported in rat ependyma (Ludwin et al., 1976) but the results shown in this study suggest that a population of ependymal cells in lamb brains express GFAP.

This study showed a population of peripheral glia morphologically similar to Schwann cells which expressed GFAP. Satellite cells of dorsal root ganglia also expressed GFAP. Expression of GFAP was weaker than in CNS but that staining should occur at all in paraffin wax embedded tissue is contrary to observations in rodent and human tissue. Immunohistochemistry of frozen tissue has, however, demonstrated GFAP in glia of the enteric portion of the rat autonomic nervous system (Jessen & Mirsky, 1980); in glia of rat and human peripheral nerve (Fields & Yen, 1981; Jessen & Mirsky, 1985) and in satellite cells of autonomic ganglia of rats (Jessen & Mirsky, 1985). The GFAP positive glia of peripheral nerves also express the antigens Ran 2 and A5E3 which define them as non-myelinating Schwann cells (Jessen & Mirsky, 1985). It is therefore probable that GFAP expression in glia of sheep ovine PNS are also non-myelinating Schwann cells.

In man GFAP is expressed more abundantly in dorsal than ventral roots of spinal nerves (Jessen & Mirsky, 1985) reflecting the relative abundance of non-myelinated fibres in dorsal versus ventral nerve roots (Coggeshall et al., 1975). In ovine tissue we found no difference in expression of GFAP between dorsal and ventral roots.

GC

Immunoperoxidase staining shows that GC is expressed by sub-pial glial cells.

Previous studies of developing chick brain shows that sub-pial glial cells or glioblasts derive from para-ependymal matrix cells which

migrate to the periphery of the spinal cord. These cells give rise to both oligodendrocytes and astrocytes (Fujita, 1965). In cultured cells GC may be expressed on pre-myelinating oligodendroglia (Bologa-Sandru et al., 1981a) and on glioblasts which may develop into either astrocytes or oligodendroglia (Raff et al., 1983a). Thus, the occurrence in ovine spinal cord of immunocytochemical expression of GC in sub-pial glial cells of an indeterminate cell type is consistent with such cells being pre-myelinating oligodendroglia or a progenitor cell type.

Apparent expression of GC by neurons in ventral horn is probably non-specific staining brought about by use of unpurified antiserum. Ammonium precipitation of the IgG fraction of the antiserum or antigen affinity chromatography may have been helpful in improving the specificity of the antiserum.

MBP

In the newborn lamb, myelin throughout the CNS expresses MBP. Tinctorial (LFBCV) and immunohistochemical demonstration of myelin in tissues fixed in formalin and modified Karnovsky's fixative show a comparable distribution but in tissues fixed in formol sublimate MBP expression was more widely distributed than LFBCV staining. The reason for such a difference was not found; possibly in sublimate fixed tissues MBP recognises less mature myelin or in some other undetermined way is a more sensitive detector of myelin than the LFBCV method. The distribution of myelin is an agreement with the findings of Barlow (1969) confirming that white matter of the cerebral cortex and corpus callosum are the last structures to be fully myelinated in ovine brain.

Intracytoplasmic expression of MBP has previously been described for premyelinating and myelinating oligodendroglia in developing rat brain (Sternberger et al., 1978b). Intracytoplasmic expression of MBP is also present in oligodendroglial cells of fetal (90 days gestation) ovine brain (see following chapter). Granular intracytoplasmic expression of MBP in corpus callosum and cerebro-cortical white matter of newborn lambs probably represents residual myelinating oligodendroglia. Poor affinity of myelin for LFBCV in these structures further suggests that myelination is incomplete in these areas.

PNS myelin also expressed MBP but showed less intensity of staining than CNS myelin. PNS myelin has some, but not all, of the same proteins as CNS myelin (Brostoff, 1984) and since antisera used in these studies was polyclonal it is likely that antibodies present recognise only epitopes of PNS myelin which are common to CNS myelin.

CAII

The localisation of CAII in choroid plexus epithelium is consistent with the distribution of this isoenzyme in human and mouse brain (Kupulainen & Nystrom, 1981; Ghandour, et al., 1980). However, in these species CAII is also demonstrated in outer lamellae of myelin and in cells with morphologic, ultrastructural and immunological characteristics of oligodendroglia (Ghandour et al., 1980; Langley et al., 1980).

Ghandour et al. (1980), using a combined peroxidase and immunofluorescence technique, showed that GFAP and CAII were present in discrete non-overlapping populations of glia in mouse brain. The immunoperoxidase method of double labelling applied in this study shows that, in mouse brain, GFAP expressing cells are morphologically similar to

astroglia and distinct from CAII expressing cells which resemble oligodendroglia. In ovine brain both CAII and GFAP are expressed by a population of white glial matter cells morphologically similar to astroglia while astroglia of grey matter express GFAP alone. Peri-capillary membranes of grey matter also express both GFAP and CAII. In the ovine neonate CAII is not expressed in the neuropil and is only expressed in parts of the spinal cord in 29-day-old sheep. CAII is perhaps only fully expressed in the ovine brain between 29 days and adulthood. These results suggest that in sheep CAII is expressed in fully differentiated fibrous astrocytes.

The origin of cells expressing CAII in ventral horn grey matter was not determined. Some cells and their processes expressed both GFAP and CAII, suggesting an astroglial origin, while others expressed only CAII. Astroglia of dorsal horn grey matter invariably expressed GFAP alone.

In conclusion these studies show that the proteins GFAP, GC, MBP and CAII are conserved in ovine CNS and that GFAP and MBP are expressed in PNS. GFAP is expressed by fibrous astroglia, protoplasmic astroglia, Bergmann glial cells, satellite cells of spinal ganglia and subpopulations of ependymal cells and Schwann cells. In fetal spinal cord GC is expressed in sub-pial glia cells (possibly undifferentiated glioblasts). In newborn lamb brains MBP is expressed in mature and developing myelin sheaths of CNS and is present in the cytoplasm of myelinating oligodendroglia of the cerebrum. The polyclonal MBP antibody used in this study also shows immunohistochemical affinity for PNS myelin. CAII is expressed in choroid plexus epithelium and, in mature nervous system in fibrous astrocytes. It is also expressed in unidentified cells and processes of the ventral horn of spinal cord.

TABLE X

DETAILS OF SHEEP AND FIXATION METHODS

Age	Breed	Fixative
<1 day	Dorset horn	Formalin
<1 day*	Suffolk cross	Formalin
<1 day	Cheviot cross	4% paraformaldehyde 0.1% glutaraldehyde
<1 day+	Border Leicester	Carnoys
<1 day	Dorset horn	Formol sublimate
29 days	Dorset horn	Carnoys
Adult	Dorset horn	Carnoys
80 day fetus	Dorset horn	Liquid N_2
<1 day	Dorset horn	Liquid N^2

+ died at birth following dystokia.

* fixed in formalin and preserved for 18 months prior to paraffin wax embedding.

TABLE XI

GALACTOCEREBROSIDE RABBIT INOCULATIONS

		Date of inoculation					
		21.11.86	28.11.86	5.12.86	12.12.86	19.12.86	21.1.87
Rabbit 1	Route	I/M	S/C	I/M	S/C	S/C	S/C
	Dose	1.0ml	1.0ml	1.0ml	1.0ml	1.0ml	1.0ml
Rabbit 2	Route	I/M	S/C	I/M	S/C	S/C	S/C
	Dose	1.0ml	1.0ml	1.0ml	1.0ml	1.0ml	1.0ml
Rabbit 3	Route	I/M	S/C	I/M	S/C	S/C	S/C
	Dose	1.0ml	1.0ml	1.0ml	1.0ml	1.0ml	1.0ml

I/M Intramuscular

S/C Subcutaneous

TABLE XII

RESULTS OF IMMUNOPEROXIDASE STAINING USING SERA FROM CEREBROSIDE INOCULATED RABBITS

Rabbit Number	Date of Blood Sample	Antisera Dilution				
		1:40	1:80	1:120	1:160	1:200
2	16.12.86	-	-	-	-	-
Pooled sera from all 3 rabbits	20.1.86	-	-	-	-	-
1	5.2.87	+	-	-	-	-
1	12.3.87	++	+	±	±	±
2	12.3.87	±	-	-	-	-
3	12.3.87	+	N/A	±	-	-

Legend ++ moderate
+ weak
- absent
N/A not available for examination

Antiserum taken from rabbit 1 on the 12.3.87 was used for subsequent immunocytochemical investigations.

TABLE XIII

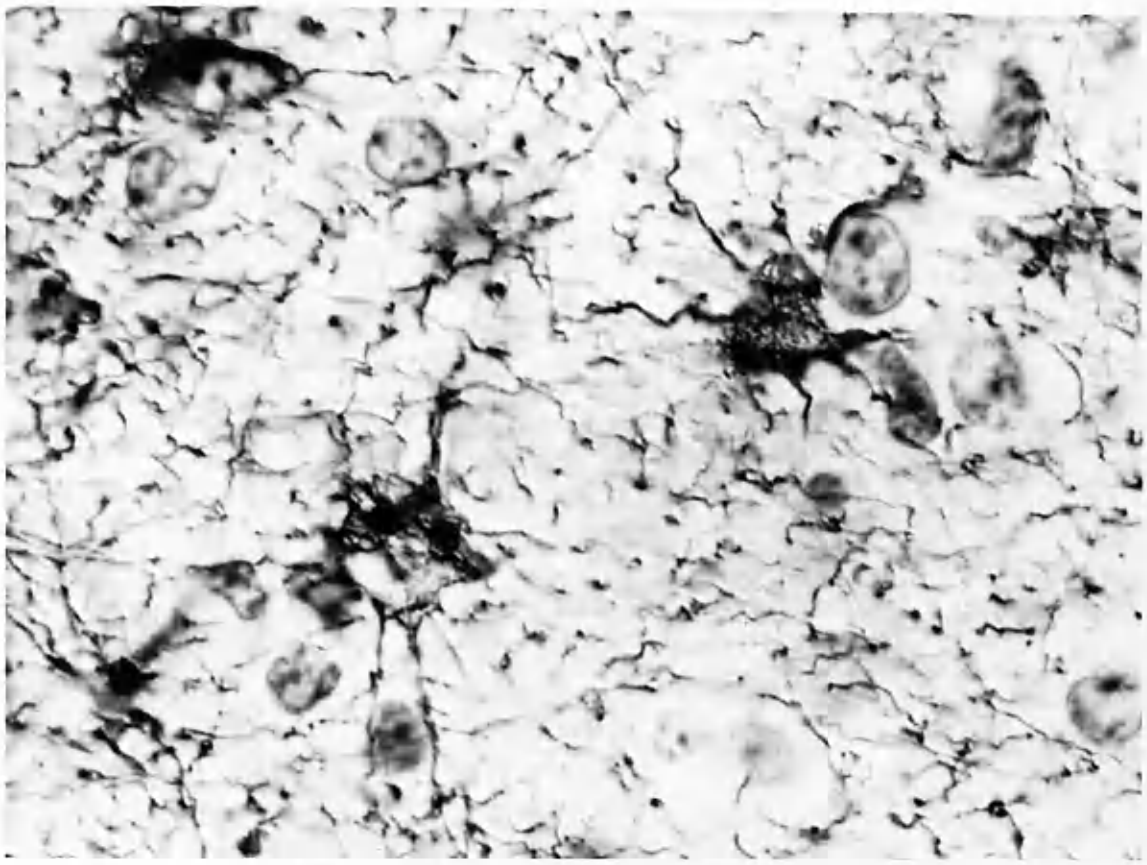
PRIMARY ANTISERA DILUTIONS

Primary antiserum	Incubation period	
	30 mins.	24 hrs.
GFAP (monoclonal)	N/A	1:100
GFAP (polyclonal)	1:500	N/A
MBP	1:120	1:400
CAII	N/A	1:360 or 1:200
GC	1:40	N/A

N/A not applicable

Fig 23. Cerebral cortex. GFAP expression in white matter glial cells showing numerous tortuous processes. Peroxidase-anti-peroxidase (PAP) GFAP stain. Differential interference contrast (DIC) x 1.400.

Fig 24. Cerebral Cortex. GFAP expression of grey matter glial cells showing fewer and finer processes that white matter GFAP expressing glial. One glial cell process connects with a pericapillary membrane. PAP. GFAP stain DIC. x 1.400.



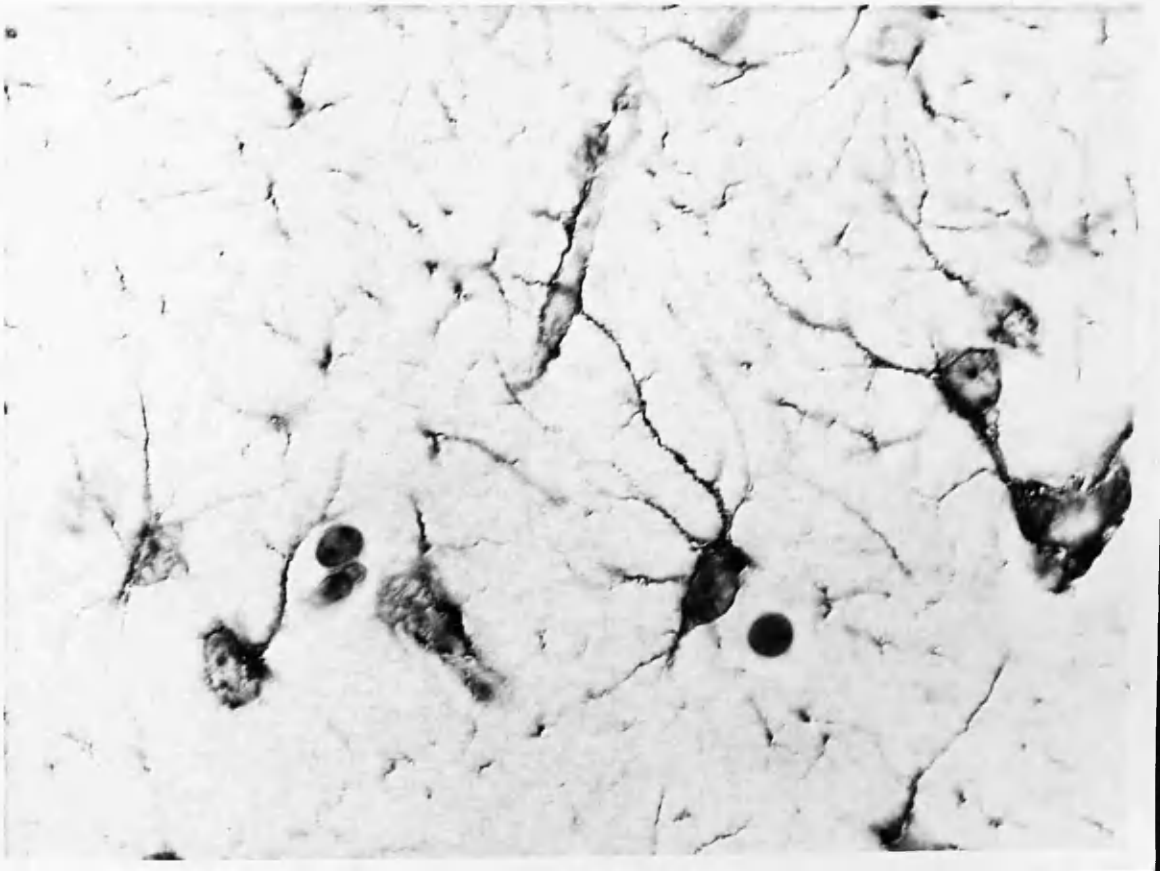
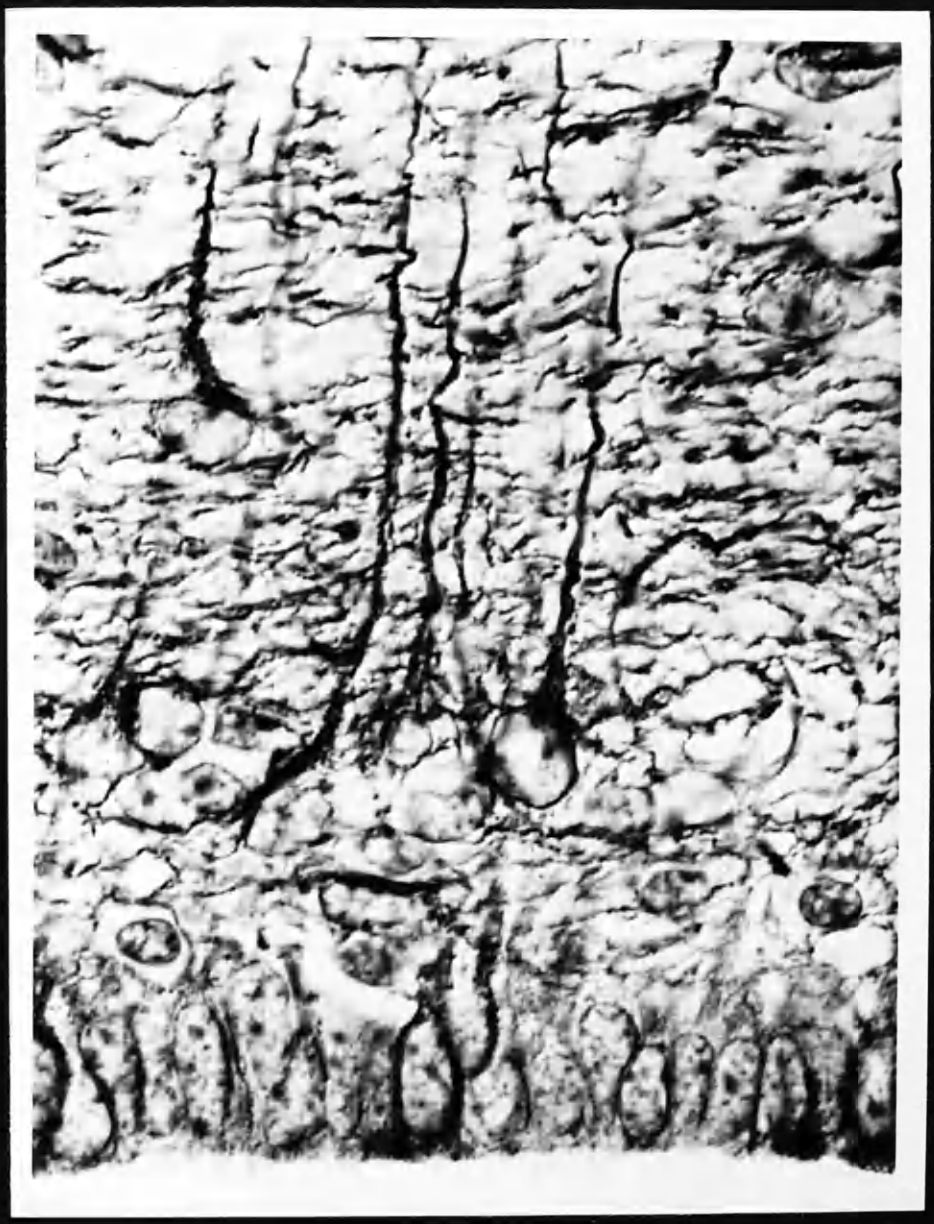
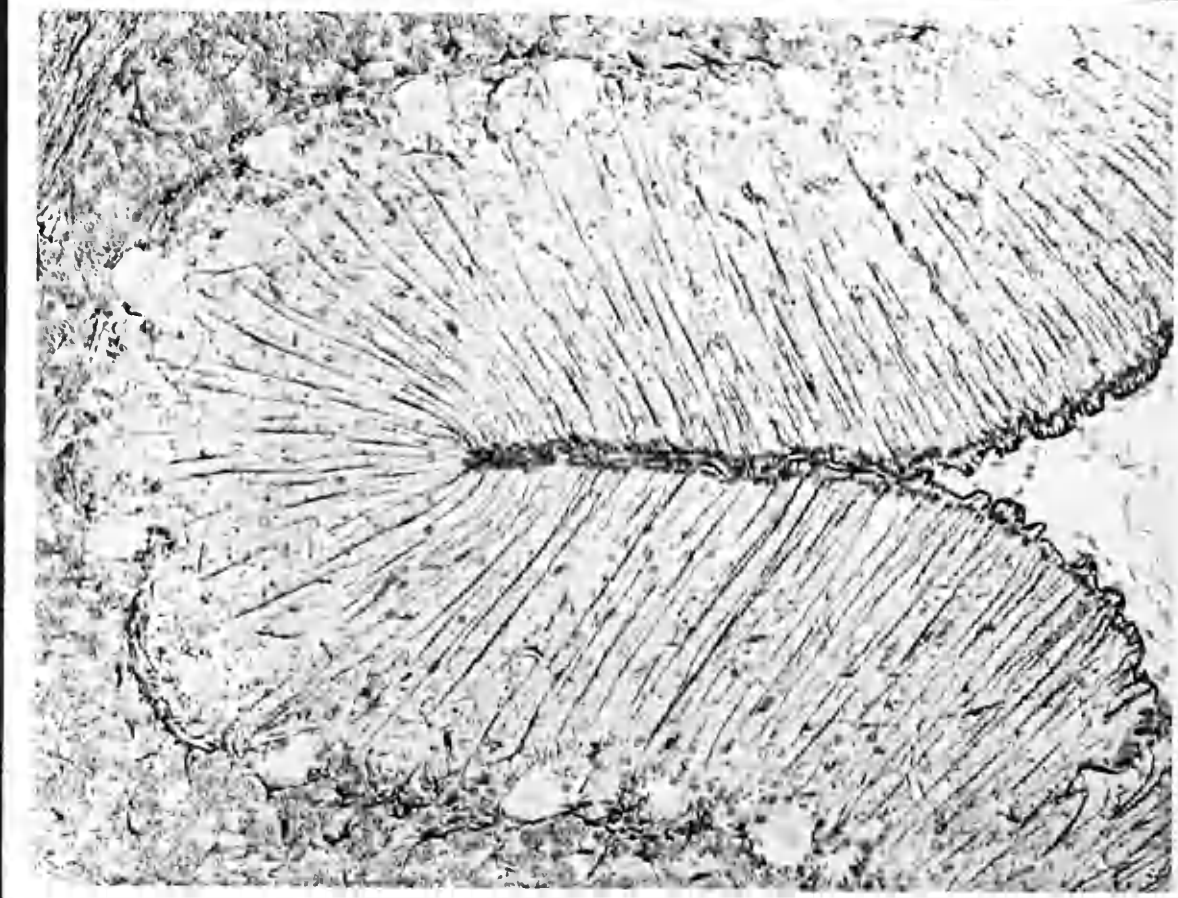


Fig 25a and b Lateral ventricle. Selective GFAP expression of unipolar ependymal (a) and sub-ependymal cells (b) showing parallel arrangement of processes traversing the corpus callosum. PAP. GFAP stain DIC. x 1,400.





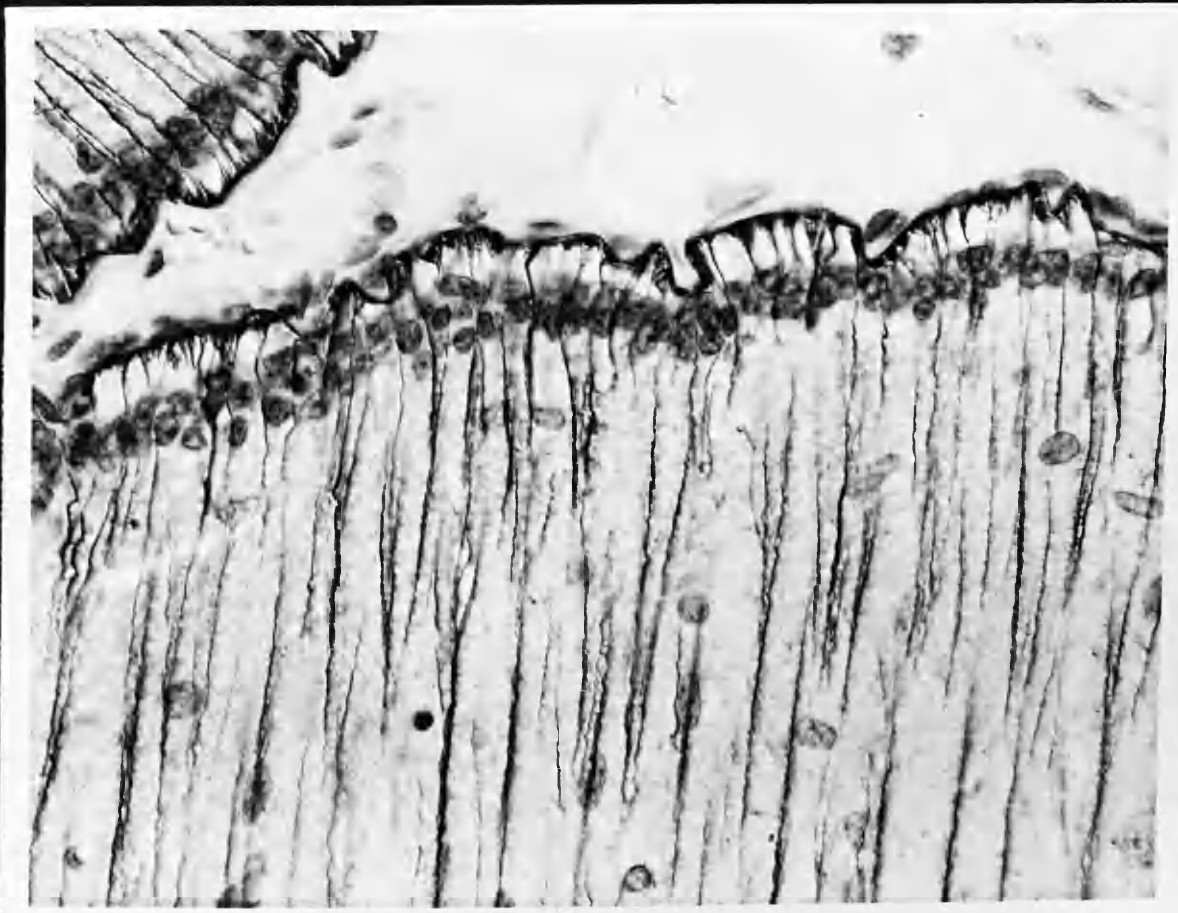


Fig 26. Cerebellum. GFAP expressing parallel cell processes crossing molecular layer. PAP. GFAP stain DIC. x 250.

Fig 27. Cerebellum. GFAP expressing cell processes of molecular layer showing terminal divisions and arrangement of endfeet. PAP. GFAP stain DIC. x 980.

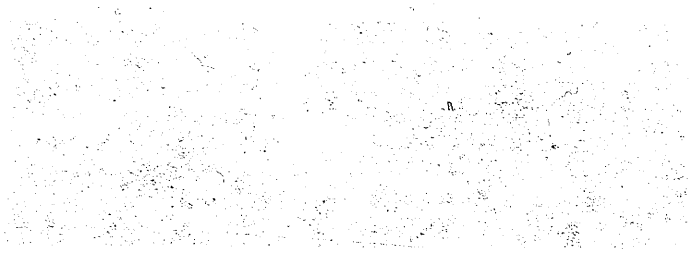
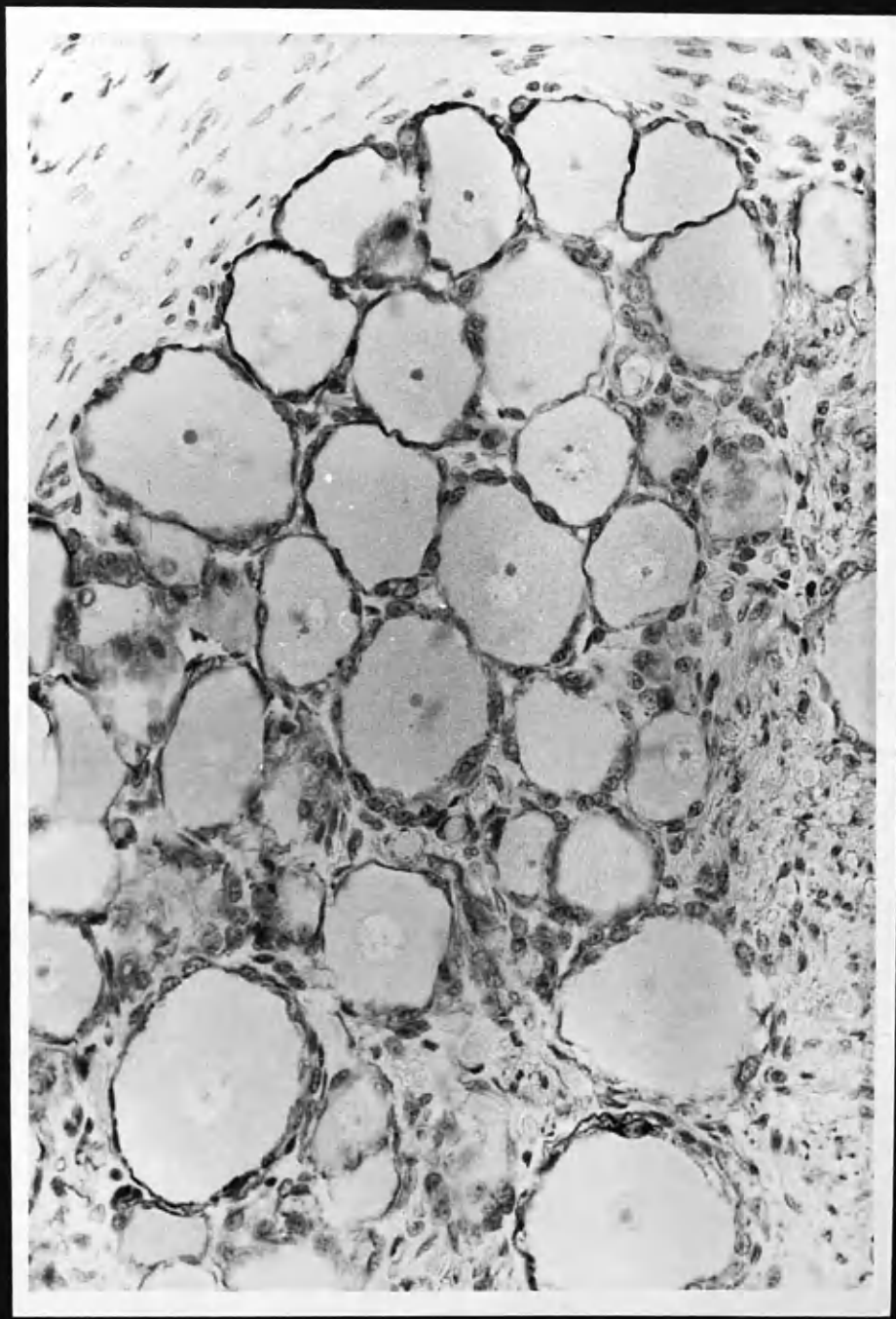
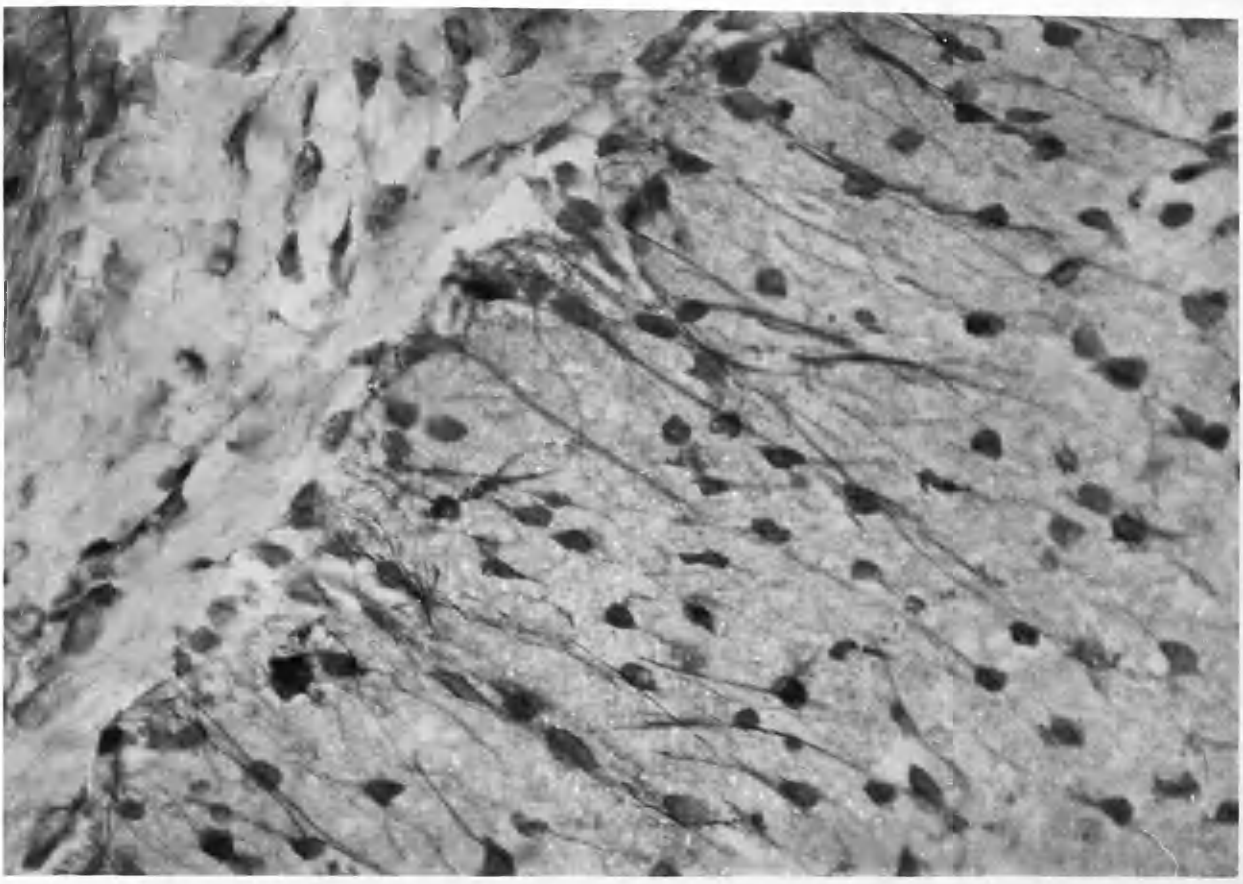
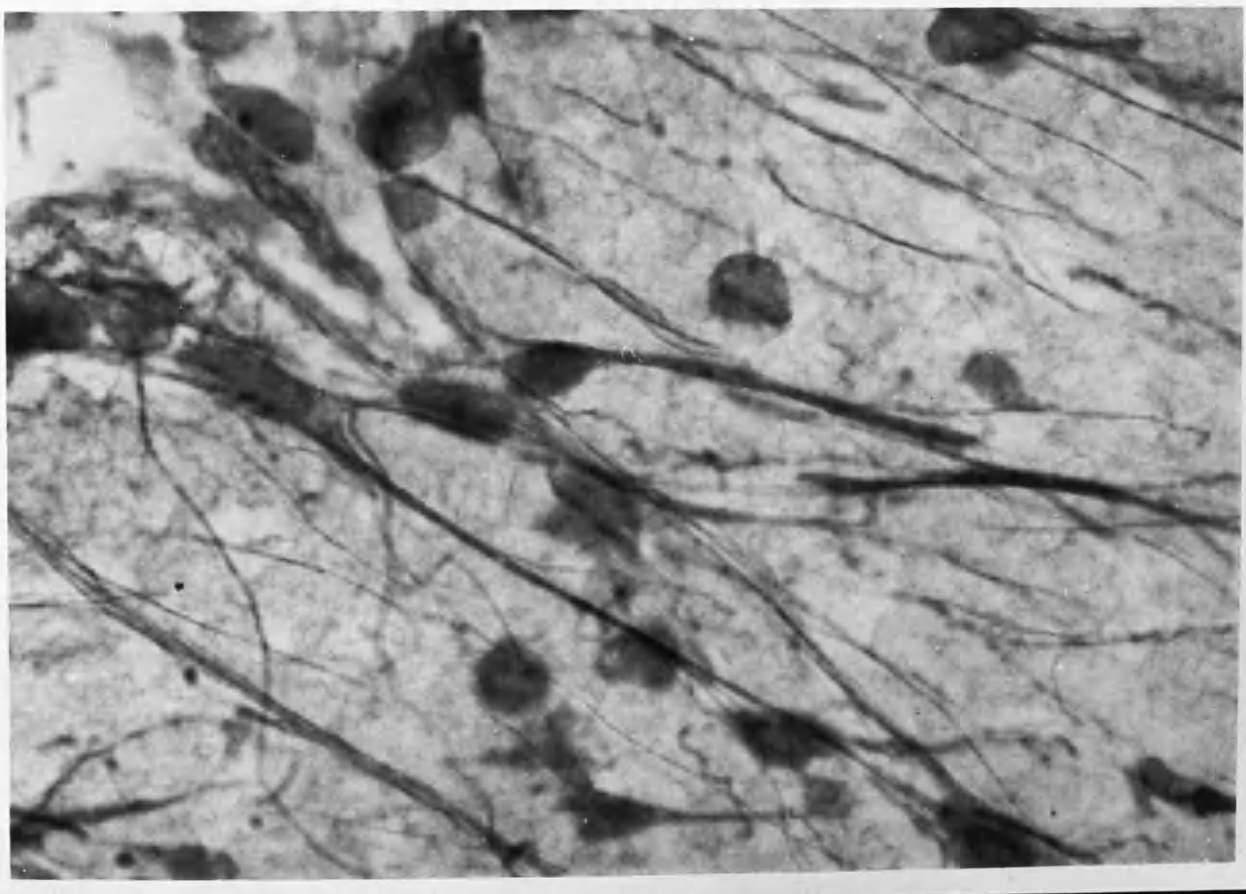


Fig 28. Dorsal root ganglion. GFAP expression in amphicytes. PAP. GFAP stain x 400.







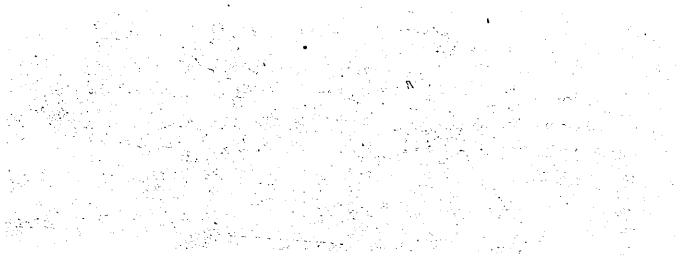
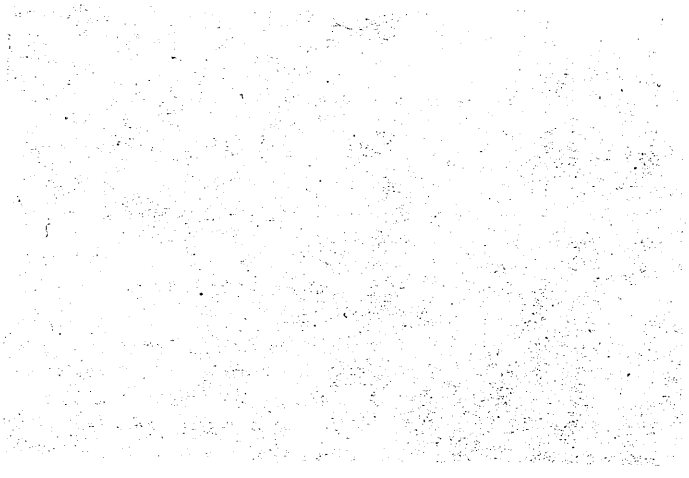


Fig 29a and b Spinal cord of 80 day fetus. (a) GC expression of sub-pial glia, showing long thin unbranched processes. (b) GC expression of unipolar & bipolar sub-pial glia. Cryostat sections PAP. GC stain x 1000 (a) and x 230 (b).



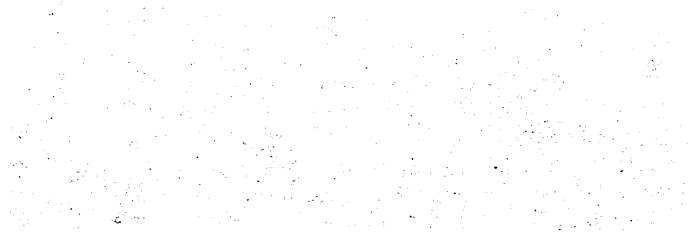


Fig 30. Corpus callosum of newborn lamb. Weak intracytoplasmic expression of MBP (arrow) and myelin MBP expression. PAP. MBP stain. PAP MBP x 1,100.

Fig 31. Choroid plexus. CAII expression in cytoplasm and microvillous border of choroid plexus epithelium PAP. CAII stain x 1,100.

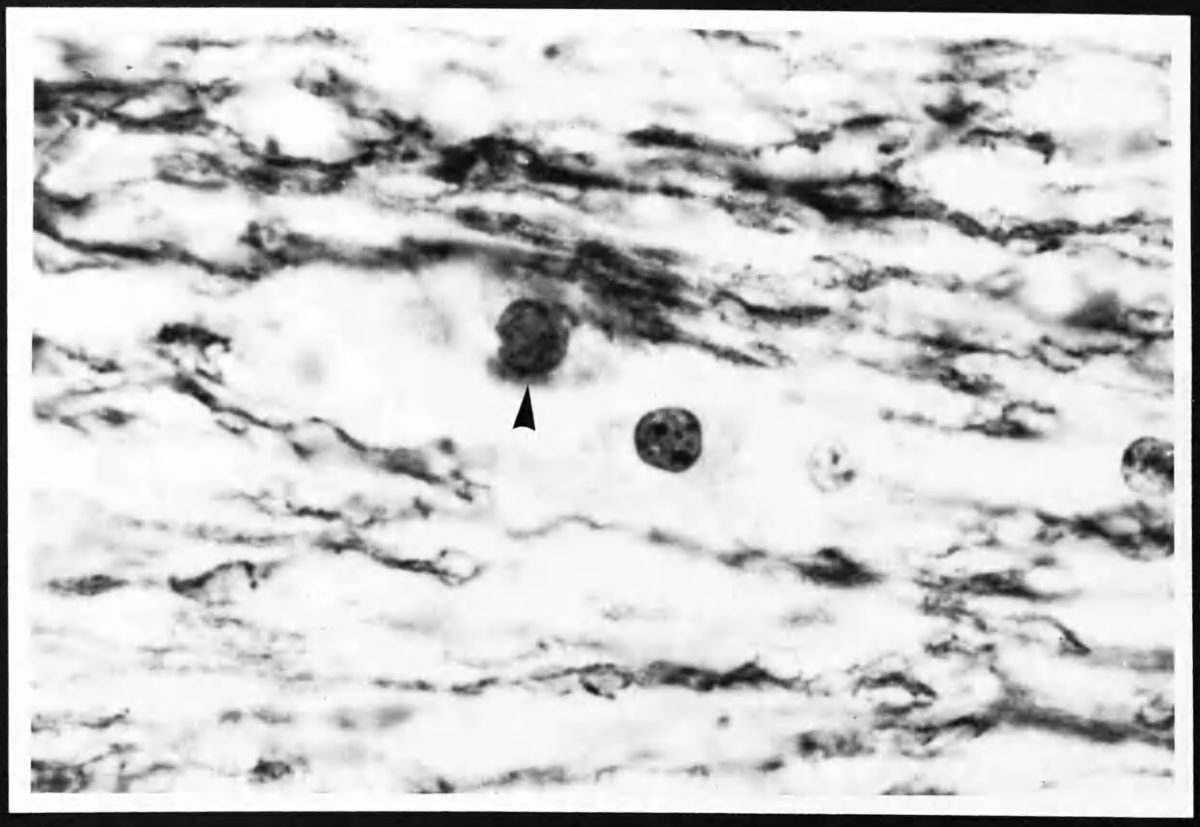


Fig. 3a

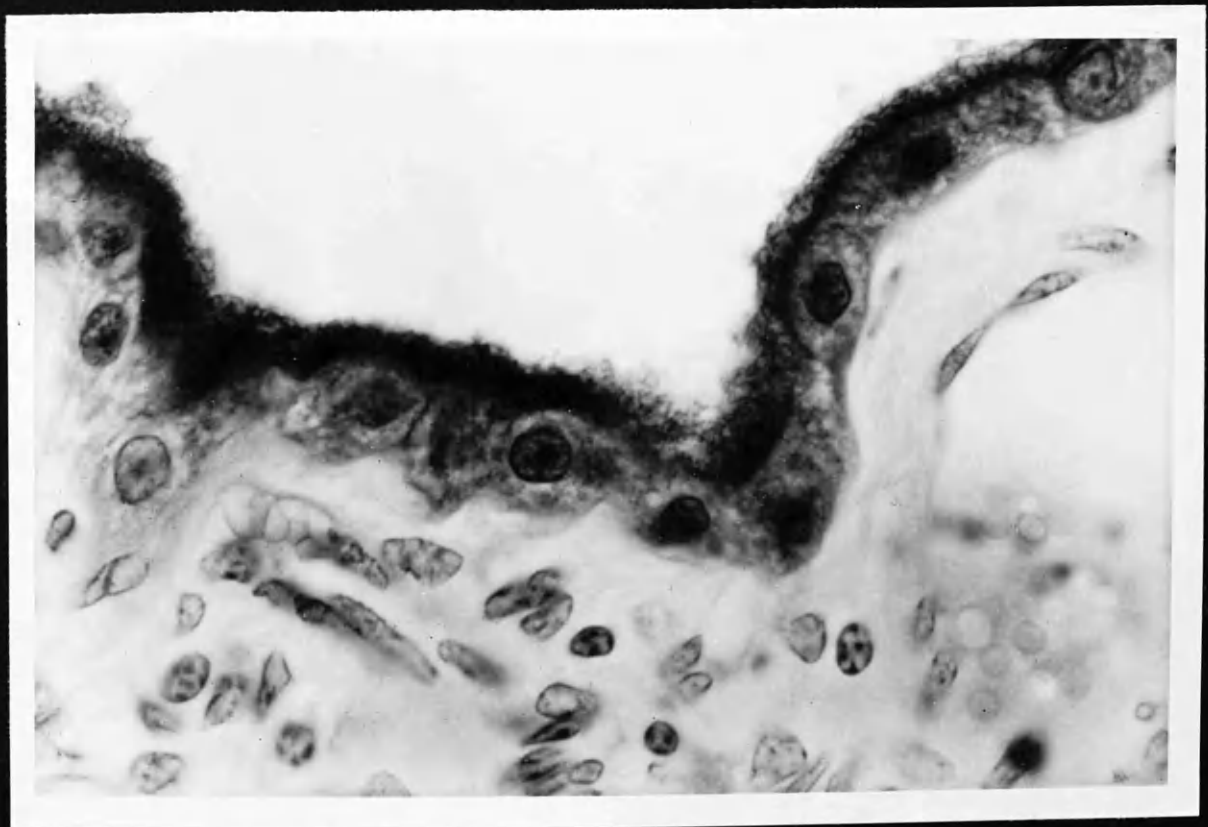
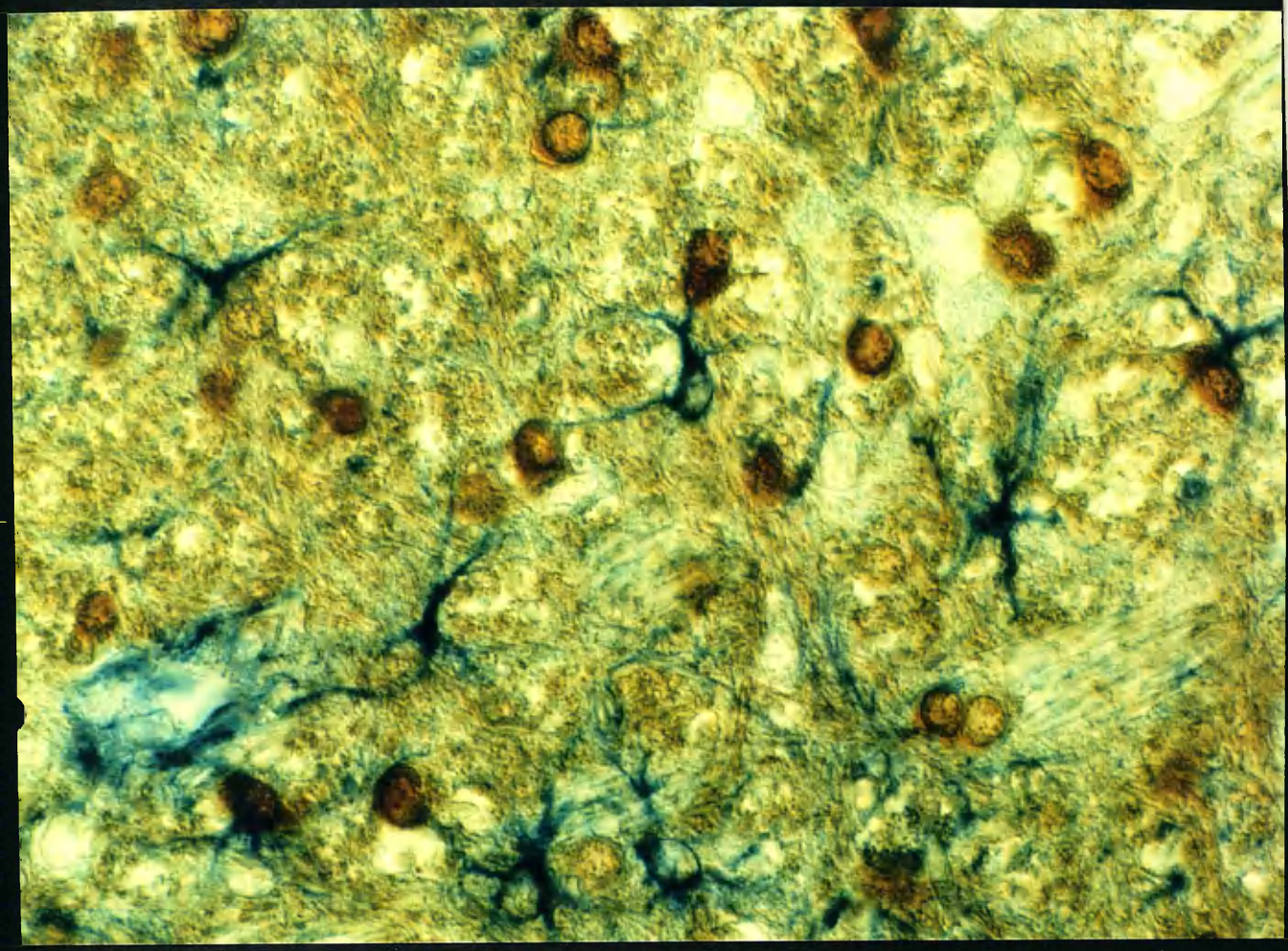
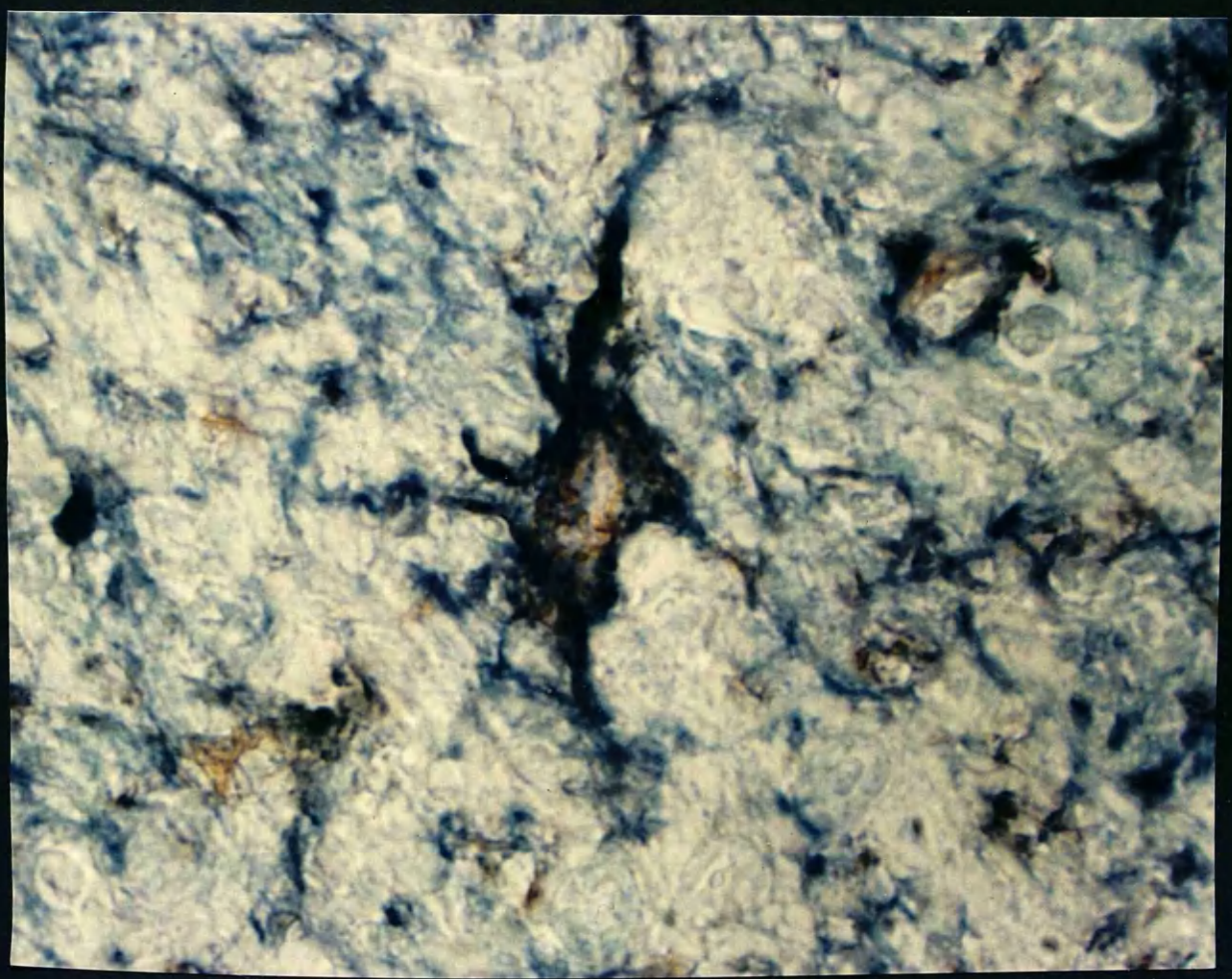


Fig 32a. Mouse cerebrum. Double immunohistochemical stain showing peroxidase labelled CAII expressing cells (brown) and alkaline phosphatase labelled GFAP expressing cells (blue). PAP. CAII and alkaline phosphatase anti-alkaline phosphatase GFAP stain x 1,200.

Fig 32b. Adult sheep cerebellum. Double immunohistochemical stain showing combined expression of peroxidase labelled CAII and alkaline phosphatase labelled GFAP in glia of white matter. Brown, blue and indigo (brown + blue) elements of the reaction product can be seen in the glial cell at the centre of this field. PAP. CAII and alkaline phosphatase-anti alkaline phosphatase GFAP stain x 1,800.





CHAPTER SEVEN

AN IMMUNOHISTOCHEMICAL STUDY OF THE CELLULAR LOCALISATION OF BORDER DISEASE VIRUS IN SPINAL CORD OF FETUSES AND NEWBORN LAMBS INFECTED IN UTERO AT 50 DAYS GESTATION.

INTRODUCTION

A wide spectrum of pathological effects of BD virus infection on the developing CNS have been described of which the most consistent and characteristic defect is hypomyelinogenesis. Extensive research has been performed on virological and morphological aspects of the disease but the pathogenesis of any of the described abnormalities is still unknown.

The qualitative, quantitative and neurochemical aspects of hypomyelination have been most extensively studied in the spinal cord and these studies suggest that hypomyelination may be caused by defects in the differentiation of oligodendroglia. The principal observation upon which this hypothesis is based is the finding of increased numbers of "type III glia" in white matter and the characterisation of such cells as glial progenitor cells (Barlow & Storey, 1977a). However, the interpretation of these results is controversial due to the lack of satisfactory morphologic criteria for the identification of glial cells, especially during early stages of development and the recent re designation of type III glia as microglia (Kitamura, 1980).

Virus infections of animals and man cause demyelination by different mechanisms including virus induced cytolysis of oligodendrocytes (mouse hepatitis virus; progressive multifocal leucoencephalopathy) and immunologically mediated destruction of virus infected cells (sub-acute sclerosing panencephalitis; Theiler's mouse polioencephalomyelitis) (Lampert & Rodriguez, 1984). The possibility that virus infection of oligodendrocytes or their precursor cells also causes hypomyelination in BD is an appealing hypothesis but virus infection of oligodendrocytes has not yet been demonstrated.

The purpose of this study is to demonstrate the cellular localisation of BD virus within the 90 day fetal and newborn lamb spinal cord using double immunoenzymatic labelling techniques. The neuroglial cell markers GFAP, GC and MBP were selected for these studies because, as discussed in the previous chapter, in the sheep these markers are expressed in astrocytes, developing glial cells and in myelin and myelinating oligodendroglia respectively.

MATERIALS AND METHODS

Source of animals and experimental design.

The source of animals for this Chapter and for Chapter eight are the same. For convenience a complete description of the experimental design and source of animals is included here.

Nine Dorset horn ewes, non-viraemic and seronegative to BD virus (group A), were mated to two Dorset horn rams which were also non-viraemic and seronegative to BD virus. A further nine Dorset horn ewes were also mated to these rams and were used as controls (group B). These sheep were non-viraemic and seropositive to BD virus. Rams were raddled and raddle marks were noted on ewes. Where ewes did not subsequently return to service the date of the first raddle mark was taken as the first day of pregnancy. All sheep were maintained in one group in disease secure accommodation until, at 34-48 days gestation, the sheep from group A were separated from group B and inoculated with BD virus. Four sheep from group A and four sheep from group B were killed at 93-96 days gestation and their fetus(s) removed. The remaining sheep (five from group A and five from group B) were allowed to go to term. For practical reasons, lambs were obtained at term by hysterotomy or lambing was induced using corticosteroid (Betsolan, Glaxo Ltd). The lambs so derived has gestational ages from 143-145 days.

Source of inoculum

The virus (Weybridge strain) was originally isolated from a pool of brain and spleen tissue taken from cases of BD in newborn lambs. It had been passaged six times in calf testes, five times in calf kidney, once in calf testes and once in choroid plexus cell cultures. The mean titre of the virus inoculum was $10^{4.86}$ TCID 5 ml.

Each of the sheep in group A was inoculated intramuscularly with 5ml of the inoculum.

Pathological procedures

At birth live lambs were, when possible, immediately separated from their dams and examined clinically. Lambs and viable fetuses were killed with an overdose of barbiturate. Lambs and fetuses were sexed, weighed, crown anus length measured and blood samples collected. The brain and spinal cords were removed and brains placed in saturated mercuric chloride formaldehyde (formol sublimate). Three 0.5cm thick serial sections of spinal cord segments C1, T9 and L1 were sampled and the remainder of the spinal cord placed also in formol sublimate. The samples of spinal cord taken from C1, T9 and L1 were then placed on pre-labelled cork disks, covered with talc and quenched in liquid nitrogen. Statistical examinations (Student's T-test) was performed on the data from twin infected lambs (n=4) and fetuses (n=8) and twin control lambs (n=3) and fetuses (n=4). There were insufficient numbers of single lambs and triplets in the relevant groupings to permit meaningful statistical analysis or to adjust the twin data to allow for sex differences. The following parameters were tested: whole brain weight, cerebellar weight, whole brain weight/cerebellar weight ratio, whole brain weight/body weight ratio, whole body weight, crown anus and tibial length.

Spleen, kidney, mesenteric lymph node, thyroid and thymus were aseptically taken into a sterile container and stored at -20°C prior to virological examination. The right tibia was also removed and placed in formalin for radiography.

Serology and Virology

See Chapter 1.

Source of antisera for immunohistochemistry

Antisera to GFAP, GC and MBP are as described in Chapter Six. Pooled monoclonal antibodies to BVD-MD virus were raised from BVD-MD virus isolates from clinical cases of mucosal disease and were used to demonstrate BD virus in tissue sections.

Histology

Following fixation, coronal sections of brain and spinal cord segments C3, T1 and L5-6 were processed in paraffin wax. Selected samples were sectioned at 10μm and stained by LFBCV. Samples of spinal cord quenched in liquid N_2 were cut on a cryostat at 15μm and stained by single and double labelled immunoenzymatic techniques as follows.

Following a wash in tris-buffered saline (TBS) sections were incubated with normal serum diluted 1 in 5 or 1 in 10 in TBS for 15 minutes. To reduce background staining bovine serum albumen was added to give a final concentration of between 2-5%. Serum was drained off and primary antiserum applied. Primary antiserum to GFAP (polyclonal) was raised in rabbits and used at a dilution of 1 in 500. Primary antiserum to MBP (polyclonal) was also raised in rabbits and used at a dilution of 1 in 400. BVD antiserum used was a pool of three monoclonal antisera (and therefore of mouse origin) and was used at 1 in 100 dilution. For double staining two primary antisera (GFAP and BVD-MD; MBP and BVD-MD; GFAP and MBP) were mixed in TBS and applied to the section.

After three washes in TBS for 10 minutes the bridging antiserum was

then applied in excess at dilution of 1 in 50. For MBP and GFAP immunostaining swine anti-rabbit antisera was used and for BVD-MD immunostaining goat antimouse was used. For double stains two bridging antisera were mixed in TBS. A further TBS wash was followed by application of the PAP and alkaline phosphatase-anti-alkaline-phosphatase complexes (APAAP). TBS wash, and finally the substrate was applied. DAB was used for the PAP reaction and fast red or fast blue was applied for APAAP reaction. The fast red and fast blue substrates contained levamisole to inhibit endogeneous peroxidases. Various combinations of double enhancement of primary and secondary antisera were also prepared to facilitate photomicroscopy. Double enhancement involved re-applying antisera following a TBS wash.

For antisera controls sections were reacted with swine fever antiserum in place of BVD-MD antiserum and sections were also prepared following omission of primary antiserum (or omission of primary antisera in double immunoenzyme preparations). Tissue control for BVD-MD was uninfected lamb brains and, for GFAP and MBP, human or mouse brains. Antisera controls and tissue controls were prepared for each fetus/ lamb.

Sections were counterstained with Meyers' haematoxylin and mounted with aquamount (Dako Ltd). Fast blue sections were mounted without a counterstain.

For the purpose of this investigation tissues from three infected fetuses, two control fetuses, three infected new-born lambs and one control newborn lamb were selected for further immunocytochemical studies.

RESULTS

The five BD naive dams of Group A used in this experiment infected with virus seroconverted to BD virus and showed titres of more than 1 in 453. The control ewes (Group B) showed static titres of more than 1 in 453 when sampled before, during and at completion of the experiment. Clinical examination of Group A new-born lambs 33 and 34 (twins, see Table XIV) showed both had a marked tremor and hairy fleeces. Lamb 32 (Group A) showed a precolostral antibody titre of more than 1 in 453 and virus was not isolated from blood serum, clot and viscera. All five remaining infected fetuses and lambs showed no precolostral antibody to BD virus and virus was not isolated from either serum or blood clot (Table XIV). Control lambs showed neither precolostral antibody titre and nor was virus isolated.

Gross examination and radiography

No significant gross lesions were identified in control lambs or fetuses or, except for lamb 32, infected fetuses and lambs. Coronal slices of fixed brain of lamb 32 showed severe bilateral cystic cavitation (porencephaly) of the cerebrum affecting the frontal parietal and occipital cortices. Cavities were centred on white matter and were traversed by numerous fine filamentous strands (Fig. 33). Linear radiographic densities of tibial bones consistent with growth retardation lines were present in the infected newborn lambs 32 and infected fetuses 14 and 12 (Fig.34). No such densities were present in tibia of control lambs and fetuses.

A statistically significant difference ($P<0.005$) between the cerebellum whole brain weight ratio of affected twin lambs ($\bar{x} = 8.77 \pm 0.665$) and that of control lambs ($\bar{x} = 9.95 \pm 0.58$) was found. Statistical differences ($P<0.05$) were also obtained for tibial length (affected

lambs $\bar{x}$ = 84.0 mm ± 3.37; control lambs $\bar{x}$ = 90.86 mm ± 6.39). No statistically significant differences were found for other parameters measured.

Histopathology

LFBCV staining of brain and spinal cord showed deficiencies of stainable myelin in both persistently infected lambs and all three viraemic fetuses. No abnormalities of myelination were evident in control fetuses or in the porencephalic lamb 32.

Immunohistochemistry

GFAP expressing glia of fetal spinal cord were evident in both grey and white matter. GFAP expressing sub-pial glia and cells adjacent to the ventral longitudinal median fissure were unipolar with cell nuclei located on the pia. These cells were morphologically similar to GC expressing cells described in the previous chapter and possessed long thin radially arranged processes which extended for considerable distances into white matter. Bipolar cells also with radially arranged processes and stellate GFAP expressing cells (Fig. 35) were present throughout all white matter columns. GFAP expressing cells of grey matter were morphologically indistinguishable from these latter cells. GFAP expression was also present in some ependymal cells and subependymal glia and in cells of dorsal and ventral nerve roots. In newborn lambs GFAP expression was similar to that described for paraffin wax embedded tissue.

In fetal tissue MBP expressing glia was present mainly in white matter but sparse MBP expressing glia were also present adjacent to myelinated fibres in grey matter. As with GC expression, most MBP expressing cells were unipolar or bipolar and were situated on or near the pia

matter (Fig.36). Stellate MBP expressing glia were also present in white matter columns adjacent to grey matter. MBP expression of myelin presented as an indistinct, evenly distributed, mottled peroxidase deposit in white matter. MBP expression was also present in dorsal and ventral nerve roots. In new-born animals only fine staining of processes and indistinct staining of myelin was seen.

GC expression was as described in the previous Chapter.

No significant differences between infected animals and controls was seen in the distribution or cellular localisation of expression of GFAP, GC or MBP.

BD virus infected cells (Fig.37) were present in all infected fetuses and lambs. In lamb 32 grey matter was conspicuously devoid of BD virus infected cells but numerous cells in white matter were infected. In the remaining viraemic animals infected cells were present throughout grey and white matter. Neuronal perikarya, grey and white matter glia, ependymal cells, vascular endothelial cells, fibrocytes within dura and cells within the dorsal and ventral nerve roots were all infected with virus. BD virus was not demonstrated in spinal cords of control lambs. Sections taken from infected lambs and fetuses and reacted with swine fever antiserum or following omission of the primary antiserum showed no immunostaining for BD virus.

Double labelled immunohistochemistry

Sections of fetal and new-born lamb spinal cord stained for BD virus and GFAP also showed the presence of BD virus in GFAP expressing cells. Alkaline phosphatase labelled cells (BD virus expressing) were blue, peroxidase labelled cells (GFAP expressing) were brown and cells interpreted as showing combined localisation of GFAP and BD

virus were dark brown, black or dark purple (Fig. 38). Cells which expressed GFAP alone (brown) or BD virus alone (blue) were also recognised.

Using the double labelling techniques and cryostat sections employed in this study the fine processes of GC expressing glia did not lend themselves to accurate co-localisation studies. No conclusions were reached regarding the localisation of BD virus in GC expressing glia.

Sections of fetal and new-born lamb tissue stained for BD virus and MBP showed the presence of BD virus in MBP expressing cells. In such sections alkaline phosphatase labelled cells (BD virus expressing) were blue, peroxidase labelled cells (MBP expressing) were brown and cells and processes which were dark brown or purple or black were interpreted as demonstrating co-localisation of both MBP and BD virus in the same cell (Fig. 39). In addition to cells showing simultaneous MBP and BD virus expression, non-MBP expressing BD virus infected glia (BD+ MBP-) and MBP expressing BD virus uninfected glia (BD- MBP+) were also recognised (Fig. 39).

Sections of fetal spinal cord embedded in paraffin wax and double immunolabelled with GFAP and MBP showed glial cell GFAP expression as previously described but MBP expression was restricted to myelin. Some subpial GFAP expressing cells had processes which contributed to MBP expressing myelin sheaths (Fig.40).

DISCUSSION

The results of this investigation show that BD virus infects a wide range of neural and non-neural cells in the central nervous system. Previous immunofluorescence studies have shown inconsistent staining of neurons of cerebral cortex (two of eight lambs) and cerebellum

(one of six lambs) (Terpstra, 1978). Gardiner (1980) also showed that neurons, cells adjacent to ependyma and subependyma, perineuronal cells of dorsal root ganglia and cells in peripheral nervous system may be infected by BD virus. In a further study Anderson et al. (1987a) reported that small numbers of cells of the spinal cord are infected. The difficulties of cell identification inherent in the staining methods employed in these studies permitted no accurate information regarding the location of virus in glial cells, endothelial cells or ependymal cells.

The results of this study confirm and extend those of Gardiner (1980), Terpstra (1978) and Anderson et al. (1987a). Using the peroxidase-anti-peroxidase method, virus was demonstrated in cells morphologically identified as neurons, glia, ependymal cells, endothelial cells and presumed fibrocytes of the dura matter. In contrast to the findings of Anderson et al. (1987a) marked neuronal and glial cell infection was seen. No significant differences in the cellular localisation of virus or severity of infection was seen in persistently infected fetuses or lambs. In lamb 32, however, virus was demonstrated only in glia of white matter, ependymal cells and cells within or adjacent to blood vessels (pericytes or possibly endothelial cells). No staining of grey matter was seen in any of ten sections examined.

Previous studies have shown that gliogenesis in man and rat follows a common pattern. Glioblasts migrate from matrix cells adjacent to the central canal to the periphery of the spinal cord (Fujita, 1965). These cells which initially accumulate in the subpial region already possess biochemical characteristics of astroglia and express GFAP (Choi, 1986). Here, just prior to the onset of myelination, there is

a rise in the mitotic rate of subpial glioblasts. There follows an abrupt occurrence of oligodendrocytes in the peripheral presumptive white matter. At least some of these subpial glia possess biochemical characteristics of both oligodendroglia and astroglia and express both GFAP and MBP. These cells have been dubbed "transitional" cells (Choi, 1986).

This study shows that subpial glia of ovine fetal spinal cord express GC, MBP or GFAP. Furthermore, double labelling of paraffin wax embedded tissues with MBP and GFAP shows that cells with morphological and biochemical characteristics suggestive of transitional cells are present in this location. Thus, gliogenesis in the lamb appears to follow the same pattern as that of rat and man. In fetal spinal cord BD virus was detected in both GFAP expressing and MBP expressing glia. Nuclei of cells of each of these two biochemical types were situated at or subjacent to the pia, and are therefore probably GFAP-MBP-BD virus expressing cells. This suggests that, in fetal spinal cord, BD virus infects differentiated and differentiating astroglia and oligodendroglia and probably also transitional cells or pluripotential subpial glia. The widespread occurrence of BD virus infected GFAP and MBP glia in new-born lambs suggest virus infection persists in fully differentiated astrocytes and oligodendrocytes. These results are in agreement with those of two recent in vitro studies (Elder & Potts, 1987; Anderson et al., 1987b) which showed the occurrence of BD virus in cultured ovine astroglia and oligodendroglia.

No significant differences in the distribution of GC expressing cells was obtained in infected lambs as compared with controls. Also localisation of BD virus in GC expressing cells was not resolved.

It is now well established that CNS myelin is formed solely by oligodendroglia. A comparison of infected lambs compared with that of controls did not show any significant differences in morphology of oligodendroglia. However, it is possible that virus infection of oligodendroglia, transitional cells or pluripotential glia may affect the differentiation and/or function of oligodendrocytes which may then give rise to hypomyelination.

Previous studies of BD infected fetuses have shown a reduced rate of myelination, a reduced thickness of myelin related to cross sectional axonal diameter and incomplete compaction of myelin (Barlow & Storey, 1977a). Also described are increased numbers of type III glia (Barlow & Storey, 1977a & b). These cells were interpreted according to a classification of Vaughn et al. (1970) as multipotential glial cells. As the total number of glia and astrocytes in normal fetuses compared with that of controls were similar, Barlow and Storey (1977a&b) concluded that total numbers of oligodendroglia of BD fetuses must be diminished. They suggest that BD virus infection deflects differentiation of glioblasts aways from oligodendrocytes and towards type III glia.

The original glial cell classification of Vaughn et al. (1970) is no longer widely accepted. Cells with ultrastructural features of type III glia have been variously described as glioblasts, oligodendroglia and "pericytal microglia" but these cells are now generally accepted as resting microglia (for review see Kitamura, 1980). The histogenesis of microglia remains poorly understood. Combined tritiated thymidine autoradiography, electron microscopy and immunohistochemical studies suggest that resting microglia are a third ectodermal neuroglial cell arising from glioblasts (for review see Fujita and

Kitamura, 1976). Specific immunohistochemical markers for identification of microglia are not available for sheep at present but several recent studies have shown that some lectins bind specifically to human and murine microglia (Mannoji et al., 1986; Streit & Kreutzberg, 1987). Identification of suitable lectins as markers for ovine microglia may help clarify the nature of the increased glia cells density described by Barlow and Storey (1977b).

Thus, although there is some doubt regarding the identity and role of type III glia in the CNS of BD affected lambs, the hypothesis of Barlow and Storey (1977a & b): BD virus infection modulates differentiation of glial cells, remains attractive in view of the demonstration in this study of BD virus in myelinating oligodendroglia and probably also transitional cells. Morphometric studies to determine populations of oligodendrocytes and astrocytes using immunohistochemical criteria for identification of cell type (particularly myelinating oligodendroglia) are therefore warranted and are investigated in the following Chapter.

One animal in this study showed lesions of so-called alternative pathology. In this animal virus was not isolated from blood or viscera but was demonstrated immunohistologically in CNS, although virus was conspicuously absent from grey matter. A precolostral antibody titre of more than 1 in 453 indicated that the lamb had responded immunologically. It is likely that virus persisted in the CNS in this case because of its immunologically privileged site. At the developmental stage at which infection occurred in this fetus the only available susceptible cells may have been restricted to white matter in oligodendrocytes and type II astrocytes. These cells are known to have the same progenitor cell which is different from the

progenitor cell of Type I astrocytes (Raff et al., 1983b). The possibility that porencephaly is related to a localised host immune mediated destruction of virus infected cells should be considered. That an emerging immune response may initiate nervous disease has previously been suggested for Borna disease, an unclassified agent, infecting rats and horses. Neonatal rats may acquire a persistent, tolerant infection and expression of disease is mediated by immunological factors (Hirano et al., 1983).

Although hypomyelination was not evident in the single porencephalic lamb studied here, double labelling with BD virus and MBP showed that oligodendroglial infection had taken place. Clearly oligodendroglial infection is not the sole factor required for hypomyelination to occur. Oligodendroglia may require to be infected prior to some critical event in their differentiation, or their synthesis or maturation or compaction of myelin. The possibility that hypomyelination is independent of oligodendroglial infection should also be considered. A recent report described decreased thyroid hormone levels in BD infected lambs (Anderson et al. 1987c). These authors draw parallels with the hypothyroid mouse which also shows hypomyelination and suggest that infection of the thyroid may be important in the pathogenesis of hypomyelination in BD.

In summary this study shows that BD virus infects a wide range of cell types in the CNS of fetal and new-born lambs. Neurons, ependymal cells, macroglia and unidentified cells within the PNS as well as non-neural cells including endothelial cells and presumptive fibroblasts are all infected by BD virus. Virus infected cells with immunohistochemical characteristics of myelinating oligodendroglia (MBP expressing) and astroglia (GFAP expressing) were recognised.

Virus was also demonstrated in cells whose anatomic localisation, morphology and biochemical characteristics are consistent with transitional cells or subpial pluripotential glioblasts.

The relationship between infection of specific cell types and hypomyelinogenesis was not resolved. However, infection of transitional cells and myelinating oligodendrocytes permits the possibility that perturbations of oligodendrocyte function not morphologically evident by light microscopic examination may lead to hypomyelinogenesis. In a porencephalic lamb serological evidence of immunological responsiveness combined with elimination of virus from viscera but viral persistence in CNS white matter was interpreted as suggesting that the destructive cerebral lesions seen in the alternative pathology of BD are mediated by the host's immune response.

Table XIV Summary of serology, virology and pathology results for infected and control fetuses/lambs.

	Fetus/lamb identification No.	Gestational age of infection	Gestation age of examination	Pre colostral antibody titre of fetus/lamb	Virus isolation	Presence of hypomyelination
FETUSES	*3	N/A	96	<10	-	-
	*7	N/A	95	<10	-	-
	9	37 days	95	<10	+	+
	12	37	95	<10	+	+
	14	34	93	<10	+	+
LAMBS	†32	48	144	>453	-	-
	ǂ33	42	144	<10	+	+
	ǂ34	42	144	<10	+	+
	*36	N/A	144	<10	-	-

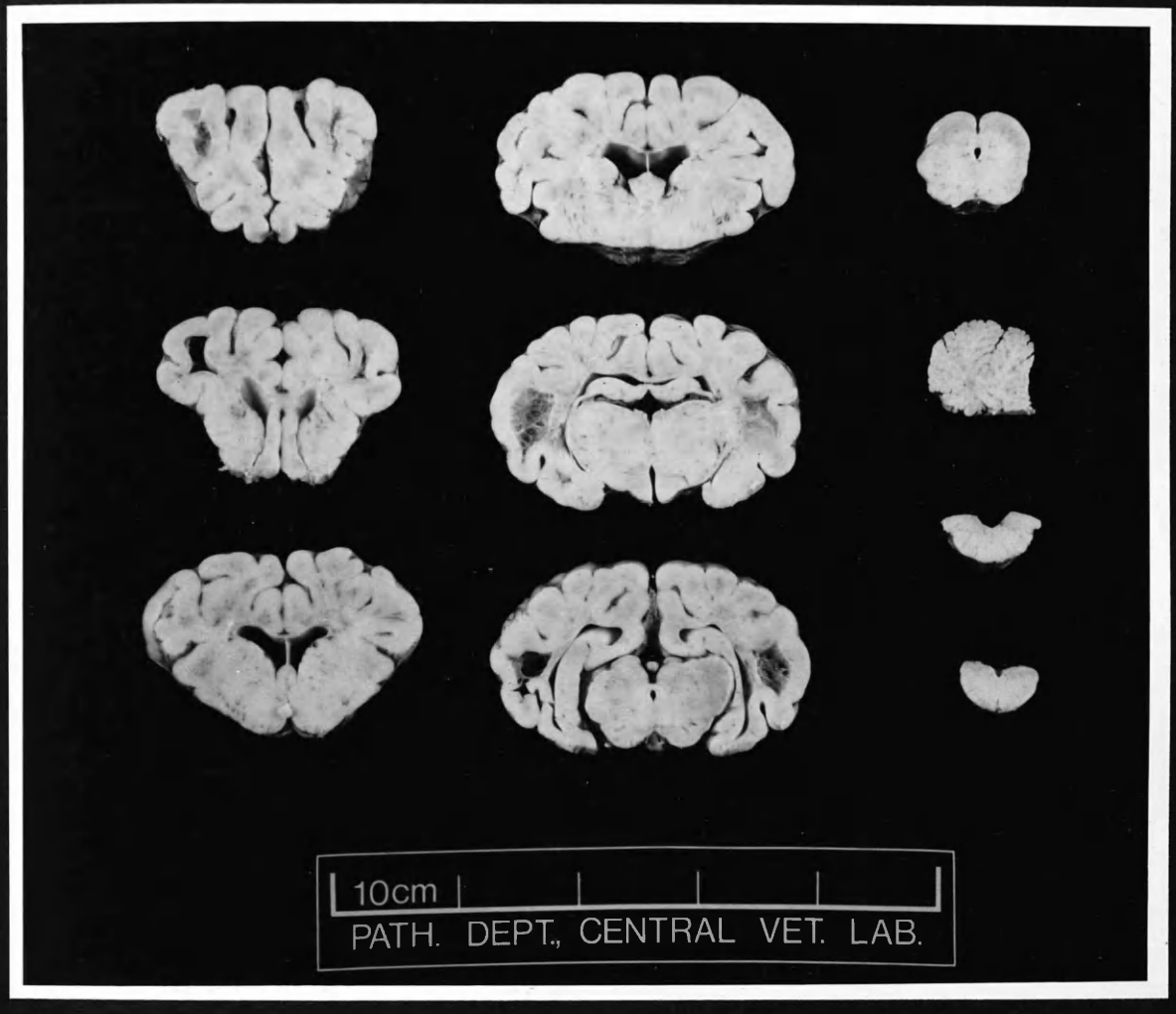
* Control fetuses/lambs.

† Porencephaly was also present.

N/A not applicable

ǂ Twin lambs

Fig. 33. Lamb 32. Coronally sliced brain showing bilateral cavitation of the cerebral cortices. Cavities are traversed by numerous fine filamentous strands of tissue.


10cm
PATH. DEPT., CENTRAL VET. LAB.

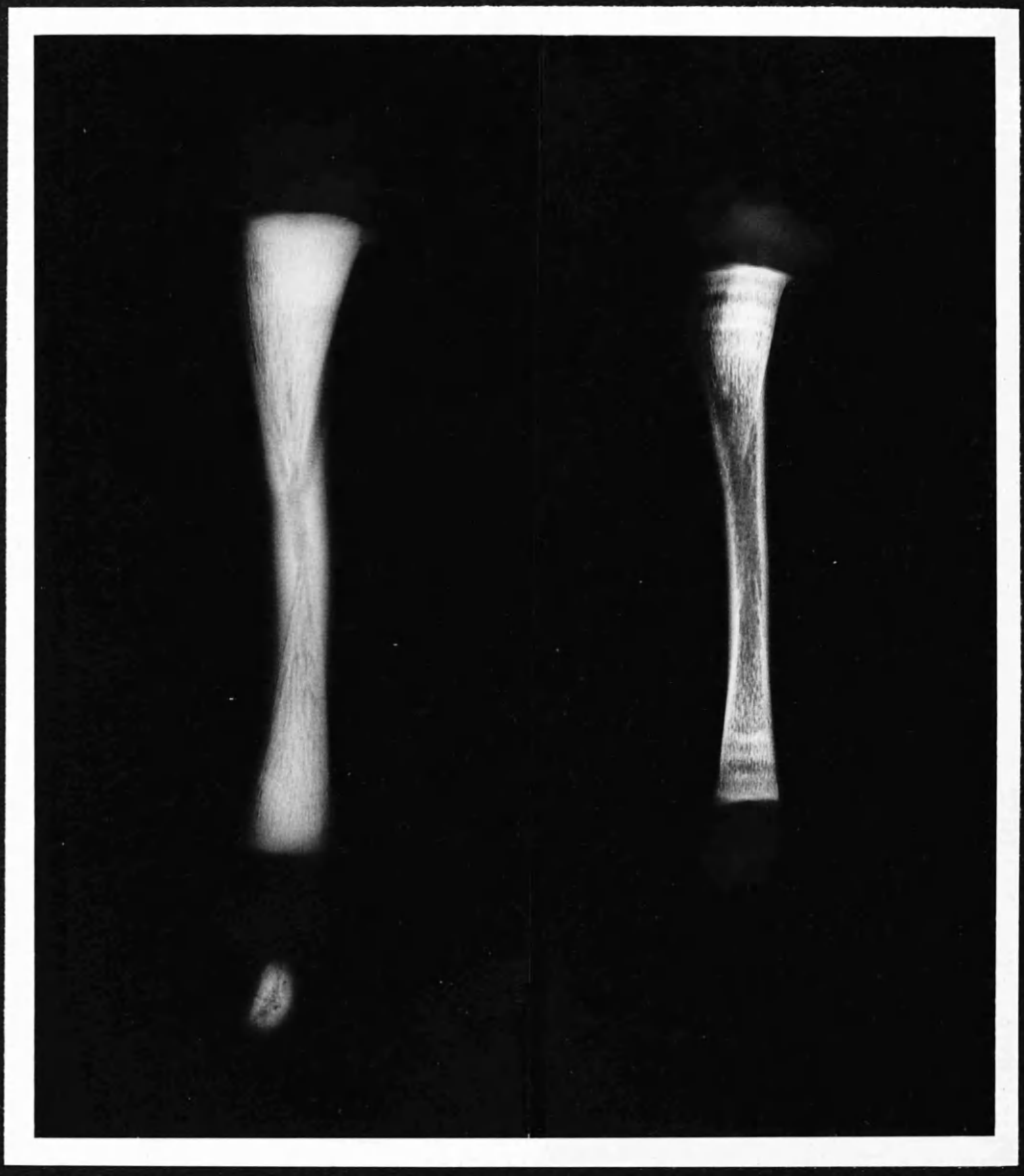
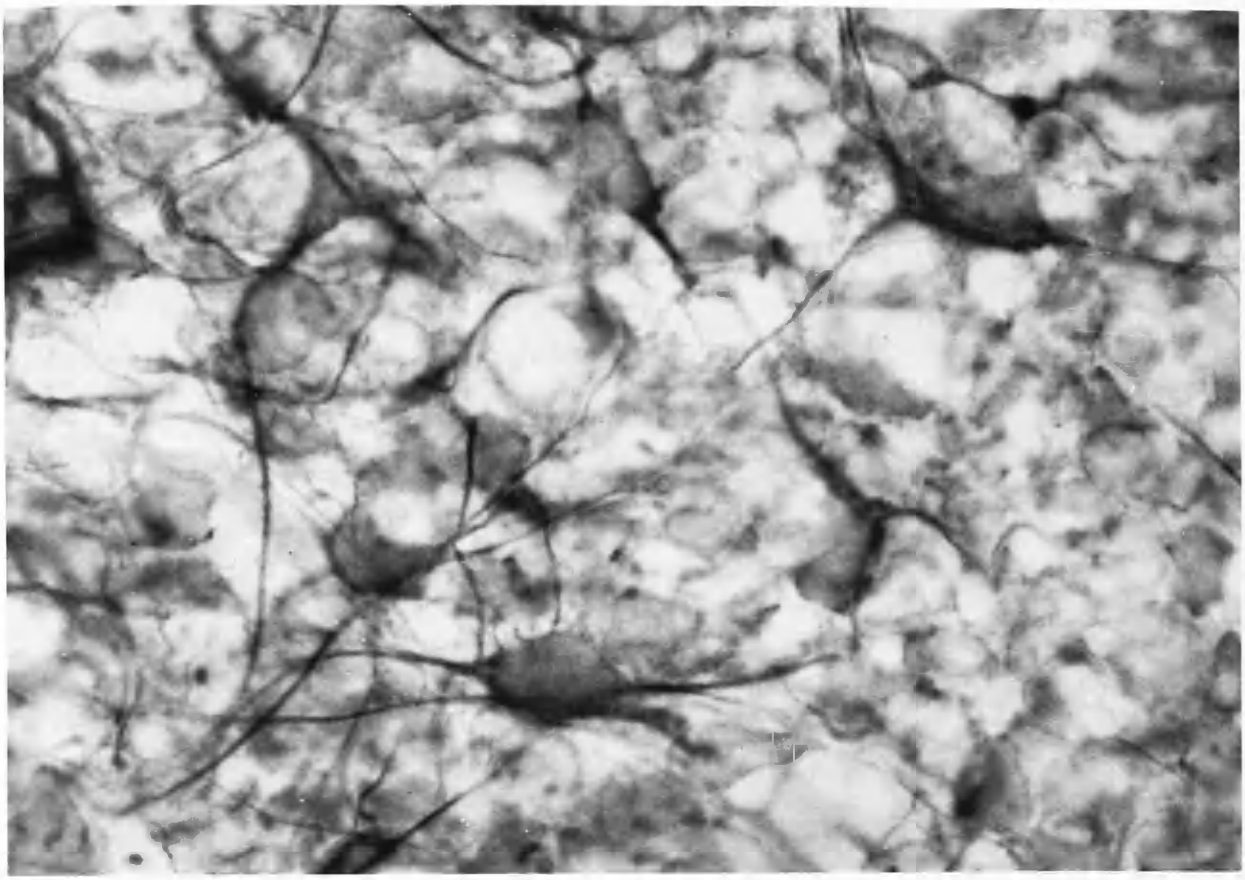


Fig. 34. Radiograph of normal (control) fetal tibia (left) and a tibia of an infected fetus (right). The tibia of the infected fetus shows multiple growth retardation lines.

Fig. 35. Spinal cord of Fetus 12. Cells showing several processes expressing GFAP and perinuclear cytoplasmic expression of GFAP. Cryostat section. PAP GFAP stain x2000.

Fig. 36. Spinal cord of Fetus 14. MBP expression of unipolar and bipolar sub-pial cells. Occasional cells with several MBP expressing fine processes can be recognised in deeper parts of white matter (arrows). Cryostat section. PAP. MBP stain x750.



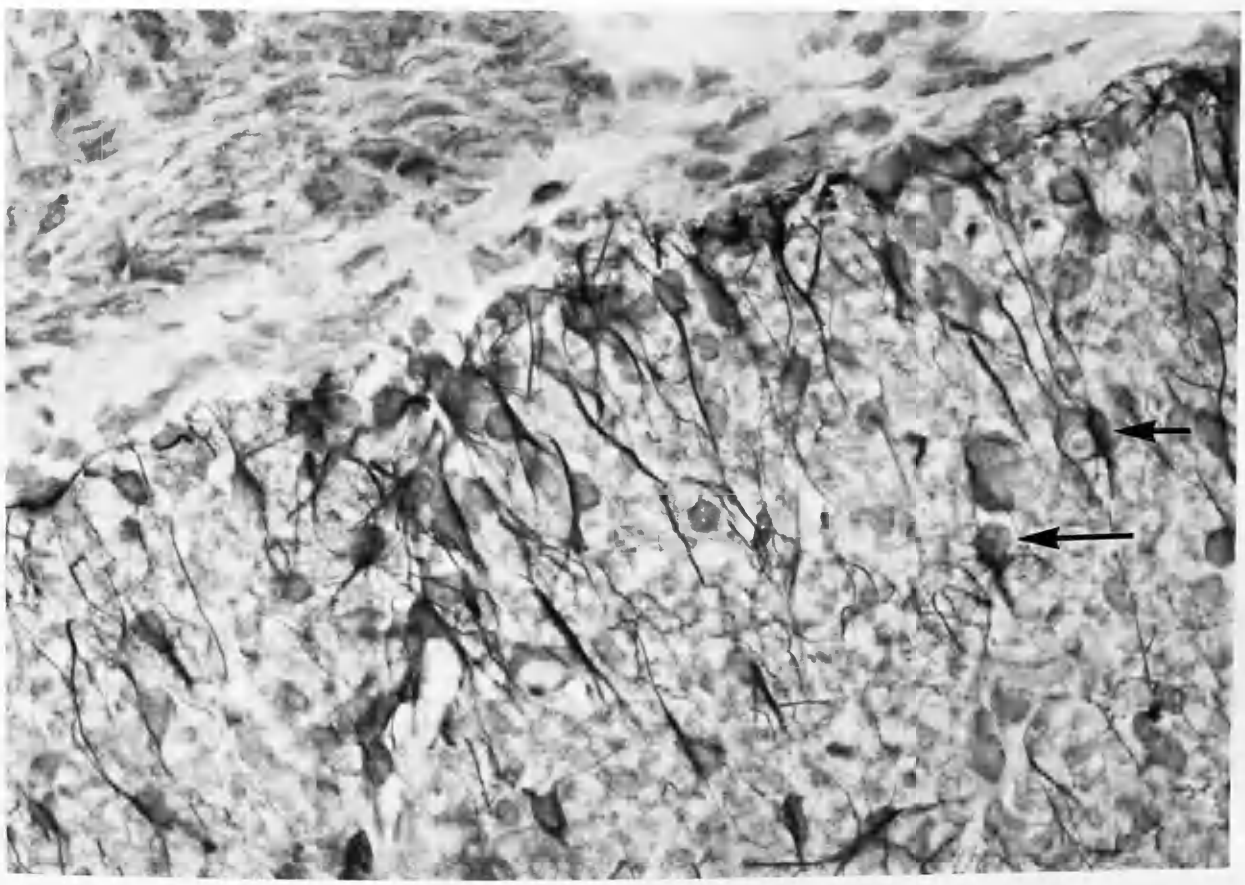
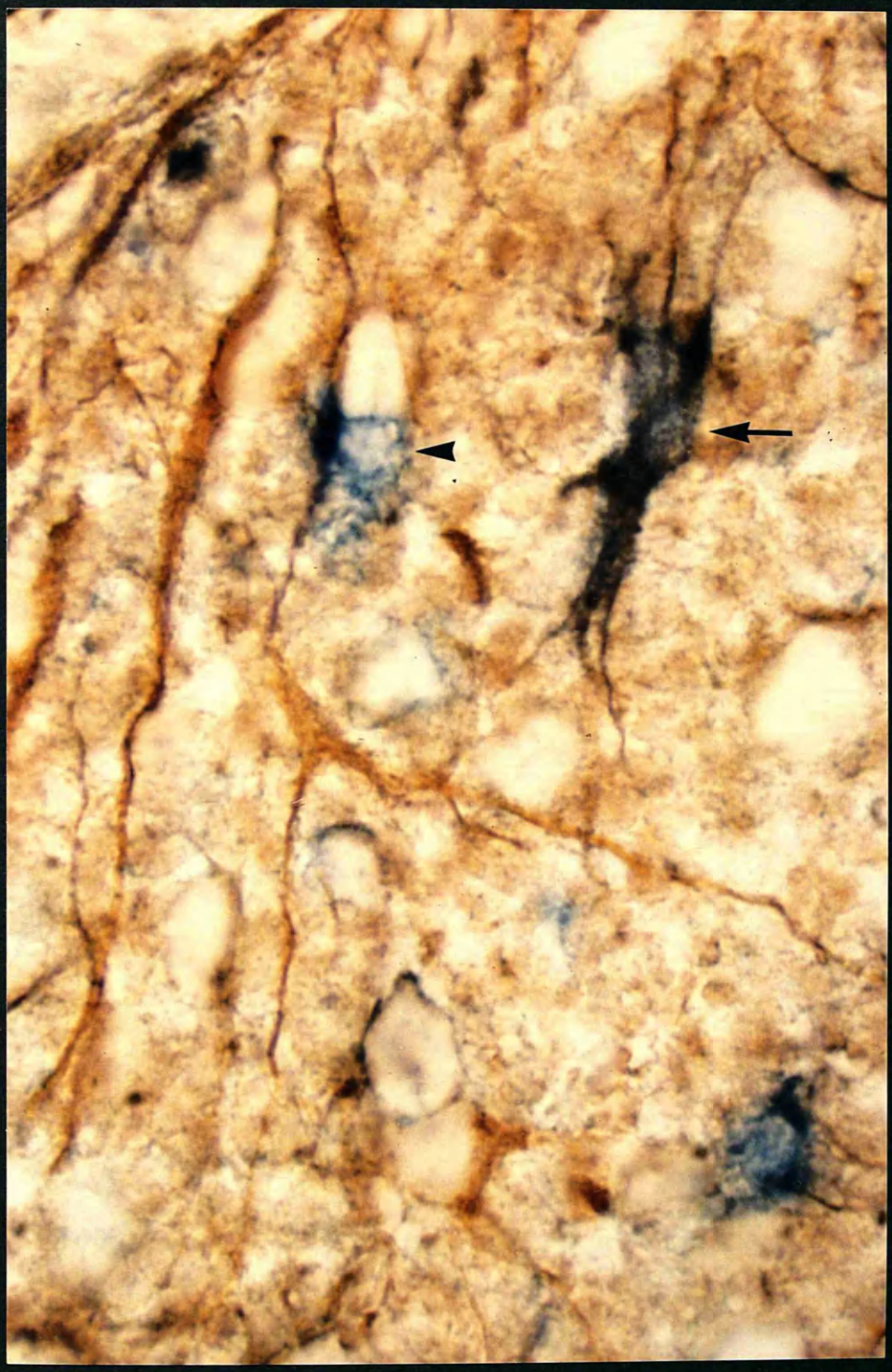


Fig. 37. Spinal cord of Fetus 12. BD virus infected ventral horn neurons (arrows) and numerous infected glial cells of ventral column white matter are shown. Cryostat section PAP for BVD-MD virus x200.

Fig. 38. Spinal cord of Fetus 12. A section double stained for GFAP (labelled brown with DAB) and BVD-MD virus (labelled blue with fast blue) is shown. Brown GFAP expressing processes and a blue BVD-MD infected cell (arrowhead) are present. A single cell with several processes stained indigo or black is interpreted as showing combined GFAP expression and BD virus infection (arrow). Cryostat section PAP and APAAP double stain for GFAP and MBP respectively x800.



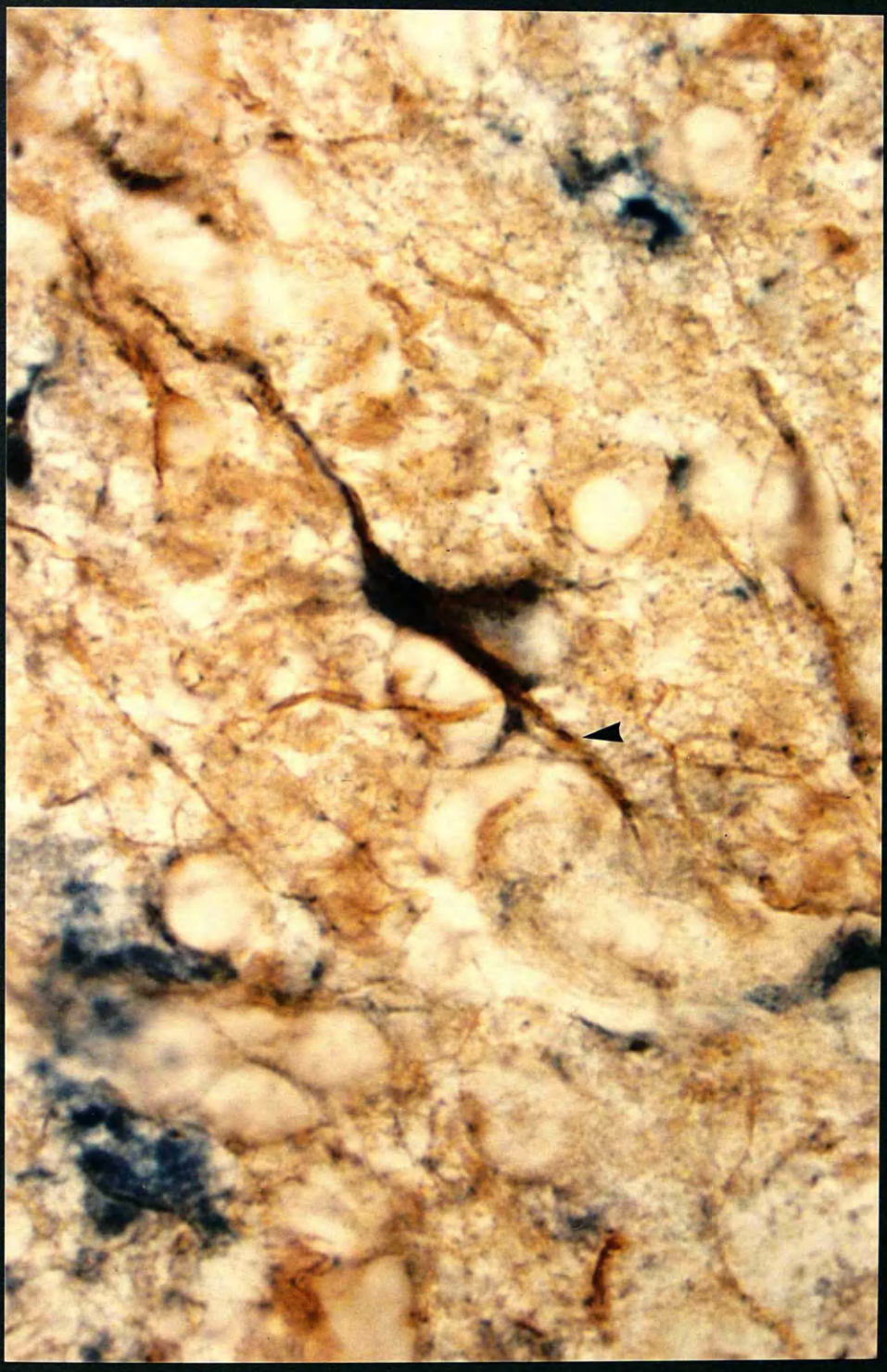
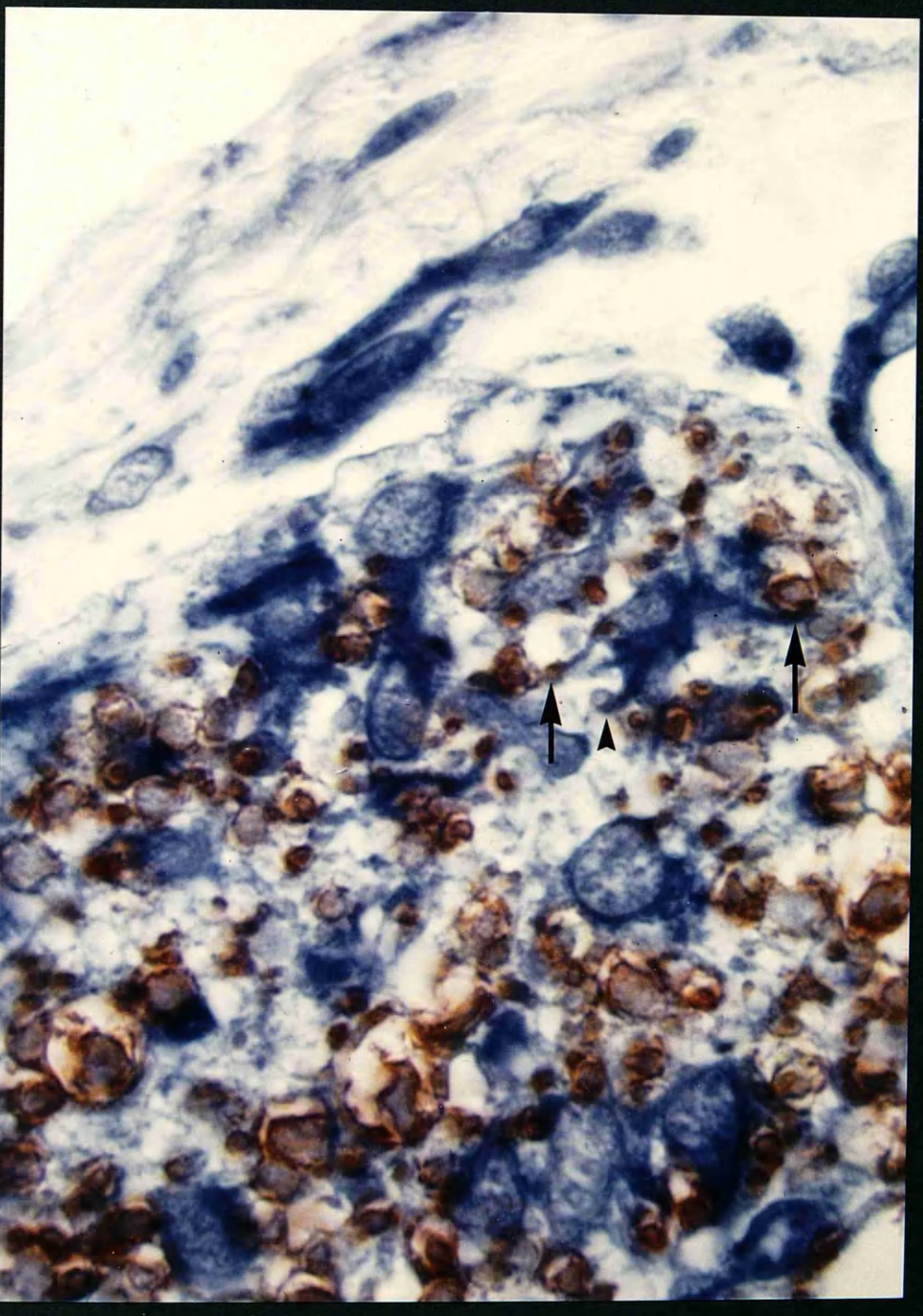


Fig. 39. Spinal cord of Fetus 14. A section double stained for MBP (labelled brown with DAB) and BVD-MD virus (labelled blue with fast blue) is shown. Several segments of brown MBP expressing processes are present. A single cell with several processes stained black is interpreted as showing combined MBP expression and BD virus infection. One process shows only brown staining at its periphery (arrow-head) indicating a loss of virus expression in distal parts of this cell process. Indistinct areas of blue staining may represent part of a BD virus infected cells. Cryostat section PAP and APAAP double stain for MBP and BVD-MD respectively x 800.

Fig. 40. Spinal cord of Fetus 3. A GFAP expressing cell (blue) shows processes extending towards and in close proximity to MBP expressing (brown) myelin sheaths (arrows) and an unmyelinated process (arrowhead). Paraffin wax embedded. PAP and APAAP double stain for MBP and GFAP respectively. x1500.



CHAPTER EIGHT

A MORPHOMETRIC ANALYSIS OF WHITE MATTER GLIA OF FETAL AND NEWBORN BORDER DISEASE VIRUS INFECTED SHEEP USING GLIAL FIBRILLARY ACID PROTEIN AND MYELIN BASIC PROTEIN.

INTRODUCTION

The previous chapter showed that early in-utero infections with BD virus causes hypomyelination and persistent infection of GFAP expressing glia (presumed astroglia) and MBP expressing glia (presumed myelinating oligodendroglia) of the CNS. In fetuses, so-called transitional cells may also be infected. No consistent morphologic defect of oligodendroglia or astroglia which might account for hypomyelinogenesis was seen in newborn lambs persistently infected with BD virus. Infection of oligodendroglia with BD virus (and also transitional cells) raises the possibility that infection may lead to disturbances of oligodendroglial differentiation, maturation or function not discernable by light microscopic immunohistochemical methods.

Gliosis of white matter is also an important feature of many cases of CNS pathology caused by BD virus. These glial cells are morphologically similar to type III glia and it has been suggested that these cells arise from small glioblasts (Barlow & Storey, 1977 a & b). This interpretation is no longer completely acceptable using modern glial cell classification criteria and theories of ontogenesis (Kitamura, 1980).

The purpose of this study is to investigate the populations and distributions of GFAP and MBP expressing glia using morphometric methods. The aim of the study is to characterise the cell types involved in gliosis and establish whether oligodendroglial populations are affected by viral infection.

MATERIALS AND METHODS

The source of animals, experimental design, source of inoculum, pathological procedures and source of antisera are the same as specified in the previous chapter.

Immunohistochemistry was performed on paraffin wax processed tissues from all but one fetus and one lamb produced by the experiment. Control fetus (number seven) was excluded as it had severe bilateral renal dysplasia and was small (460 grams). Two infected lambs (32 and 35) was also excluded because lesions of porencephaly were present in one (32) and neither lamb showed brain or spinal cord hypomyelination when brain tissue was stained with luxol fast blue cresyl violet (LFBCV).

The levels of brain and spinal cord selected for immunohistochemistry were full transverse sections of spinal cord segment T_1, medulla at the level of the obex and middle cerebellar peduncles; midbrain at the level of the rostral colliculus; half coronal sections of left occipital cortex; parietal cortex taken rostral to the level of the optic chiasm; frontal cortex at the head of the caudate nucleus; sagittal sections of cerebellum taken through vermis.

Morphometric Methods

Using an eyepiece graticule consisting of a square subdivided into 25 smaller squares, the number of GFAP expressing cells and the total number of glial cell nuclei were counted at 12 different sites of white matter of frontal and parietal cortex at a magnification of 400x (see Fig. 41a); at one site of cerebellar vermis lobule six at a magnification of 1000x and at four sites of spinal cord (Fig. 41a) at a magnification of 400x. Sections of newborn lamb cerebrum and cerebellum were often torn or incomplete and quantification of GFAP expressing cells was evaluated

only in spinal cord. Twelve sites were counted (Fig. 41a). Trypsinised sections of spinal cord showed a dense network of GFAP expressing cell processes which inhibited accurate counting of cell nuclei. Counts of GFAP expressing cells of spinal cord were therefore performed on untrypsinised sections which reduced the number of processes staining with GFAP and permitted more readily the recognition of nuclei of GFAP expressing glia. In fetal tissue MBP expressing cells were counted in lobules eight and nine and at two sites of lobule six of the cerebellar vermis (Fig. 41).

Intracytoplasmic expression of MBP was not seen in fetuses three or four and these fetuses were excluded from morphometric analysis of MBP expressing cells. The reason for this lack of expression is not fully understood but may be related to inadequate penetration of fixative. MBP expression of myelin was also weak in these two fetuses. Insufficient MBP expressing cells were present in newborn lambs to perform morphometric analyses.

Nuclei of cells which showed immunoperoxidase expression of MBP or, GFAP in perikarya and cell processes were counted. Any nucleus which touched the boundary of the graticule was counted in. All counting was performed on 7 μm thick sections.

In fetal lambs moderate densities of MBP expressing glia were found only in peripheral parts of cerebellar white matter. MBP expressing cells were counted along the entire length of visible white matter for lobules eight, nine and the rostral parts of lobule six (see Fig. 41b site 6) by moving the eyepiece graticule sequentially from one field to the next until the entire length of the lobular white matter had been examined. MBP expressing glial cell counts were

expressed as the number of MBP expressing cells per eyepiece graticule field area (2.5×10^{-9} m^2). For posterior parts of lobule six MBP expressing cells were counted for 50 sequential fields starting from the point at which white matter narrowed to the width of the eyepiece graticule.

MBP expressing cell densities were evaluated at the middle of lobular white matter for lobules eight and nine and, for lobule six, at the rostal aspect of the white matter/granule cell layer interface.

Statistical analysis

A Student's T test was used to compare populations of glial cells for controls and infected sheep. For GFAP expressing glia and glial cell nuclei the percentage of GFAP expressing glia was compared at each site sampled and cumulative counts of GFAP expressing glia and total glial cell nuclei were also compared. An attempt to correct these cell populations for the size of the lamb/fetus was made by comparing the above populations of cells expressed as a ratio of brain weight. Densities of MBP expressing glia (average number of MBP expressing cells per $2.5 \times 10^{-9} m^2$) were also compared at each of the sites counted.

In the middle of cerebro-cortical white matter (Counts 'B' on Fig. 41a) clusters of so-called myelinating glia were occasionally found. Cell nuclear counts at such sites were very high. For statistical analysis the counts at these levels for GFAP expressing cells and total glial cell nuclei were excluded because one way analysis of variants showed highly significant degrees of dissimilarity compared with counts taken at the edge of the gyrus.

RESULTS

Fetal MBP expression

No significant differences were seen in the distribution of myelinated tracts or MBP expressing glia of infected fetuses compared with controls. Presumptive white matter tracts of spinal cord, brainstem, and corpus medullare of cerebellum were well myelinated and most tracts showed evidence of active initiation of myelination by the presence of glial cells containing intracytoplasmic MBP.

In midbrain (with the exception of oculomotor fibres and optic tract, which were both well myelinated), peripheral parts of cerebellar folia, internal capsule and fibres of the corpus striatum, fibres showing myelin MBP expression were less densely grouped than in brainstem and spinal cord. Moreover, the intensity and thickness of myelin MBP expression decreased in rostral white matter tracts. MBP expressing glia were present throughout white matter. Some cells showed cytoplasmic MBP expression extending to fine, linearly arranged processes (Fig. 42) Other cells with diminished cytoplasmic staining showed MBP expressing processes which extended to involve irregular myelin sheaths (Fig. 43). The MBP immunoperoxidase method was considerably more sensitive in demonstrating immature myelin sheaths than was the LFBCV method. MBP expressing cells, although well separated, were frequent, readily seen and evenly distributed throughout these tracts MBP expression was not associated with abnormal nuclear forms. No MBP expression of myelin was seen in gyral white matter or corpus callosum throughout of frontal, parietal or occipital cortex. In contrast to MBP staining LFBCV showed weak myelin staining for brainstem and spinal cord and a virtual absence of myelin staining in cerebellum, midbrain and cerebrum. The low densities of MBP express-

ing glia made it difficult to accurately define a consistent location for morphometric examination and counting of these cells was confined to the cerebellum. The results of these counts are shown in Table XV. The density of MBP expressing glia of lobules eight and nine was significantly less (Lobule 8 $P<0.01$) than in controls (for Lobule 8 $P<0.01$; for Lobule 9 $P<0.05$). Only two control tissues were available for counting at each of the sites for lobule six. No significant difference was achieved at these sites.

Newborn lamb MBP expression

The distribution of myelinated fibre tracts of both control and infected lambs determined by the immunoperoxidase method for MBP was as described in chapter six. Control lambs showed a similar pattern of myelinated fibre tracts with both LFBCV and with the MBP immunoperoxidase method but all the viraemic sheep showed a marked deficiency of stainable myelin in spinal cord, cerebellum (Fig. 44a & b) and cerebrum when LFBCV staining was employed.

Insufficient numbers of MBP expressing cells were present to permit morphometric studies of these cells in newborn lambs' brains.

Fetal GFAP expression and total glial cell nuclei

GFAP expressing glia were present throughout white and grey matter of spinal cord and brainstem. GFAP expressing cells were also present in ependymal cells and Bergmann glial fibres, although these latter structures were fewer and well separated when compared with density of such fibres in newborn lambs. Fibrous (type II) and protoplasmic (type I) astrocytes could also be recognised. As in newborn lamb brains protoplasmic astrocytes had fewer and finer processes than fibrous astrocytes.

GFAP expressing cells of infected lambs and fetuses tended to be more fusiform and have fewer and shorter processes than those of controls. A minority of GFAP expressing cells of infected fetuses had vesicular and irregular nuclei which contained little nuclear chromatin. Not all cells with abnormal nuclear forms showed GFAP expression. Cells with dense nuclei, irregular rod shaped nuclei and nuclei containing fragmented chromatin showed no GFAP expression. The frequency of GFAP expressing cells and total numbers of glial cell nuclei are shown in Table XVI. Statistical examination showed that the percentage of GFAP expressing glia of white matter was increased ($P<0.01$) at all sites examined in BD infected sheep. Cumulative totals for GFAP expressing cells were also increased ($P<0.001$). These results remained statistically significant when corrected for fetal brain weights (GFAP expressing cells/brain weight ratios). The cumulative totals for glial cell nuclei of white matter were not significantly different. This result was not altered where twins alone were compared and nor was it altered when cell totals were corrected for brain weights.

Newborn lamb GFAP expressing cells and total glial cell nuclei

GFAP expressing cells of newborn lambs were morphologically indistinguishable from, and had a similar distribution to those previously described in chapter six. Infected fetuses also showed a minority of GFAP expressing cells which, like those of fetal lambs, possessed a large, vesicular nucleus with sparse nuclear chromatin. Such cells were often in closely apposed pairs.

The results of counts of GFAP expressing cells in spinal cord of newborn lambs is shown in Table XVII. Total numbers of GFAP expressing glia of spinal cord were increased ($P<0.05$) in infected fetuses

compared with controls. No significant differences were seen for total glial nuclei counts but there were insufficient numbers of comparable classes of animals to permit statistical analysis of singles, twins or triplets.

DISCUSSION

Statistically significant increased percentages of GFAP expressing glia were found at all locations examined for infected lambs and fetuses. Cumulative totals of GFAP expressing glia were also increased in BD infected sheep. Not only were increased densities of astrocytes found but also in fetal sheep total astrocyte numbers were increased when a correction for brain size (total astrocyte numbers/brain weight ratio) was made. These results are interpreted as indicating an astrogliosis is present in BD virus infected sheep. Some abnormal nuclear forms showed GFAP expression of perikarya suggesting that some of the abnormal glia seen in BD are of astroglial lineage.

Subjectively assessed gliosis was not a feature of the brains of BD virus infected sheep in this study and total numbers of glial cell nuclei were not increased for BD infected fetuses or newborn lambs. Also a Students' T test performed on total numbers of glia minus astroglial totals did not give a statistically significant difference. Thus, gliosis was not a pathological feature of the Weybridge strain of BD virus infection.

Barlow and Storey (1987 a & b) considered that type III glia were markedly increased in BD. Using a glial cell classification of Vaughn (1969) these authors suggested that such cells represented a "deflection of differentiation of small glioblasts from oligodendrocytes to the type III cells". A reinterpretation of their electron

microscopic findings in the light of more modern classifications would suggest that type III glial cells are microglia (Kitamura, 1980). That such cells also contribute to the gliosis seen following infections with some virus strains would be consistent with the observations in this study of abnormal nuclear forms which show expression of neither GFAP nor MBP. Modern theories of glial cell ontogenesis show that microglia do not differentiate from the same stem cells as astroglia or oligodendroglia (Choi, 1986a&b; Kitamura, 1980). Microgliosis occurs in a wide range of neuropathological conditions. It is likely that the frequent occurrence of Type III glial cells reported by Barlow and Storey (1977b) corresponds to non-GFAP-non-MBP expressing glia with abnormal nuclear forms or rod shaped nuclei reported in this study and are thus microglia and represent a non specific mild inflammation which may be more evident with some viral strains than others.

Cells expressing MBP, especially noticeable in areas undergoing active myelination, were interpreted as pre-myelinating or myelinating oligodendroglia (Sternberger et al., 1978 a & b). The low densities of MBP expressing cells limited the sites suitable for morphometric examination and the failure of tissues of two control sheep to stain for MBP inhibited accurate statistical analysis of data. Nevertheless, a statistically significant decrease in density of oligodendroglia was seen in cerebella of infected fetuses at lobules eight ($P<0.05$) and nine ($P<0.01$). MBP expressing glia showed nuclear configurations similar to those of "light oligodendroglia" described by Ling et al. (1973). Abnormal nuclear forms were not recognised for MBP expressing glia.

It is now well established that oligodendroglia are the sole myelin

forming cells of the CNS. Deficiences of myelinating oligodendroglia in fetal nervous system may well contribute to, or be the main cause of hypomyelination in classical BD. Immunocytochemical staining (chapter seven) showed that oligodendroglia and probably also transitional cells are infected with virus. These observations suggest that viral infection of fetal oligodendroglia and transitional cells may lead to delayed differentiation or maturation of oligodendroglia with a subsequent deficiency of myelin at birth. Support for this hypothesis can be found in previous studies with the electron microscope. Ultrastructure shows that myelin sheaths in spinal cord are thin and have disproportionally few myelin lamellae for a given axonal diameter (Cancilla & Barlow, 1968) and that the periodicity of myelin tends to be higher than is normal for mature myelin (Barlow & Storey, 1977b). Neurochemical studies show that myelin lipid composition and total myelin has returned to near normal 20 weeks post partum. (Sweasy & Patterson, 1979). Thus, ultrastructural and neurochemical observations are consistent with delayed differentiation or maturation of oligodendroglia following fetal viral infection Additional quantitative studies using markers of oligodendroglia expressed at earlier stages of differentiation, such as GC or A2B5, may shed further light on the maturation of viral infected oligodendroglia.

No morphometric analysis of MBP expressing glia was possible on newborn lamb tissues. Surprisingly, although LFBCV showed extensive deficiencies of stainable myelin throughout the CNS, MBP expression of myelin sheaths showed no appreciable differences when control and infected tissues were compared. Studies of developing rat brain also show that the MBP immunoperoxidase method is superior to

conventional histological myelin stains in terms of sensitivity and precision with regard to detection of initial stages of myelination but this method did not allow quantitative evaluation of subsequent advancement of myelination (Rozeik & von Keyserlingk, 1987).

Newborn lambs of this study showed increased numbers of spinal cord astrocytes but no increase of total glial cell nuclei. As the majority of cells of spinal cord white matter are astrocytes and oligodendroglia this observation provides indirect evidence that oligodendroglia may also be reduced in newborn lambs.

In summary the morphometric analysis presented here shows that astrogliosis occurs following infection by the Weybridge strain of BD virus. The study also shows that myelinating oligodendroglia are reduced in the 90-96 day fetus and myelination is severely retarded in newborn lambs following BD virus infection at 37-52 days gestation. It is suggested that infection of glioblasts and/or incompletely differentiated oligodendroglia may delay maturation of oligodendroglia or inhibit production of myelin. The use of markers for less well differentiated (less mature) oligodendroglia may be helpful in further unravelling the pathogenesis of the observed myelin deficiency.

TABLE XV

Numbers of MBP expressing cells counted in Lobules 6, 8 and 9 of fetal cerebellar vermis

	Case Number	Cerebellar Lobule			
		Lobule 6/ (rostral)	Lobule 6* (caudal)	Lobule 8*	Lobule 9*
CONTROL	18	75	37/35	39/30	109/48
	17	57	NE	40/38	79/39
	16	NE	19/32	25/29	86/45
INFECTED	8	58	33/32	7/21	31/34
	9	55	31/28	14/21	65/42
	10	33+	5/14	NE	NE
	11	57	40/28	31/32	56/38
	12	18	21/27	3/28	NE
	13	42	46/35	12/13	46/42
	14	NE	32/28	18/25	49/37
	15	40	16/19	N/E	47/37
	Statistical significance	NS	NS	P<0.05	P<0.01

NE : Not examined

***** : MBP expressing cells are shown as numbers of cells per number of graticule squares examined

+ : only 39 graticule squares were available for counting.

/ : MBP expressing cells per 50 graticule squares.

TABLE XVI Frequency distribution of GFAP expressing cells and total glia cell nuclei counts of fetuses

	Case No	Twin/ single fetus	% GFAP expressing cells			Total GFAP cells	Total GFAP cells/Brain wt	Total glial cells	Total glial cells/Brain wt
			Parietal Cortex	Frontal Cortex	Spinal Cord				
CONTROL	3	T	22.36	17.78	5.54	144	5.55	985	37.98
	4	T	25.49	25.52	5.29	170	6.21	950	34.73
	16	S	22.98	25.20	5.5	223	10.41	1382	64.54
	17	T	23.38	23.79	3.96	167	8.13	1205	58.69
	18	T	15.03	21.01	3.3	149	6.81	1205	55.09
INFECTED	8	T	30.46	33.15	7.94	285	13.04	1228	56.20
	9	T	24.35	30.23	7.73	NE	NE	NE	NE
	10	T	27.94	28.76	10.52	295	14.43	1363	66.71
	11	T	31.59	26.07	8.5	283	13.43	1286	61.03
	12	T	29.9	37. 3	10.74	295	12.48	1279	54.13
	13	T	35.74	30.07	9.36	250	11.79	1202	56.69
	14	T	27.24	22.44	12.8	244	12.15	1290	64.15
	15	T	30.36	30.62	7.1	265	13.4	1266	64.03
	Statistical significance		P<0.01	P<0.01	P<0.001	P<0.001	P<0.01	NS	NS

NE not examined NS not significant

TABLE XVII

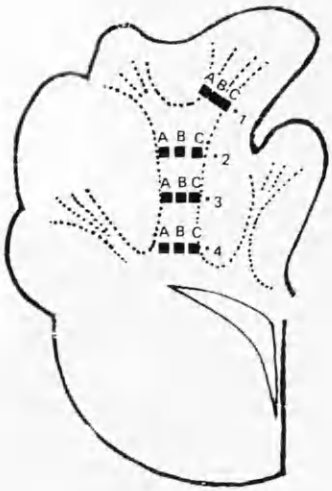
Numbers of GFAP expressing cells and total glial cell nuclei of newborn lambs

	Case	Single/ twin/ triplet	% GFAP cells	Total GFAP cells	Total GFAP cells/brain wt.	Total glial cells	Total glial cells/brain wt.
CONTROL	26	Twin	21.2	81	1.36	382	6.4
	27	Twin	22.8	65	1.08	285	4.75
	28	Trip	21.0	66	1.26	312	5.97
	29	Trip	14.5	55	0.97	378	6.7
	30	Trip	14.5	60	1.24	411	8.54
INFECTED	31	Twin	21.6	80	1.25	369	5.77
	33	Twin	27.5	128	2.06	464	7.48
	34	Twin	30.4	99	1.64	325	5.38
	35	Twin	31.7	100	1.57	317	4.97
	STATISTICAL SIGNIFICANCE		N.S	P<0.05	NS	NS	NS

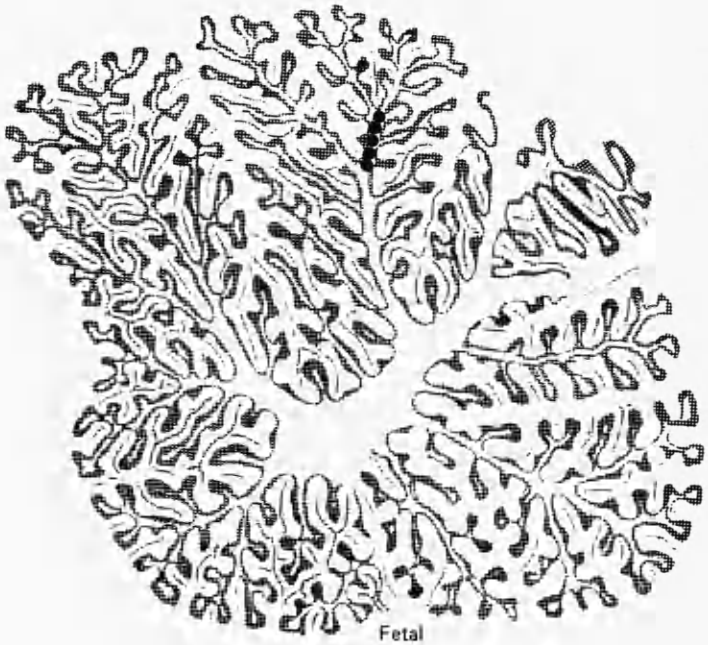
NS Not significant

Trip = triplet

Fig 41a Diagram showing sites of sampling of fetal and neonatal lamb brain for morphometric analysis of GFAP expressing cells and glial cell nuclear densities.



Fetal



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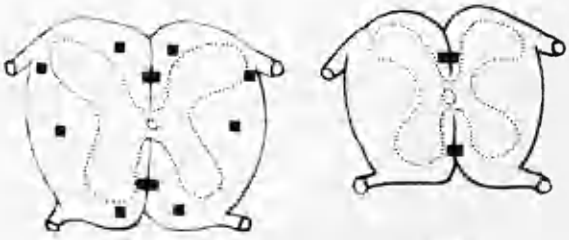


Newborn

Fetal

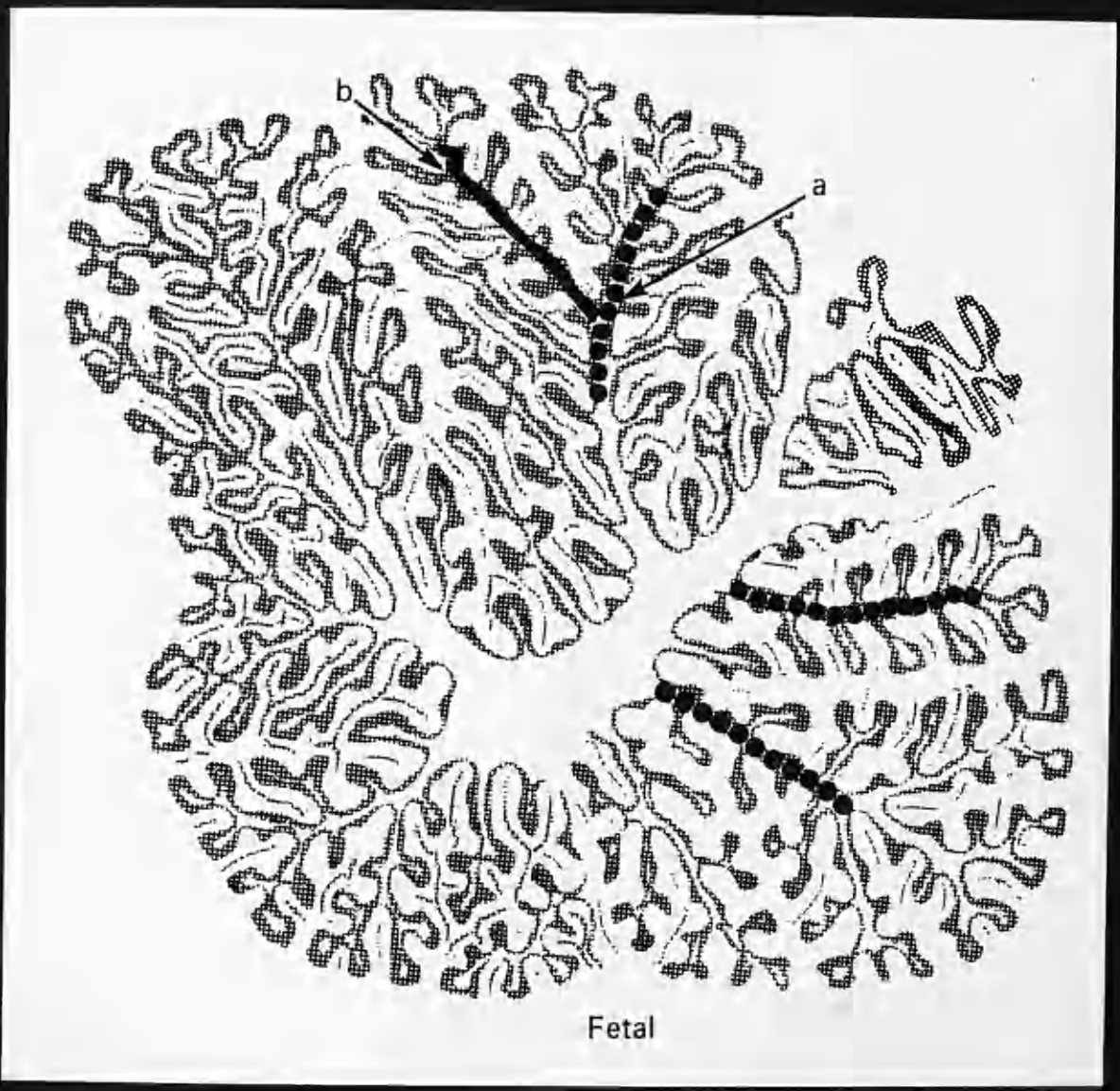

b
a
Fetal

Fig. 41b Diagram showing sites of sampling of fetal brain for morphometric analysis of MBP expressing cells. The site at lobule 6 marked 'a' is measured for 50 consecutive graticule squares.

Fig. 42 Cerebellum of fetus 14 showing cellular intracytoplasmic expression of MBP and myelin MBP expression. One cell shows a single long fine process (arrows) extending from perikarya. IPX DIC MBP stain X 1700.

Fig. 43 Corpus striatum of fetus 14 showing intracytoplasmic expression of MBP, one cell shows a narrow rim of cytoplasmic MBP expression which extends to involve a highly irregular myelin sheath. IPX DIC MBP stain X 1700.

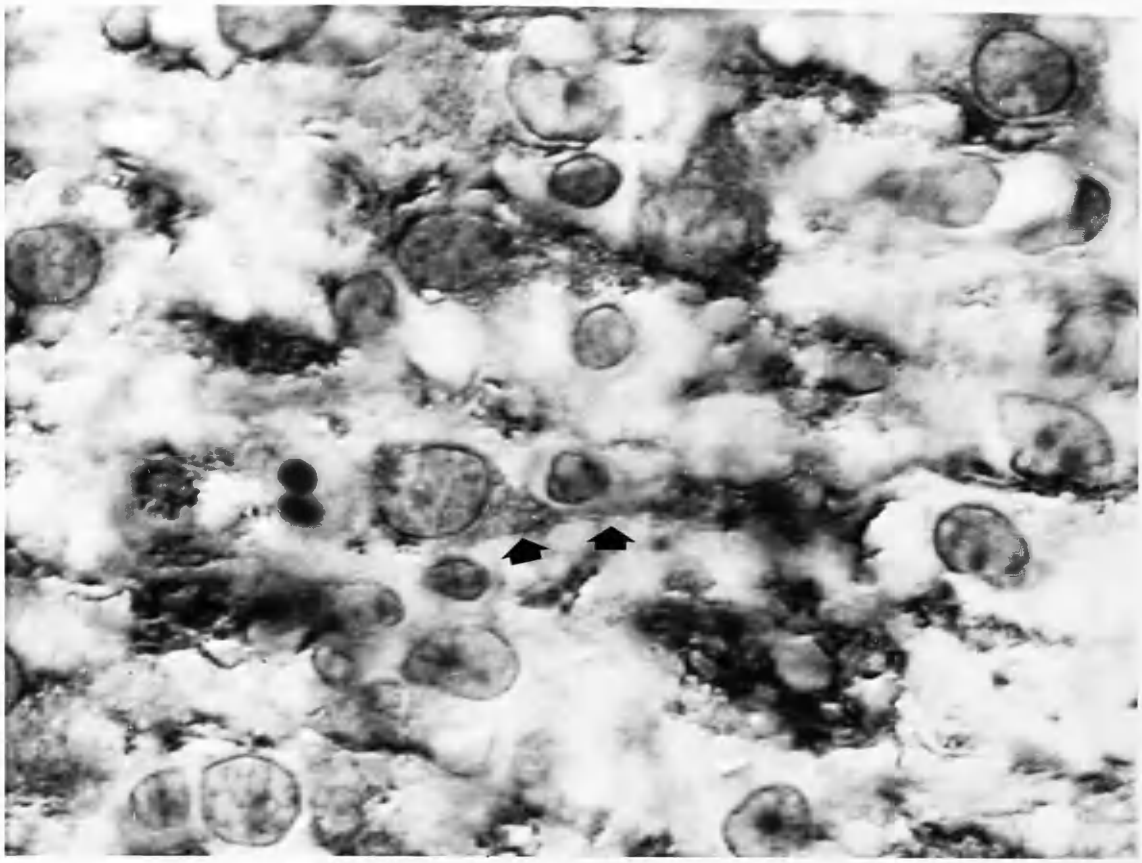
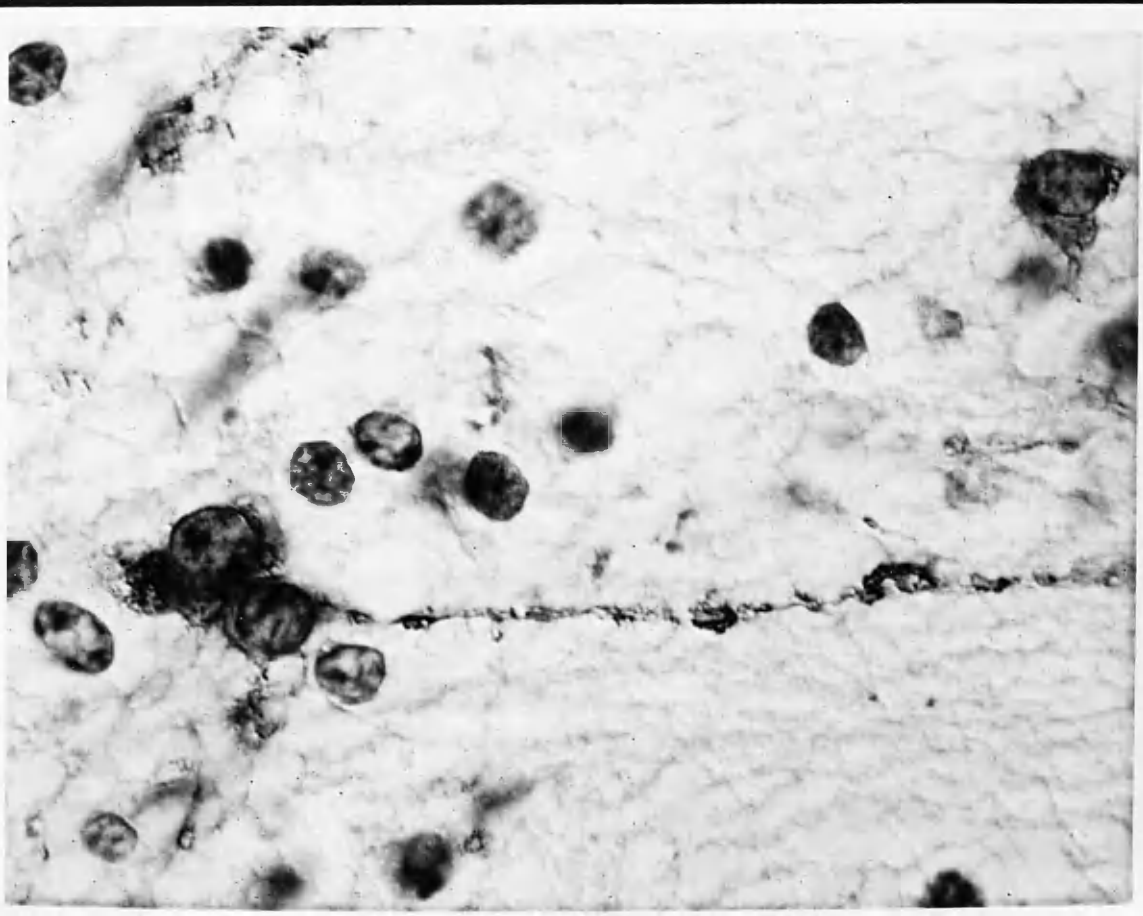
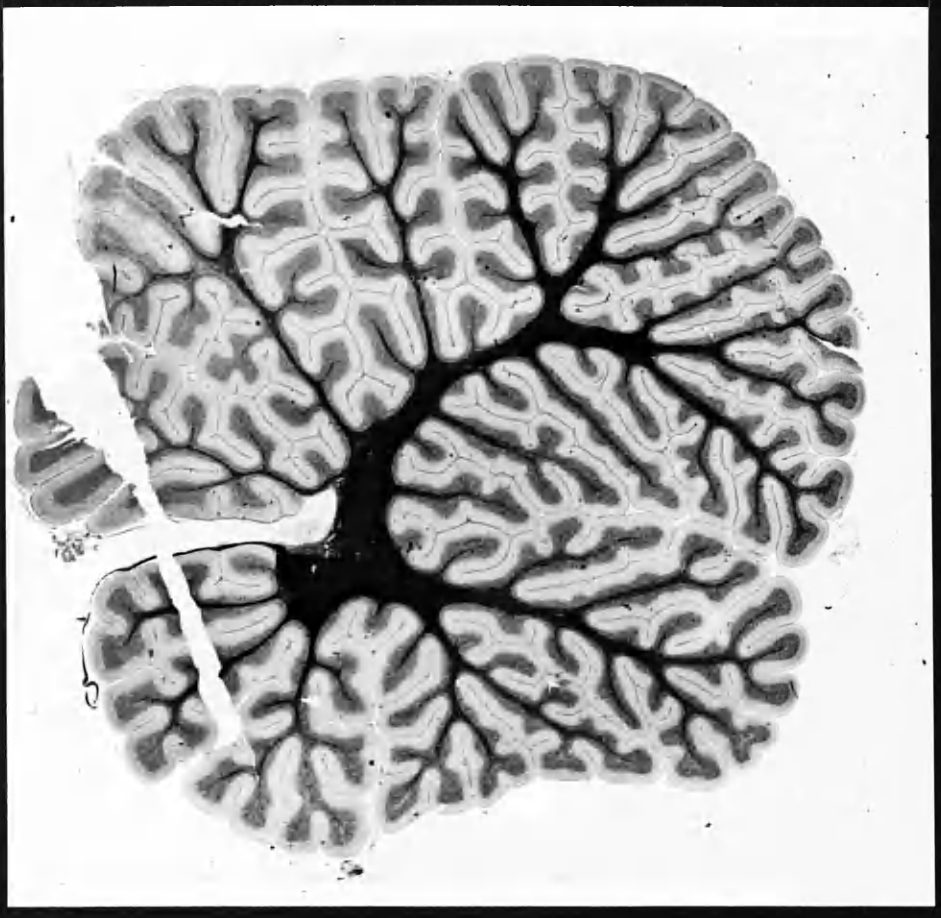


FIG 42





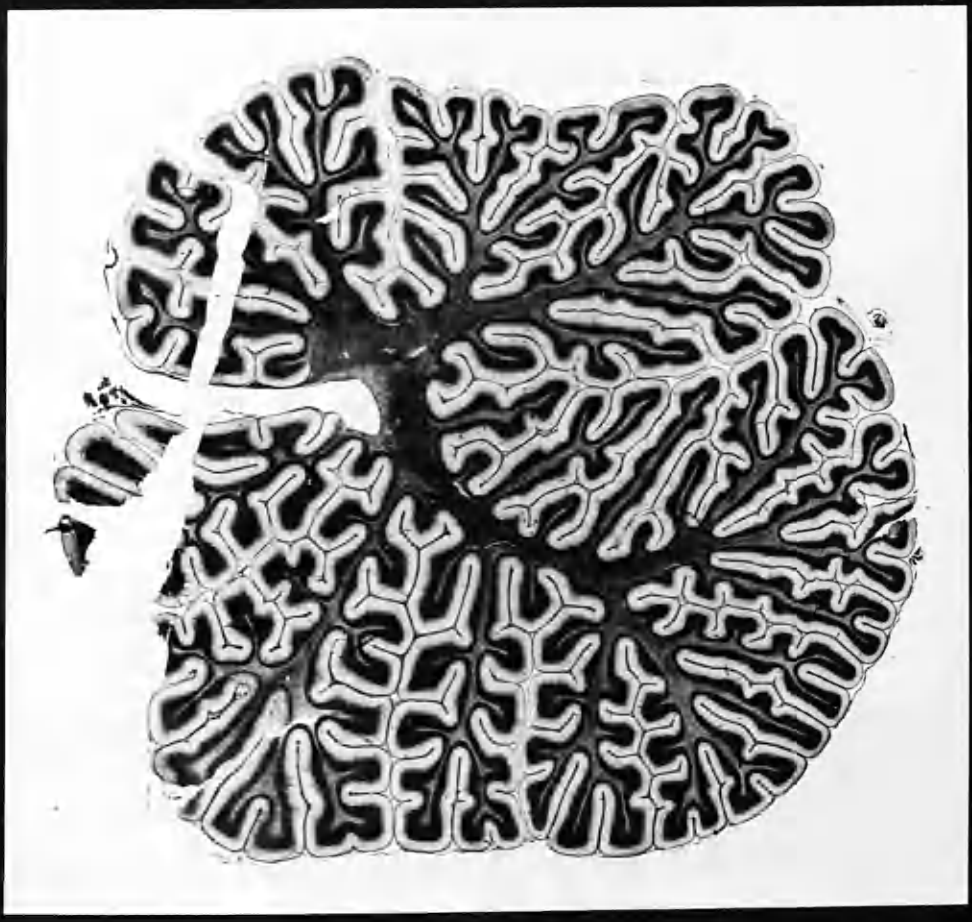


Fig. 44 a and b Sagittal sections of cerebellum from lamb 37 stained by the immunoperoxidase method (Fig. 44a) showing strong MBP expression of corpus medullare, primary and secondary folia. Fig. 44b shows the same site stained by LFBCV. There is a severe diffuse deficiency of stainable myelin. 44a IPX MBP stain x12; 44b LFBCV x12.

CHAPTER NINE

ULTRASTRUCTURAL OBSERVATIONS OF THE SECOND MUSCULAR BRANCH OF THE SCIATIC NERVE OF A 90 DAY FETUS AND NEWBORN LAMBS INFECTED WITH BORDER DISEASE VIRUS.

INTRODUCTION

Many studies of BD have been directed towards characterisation of the morphologic pathology of the CNS but the peripheral nervous system (PNS) has received scant attention. The initial report of BD virus infection (then identified as hypomyelinogenesis congenita) described absence of lipid in a small number of myelin sheaths of the sciatic nerve (Markson et al., 1959). This remains the sole description of BD PNS pathology. In a retrospective study, Barlow (1982) found no PNS lesions of fetuses infected with virus at 25-31 days gestation but only paraffin and celloidin embedded blocks of tissue were available for examination.

The paucity of published, detailed studies of the PNS of BD virus infected sheep prompted the selection of material for ultrastructural examination from the experiment detailed in chapter seven. Evidence obtained from the immunohistochemical distribution of virus reported in Chapter seven shows that cells of dorsal and ventral nerve roots of spinal cord may also be infected. This observation further indicates the relevance of a detailed examination of the PNS with particular regard to myelin sheaths.

MATERIALS AND METHODS

The source of animals and experimental methods are as described in Chapter seven. Within 30 minutes of death the second muscular branch of the sciatic nerve was carefuly dissected from the left leg of all fetuses and lambs and suspended under tension using a 2.5g weight in 2.5% glutaraldehyde in 0.1 molar Sorensen's phosphate buffer at pH 7.4. Tissues remained in glutaraldehyde for periods of up to six months. Segments of nerve were diced for transverse or longitudinal orientation, washed in buffer, post-fixed in one per cent OsO_4 for three hours, rapidly dehydrated in alcohol followed by inhibisol over a period of two hours 40 minutes and infiltrated with araldite MY 753 (Polaron Ltd). Transverse and longitudinal sections were cut 1 µm thick and stained with toluidine blue. Ultrathin sections were cut 70 to 90 nm thick, contrasted with uranyl acetate and lead citrate and examined in a Jeol 100b electron microscope. An accident occurred during transportation of tissues to the Lasswade Laboratory and tissues from only four animals were available for examination. These originated from an infected 90 day fetus (11/87), two newborn infected lambs (34/87, 35/87) and a control lamb (28/87). Only one of the infected lambs was viraemic and showed CNS hypomyelination (PG34/87). The other had an antibody titre of 1:80 and showed no CNS hypomyelination. Samples of semitendinosus muscle were orientated transversely, placed on cork disks, covered in powdered talc and quenched in liquid nitrogen. Muscle samples were subsequently stained for HE and reacted for ATPase at pH9.5.

Morphometric analysis

At a microscope magnification of 2000, non-overlapping photographs were taken of a fascicle containing large and medium diameter myelinated fibres until 12 photographs or at least 150 fibres had been

taken. A calibration grid with 1200 lines/mm was also photographed and printed with each series of samples to allow accurate calibration. On the final photographic prints a dissecting binocular microscope was used to measure the maximum and minimum diameters of axons and fibres and to measure the thickness of myelin sheaths. The fibre diameter, axonal diameter, myelin thickness and G ratio (ratio of axonal diameter to total fibre diameter) was calculated from these figures. Fibres excluded from measurement were those at nodes, paranodes, Schmidt-Lanterman incisures, and where the shape was distorted by Schwann cell nuclei, inadequate fixation or myelin bubble formation. Myelinating fibres showing highly irregular myelin configurations were also excluded. All measurements were carried out by a single operator on numbered photographs. The operator did not know from which animal the photograph had been taken.

Using a minitab computer programme a one way analysis of variance was performed on each parameter. The values for the axonal diameters, myelin sheath thickness and fibre diameter were compared for affected and control sheep using the Mann-Whitney test. The thickness of myelin sheath in relation to its axon was assessed by plotting, myelin thickness, fibre diameter and G ratio against axonal diameter.

The frequency of certain pathological features was counted in each of the above photographs. These features were axoplasmic accumulation of organelles, features consistent with early myelin sheath formation and myelin sheath degeneration. Control and BD infected lambs were compared.

RESULTS

Fetal tissue

1 µm thick toluidine blue stained sections showed many fibres to be

in advanced stages of myelination. Fascicles were of two types; those containing predominantly larger diameter fibres and fascicles containing mainly unmyelinated fibres with a few small myelinated fibres (Fig.45a & b). Longitudinal sections showed regular variation of internodal fibre calibre (Fig.46) suggesting excess tension had been applied during fixation. Several axons showed focal nodal and paranodal increased staining density of axoplasm and occasional Schwann cells showed intracytoplasmic dense bodies (Fig. 46). Many axons were surrounded by disproportionately thin myelin sheaths suggesting active myelination.

With electron microscopy the detailed structure and nature of the formations noted at light microscopy became more clear and a number of new observations were made. In both fascicular types a large number of fibres were unmyelinated but many fibres were found at various stages of the myelination process. This included isolation of axons from unmyelinated fibre bundles by tongues of Schwann cell cytoplasm, promyelin figures (large unmyelinated fibres in intimate contact over the whole of its circumference with one Schwann cell) and formation of non-compacted myelin (where the mesaxon has completed one or more spiral turns about its axon but has nowhere condensed to give rise to period and intraperiod lines, or, where the mesaxon has completed less than one turn about an axon).

Myelin sheaths were frequently irregular and large compact membranous myelin like bodies were present external to myelin sheaths (myelinoid bodies) (Figs. 47, 48, 49 and 51). Where the lamellar structure of these bodies was preserved the periodicity of myelin approached or was equal to that of normal myelin. Some lamellar structures have a periodicity (6.5 nm) which is considerably less than that of normal

glutaraldehyde fixed peripheral nerve myelin (12-17 nm). Myelinoid bodies were frequently identified at nodal and paranodal areas but were also occasionally present at internodes. Myelin sheaths were not always of the same diameter at consecutive internodes (Fig. 48). The most extreme myelin changes involved formation of short unmyelinated segments intercalated between normal myelin sheaths (Fig. 48). One such segment was 9.5 µm long. Exceptionally, short myelinated internodes were also present. One measured 7.0 µm. Unmyelinated axons surrounded by discontinuous myelin fragments or compact balls of folded myelin were encountered (Fig. 50a&b). Many of these structures showed normal myelin periodicity. Fibres showing abnormal myelin configurations ranged in diameter from 0.2 µm - 3.5 µm (n=20), and the majority of fibres (n=14) measured between 0.8 µm and 2µm. Only one fibre measured less than 0.8 µm. In some fibres the nodal gap was enlarged and the decrement zones of paranodes were increased in length (Fig. 47 & 49).

Changes recognised in axons included focal swellings and accummulations of organelles, dense bodies and membranous structures (Fig. 48 & 50b). Although accumulations were occasionally encountered at internodal locations they were most frequently recognised at nodes and paranodes. At such locations they were sometimes present on only one side of the paranode and adjacent internodal axon. Samples were not orientated to permit recognition of proximal and distal paranodes. Sub-axonal densification, which marks the bare nodal axon, was sometime interrupted or partially absent at abnormally wide nodes (Fig. 52 a & b) and was occasionally accompanied by outpocketing of nodal axonal cytoplasm (Figs. 48, 52a & b).

Newborn lamb peripheral nerve

In newborn lambs, peripheral nerve fibre diameters, numbers of myelin lamella comprising the sheath and frequency of myelinated fibres had all increased considerably (Fig.45.c) by comparison with fetal nerve (Fig.45a). Nevertheless fibres showing active myelination were present (Fig.53 & 54). Two fascicular types were again recognised: fascicles consisting almost entirely of large myelinated fibres and fascicles consisting of a 50:50 mixture of myelinated fibres and unmyelinated fibres. In the former fascicular type artefactual myelin splitting and bubble formation was a frequent feature, such as is commonly found in immersion fixed material. The principal abnormal features were similar to those decribed for fetal nerve and can be summarised as:

a) irregularities of myelin sheaths, with formation of myelinoid bodies and sometimes associated with unmyelinated large diameter axons; and

b) focal increased densities of axonal organelles and/or membranous bodies. These features were present in both infected newborn and fetal tissue and no significant differences were noted in the frequency of occurrence of these changes in affected tissue when compared with control tissue.

These abnormal features were recognised much less frequently than in fetal nerve.

The results of morphometric evaluations are presented in Tables XVIII-XXI. Mean myelin thickness and mean G ratio of the control fell between the values for the two infected lambs but control values for axonal diameter and fibre diameter were reduced compared with values for infected lambs. One way analyses of variance showed that there

were significant differences ($P<0.5$) among the means for each animal for each parameter. Graphs of myelin thickness versus axonal diameter showed that myelin thickness was proportional to axonal diameter in each case. Regression lines drawn for fibre diameter and axonal diameter showed that fibre diameter for a given axonal diameter was closely similar for the control lamb 28 and non-viraemic lamb 35 but the viraemic lamb 34 had thinner fibre diameters for any given axonal diameter (Fig. 55). Similar observations were made on graphs of myelin thickness or G ratio plotted against axonal diameter. No significant lesions were present in muscle samples.

DISCUSSION

This study shows that the PNS of fetal sheep is already well myelinated at 90 days gestation. At birth, mean fibre diameter has increased and axons of any given diameter have increased numbers of myelin lamellae in myelin sheaths. Myelin lamellae are laid down in proportion to axonal diameter, and the relationship of myelin thickness or G ratio to axonal diameter was similar to that found for the rat (Friede & Beuche, 1985). At birth initiation of myelination is still occurring in a small number of fibres, mainly located at the periphery of fascicles.

Features consistent with normal myelination were seen in both fetal and newborn lambs infected with BD. Also present are degenerative features apparently representing myelin sheath disintegration, segmental demyelination and paranodal dysmyelination. In developing feline peripheral nerves, Berthold and Skoglund (1968) described myelin sheath disintegration which was most prominant in the paranodal regions. Some internodes, the myelin sheaths of which were highly irregular and distorted by multiple lamellated fragments, were short.

In other instances Schwann cells containing myelin bodies were adjacent to naked axonal segments. Such changes were interpreted as indicative of a degenerative process by which some internodes are removed during development as the parent axon enlarges in diameter. This permits the establishment of a mature axon diameter internodal length relationship. In mature PNS axonal diameter is closely related to internodal length, an internode being approximately 100 times the diameter of its axon (Freide & Beuche, 1985). Similar degenerative changes occur in developing feline CNS myelin (Hildebrand, 1971) and in remyelinating lapine PNS (Bonnaud-Toulze & Raine, 1980).

Degenerative myelin changes of the developing cat occur most frequently in CNS fibres with an axonal diameter of approximately 1 µm (Hildebrand, 1971) and in slightly larger fibres of the PNS (Berthold & Skoglund, 1968). Most fibres showing degenerative changes in the fetal lamb examined in this study were in the range 0.8 - 2µm.

Thus, myelin disintegration, paranodal dysmyelination or segmental demyelination of fibres of 0.8 - 2 µm of fetal and newborn lambs infected with BD virus is not qualitatively distinguished from features of normal developmental remodelling of CNS and PNS myelin in the cat.

Lamellated axonal inclusions were commonly recognised in nerves of infected fetuses and, less frequently, in normal lambs. The frequency of occurrence of such inclusions was not significantly different in infected lambs when compared with each other or when compared with the control. Similar inclusions occur, albeit rarely, in developing feline CNS (Hildebrand, 1971) but their significance has not been determined.

Morphometric analysis showed that newborn control lamb 28 and lamb 35 had thicker myelin sheaths and greater G ratios than lamb 34 with

classical BD. This lamb also had thicker axons for any given fibre diameter (and thus thinner myelin sheaths) than either control lamb 28 or lamb 35 (see Fig.55). The CNS of hypomyelinated BD infected lambs shows similar thinning of the myelin sheath (Cancilla & Barlow, 1968).

As myelination in the PNS is generally initiated earlier than in the CNS it is likely that a period of susceptibility for PNS hypomyelination following BD virus infection, assuming such susceptibility exists, would preceed or overlap that of CNS hypomyelination. CNS hypomyelination is known to occur from infections initiated between 21 and 72 days gestation (Barlow et al., 1980a; Plant et al., 1983a). Thus it is unlikely that PNS hypomyelination would be seen in the absence of CNS hypomyelination. This may explain why regression lines for axonal diameter versus fibre diameter for lamb 35 and control are virtually identical.

The control lamb had smaller axons and fibre diameters than either of the infected lambs. The significance of this observation is unclear.

These results show that myelination of the second muscular branch of the sciatic nerve is well advanced in the 90 day ovine fetus. Segmental demyelination and paranodal myelin sheath remodelling are prominant features of the peripheral nerve at this time. In the newborn lamb, active myelination and initiation of myelination of unmyelinated axons is still in progress. In this limited study myelin sheaths were thinner in the PNS of a classical BD lamb when compared with a control lamb and also a pre-natally infected but non-viraemic lamb showing no CNS hypomyelination. The possibility that BD may cause PNS hypomyelination should, therefore, be considered but examination of peripheral nerves from further virus infected fetuses and newborn lambs is necessary.

TABLE XVIII MYELIN THICKNESS

	Mean	St Dev
28 (Control)	1.6894	0.7470
34	1.0587	0.4960
35	2.2767	0.7253

TABLE XIX FIBRE DIAMETER

	Mean	St Dev
28 (Control)	13.352	3.449
34	17.043	4.088
35	15.554	2.917

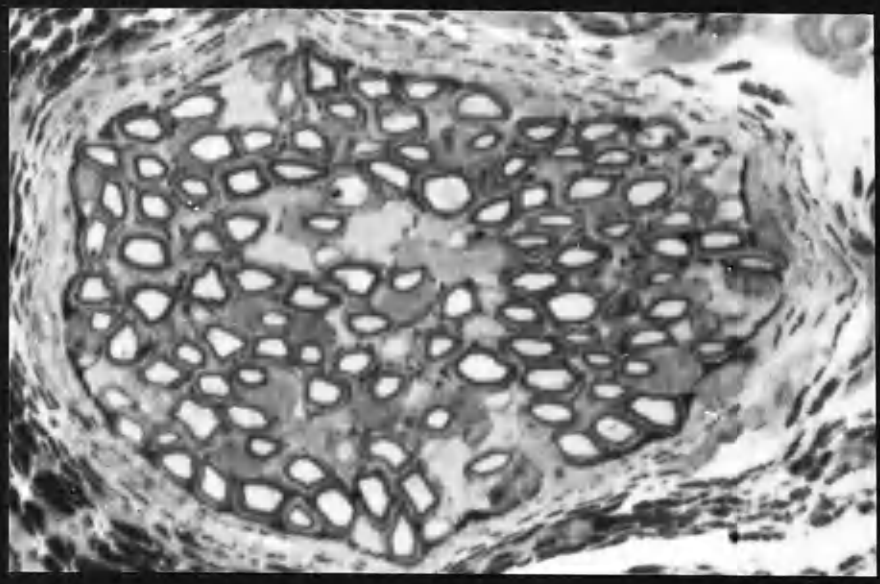
TABLE XX AXON DIAMETER

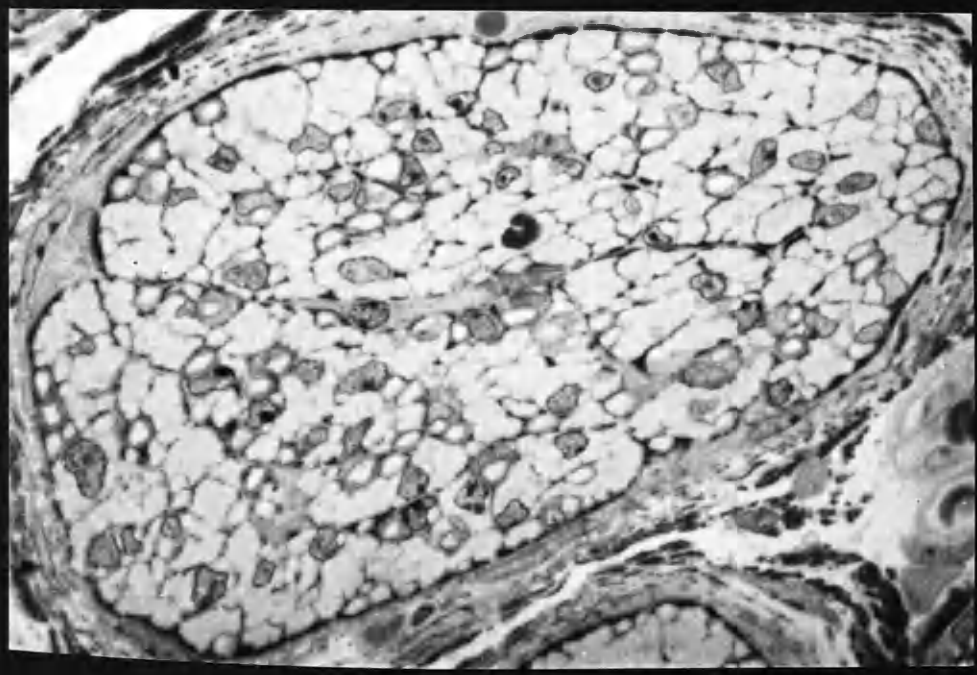
	Mean	St Dev
28 (Control)	9.973	2.254
34	14.926	3.234
35	10.954	2.110

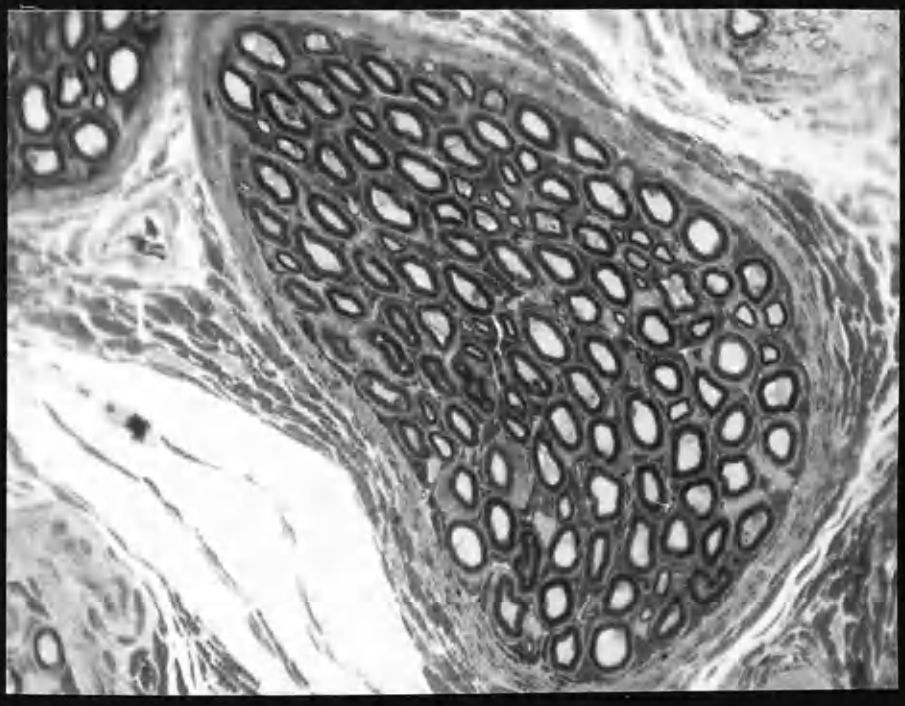
TABLE XXI G RATIO

	Mean	St Dev
28 (Control	1.3362	0.1148
34	1.1385	0.0369
35	1.4297	0.1723

Fig. 45 a,b,c. a and b, Fetal nerves showing a fascicle containing predominantly myelinated fibres (a) and a fascicle containing mainly unmyelinated fibres (b); c, nerve of newborn lamb showing large myelinated fibres.
Toluidine blue x 1,700.







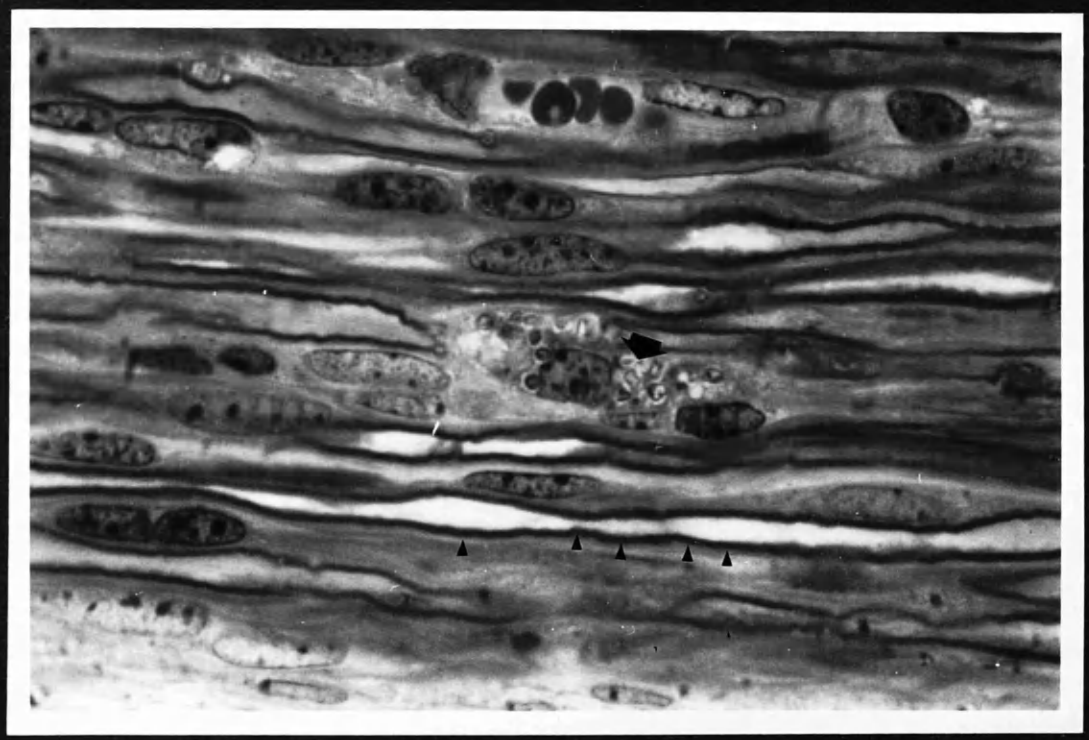


Fig 46

Fig. 46. Fetal nerve showing regular variation of fibre calibre (arrowheads) (stretching artefact) and Schwann cell inclusions (arrow).
Toluidine Blue x 1,700

Fig. 47. Fetal nerve showing irregular myelin sheath and compact myelinoid bodies at internodal and nodal regions. Intra-axonal membranous bodies are present and involve mainly the node and one side of the paranodal and internodal axon. x 2,000.

Fig. 48. Fetal nerve showing disparity of myelin sheath thickness across a node. The myelin sheath and axon on the left of the centrally placed fibre is indented by a Schwann cell nucleus and is thinner than the myelin sheath to the right of the node. The nodal gap and decrement zones are wide and there is an nodal outpocketing of axonal cytoplasm (See Fig. 52a). The fibre above is obliquely sectioned and shows intra axonal membranous bodies; Dense myelin like bodies are present in a Schwann cell. This fibre is unmyelinated at the left of the illustration. x 2,000.

Fig. 49. Fetal nerve showing fibres with nodal and paranodal myelinoid bodies and an increased nodal gap. x 3,000.

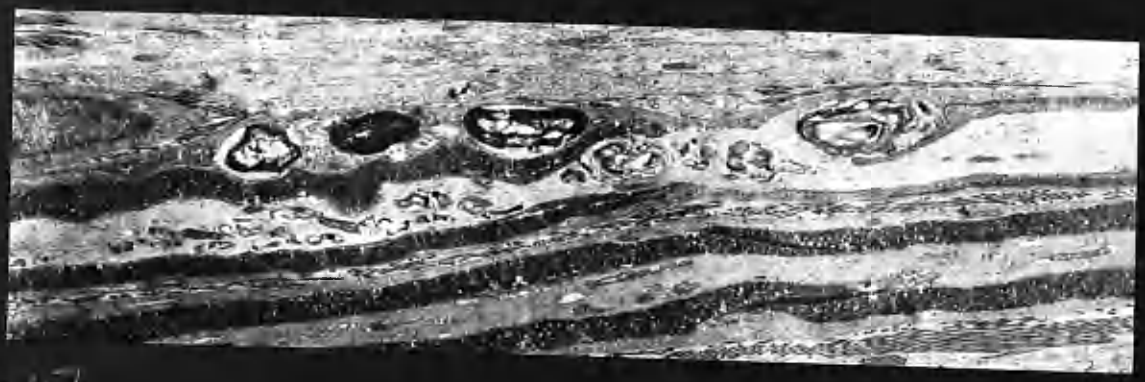
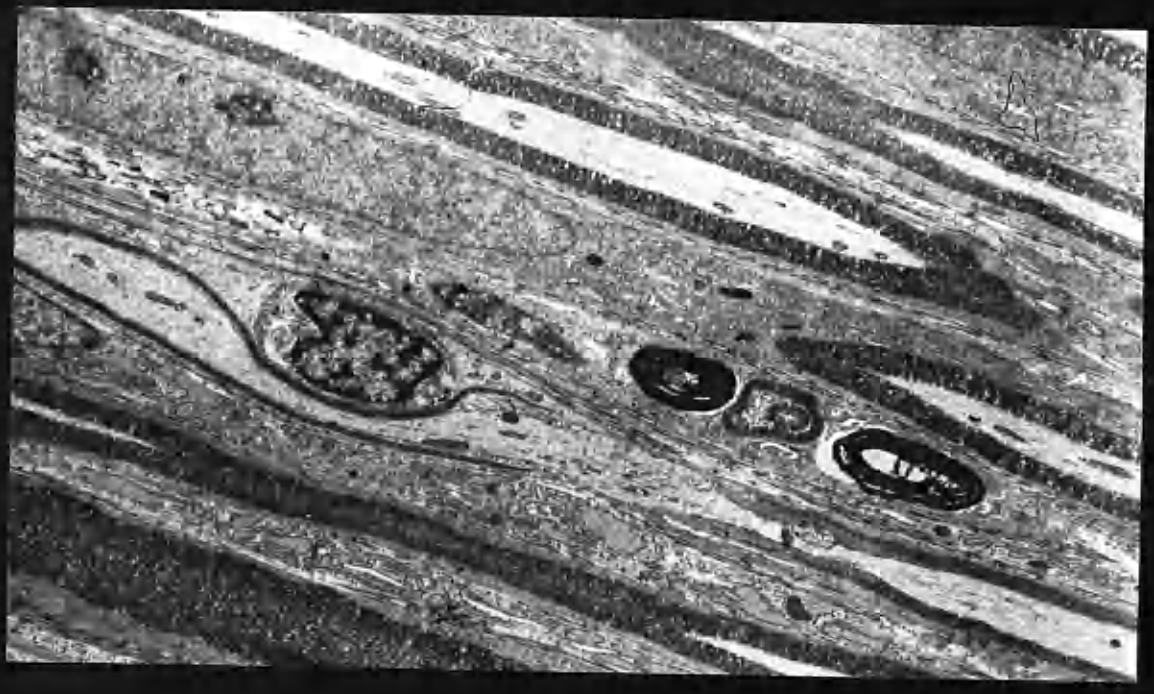
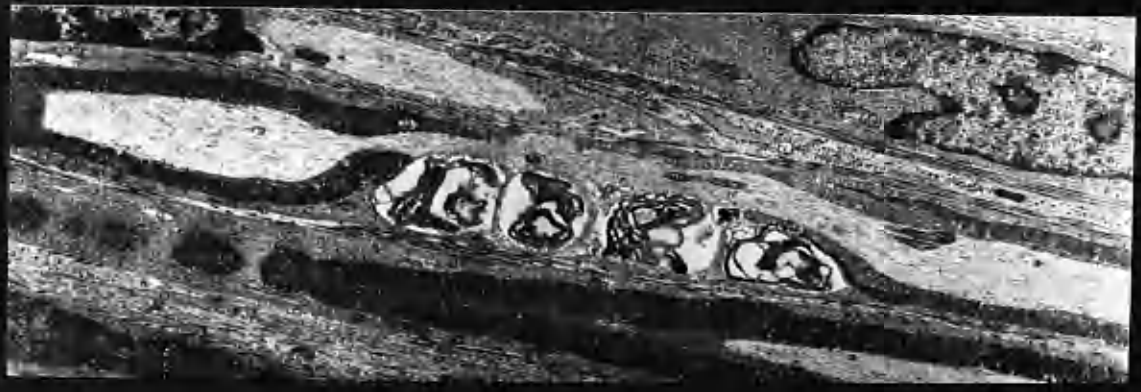


Fig 47





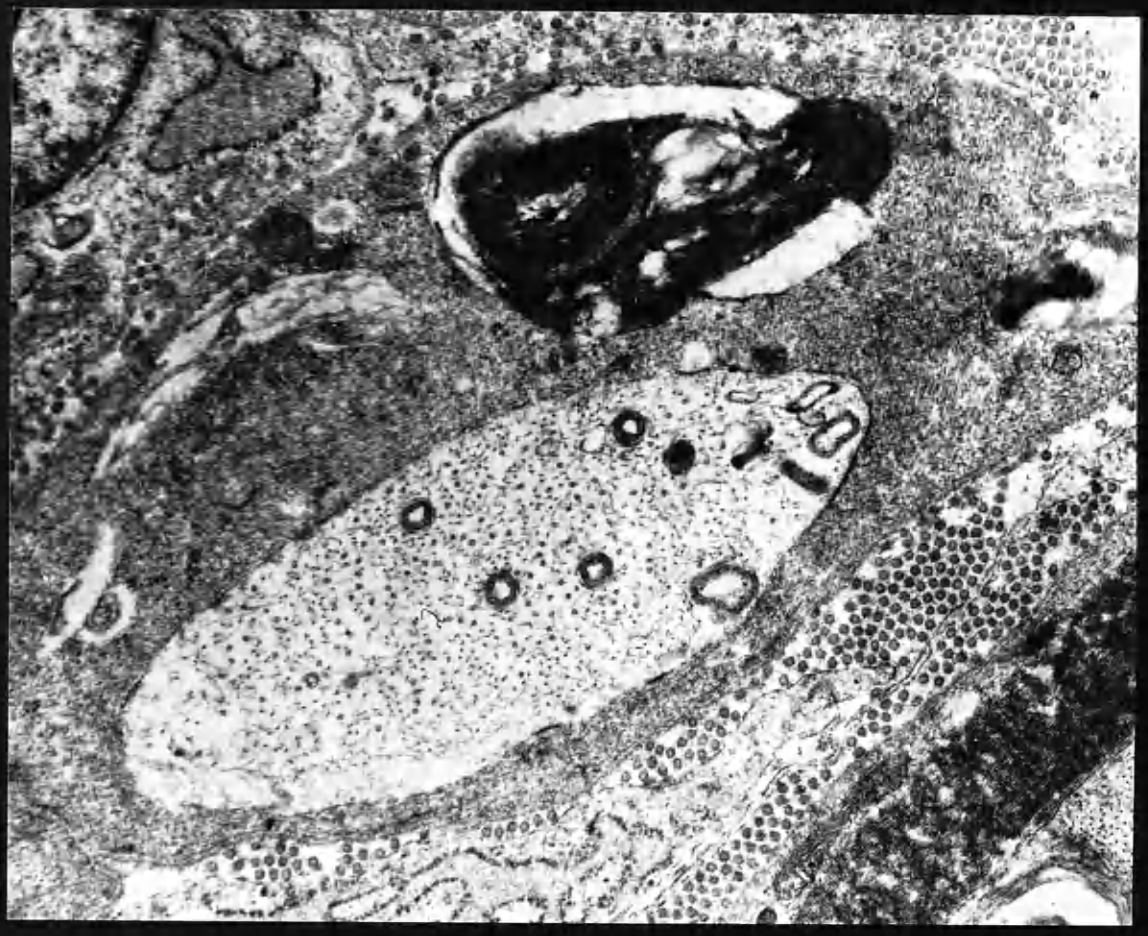
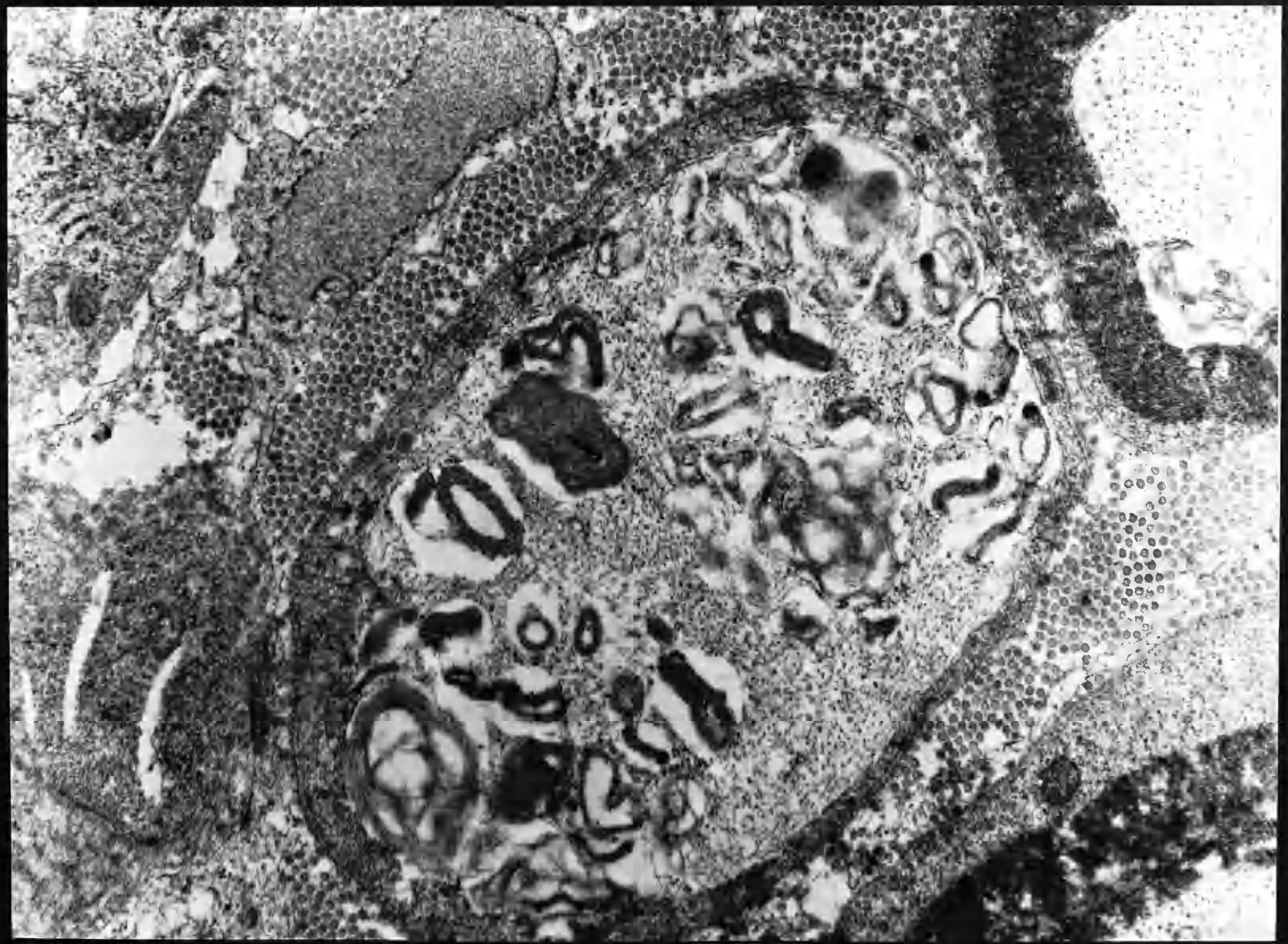
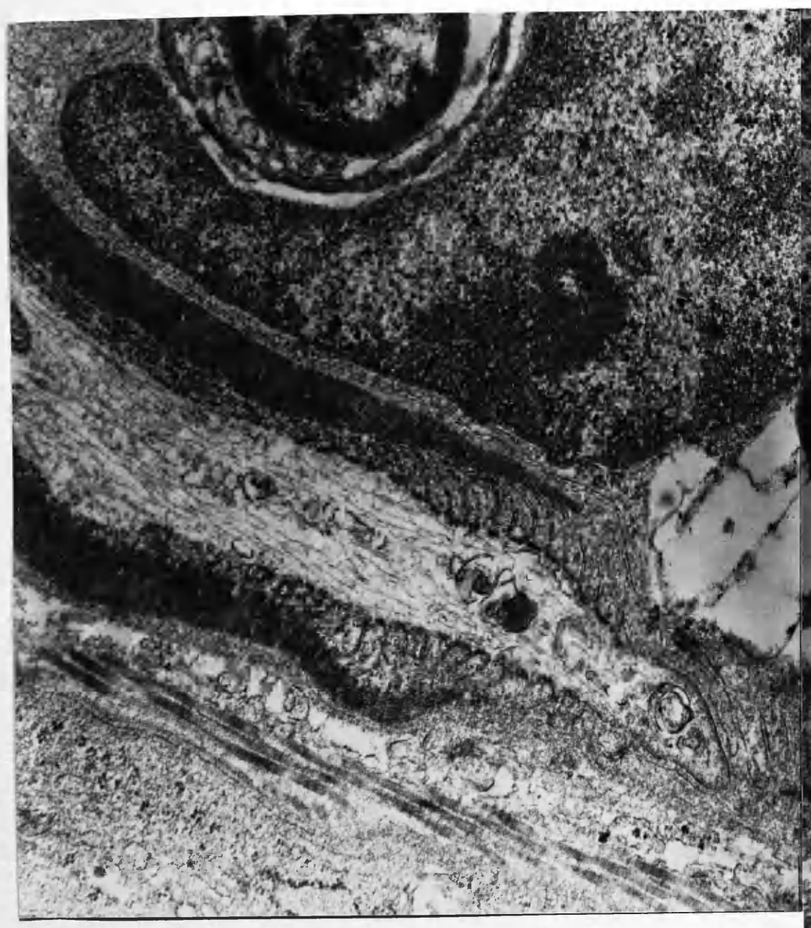
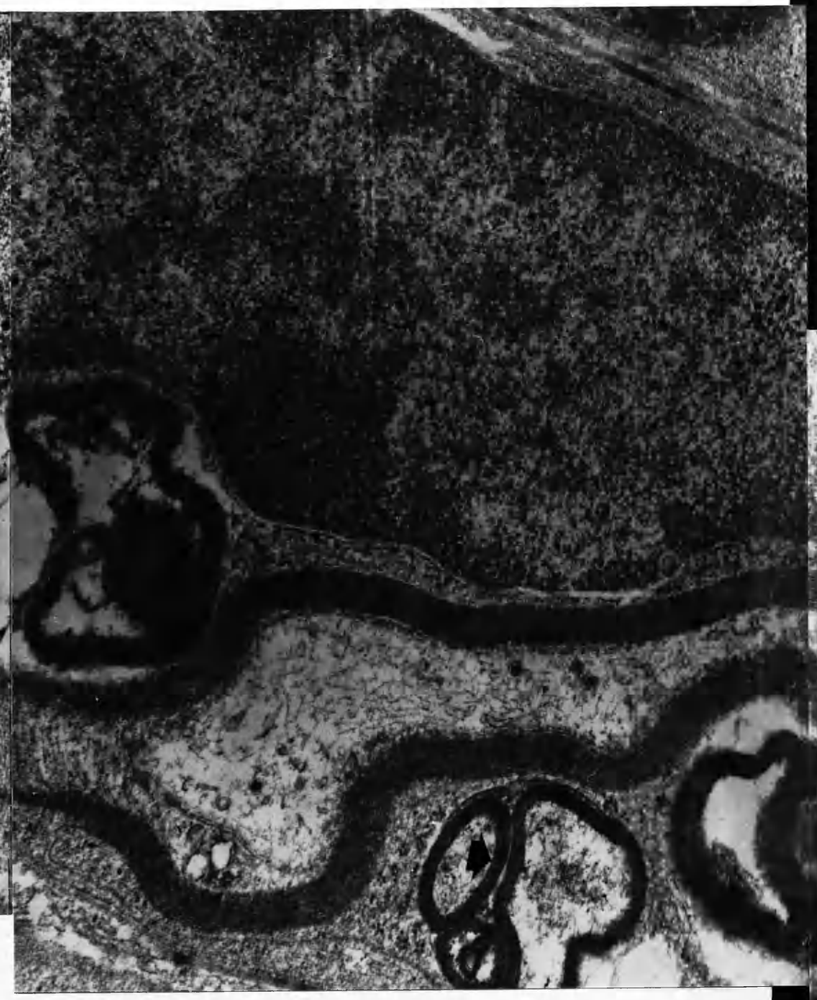


Fig. 50 a and b Transverse sections of fetal nerve showing large diameter unmyelinated fibres (a) associated with a myelinoid body in the cytoplasm of a Schwann cell and (b) intra-axonal accumulation of membranous bodies and organelles. a & b x10,000







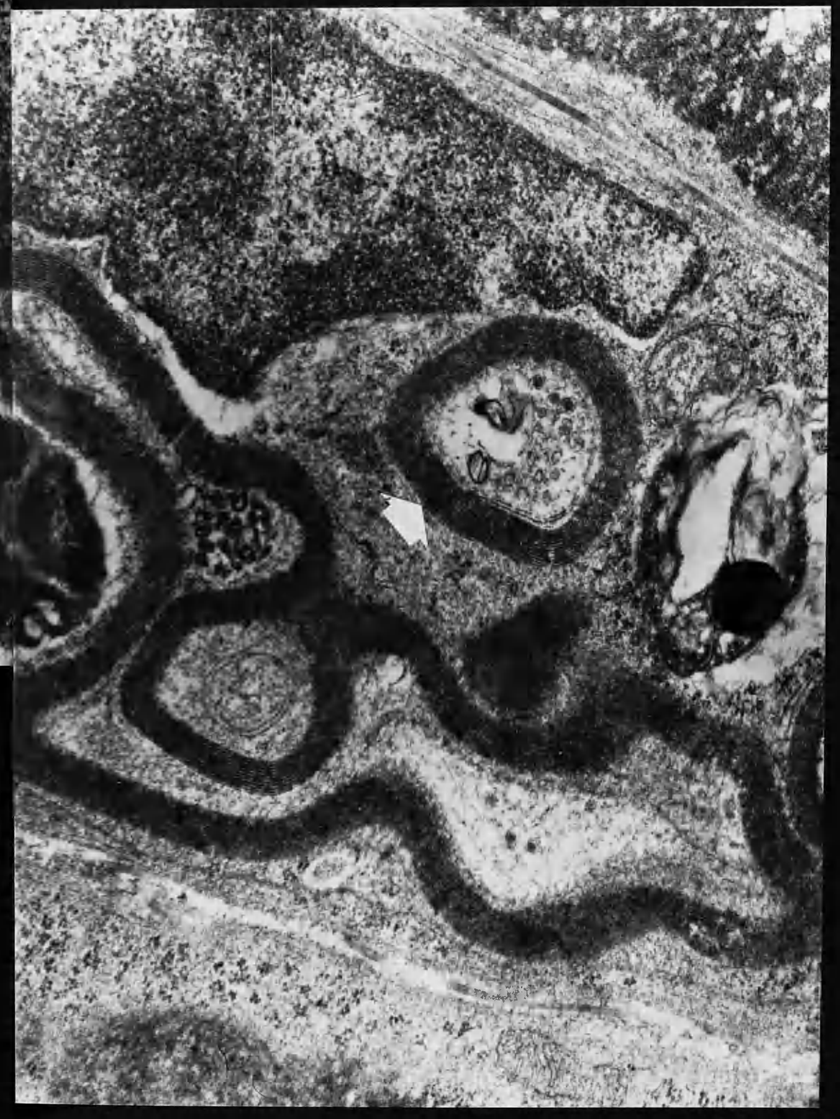


Fig. 51. Fetal nerve showing a highly irregularly-shaped fibre with Schwann cell inclusions, some of which showed normal myelin periodicity (arrows). The Schwann cell nucleus is highly reactive and spans the node.

x 15,000

Fig. 52. (a) Enlargement of node shown in Fig. 48. On one side of the node sub-axonal membranal densification is interrupted (arrowheads). On the other side there is an outpocketing of axonal cytoplasm. x 10,000.

(b) Nodal diverticulation of axoplasm containing a membranous body. x 8,000

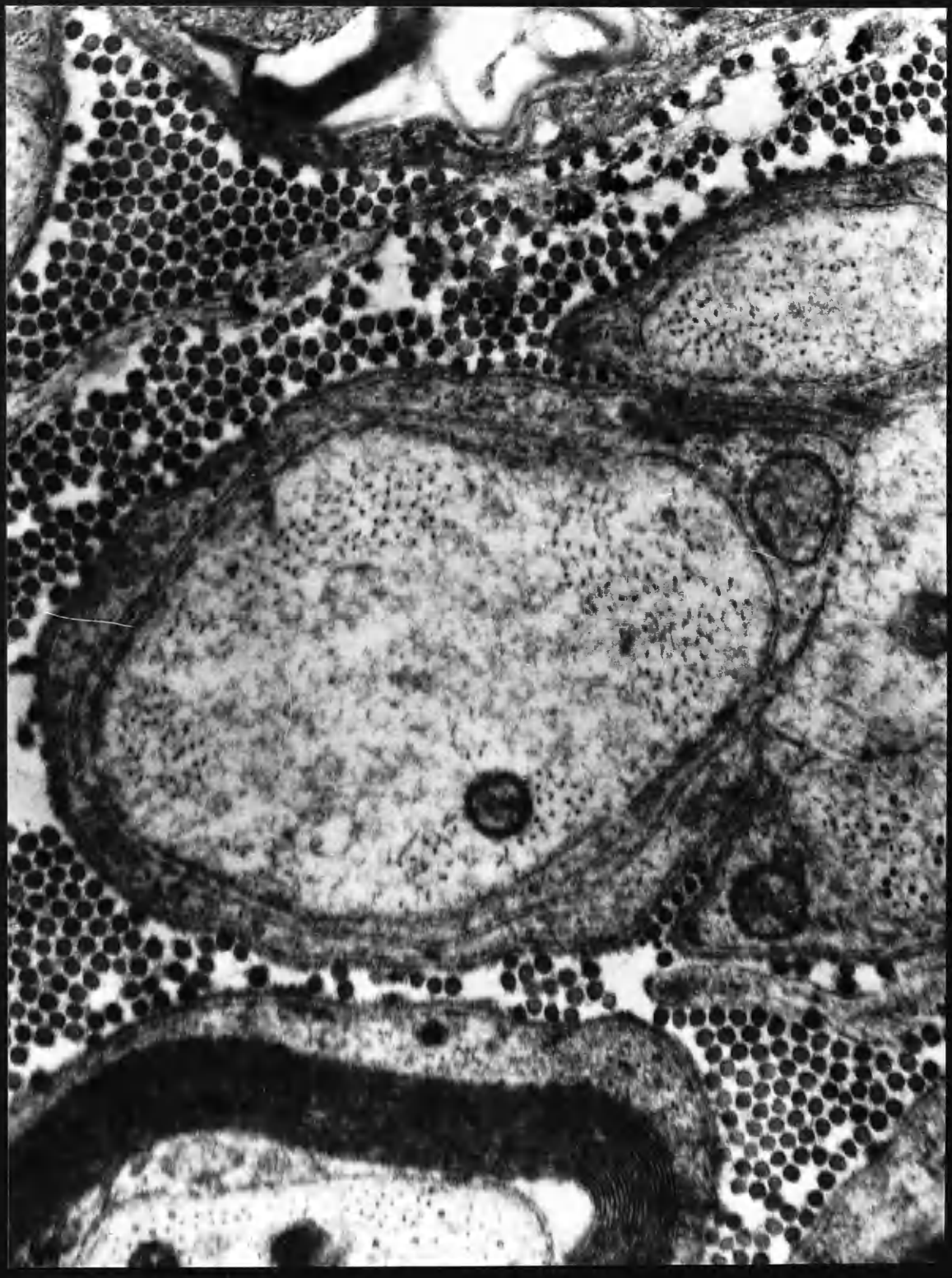
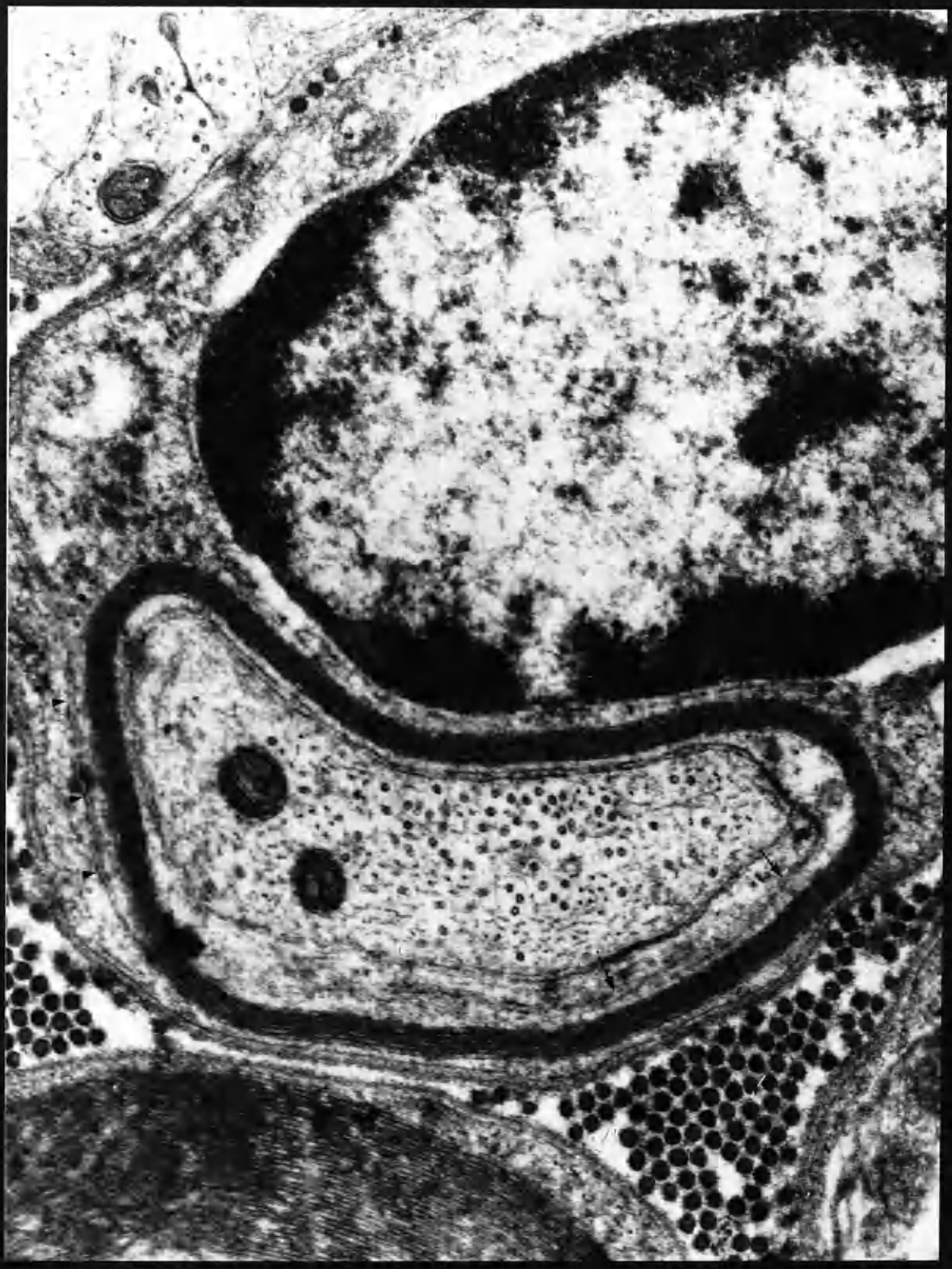


Fig. 53. Nerve from newborn lamb showing a large diameter unmyelinated fibre. This fibre is separated from an unmyelinated fibre bundle by Schwann cell cytoplasm which almost completely surrounds the fibre. Localised loss of microtubules is an artefact. x 25,000

Fig. 54. Newborn nerve showing myelinating fibre. The axon shows six turns of compacted myelin. The internal (arrows) and external mesaxon (arrowheads) are evident. x 25,000



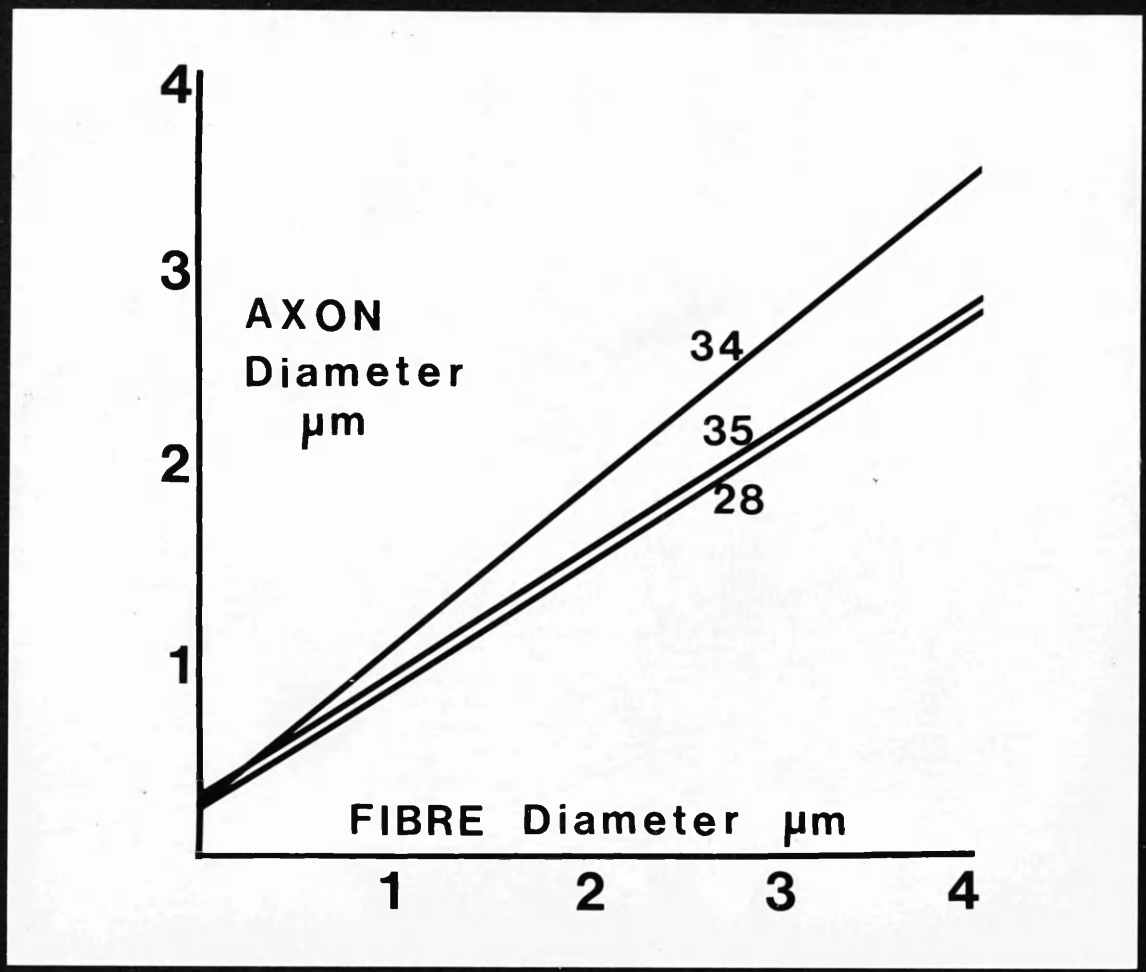

4
3
2
1
AXON
Diameter
µm
34
35
28
FIBRE Diameter µm
1
2
3
4

Fig. 55. Graph showing regression lines of axonal diameter versus fibre diameter for the newborn control and infected lambs.

CONCLUSIONS

A similar range of defects was observed in cattle and sheep as a result of naturally occurring pestivirus infections. In non-immune herds and flocks there is rapid spread of infection and a high frequency of non-viable progeny are found. These studies have shown that, in addition to classical BD lesion of hypomyelinogenesis, previously unreported hydranencephaly, porencephaly, cerebellar dysplasia and retinopathy may occur following natural infection of sheep. With the exception of hypomyelinogensis all such lesions were more frequently encountered in the newly born affected calf.

The development of the lesion is related here to the stage of gestation at which infection took place. Persistently viraemic calves, which acquire infection in-utero up at 125 days of gestation (McClurkin et al., 1984), have either no lesions, or only mild granuloprival cerebellar dysplasia. Photoreceptor cell atrophy and mild retinal dysplasia can also occasionally occur. The presence of a viraemia and the histological appearance of the lesions indicate that viral infection is occurring in the proliferative cells of the fetal external granule cell layer of the cerebellum and in retinal neuroblasts. In these studies calves with severe cystic cerebral cavitation are invariably non-viraemic and most demonstrate a serological response to BVD-MD virus. Such lesions must therefore occur after 90 days gestation at which time the calf is able to respond immunologically to BVD-MD (Liess et al., 1984; Schultz, 1973). The nature of these lesions and their association with a serological response suggests that they may be immunologically mediated. A minority of non-viraemic calves infected near midgestation may exhibit severe teratogenic defects yet not mount a detectable serological response to infection.

The same general principles governing the outcome of infection were also shown to apply to sheep. Lambs with CNS hypomyelination were invariably viraemic whereas those with retinal or cerebral changes were not viraemic. An experimentally induced porencephalic lamb (Chapter eight) showed a precolostral antibody response and virus was not demonstrated in viscera or blood. However, immunoperoxidase staining showed persistence of glial cell infection in white matter of spinal cord. These features suggest that under certain circumstances virus may be eliminated from viscera by an effective immunological response yet persist in the immunologically privileged CNS. Porencephaly may then occur following exposure of virus to the immune system, perhaps through defects in immature capillary endothelia (increased permeability of developing brain capillary endothelia has previously been described for neonatal rats compared with adults (Lefauconnier et al., 1983). These observations add further weight to the hypothesis that lesions of cystic cerebral or cerebellar cavitation may be immunologically mediated.

The results of the immunohistochemical study of the normal distribution of selected glial cell markers shows that optimal preservation of MBP and CAII required rigorous attention to fixation methods but that GFAP immunoreactivity is preserved in a wide range of fixatives. GFAP marks astrocytes and astrocyte related cells of newborn sheep. It is also expressed by amphicytes and a population of Schwann cells. In fetal lambs, cells with processes which contribute to MBP expressing myelin sheaths express GFAP. These cells are morphologically and biochemically similar to transitional cells. MBP is expressed by myelinating oligodendroglia and by myelin sheaths. Myelinating oligodendroglia are present only in corpus callosum and subcortical white matter of newborn lambs but occur throughout the white matter

of brain and spinal cord of 90 day fetuses. CAII is expressed in choroid plexus epithelium but, in contrast to rodents and man, it is not expressed in mature oligodendroglia. CAII expression is seen in a population of white matter glia which also express GFAP. These cells were identified as type II astrocytes. GC was expressed in subpial-glial cells of fetal spinal cord. It was only demonstrated in cryostat sections of fetal tissues.

Immunohistochemical studies of BD virus infected fetal and newborn lamb spinal cord showed that BD virus was widely distributed throughout white and grey matter and virus infected astrocytes, oligodendrocytes and probably also transitional cells. Morphometric examination demonstrates that, in both fetuses and newborn lambs, an astrocytosis is present. Deficiencies of myelinating oligodendroglia are also present in fetuses and myelination is retarded in newborn lambs.

These results show that virus induced lysis of oligodendroglia leading to hypomyelination or demyelination (such as has been described for mouse hepatitis virus or progressive multifocal leucoencephalomyelitis (Lampert & Rodriguez, 1984) and has also been suggested for BD) is too simple an explanation for the mechanisms involved in BD hypomyelination.

Several heritable causes of hypomyelination of rodents and dog are known. In the Samoyed dog, a reactive astrocytosis is described (Cummings et al., 1986). It has been suggested that an astrocyte hypertrophy in jimpy mice and Chow Chow dogs may interfere with oligodendrocyte contact with axons and contribute to hypomyelination (Skoff, 1976; Vandevelde et al., 1978). However, Meier and Bischoff (1977) consider that such astrocytosis and astrocytic hypertrophy are

the sequelae to hypomyelination rather than initiators of it.

Most investigations suggest that murine and canine hypomyelination results from oligodendrocyte dysfunction (Duncan et al., 1987; Knapp & Skoff, 1987; Nave et al., 1986). Delayed or abnormal differentiation of oligodendroglia has been suggested for the Weimaraner dog (Kornegay et al., 1987). Increased numbers of type III glia in BD lambs led Barlow and Storey (1977 a&b) to suggest that abnormalities of differentiation of oligodendrocytes from type III glia (interpreted by these authors as multipotential glia) may lead to reduced numbers of oligodendroglia and hypomyelination. However, type III glia are now considered to be microglia and not multi-potential glia. Type III glia do not have any ontogenic relationship with oligodendroglia. The studies reported in this thesis show that virus infection of oligodendrocytes and astrocytes and probably transitional cells occurs in association with depletion of oligodendroglia and astrocytosis. Thus, these observations provide evidence to support a hypothesis, suggested first by Barlow and Storey (1977a), that BD viral infection of the CNS of sheep may shift the balance of glial cell differentiation away from oligodendroglia and towards astroglia. The possibility that astroglial proliferation is incidental (Meier & Bischoff, 1977) cannot be excluded and further studies of the cellular basis of hypomyelination may be helpful in clarifying the role of astrocytes in hypomyelination of BD.

One further hypothesis for BD hypomyelination deserves consideration. Recent investigations of serum concentrations of thyroxine and L-3,3', 5 tri-iodothyronine have led some researchers to suggest that the pathogenesis of hypomyelination "may involve viral infection of the thyroid gland in utero, with a concomitant modulation of circulating

thyroid levels that results in retardation of myelin maturation" (Anderson et al., 1987b). However, this study fails to address several important differences between BD pathology of the newborn lamb and ovine and murine thyroid deficiency states. Thyroidectomised rats and ovine fetuses show a reduction in total amounts of cerebral myelin without altering its composition (Balazs et al., 1969; Hetzel et al., 1988) whereas features of the hypomyelinogenesis of BD include dysmyelination with presence of esterified cholesterol, raised ratios of non-hydroxy/hydroxy fatty acids and raised ratios of saturated/unsaturated fatty acids (Patterson & Sweasy, 1982). Ovine fetuses thyroidectomised at 50 days gestation show prolonged gestational lengths, a reduction in total brain weights, severe respiratory distress and cerebellar hypoplasia (Hetzel et al., 1988). None of these features are consistently associated with viraemic hypomyelinated BD virus infected lambs. The hypothesis that the deficiencies of thyroid hormone cause hypomyelination takes no account of the morphological or quantitative abnormalities of glial cells described for BD. Thus the discrepancies between published accounts of ovine thyroid deficiency and pathology of BD suggest that BD hypomyelination is unlikely to be related to deficiences of thyroid hormone.

The main conclusions of this thesis refer to the pathogenesis of BD virus and BVD-MD virus induced lesions of the CNS and may be briefly stated as follows:

The spectrum of pathological changes seen following BD or BVD-MD virus infection in their respective hosts is similar. The protean manifestations of pestivirus induced CNS lesions caused by in utero viral infections are related partly to the availability or susceptibility of target cells and partly to a nascent immunological response. Three main responses to virus infection may be recognised.

1. Lesions of hypomyelinogenesis are associated with early gestational viral infections and the resultant progeny are persistently viraemic. Infection of oligodendroglia, astroglia and probably also incompletely differentiated cells occurs. It is suggested that hypomyelination is the result of abnormal or delayed differentiation of oligodendroglia. Mild teratogenic lesions probably associated with necrosis of germinal cells of cerebellum and retina may occur in viraemic animals.

2. Lesions of cystic cerebral and cerebellar cavitation occur following midgestational viral infections and the resultant progeny are non-viraemic and most show a serological response. In some animals at least there is persistence of virus in the white matter of the central nervous system. Lesions of cystic cerebral and cerebellar cavitation probably have an immunological basis.

3. Viral infections occurring in the third trimester of pregnancy result in a rapid and effective fetal immune response and elimination of virus from tissues. No significant pathological lesions are found.

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