STUDIES ON PROLIFERATIVE KIDNEY DISEASE USING MONOCLONAL ANTIBODY PROBES

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by

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DEDICATION

I dedicate this work to my family and to Susan for her love and patience.

DECLARATION

I hereby declare that this thesis has been composed by myself and is the result of my own investigations. It has neither been accepted, nor submitted for any other degrees. All sources of information have been duly acknowledged.

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ABSTRACT

Studies on PKD using Monoclonal Antibody Probes

Four monoclonal antibody (MAb) probes were produced which reacted against PK'X' the causative agent of proliferative kidney disease in salmonids. Three of the MAbs appeared to be specific for carbohydrate epitopes whilst the fourth appeared to recognise a protein epitope. These probes were utilised to investigate the progression of the disease in rainbow trout, examine possible alternative hosts/ infective agents for the disease and to explore a novel method of purification for the PK'X' cell.

Purification of PK'X' from kidney homogenates was investigated using immunomagnetism. The success of this was hampered by lymphocytes and macrophages adhering to the parasites' surface. Incubations using detergents, trypsin, sonication and osmotic shock were employed to remove these cells with varying degrees of success.

The course of the infection in rainbow trout was studied using immunohistochemistry on tissues sampled throughout the summer. This revealed that the antigenic characteristics of PK'X' changed, with an intensification of staining of the carbohydrate binding MAbs on extrasporogonic stages and the protein binding MAb recognising secondary cells and sporogonic stages. Immunogold electron microscopy revealed that the carbohydrate binding MAbs were specific for the lysosomal membranes in the primary cell of the parasite whilst the protein binding MAb reacted to the surface of the secondary cell, vacuoles contained within it and this cell's immediate surroundings. It was speculated

that the extrasporogonic parasite undergoes autophagy with the development and release of the sporogonic stage.

Aquatic oligochaetes from PKD enzootic rivers infected with Actinosporean species were studied using immunohistochemistry, electron microscopy and transmission experiments performed. However, the alternative host for the disease was not determined. Wild fish kidneys from PKD enzootic waters were tested with immunohistochemistry to determine the possible definitive host of PK'X'. An unidentified *Sphaerospora sp.* infecting Atlantic salmon reacted with all of the MAbs indicating a possible definitive host for the parasite.

INDEX

5 STUDIES ON THE LIFE CYCLE OF PK'X' INSIDE RAINBOW TROUT

6 ALTERNATIVE HOST STUDIES

INDEX OF FIGURES

6 ALTERNATIVE HOST STUDIES

Chapter 1

Literature Review

Proliferative kidney disease (PKD) is an important economic disease affecting intensively reared rainbow trout (Clifton-Hadley, Bucke and Richards 1986a; O'Flynn and Mulcahy 1994). The disease was named PKD by Roberts and Shepherd in 1974 and this name has since received a widespread acceptance. The earliest reported cases of the disease were identified in tissue samples from 1958 (Hedrick, Kent, Foot and Rosemark 1984), although it is likely that PKD was established prior to this but was not recognised as such (Plehn 1924; Shäperclaus 1954; Besse 1956). Major review articles of the literature associated with the disease have recently been compiled by Hedrick, MacConnell and de Kinkelin (1993) and by Hoffmann and El-Matbouli (1994).

The organism believed to be responsible for PKD, referred to as PK'X', was first identified by Ferguson and Adair in 1977. After its characterisation there have been many reported incidences of PKD both in Western Europe and North America. European countries affected by the disease include Denmark (Olesen and Jorgensen 1986), Norway (Roberts 1978), Sweden (Clifton Hadley, Bucke and Richards 1984a), France (Schlotfeldt 1985), Holland (Clifton-Hadley 1986), West Germany (Hoffmann and Dangschat 1981), Italy (Clifton-Hadley *et al.* 1984a), Spain (Marin de Mateo and Sanz 1993), Eire (O'Brien, McArdle and Doyle 1977), Northern Ireland (Ferguson and Adair 1977), England (Scott 1979) and Scotland (Ferguson and Needham 1978). Smith, Morrison, Ramsey and Ferguson (1984) reported the first incidence of the disease in the United States of America. It was also identified occurring in Canada by Hoskins and Kieser (1985). Although there appears to be a rising incidence of the disease world-wide, because of its relatively late characterisation and

difficulties experienced in its correct diagnosis PKD is likely to have been endemic in some of these countries many years before it was formally described.

The PK'X' organism has been identified in a variety of species of the family *Salmonidae*. These include brown trout, *Salmo trutta*, grayling, *Thymallus thymallus* (Seagrave, Bucke, Hudson and McGregor 1981), Atlantic salmon, *Salmo salar* (Ellis, McVicar and Munro 1985), landlocked Atlantic salmon, *Salmo salar ouananiche* (Brown, Thonney, Howell and Wilson 1991), char, *Salvelinus alpinus* (Bucke, Feist and Clifton-Hadley 1991), kokanee salmon (Kent, Higgins, Whitaker and Yokoyama 1994), chinook salmon, *Oncorhynchus tshawytscha*, coho salmon, *O. kisutch* and steelhead trout, *Salmo gairdneri* (Hedrick, Kent, Rosemark and Manzer 1984). Pike, *Esox lucius* is the only known non-salmonid to harbour the disease (Seagrave *et al.* 1981). Tui chub *Gilar bicolor* and the stickleback *Gasterosteus aculeatus* were implicated as possible hosts for the PK'X' organism by Hedrick, Kent and Toth (1986) but this finding has been disputed (MacConnell and Smith 1990; Kent *et al.* 1994).

The external signs of fish infected with PKD include a distended abdomen, longitudinal swelling of the body at the level of the lateral line, melanosis, varying degrees of monolateral exophthalmia, and gill pallor (Ferguson and Needham 1978). Behavioural changes are noted such as languor, coupled with a loss in equilibrium and in the concluding stages of the disease, respiratory distress. These external manifestations of the infection however are not evident in all infected fish. Clifton-Hadley, Bucke and Richards (1987a) noted that the

external symptoms of the disease only became apparent one month after the appearance of the PK'X' organism. The majority of these signs occurring in heavily infected fish, abdominal distension being found in just 56% of severe cases. They also recorded that melanosis was rarely encountered and that most of the affected fish were lighter in colour than normal. Ecchymoses and corneal cloudiness were also reported as occasional findings.

The external symptoms of PKD are not indicative of the disease and its diagnosis can be hampered by the occurrence of secondary infections (Bucke, McGregor, Hudson and Scott 1981; Wootten and McVicar 1982). These can include such diseases as viral haemorrhagic septicaemia and nephrocalcinosis (O'Brien *et al.* 1977), infectious pancreatic necrosis (Smith *et al.* 1984), furunculosis (Hoffmann and Dangschat 1981), *Exophalia spp.* infection (Alderman and Feist 1985) and enteric redmouth disease (Rafferty 1986).

The most noticeable gross internal sign of PKD infection is the chronic enlargement of the kidney (Ferguson and Needham 1978). Other gross internal pathological signs of the disease include liver pallor, a distended gall bladder, splenic enlargement and ascites (Clifton-Hadley *et al.* 1987a). The swelling of the kidney may result in the displacement of the swimbladder, resulting in the external clinical signs previously noted. These may be exacerbated by a build up of excess ascitic fluid in the body cavity. Clifton-Hadley *et al.* (1987a) noted that as the disease developed so the kidney became increasingly swollen. These authors described the kidney changing colour corresponding to the degree of its inflammation, progressing from a uniform dark red at the beginning of the infection, to a mottled pink and grey, finally

appearing pale with greyish areas, grossly inflamed with bulbous ridges. Infected kidneys were graded according to their appearance, thus devising a system by which the severity and progression of a PKD outbreak could be assessed.

Anaemia, typically identified by gill and liver pallor in PK'X' infected fish was examined by Ferguson and Needham (1978). These authors found that haematocrit levels were significantly lowered in affected fish. Clifton-Hadley *et al.* (1987a) recorded the presence of crystallised haemoglobin in the kidneys of heavily infected fish. They suggested that this was the result of blood vessels in the kidney becoming blocked, thus preventing newly formed erythrocytes from entering the circulation, resulting in haemolysis. Clifton-Hadley, Richards and Bucke (1987b) studied the blood parameters of infected fish in detail with relation to the progression of the disease. They found that as the disease progressed so the erythrocyte levels of the blood decreased, even though haemopoietic hyperplasia was seen to occur. They suggested that the anaemia had a multifactorial causation with features reminiscent of both haemolysis and hypoplasia.

The PK'X' organism can be visualised under light microscopy by employing a variety of different staining procedures such as haematoxylin and eosin (Ferguson and Adair 1977), the Leishman-Giemsa technique, methylene blue (Klontz and Chacko 1983), Massons trichrome, Turnballs blue, haematoxylin-periodic acid-Schiff (MacConnell, Smith, Hedrick and Speer 1989) and the standard May-Grunwald-Giemsa technique, recommended by Clifton-Hadley, Richards and Bucke (1983) for the diagnosis of the disease using kidney smears. Recently, a

rapid lectin-based staining procedure, using the lectin GS-I (*Griffonia simplicifolia* agglutinin-I), was developed by Hedrick, Marin, Castagnaro, Monge and de Kinkelin (1992a) which removes much of the subjectivity encountered by previous staining techniques.

The histopathological changes that occur within the kidney are associated with the course of the PK'X' infection. Early in infection there is an increase in the number of mononuclear cells in the interstitium associated with the haemopoietic tissue. This may occur even before the parasite has been visualised within the kidney. The PK'X' organism first appears within the renal sinuses and glomeruli of the kidney and is associated with disruption of the vascular epithelium and thrombi formation (Kent and Hedrick 1986; Clifton-Hadley *et al.* 1987a), the parasite often being at the centre of such reactions. As the infection progresses, the parasites migrate into the kidney interstitium and proliferate, invoking inflammatory responses within the tissue. These responses are centred primarily around the parasites. Large numbers of macrophages and lymphocytes appear within the tissue and surround the PK'X' cells giving the tissue a 'whorled' appearance when viewed histologically (Ferguson and Needham 1978; MacConnell *et al.* 1989). Coupled with the increased hypercellularity degenerative changes are observed within the affected tissue, resulting in a reduction in the number of tubules and glomeruli present. This reduction in renal tubules is thought to severely impair the functioning of the kidney (Foott and Hedrick 1990). As the infection progresses so the inflammatory response increases, resulting in patches of haemopoietic cells and tubules surrounded by large areas of inflammation. These lesions, often several millimetres in

diameter, may be seen macroscopically resulting in the kidneys' grey and red marbled appearance (Clifton-Hadley *et al.* 1987a).

The kidney may show signs of recovery from the disease twelve weeks post-exposure to the infective agent, PK'X'. These signs are manifest with degenerative changes in the parasite such as increased vacuolation and pyknosis of its nuclei, the parasite finally appearing to totally disintegrate. Regeneration of the kidney tubules and glomeruli occurs at this time with kidneys becoming completely healed 20-25 weeks post-exposure. After the fish has fully recovered from the disease the kidney appears normal with no signs of inflammation or infection (Clifton-Hadley, Bucke and Richards 1985). Persistence of intratubular sporogonic forms of the parasite have however been reported in North America many months after the fishes' recovery from the clinical disease (Kent and Hedrick 1986).

The PK'X' parasite, although most prominent in the kidney, has been reported in a variety of other tissues. These include the liver, spleen, pancreatic Islets of Langerhans, submucosa of the pyloric caeca, gills, dorsal kidney muscle (Ferguson and Needham 1978), spinal canal (Smith *et al.* 1984), heart, striated muscle and the brain (Clifton-Hadley *et al.* 1987a). Examination of the eyes, swimbladder, gonads, ureters and gallbladder of infected fish have all failed to detect the presence of the parasite (Ellis *et al.* 1985; Clifton-Hadley *et al.* 1987a).

Light microscopy reveals that the PK'X' cells measure approximately 10-20μm in diameter and are regularly rounded (Ferguson and Adair 1977; Seagrave, Bucke and Alderman 1980),

although ovoid forms have been recorded (Smith *et al* 1984; Rafferty 1986). The cytoplasm consists of a dense granular endoplasm surrounded by a lighter ectoplasm. Ferguson and Needham (1978) noted the presence of inclusion bodies within the cytoplasm. These bodies measured approximately 4μm in diameter and amounted to as many as six in one cell. Ultrastructural examination of these bodies demonstrated that they were distinct cells some of which contained smaller cells (Ferguson and Needham 1978). The inclusion bodies were termed secondary cells while their intracellular cells were called tertiary cells. The enveloping parasite cell was named the primary cell (Seagrave *et al.* 1980).

Studies on the life cycle of PK'X' demonstrate that it appears in the intrarenal sinuses of the kidney 2-3 weeks after the exposure of the fish to the infective stage (Kent and Hedrick 1986). The parasite has two recognisable developmental forms within the kidney. The extrasporogonic and the sporogonic stages. The extrasporogonic parasite occurs within the kidney interstitium and blood vessels of the fish and is composed of a primary cell containing one or more secondary cells, some of which may contain a tertiary cell within them. The extrasporogonic stage is believed to reproduce by endogeny, plasmotomy, binary fission and budding, although no fixed developmental cycle has yet been ascertained. The sporogonic form of the parasite appears 2 weeks after the initial appearance of the extrasporogonic parasite and occurs within the lumen of the kidney tubule. It is suggested that the sporogonic parasite arises from the release of secondary cells into the tubule lumen (Kent and Hedrick 1986; Marin de Mateo, Adams, Richards, Castagnaro and Hedrick 1993). Because complete sporogenesis has yet to be described and there is a severe inflammatory response to the

parasite there has been speculation that salmonids are not the definitive host for the disease (Clifton-Hadley and Feist 1989).

A definitive host for PK'X' has yet to be established, although studies have shown a variety of myxosporean infections occurring in fish held in waters enzootic for PKD (Hedrick, Kent and Toth 1986; Odening, Walter and Bockhart 1988; MacConnell and Smith 1990). Recently Kent, Whitaker and Margolis (1993a) studying chinook salmon have suggested that complete sporulation of the parasite occurs within a species of mature salmonid after noting the concurrent presence of *Sphaerospora onchorhynchi* spores in kidneys of mature fish inhabiting PKD enzootic waters.

The ultrastructure of the extrasporogonic parasite has been examined using electron microscopy. The primary cells were found to have an amorphous cell membrane, 40-70nm thick, which possessed small pseudopodia (Seagrave *et al.* 1980; Ferguson and Needham 1978). This membrane was found to be associated with an external glycocalyx with some parasites exhibiting an ectoplasmic region devoid of organelles immediately beneath the membrane (Ferguson and Needham 1978; Smith *et al.* 1984). The presence of actin-like filaments have been reported in this region (Rafferty 1986). The cytoplasm appears granular and contains rough endoplasmic reticulum, mitochondria, phagocytic vacuoles, nucleus with well defined nucleoli and golgi bodies (Ferguson and Needham 1978; Rafferty 1986). The mitochondria appeared to differ in the amount of cristae formation they exhibited with some having "elaborate" arrangements whilst others were very "reduced" (Seagrave *et al.* 1980).

The granularity of the parasites' cytoplasm was found to be due to the presence of large numbers of electron-dense, spherical inclusion bodies, 0.1-0.2 μm in diameter. These bodies were partially bisected by an electron lucent bar and often appeared in close association with the plasmalemma. The bars of these bodies regularly orientated towards the membrane (Ferguson and Needham 1978). Seagrave *et al.* (1980) referred to the bodies as 'haplosporosomes' after similar structures described in members of the Haplosporidia and noted the presence of an electron lucent membrane surrounding them. They were renamed 'electron dense bodies' by Kent and Hedrick (1985a). Smith *et al.* (1984) recorded that some of the electron dense bodies appeared to be exocytosed from the primary cell and Rafferty (1986) noted the presence of similar bodies free in the kidney interstitium although active extrusion from the PK'X' cell was not ascertained. Smith *et al.* (1984) also reported a vesicular system as their possible production site and noted that the golgi apparatus appeared to be packaging them at their maturing faces. Some of the electron dense bodies also appeared to possess tails (Ferguson and Needham 1978; Rafferty 1986). Experiments to determine a viral aetiology connected with the bodies failed to detect any DNA within them or to successfully transmit them to rainbow trout cell lines (Smith *et al.* 1984; Rafferty 1986). The purpose of these bodies remains unclear.

Large vesicular elements with a diameter of 0.3-1.9μm were reported by Seagrave *et al.* (1980) and Rafferty (1986). These structures consisted of a single membrane enclosing electron dense granules, amorphous material and occasionally associated electron dense

bodies. Seagrave *et al.*(1980) reported that as the fish began recovering from the effects of PK'X' infection so these structures increased in size.

The ultrastructural studies of the extrasporogonic parasite usually only permit the examination of one or two secondary cells within the primary cell at one time (Seagrave *et al* 1980). These are delimited from the primary cell by a double membrane and contain all of that cell's features except for the electron dense bodies and the large vesicular elements (Seagrave *et al* 1980; Rafferty 1986). Tertiary cells contain all of the elements of their enclosing secondary cell and are delimited from them by a double membrane (Seagrave *et al* 1980). Rafferty (1986) reported expulsion of the tertiary cells from the secondary cells to form a new secondary cell. However Feist and Bucke (1987) suggested that instead of being expelled, one secondary cell was being enveloped by another to create the tertiary cell, possibly as a stage of early sporogenesis with later development occurring in the renal tubules.

The precise taxonomic status of PK'X' remains unclear. Ferguson and Adair (1977) suggested that due to the presence of small pseudopodia the parasite was possibly an amoeba or species of myxosporidean. Ferguson and Needham (1978), concluded that the absence of typical myxosporidean spores indicated that it was more likely to be a parasitic amoeba. Seagrave *et al.* (1980) formally named the infective protozoan PK'X'. They noted similarities between the PK'X' organism and *Marteilia refringens*, a haplosporean parasite of the oyster *Ostrea edulis*. Because of the analogies drawn between the two parasites, notably internal

cleavage, the presence of "haplosporosomes" and an amorphous cell wall, they placed the PK'X' organism in the protozoan class Haplosporea of the phylum Ascetospora.

Kent and Hedrick (1984) recorded spore formation in fish recovering from PKD as well as possible forms of the parasite in the lumen of the kidney tubules. Both of these observations are indicative of a myxosporidean aetiology. Further investigations on the intraluminal parasites were conducted by Kent and Hedrick (1985a) and were found to be later stages of the PK'X' organism. These findings lead to classifying the parasite as a myxosporidean. Comparisons between PK'X' and the Phylum Myxozoa were drawn by Kent and Hedrick (1985a) and affinities were observed between the parasite and the sphaerosporean, *Sphaerospora renicola*, suggesting that it may be classified in the genus Sphaerosporidea. Rafferty and Mulcahy (1988) cast doubt on this classification when it was found that antisera raised against PK'X' did not react with *S. renicola*, implying that they were not related. Clifton-Hadley and Feist (1989), however, studying the disease in brown trout, again recorded similarities with members of the *Sphaerospora* and with the *Parvicapsula* although no mature spores could be discerned. This apparent absence of mature spores prevents a precise taxonomic classification being achieved and suggests that immature brown trout, as with other salmonids, such as rainbow trout, may not be the definitive hosts for PK'X' (Seagrave *et al.* 1980; Kent and Hedrick 1987; Clifton-Hadley and Feist 1989).

Ferguson and Ball (1979) indicated that fish that had recovered from PKD developed an immunity against reinfection in subsequent years. This suggests that there is an immune

response against the parasite. Klontz, Rourke and Eckblad (1986) noted that the PK'X' organism caused a massive proliferation of small lymphocytes, a decrease in serum albumins and an increase in sera beta-globulins in the fish. They noted that ultrastructurally PK'X' appeared to be engulfed by macrophages surrounded by lymphocytes and proposed that the PK'X' cell was highly antigenic, stimulating a cell mediated immunity. Hypergammaglobulinaemia was observed in infected fish by Olesen and Jorgensen (1986), with IgM levels being 34% of the total serum protein compared with 5-6% in healthy fish. Infected fish have further been reported as exhibiting neutrophilia and leucopenia when challenged with PKD (Clifton-Hadley *et al.* 1987b). Angelidis, Baudin-Laurencin, Quentel and Youinou (1987) reported that PK'X' infected fish were more susceptible to mortalities when challenged with *Vibrio*. They suggested that this was due to the parasite lowering the fishes' immune response. However, Foott and Hedrick (1990) demonstrated that fish infected with PK'X' exhibited a greater resistance to bacterial challenge and possessed heightened non-specific immune responses in contrast to uninfected fish. Using cortisol, an immunosuppressant, implanted into PK'X' challenged fish, Kent and Hedrick (1987) noted that there was a suppression of the interstitial inflammatory response and that the parasite was able to develop further into a sporogenic form, although complete sporogenesis was not observed. They postulated that differences in levels of interstitial inflammation of infected kidney may be associated with stressful conditions so increasing cortisol levels resulting in immunosuppression.

The severity of an outbreak of PKD on a fish farm is related to environmental stresses such as water temperature and the presence of any secondary infections, and also to the innate susceptibility of the fish to the disease (Seagrave *et al.* 1981; Ellis, McVicar and Munro 1982). Therefore mortalities attributable to the disease may vary significantly between individual farms, with some farms reporting no losses whilst others experience up to 90% mortalities (Seagrave *et al.* 1981). Examination of infected stocks have shown that even though there may be a low mortality rate, morbidity is usually high, with up to 100% of the fish being infected (Ferguson and Ball 1979; Ellis *et al.* 1985; Clifton-Hadley *et al.* 1987a). With careful management of diseased stocks it should be possible to limit losses to around 10% (Clifton-Hadley, Richards and Bucke 1986b).

PKD is recognised as a seasonal disease, with the peak time for infectivity being May (Ferguson and Ball 1979), resulting in mortalities occurring from mid July through to mid September (Ferguson and Adair 1977; Ellis *et al.* 1985). The seasonality of the disease was thought to be due to rising water temperatures or the abundance of the infective agent during May (Ferguson and Ball 1979). It was noted that fish moved in July to PK'X' enzootic rearing facilities, although becoming infected, did not die. Ferguson (1981) demonstrated that the development of the disease was temperature dependant and that low temperatures (5-7°C) inhibited its progression and increased the speed of the fishes' recovery. These findings were corroborated by Clifton-Hadley *et al.* (1986b) who noted that the disease developed at temperatures from 12°C to 18°C but not at 9°C. These authors also noted that the fish held at 9°C did not develop a protective immunity against the disease in subsequent years and

suggested that reducing the water temperatures to 12°C at the initial stages of infection may be used as a method of control for the disease, limiting mortalities to a minimum.

As well as temperature, water quality also affects the severity of an outbreak of PKD. Initially it was thought that the disease occurred only in soft, acidic, oligotrophic conditions (Roberts 1974; Ferguson and Adair 1977; Ferguson and Needham 1978). However Scott (1979) recorded an outbreak in a farm supplied by an alkaline, highly eutrophic, chalk stream and proposed that extremes of pH cause stress to the fish making them more susceptible to infection. Seagrave *et al.* (1981) attributed stress, caused by husbandry practices such as grading, secondary infections and low dissolved oxygen content of the water, as important components to high mortality on PKD infected farms. Other causes of stress related PKD mortality include pollution (Hoffmann and Dangschat 1981) and overfeeding (Ferguson and Ball 1979). Osmotic stress in PKD affected Atlantic salmon was suggested as a significant cause of mortality by O'Hara (1985).

The mode by which PKD is transmitted in the wild is not yet known. Kent and Hedrick (1985b) managed to successfully transmit the disease by the intraperitoneal injection of infected blood and spleen into healthy fish and proposed that the infective agents were possibly PK'X' spores or trophozoites. By passing infected kidney suspensions through filters it was ascertained that the infective agent within the inoculum was 10μm in diameter. Rafferty (1986), by using intraperitoneal injections, ascertained that experimental transmission failed after August and suggested a seasonality of the infectivity or availability

of the infective agent. Clifton-Hadley (1986) performed a series of experiments to determine if the disease was transmitted directly from fish to fish. These experiments included holding healthy fish with diseased fish, the feeding of fish with infected fish, immersion of fish in infected kidney suspensions and the injection of kidney suspensions into fish. All of the experiments failed to transmit the disease and suggested that an alternative host was required in the parasite's life cycle. Ferguson and Ball (1979) noted that the peak time of infectivity in Ireland was during May and concluded that successful transmission in the wild was temperature dependent. They were also able to infect susceptible fish with the parasite by exposing them to river water from an affected farm, thus demonstrating that the infective agent was waterborne. Experiments performed by Rafferty (1986) to determine the transmission of the disease from possible alternative hosts included the manual abrasion of naive fish with infected ones, feeding of tubificid worms from infected waters to the fish and infecting naive fish with the ectoparasites of diseased fish. All of these experiments were unsuccessful.

Alderman and Rodgers (1984), using precision meshes to filter infected water, failed to prevent the disease from occurring and proposed that the infective agent was smaller than 60μm and free living. Chilmonczyk, Thomas and de Kinkelin (1989) reported successful transmission of the disease from infected sediments to rainbow trout at an indoor recycling unit and suggested that the infective form was either free living or inhabited an intermediate host such as another fish species or possibly invertebrates such as tubificid worms. Further filtration experiments of enzootic waters have ascertained that the infective agent of PK'X' is

smaller than 30μm and possibly represents a species of *Aurantiactinosporea* of the class Actinosporea (Hoffmann and El-Matbouli 1994). Species of Actinosporea have been implicated in the life cycles of several other myxosporean infections including the *Sphaerospora* (Hedrick, Monge and de Kinkelin 1992b; El-Matbouli, Hoffmann and Madnok 1995).

A satisfactory treatment for PKD has yet to be developed. Because of the seasonality of the disease, Ferguson and Ball (1979) suggested that the placement of fish to rearing facilities should be delayed until July and this could be used as a possible control measure. However Bucke *et al.* (1981), reported that this method proved to be ineffectual in combating the disease in southern England. O'Hara (1985) noted that salination of the water supply at a salmon smolt unit successfully alleviated the symptoms of PKD and suggested such treatments lead to the *in vivo* destruction of the parasite in this species of salmonid. However Hedrick and Aronstien (1987) could find no evidence of this occurring with infected chinook salmon and proposed that any improvements in the mortality rate were due to the reduction in complicating bacterial and protozoan infection. Recently Kent, Whitaker, Higgins, Blackburn and Dawe (1995) have shown that chinook salmon can still possess subclinical PKD in sea water and suggested that it may have an impact on the ocean survival of infected salmon.

Chilmonczyk *et al.* (1989) reported that trout supplied with PKD infected water treated with ultraviolet light at a dose of 5x10 mwatt/cm^3/sec^{-1}, did not contract the disease, however such measures are impractical on a large scale. Periodic flush treatments and immersion in the

arylmethane dye, malachite green, were found by Alderman and Clifton-Hadley (1988) to control both the development and presence of the PK'X' parasite. However this dye has been shown to cause liver and nuclear damage in treated fish and its use has now been restricted in the USA (Gerundo, Alderman, Clifton-Hadley and Feist 1991). The fungicide Fumagillin DCH has also been reported as an effective control for several myxosporean diseases including PKD (Wishkovsky, Groff, Lauren, Toth and Hedrick 1990; Sitja-bobadilla and Alvarez-Pellitero 1992). The administration of this chemical may have deleterious effects on the fish resulting in grossly reduced sizes and in some cases mortalities (Lauren, Wishkovsky, Groff, Hedrick and Hinton 1989). However, the toxicity of Fumagillin DCH can be regulated by its careful administration resulting in substantially increased survival rates of infected fish (le Gouvello, Blanc and de Kinkelin 1993; le Gouvello and Pobel 1995)

For the successful development of future vaccines against the PK'X' organism it is important to gain an understanding of the molecular biology of the parasite. The resistance of salmonids which have recovered from clinical PKD to reinfection suggests that there is a strong immune response to the parasite (Ferguson and Ball 1979; Kent and Hedrick 1984; Foott and Hedrick 1990). Studies on the antigenic properties of the parasite using immunohistochemistry with the lectin GS-I and a monoclonal antibody (MAb 18) raised specifically against the disease have demonstrated that the parasite possesses antigens common to the epithelial lining and cells of the kidney tubules, maybe as an aid to avoid host detection (Castagnaro, Marin, Ghittino and Hedrick 1991; Adams, Richards and Marin de Mateo 1992). However, MAb 12, a further monoclonal antibody raised against the parasite, suggests that it also possesses

unique antigens which may explain its immunoreactivity and may be exploited in vaccine development (Adams *et al.* 1992).

The successful production of monoclonal antibody probes against PK'X' may not only aid in the development of vaccines but also permits study of the parasites' life cycle, identifying any unknown parasite forms, detecting possible intermediate hosts and may be used in the production of diagnostic kits.

The objective of this research project was to produce and utilise a panel of monoclonal antibody probes to study aspects of PKD. In particular, the antibodies were used to examine the life cycle of the disease both inside and outside the rainbow trout. Immunogold localisation of the probes was used to study the ultrastructure and cell biology of the PK'X' organism. A novel purification procedure, utilising the monoclonal probes, to obtain pure suspensions of the parasite for future study was also examined.

Chapter 2

Development of Monoclonal Antibodies against PK'X'

2.1 **Introduction**

Antibodies are circulating proteins involved in the immune response of vertebrates. Their function is to specifically attach to foreign molecules and alert the host's cellular immune response to these molecules.

In mammals there are five different classes of antibody. These are referred to as IgA, IgD, IgE, IgG and IgM respectively. The IgG and IgM classes have been the most extensively studied. IgM antibodies are polymeric molecules with a low affinity and are made during the primary immune response whilst the secondary immune response is regulated by the IgG group of antibodies which are monomeric and have a high affinity (Harlow and Lane 1988). The piscine immune response is believed to only involve IgM-like antibodies (Evans and Gratzek 1989).

Monoclonal antibodies (MAbs) were first produced *in vitro* by Köhler and Milstein in 1975. They were produced by fusing myeloma cells with spleen cells from mice using a virus, and by successive screenings these researchers were able to produce a single immortal cell line that produced one specific antibody. Such cell lines are referred to as hybridomas and the antibodies produced referred to as monoclonal. Because the cell line is immortal MAbs can be produced in large quantities. Polyclonal antibodies can also be produced by collecting the sera from an immunised animal such as a rabbit, however unlike MAbs these contain many different antibodies and only a limited amount can be produced.

The ability to produce MAbs has been invaluable in the diagnosis and investigation of many diseases of both man and other animals including fish (Adams, Thompson, Morris, Farias and Chen 1995). Myxosporean parasites investigated using MAbs include *Ceratomyxa shasta* and *Henneguya exilis* (Bartholomew, Rohovec and Fryer 1989; Belem and Pote 1994). Two MAbs, termed MAb 12 and MAb 18, that reacted with PK'X' were produced (Adams *et al.* 1992). Initial studies using MAb 12 demonstrated an antigenic relationship between the extrasporogonic and intratubular sporogonic stages of the parasite, whilst MAb 18 indicated the presence of a shared antigen between the parasite and the host kidney tubule. (Adams *et al.* 1992; Marin de Mateo *et al.* 1993). Such MAbs prove to be effective tools for the further study of PK'X'.

2.2 **Objectives**

The objectives of this chapter were to produce a range of MAbs against PK'X' following a modified method of Adams *et al.* (1992). Initial studies to characterise these antibodies and the epitopes to which they bind were also performed.

2.3 **Materials and Methods**

2.3.1 *Purification of Antigen*

Purification of PK'X' has so far not been possible and so a partially pure suspension prepared by differential centrifugation was used to inject mice.

A heavily infected rainbow trout with PKD was killed with a sharp blow to the head and the kidney excised. The kidney was kept at 4°C for 3 hours to reduce macrophage activity (Prof. R. Hedrick, University of California pers. comm.). It was placed upon a 40µm nylon mesh, on top of a 300µm sieve screen, in a petri dish on ice. 10ml of ice cold sterile phosphate buffered saline (PBS: 0.02M phosphate, 0.15M sodium chloride pH 7.2), was gently pipetted over the kidney into the petri dish. The kidney was pushed through the gauze using the plunger from a 5ml disposable syringe to disassociate the cells. The remaining cells were washed through the gauze into the petri dish using a further 10mls of PBS.

The kidney homogenate was transferred to a 25ml universal on ice. After 5 minutes, to allow large aggregates to settle, the supernatant was pipetted to a fresh universal and centrifuged for 4 minutes at 500g in a Wifug 500E centrifuge. The subsequent supernatant was transferred to a fresh universal and centrifuged again for 4 minutes at 500g. This supernatant was discarded and the two pellets combined and resuspended in 1ml of cold PBS. The concentration of PK'X' parasites in the cell concentrate was

assessed by viewing a drop of the suspension under a coverslipped slide using an Olympus CH-2 microscope with phase contrast illumination at x400 magnification. The PK'X' parasites are easily recognised under phase contrast by the appearance of refractile granules within them and their comparatively large size.

Cell concentrates containing relatively high levels of PK'X' (over 10% of the concentrate comprising PK'X' cells), were stored at -70°C.

2.3.2 *Immunisation Schedule*

PK'X' cell concentrate was mixed with an equal volume of Freunds complete adjuvant by placing the adjuvant and the concentrate into two glass syringes connected to each other by a Luer lock. The solutions were mixed by passing them from one syringe to the other until a stiff white emulsion was formed. The syringes were disconnected and a 25G needle placed on the syringe containing the suspension. This suspension was used to inoculate the mice.

Six balb/c mice were each immunised by an interperitoneal injection with 0.2ml of the emulsion. After 4 weeks the mice were given a booster injection of 0.2ml PK'X' suspension mixed (50:50) with Freunds incomplete adjuvant. Tail bleeds were taken 7 days later to assess which of the mice produced the most specific antibody response against PK'X'.

2.3.3 *Tail Bleeds and Sera Preparation*

0.2ml of blood was taken from each mouse by swabbing the tail with a small amount of alcohol before nicking a prominent vein with a sterile scalpel, 1-2 inches from the mouse's body. The blood was collected into a 0.5ml eppendorf tube, left for 1 hour at room temperature before being incubated at 4°C for a further 2 hours. The clot formed was pelletted by centrifuging at 6,000g for 10 minutes in a microfuge centrifuge. The supernatant was removed, placed into a fresh eppendorf and serially diluted 1/10, 1/100, 1/1000, 1/10,000 and 1/100,000 with PBS. These dilutions were used as the primary antibody on PK'X' infected rainbow trout tissue during testing by immunohistochemistry.

2.3.4 *Preparation of Samples for Immunohistochemistry*

Uninfected and PK'X' infected rainbow trout kidneys were fixed in 10% neutral buffered formalin overnight. The tissues were processed through a histinokette processor and embedded in paraffin wax using an embedding centre. Sections were cut at 5μm, floated onto poly-l-lysine coated slides and dried in a 60°C oven for 2 hours.

2.3.5 *Immunohistochemistry of Sections*

Immunohistochemistry was performed on the sections following the method of Adams and Marin de Mateo (1994) as follows.

2.3.5.1 *Dewaxing of Sections*

Sections were dewaxed and rehydrated by placing in xylene for 5 minutes followed by 100% alcohol for 5 minutes, 70% alcohol for 3 minutes and finally rinsed in distilled water.

2.3.5.2 *Immunostaining of Sections*

Sections to be stained with immunohistochemistry were circled with a PAP pen (BDH Ltd) to localise incubations directly onto sections. Endogenous peroxide activity was blocked by incubating the sections for 10 minutes with 10% (v/v) hydrogen peroxide in methanol. The sections were washed by briefly holding the slides under a stream of Tris buffered saline (TBS: 20mM Tris, 0.5M Sodium chloride, pH 7.6). Non specific binding was blocked by incubating the slides with 10% normal goat serum (SAPU: Scottish Antibody Production Unit) in TBS for 10 minutes. The goat serum was tapped off the slides and the primary antibody incubated on the sections for 1 hour in a moist chamber. After incubation the primary antibody was washed from the sections as previously described and the slides incubated with goat anti-mouse horse radish peroxidase conjugate (HRP: SAPU), diluted 1/50 in TBS for 30 minutes, again in a moist chamber. The slides were washed with TBS and sections incubated for 10 minutes with 3'3' diaminobenzidine tetrahydrochloride (20μM DAB, 5ml TBS, 100μl of 1% Hydrogen peroxide). The DAB was washed from the slides by immersing them in tap water for 3 minutes. The primary antibody controls were MAb 12 (a PK'X' specific antibody

previously developed) and Dulbecco's modified essential medium (DMEM: Sigma) tested on infected and uninfected tissue.

2.3.5.3 *Counterstaining of Sections*

The sections were counterstained for 3 minutes in Meyers Haematoxylin (3mM haematoxylin, 1mM sodium iodate, 0.1M aluminium potassium sulphate dodecahydrate, 5mM citric acid, 30mM trichloroacetaldehyde hydrate), washed for 10 minutes in running tap water, dehydrated through an alcohol series and rinsed twice for 5 minutes in xylene. The slides were coverslipped using Pertex (Sigma) and viewed at x400 under bright field using an Olympus CH-2 microscope.

2.3.5.4 *Evaluation of Mouse Sera for Antibody Production and Preparation for Fusion*

All of the mouse sera tested reacted positively in immunohistochemistry. However only one mouse showed good specificity to PK'X' with low concentrations of sera (1/10,000) and relatively little background staining. Four weeks after the previous injection this mouse was given a final intravenous injection into a tail vein with 100µl of PK'X' concentrate in preparation for fusion.

2.3.6 *Fusion*

2.3.6.1 *Production of Myeloma Cell Line*

A mouse myeloma cell line, SP2/0-Ag-14, (ICN Biomedicals) was held in storage in liquid nitrogen. One week before the fusion the myeloma cells were thawed out as

described in section 2.28 and transferred to two $25cm^3$ tissue culture flasks (Corning) each containing 10ml of DMEM (all DMEM contained 1% Sodium pyruvate, 1% Penicillin/ Streptomycin, 2% L glutamine and was warmed to 37°C) with 20% Foetal calf serum (FCS: heat inactivated by incubating at 56°C for 30 minutes). The flasks were placed in a humidified, water jacketed incubator (Forma scientific) at 37°C with a 5% CO_2 atmosphere.

After two days the cells were counted using a haemocytometer (Hawksley Ltd) and the cell concentration adjusted to 1.5×10^5 cells ml^{-1} with the addition of fresh medium, so maintaining the cells in mid-log phase of growth.

On the day of the fusion the number of viable cells was counted. This was achieved by using the vital dye trypan blue to stain dead cells. 1ml of 0.4% trypan blue (Sigma) was mixed with 1ml of cell suspension and the cells counted immediately under a haemocytometer. The contents of the flasks were combined into a 25ml universal and centrifuged for 10 minutes at 125g using a Wifug 500E centrifuge. The supernatant was carefully aspirated and the cell pellet resuspended in 20ml of DMEM. The universal was sealed and replaced in the incubator.

2.3.6.2 *Preparation of Mouse Spleen Cells*

Seven days after the final intravenous injection, the mouse was killed by placing it in a sealed chamber and exposing it to gradually increasing levels of CO_2. The mouse was immediately bled out by cardiac puncture using a 20G needle connected to a 2ml

syringe. The serum was collected from the blood as previously described (section 2.3.3) and frozen in 0.5ml aliquots at -20°C.

The mouse was transferred to 70% alcohol before being placed onto a clean paper tissue. The skin covering the spleen was removed aseptically using sterile forceps and scissors. The spleen was carefully removed from the mouse using a fresh pair of sterile scissors and forceps, placed into a small petri dish containing 5ml of DMEM and transferred to a tissue culture hood. It was then placed into a fresh petri dish containing 5ml of DMEM.

Using two 25G needles the spleen was teased apart until no large cell aggregates remained. The cell suspension was drawn through a 21G needle connected to a 10ml syringe and pipetted back into the petri dish. This procedure was repeated and the suspension drawn into a fresh 10ml syringe connected to a sterile 25G needle before being carefully pipetted into a 25ml universal containing 5ml of DMEM.

The suspension was allowed to settle for 1 minute before the supernatant was removed to a fresh universal and made up to 20ml using DMEM.

2.3.6.3 *Preparation of Blood Feeder Cells*

To prepare mouse blood feeder cells, 200ml of DMEM medium containing 20% FCS was warmed in an incubator to 37°C. An unimmunised balb/c mouse was killed as previously described (section 2.3.6.2). It was bled out by cardiac puncture using a 1ml

syringe containing 10 i.u. of heparin in DMEM connected to a 23G needle. The needle was removed and a fresh needle attached to the syringe. 0.6ml of the blood was added to the flask containing the DMEM/ FCS in the incubator.

2.3.6.4 *Fusion of Mouse and Myeloma Cells*

The myeloma and spleen cells were centrifuged at 125g for 10 minutes. The supernatants were removed and 10mls of DMEM added to resuspend the cells.

The spleen cells were counted using a haemocytometer. The volumes of the myeloma and spleen suspensions were adjusted using DMEM to give a cell ratio of 1:10 myeloma to spleen cells. The suspensions were combined and the resultant suspension centrifuged at 75g for 10 minutes. The pellet thus formed was aspirated dry and resuspended with 1ml of a warmed solution of Polyethylene Glycol (PEG: 50% w/v polyethylene glycol, DMEM, obtained from Sigma). This was added drop wise to the pellet over 15 seconds with gentle shaking. The universal was subsequently left to stand for 30 seconds. 5ml of DMEM was added dropwise over 2 minutes again with gentle agitation of the universal. Another 5ml of DMEM was added and the universal centrifuged at 75g for 10 minutes. The pellet was aspirated dry and resuspended in 20ml of blood feeder cells containing 5mM Hydroxanthine, 50μM Aminopterin and 0.8mM Thyamine (HAT: obtained from Sigma). This suspension was added to a flask containing 80ml of warmed HAT blood feeder cells.

The HAT blood feeder cells were plated out onto 5 x 24 well tissue culture plates (Corning) using 1ml of suspension per well. Three wells were used as controls containing HAT blood feeder cells, HAT blood feeder and spleen cells and HAT blood feeder and myeloma cells respectively.

After 2 days incubation the cell wells were checked under a Zeiss Telaval 3 inverted microscope for bacterial or fungal contamination. The plates were re-examined five days later for hybridoma growth. Hybridoma growth was determined by the presence of aggregations of cells in the fused cell wells and cell death in the control wells.

The screening of growing hybridomas for antibody production was performed 10 days after the fusion when the growing hybridomas were easily visible to the naked eye (~1mm^2 across).

2.3.6.5 *Screening of Hybridomas*

The hybridomas were screened by removing 50µl of the supernatant of each cell well and transferring it to a corresponding well on a 96 well plate (Corning). The harvested supernatants were used as the primary antibodies for immunohistochemistry on sections of PK'X' infected kidney as described in section 2.3.5. Additional control primary antisera to MAb 12 and DMEM was obtained from the myeloma control supernatant and the mouse sera (1/10,000 dilution in PBS). 50µl of HT medium (HT medium: 5mM Hydroxanthine, 0.8mM Thymidine, 20% FCS in DMEM) was added to the positive wells and they were marked for cloning. All of the medium from the 24 well plates was

replaced with HT medium after the initial screening and the plates tested again for PK'X' specific antibody production 5 days later.

2.3.6.6 *Maintenance of Cells*

All of the cell wells were regularly checked for contamination and build up of acidic conditions. This was determined by a colour change in the medium from red to yellow. Acidic media was replaced initially with HT medium and then with DMEM containing 20% FCS.

2.3.6.7 *Initial Cloning of Positive Clones Using Doubling Dilutions*

300ml of red blood feeder cells were prepared in DMEM as previously described (section 2.3.6.2). Using an 8 well multichannel pipette 100µl of blood feeder cells was added to all of the wells of a 96 well tissue culture plate (Corning) and incubated at 37°C. 5 plates were used with each hybridoma to be cloned.

Cells within PK'X' positive wells as determined by immunohistochemistry were suspended by gently drawing them up and down a 200µl Gilson pipette. 100µl of the suspension was added to the top left hand well of each of the 96 well plates and mixed with the feeder cells. Doubling dilutions were made by removing 100µl from the first well and introducing it into the next well down mixing the cells together. This was repeated until the first row of the plate contained a sequential dilution of hybridoma cells. Using an 8 well multichannel pipette 100µl from each of wells in the first row was added to the second row and mixed. This was repeated across the plate resulting in

doubling dilutions over the whole of the plate. When all 5 plates had been seeded with the chosen hybridomas in this way, the plates were transferred to the incubator and monitored for contamination and hybridoma growth. When the hybridomas had grown $1mm^2$ across they were ready for screening.

2.3.6.8 *Screening of Hybridomas after Initial Cloning*

Due to the large number of wells to be screened using immunohistochemistry it was impractical to screen all of the wells. Thus only wells containing one or two growing clones were marked for screening. From the marked wells 50µl of supernatant was removed, and transferred to a fresh 96 well plate. The supernatant was replaced by 50µl of DMEM. Immunohistochemistry was performed using PK'X' positive kidney sections with the collected supernatants as the primary antibody as described in section 2.3.5. Wells producing PK'X' antibodies were examined using a Zeiss Telaval 3 inverted microscope. The well containing the least number of clones, ideally one, was selected for further cloning.

2.3.6.9 *Second Cloning of Positive Clones Using Limiting Dilution*

Wells selected for further cloning were allowed to grow to $2mm^2$ across before cloning. The cells were put into suspension by gentle pipetting and then transferred to a bijou before being counted using a haemocytometer. The cell concentration was adjusted to $1x10^{-5}$ cells ml^{-1} using DMEM containing 20% FCS and serially diluted to achieve final dilutions of 25, 12 and 3 cells per ml. These three dilutions were plated, 100µl per well, onto 96 well tissue culture plates containing 100µl per well of blood feeder cells. The

plates were placed in an incubator and 10 days later, wells containing single clones were screened using immunohistochemistry (section 2.3.5).

When all of the wells tested were positive the cell line was said to be monoclonal and expanded into flasks for monoclonal production. The presence of any negatively tested wells containing clones would suggest the presence of more than one cell line and therefore further clonings would be necessary.

2.3.6.10 *Expansion into Flasks*

A well, containing a single clone that was determined to be homogenous by limiting dilution was incubated until the clone was $2mm^2$ in diameter. It was put into suspension using a pipette and 100µl used to seed 5 wells on a 24 well plate each containing 1.5ml of blood feeder cells. After a further 10 days 50µl of supernatant from each well was tested using immunohistochemistry (section 2.3.5), to determine continued monoclonal production. The contents of the wells were put into suspension and pooled. This cell suspension was added to two 75ml tissue culture flasks (Corning) each containing 10mls of DMEM, 20% FCS.

Cell growth and death in the flasks was checked using a Zeiss Telaval 3 inverted microscope. When cell density reached an optimum (judged by the colour of the medium and the visible condition of cells) 5mls of cell suspension were removed and added to 45mls of DMEM, 20% FCS in a 200ml tissue culture flask (Corning). Antibody production was routinely assessed using immunohistochemistry.

2.3.6.11 *Antibody Harvesting*

Antibodies were harvested by carefully pipetting the old medium from the flasks and placing it into universals. The flask medium was replaced immediately with 50mls of fresh DMEM/ 20% FCS. The universals were centrifuged for 10 minutes at 125g. The supernatant was collected, placed into a fresh universals, and stored at -70°C.

2.3.6.12 *Cryopreservation of Cell Lines*

Cell lines were routinely cryopreserved throughout the procedure to prevent possible contamination from permanently destroying the cell line and for the permanent storage of cell lines of interest. The cells were harvested by pipetting the cells into a suspension and counting the number of viable cells within the flask using trypan blue and a haemocytometer.

The suspension was centrifuged for 10 minutes at 75g and resuspended in DMEM containing 20% Demethyl sodium oxide (DMSO: Sigma) to give an adjusted cell volume of 1 x 10^5 cells/ml. 2mls of this cell suspension was transferred to cryovials (Linbro cryogenic vials, Flow laboratories) placed in a neuamberg chamber (Nalgene ltd) surrounded by propylene oxide and placed overnight at -70°C. The following day the vials were transferred to liquid nitrogen for permanent storage.

2.3.6.13 *Thawing Cell Lines*

Frozen vials were transferred from liquid nitrogen storage and immediately placed into a 37°C water bath. When the contents of the vials had nearly completely thawed the tops

of the vials were wiped with 70% ethanol and the cell suspension pipetted onto 9mls of DMEM layered upon 1ml of FCS. The suspension was centrifuged at 75g for 10 minutes, resuspended in 10 mls of DMEM, 20% FCS, placed in a $25mm^3$ tissue culture flask (Corning) and incubated for 5 days whereupon antibody production was determined using immunohistochemistry (section 2.3.5).

2.3.7 *Classification of Antibodies*

Initial studies on the MAbs produced in section 2.3.6 were performed to determine their isotype, specificity to carbohydrate or protein epitopes and the molecular weight of the antigens to which they bind. The possible steric relationships between their epitopes was also examined by double immunolabelling.

2.3.7.1 *Isotyping of antibodies using an ELISA (Enzyme Linked Immunosorbent Assay)*

The antibodies were isotyped using Sigma ISO-2 mouse monoclonal antibody isotyping reagents in an ELISA as specified by Sigma product instruction manual 073H-4810. Goat anti-mouse immunoglobulins (IgM, IgG, IgG2a, IgG2b and IgG3) were diluted 1/100 in bicarbonate buffer (15mM Sodium carbonate, 35mM Sodium bicarbonate pH 9.6 adjusted with HCl) and incubated on a 96 well ELISA plate (Corning) overnight at 4°C.

The plate was washed three times with low salt wash buffer (0.02M phosphate, 0.38M Sodium chloride, 0.5% Tween 20 pH 7.3) and coated with blocking buffer (1% w/v

bovine serum albumin in low salt wash buffer) for 2 hours at room temperature. The plate was washed again three times with low salt wash buffer. 100µl of hybridoma supernatant (obtained from section 2.3.5) was added to each of the wells containing a different goat anti-mouse antibody and incubated for 60 minutes at room temperature. The plates were washed with five washes of high salt wash buffer (0.02M phosphate, 0.5M Sodium chloride, 0.5% Tween 20 pH 7.3) with a final 5 minute wash with low salt wash buffer. 100µl of conjugate (goat anti mouse IgG HRP, SAPU, goat anti mouse IgM HRP) diluted 1/1000 in PBS was incubated in the wells for 60 minutes. After washing with high salt wash buffer, as previously described, 100µl $well^{-1}$ of the chromogen tetramethylbenzidine dihydrochloride (0.04M tetramethylbenzidine dihydrochloride in 2M acetic acid solution) diluted 1/10 in substrate buffer (0.1M citric acid, 0.1M sodium acetate) was added to each well and incubated for 10 minutes. The reaction was stopped by the addition of 50µl of stop solution (2M sulphuric acid) to the wells and the plate read at 450nm using a Dynatech MR5000 ELISA reader. A negative control of low salt wash buffer and positive controls of IgM, IgG, IgG2a, IgG2B and IgG3 antibodies (Sigma) were included in the assay in place of the hybridoma supernatant.

2.3.7.2 *Classification of Antibodies Using Periodate Oxidation*

Incubation of antigens with periodate has been reported to indicate whether antibodies are specific for protein or carbohydrate epitopes (Woodward, Young and Bloodgood 1985). The method used was adapted for immunohistochemistry from the protocol of Woodward *et al.* (1985) to determine the specificity of the anti-PK'X' MAbs.

Tissues infected with PK'X' were dewaxed and treated with 10% hydrogen peroxide (section 2.3.5). The sections were then incubated for 30 minutes in the dark with varying concentrations of periodic acid (0, 5, 10, 20mM). Following three washes in PBS the sections were incubated for 1 hour in 0.4M sodium borohydride. The sections were rinsed again with PBS and incubated as previously described for immunohistochemistry (section 2.3.5) using the monoclonal antibodies obtained from the fusion. Control sections using MAb 12 and DMEM were also included in the procedure. The antibodies tested using periodate oxidation stain the PK'X' cells if they are to a protein epitope and remain unstained if they are to a carbohydrate epitope.

2.3.7.3 *Analysis of Antibodies Using Double Immunolabelling*

To determine whether the MAbs produced were to related epitopes, PK'X' infected tissue was double immunolabelled using DAB and fluorescein isothiocyanate following a modified procedure of Marin de Mateo *et al.* 1993.

Infected kidney sections were labelled with each of the antibodies with DAB using immunohistochemistry (section 2.3.5). After washing in distilled water the slides were incubated with a different hybridoma supernatant for 2 hours, washed in PBS, followed by goat anti-mouse IgG conjugated to fluorescein isothiocyanate (SAPU) diluted 1/50 in PBS for 1 hour. The slides were then washed in PBS, followed by distilled water and mounted under 50% glycerol. The slides were examined under oil immersion using an Orthomat fluorescent microscope at x400 under UV. light.

2.3.7.4 *Analysis of Antibodies using Western Blotting*

The molecular weights of the antigens to which the MAbs bound were determined using Western blotting.

Samples were resolved using a mini-Protean II electrophoresis system (Biorad Ltd) following a modified method of Laemmli (1970). The SDS-PAGE gels were prepared thus.

Separating gel 15%

Separating gel buffer	5ml
Acrylamide stock	10ml
TEMED	10µl
10% ammonium persulphate	70µl
Distilled water	5ml

Stacking gel

Stacking gel buffer	2.5ml
Acrylamide stock	1.34ml
TEMED	10µl
10% ammonium persulphate	50µl
Distilled water	6.1ml

TEMED = N N N' N'-tetramethyl ethylene diamine (Sigma). Separating gel buffer = 1.5M Tris, 0.4% SDS, pH 8.7 with HCl. Stacking gel buffer = 0.5M Tris, 0.4% SDS, pH 6.4 with HCl. Acrylamide stock = 30% acrylamide, 0.8% bis-acrylamide, filtered through a Whatman No.1 filter paper.

Stacking and separating gels were made up as above without the TEMED and ammonium persulphate and degassed in a Buchner flask *in vacuo* for 15 minutes. The TEMED and ammonium persulphate were added to the gels immediately before they were poured between the gel plates. The separating gel was poured first between the plates with 100µl of butanol layered over it. When it had set the butanol was washed off using distilled water and the stacking gel applied.

Samples of kidney infected with PK'X' and uninfected kidney were prepared as in section 2.2.1. The samples were diluted 1:4 with sample buffer (4ml distilled water,1ml 0.5M Tris HCl pH 6.8, 0.8ml glycerol, 1.6ml 10% (w/v) SDS, 0.4ml 2-B-mercaptoethanol, 0.2ml 0.05% (w/v) bromophenol blue) and heated in a waterbath at 98°C for 4 minutes. The samples were centrifuged at 6,000g in a Mistral 3000i MSE centrifuge for 5 minutes before applying to the gels with a clean hamilton syringe. 150µl of sample was loaded onto each gel against 5µl prestained broad range molecular weight markers (Biorad). Gels containing PK'X' and control kidney were run concurrently on the same apparatus. Gels were run at 120 volts using a Biorad model 200/2.0 power supply until the dye front reached the bottom of the gel.

After electrophoresis the samples were transferred onto 0.45µm nitrocellulose paper (Hoeffer) following the method of Wiens, Prasod, Turaga and Kaattari (1990). Briefly the gels were removed from the electrophoresis plates and placed into transblot buffer (0.2M Glycine, 25mM Tris, 20% methanol) for 10 minutes with 2 nitrocellulose sheets and 12 filter papers (Whatman ashless size 12). Three filter papers were placed on the

cathode side of a transblotter (Biorad, transblot SD semidry transfer cell) followed by a nitrocellulose sheet, the gel and a further three filter papers on top avoiding air bubbles. The gel was transferred onto the nitrocellulose sheets for 20 minutes at 15v.

The nitrocellulose sheets were blocked overnight at 4°C in TBS (20mM Tris, 0.5M NaCl, pH 7.5) containing 1% BSA. The sheets were then washed three times with 5 minute rinses in TBS containing 0.1% Tween-20 and placed in a mini protean II multiscreen (Biorad). The lanes were incubated with each of the antibodies produced with control lanes of DMEM overnight at 4°C. After incubation the sheets were removed and washed as previously described. The sheets were then incubated with a 1/100 dilution of sheep anti mouse IgG HRP (SAPU) in TBS for 60 minutes at room temperature with gentle agitation. The sheets were washed again followed by an additional rinse in TBS only for 1 minute. The immunoblot was developed by incubation with DAB for 10 minutes. Immunoblots were also developed using immunogold conjugates. Briefly after the incubation with the primary antibody the blot was extensively washed with 4 rinses in TBS containing 0.1% Tween20 and incubated with 1/150 dilution of goat anti-mouse gold conjugate (10nm) overnight at 4°C. The blot was washed as previously described followed by 4 rinses in distilled water. The immunoblot was developed by incubating the blot for 30 minutes in immunogold silver enhancing solution (Biocell).

2.4 **Results**

2.4.1 *Results of Fusion*

Two fusions aimed at producing MAbs against PK'X' were performed. The first fusion was deemed unsuccessful as hybridomas synthesising antibodies specific for PK'X' could not be isolated. However, four hydridomas, synthesising antibodies that reacted against PK'X' in rainbow trout kidney were produced as a result of the second fusion. These were all successfully isolated and cryopreserved. These cell lines were termed A3, B4, C5 and D4 respectively corresponding to the cell wells from which they were isolated. The hybridoma supernatants derived from these cell lines containing antibodies were termed MAb A3, MAb B4, MAb C5 and MAb D4.

Initial observations from immunohistochemistry indicated that MAbs A3, C5 and D4 reacted with the extrasporogonic parasite and the intratubular sporogonic parasite. MAb B4 however appeared to stain intratubular sporogonic parasites and secondary cells within the extrasporogonic parasites. No cross reaction was observed with any of the MAbs and the kidney tissue.

2.4.1.1 *Isotyping of Antibodies by Enzyme Linked Immunosorbent Assay (ELISA)*

ELISA determined that MAbs A3, B4 and D4 were all IgM whilst MAb C5 was IgG1.

2.4.1.2 *Periodate Oxidation*

Periodate oxidation of infected tissue sections indicated that MAbs A3, C5 and D4 were specific for carbohydrate epitopes losing their reactivity with PK'X' after the PK'X' infected tissues were incubated with 5mM periodic acid. MAb B4 retained reactivity with the parasite even with 20mM periodic acid indicating that it was specific for a protein epitope. The control slide of MAb 12 lost its reactivity with PK'X' after incubation with 5mM of periodic acid confirming the results of Marin de Mateo *et al.* (1993) performed on this MAb.

2.4.1.3 *Double Immunolabelling*

Double immunolabelling studies determined that MAbs A3, C5 and D4 inhibited each other's binding to the parasite whilst MAb B4 bound irrespective of the presence of the other MAbs.

2.4.1.4 *Western Blotting*

The results of the western blot are shown in figure 2.1. Western blotting analysis of the antibodies indicated that MAbs A3 and D4 bound to antigens of molecular weights 11 Kd , 9 Kd and a broad band less than 5 Kd. MAb C5 appeared to bind only to antigens less than 5 Kd and MAb B4 bound to antigens with molecular weights 180 Kd , 140 Kd

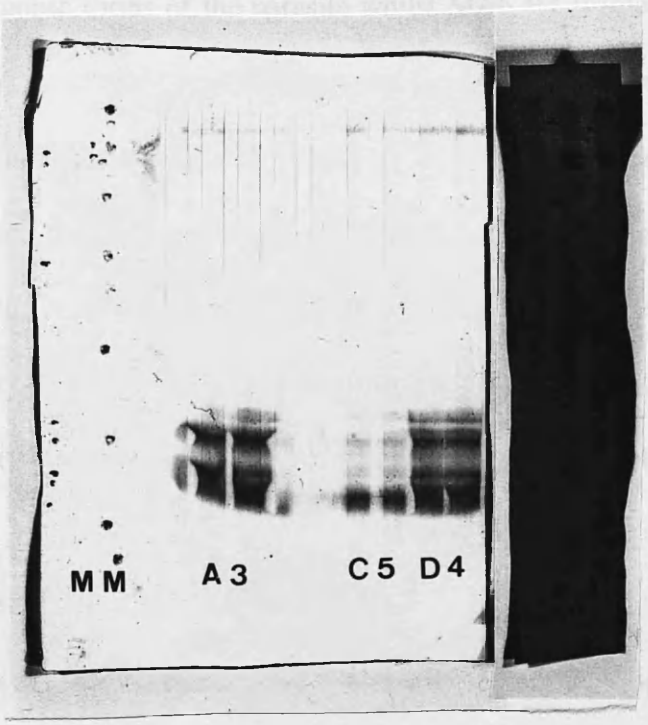


Figure 2.1
Photograph of Western Blots performed using MAbs A3, B4, C5 and D4 against PK'X' infected rainbow trout kidney homogenate. MAb B4 binding was enhanced using immunogold with silver enhancing. Molecular weight markers (MM) indicated on the left hand side of the photograph.

and 37.5 Kd. MAb B4 could only be detected using immunogold labelling and silver enhancing.

2.5 **Discussion**

The fusion of mouse spleen cells to myeloma cells resulted in the production of four hybridoma cell lines producing antibodies which reacted against PK'X' parasites within the kidney of rainbow trout. Characterisation of these MAbs by immunohistochemistry demonstrated that MAbs A3, C5 and D4 all reacted with extrasporogonic and intratubular sporogonic forms of the parasite whilst MAb B4 reacted against secondary cells and the sporogonic parasites. These results confirm that an antigenic relationship exists between the extrasporogonic parasites and the sporogonic parasites. This relationship was first demonstrated by Marin de Mateo *et al.* (1993) with observations on the staining of MAb 12 and the lectin GSI. The results also indicate a relationship between the intratubular sporogonic forms of the parasite and the secondary cells contained within the extrasprogonic parasite. This relationship will be further discussed in chapter 5.

MAbs A3, C5 and D4 all appeared to react with carbohydrate epitopes whilst MAb B4 appeared to react with a protein epitope as determined by periodate oxidation. Periodate oxidation on sections however should only be taken as an indication of the affinity of an antibody to a carbohydrate or protein epitope because carbohydrate determinants located on non-terminal domains of carbohydrate chains are unaffected by the procedure

(Woodward *et al.* 1985). It is also doubtful whether peptide determinants always remain resistant to mild periodate treatment (Feizi and Childs 1987). Thus results using the periodate procedure should be interpreted with caution. Conclusive proof of the nature of the epitopes may require treating PK'X' infected tissue with proteinases prior to Western blotting.

Western blot analysis of infected rainbow trout kidney demonstrated that MAbs A3 and D4 reacted with a range of antigens, below 11 Kd, whilst MAb C5 reacted with a relatively broad band below 5 Kd. The large number of antigens detected suggest that the epitope may represent an oligosaccharide carried by a range of different glycoconjugates. The Western blot analysis indicated that MAb B4 bound onto three antigens of molecular weights 180 Kd, 140 Kd and 37.5 Kd. This may indicate that the antigenic determinants reacting with MAb B4 are present in several distinct molecules within the parasite or, more likely, that the bands may represent the breakdown products of a larger molecule.

Double immunolabelling demonstrated that MAbs A3, C5 and D4 inhibit each other's binding to the parasite. This suggests that they bind onto related structures or epitopes within the parasite causing either a conformational change in the epitope recognised by the subsequent antibody or by physically blocking that antibody to its epitope. The analysis of the Western blotting performed on these antibodies suggests that MAbs A3 and D4 bind to antigens of the same molecular weights whilst the binding of MAb C5 only affects a proportion of these antigens. Double immunolabelling studies using the

lectin GS-I and MAb 12 by Marin de Mateo *et al.* (1993) indicated that the lectin GS-I inhibited the binding of MAb 12 to the parasite. However, the lectin also reacted with antigens not recognised by the MAb suggesting that only some of their antigenic determinants were shared. The results of double immunolabelling and Western blotting imply that MAbs A3 and D4 may be specific for the same epitope, but they do not conclusively prove this. Further work is needed to determine the molecular structure and location of the antigens that the MAbs react against.

The production of MAbs specific for PK'X' in this study proved to be difficult with only one mouse producing a sufficiently specific response to attempt a fusion. The use of 24 well plates containing many growing hybridomas in the initial screening of the fusion, although practical for immunohistochemistry, is not satisfactory for the detection of relatively slow growing hybridomas. Any future production of PK'X' MAbs could be greatly enhanced by using pure suspensions of parasite throughout the procedure. This would not only result in a more specific response being elicited from inoculated mice but also for the screening of hybridomas using an ELISA. Such an ELISA would greatly increase the number of cell wells that could be screened for antibody production at any one time.

Chapter 3

Purification of PK'X' using Immunomagnetic Separation

3.1 **Introduction**

Studies on PK'X' and other myxosporean species have largely remained descriptive concentrating on the parasites' structure or life cycle. To gain fresh insights into myxosporean biology it is desirable to obtain pure suspensions of parasites. Purification of the parasite will aid in the development of diagnostic ELISA kits, production of MAbs, identification of antigens for possible vaccine production and for its *in vitro* culture and study.

So far it has not been possible to extract a pure suspension of PK'X' cells from infected kidneys, with only partially pure separations being achieved through centrifugation (Hedrick, Monge, Kazanji, Perry, Marin and de Kinkelin 1991 cited by Adams *et al.* 1992). Purification attempts of other species of Myxozoa have relied primarily on the isolation of mature spores by mechanically or enzymatically attacking surrounding tissues (Markiw and Wolf, 1974; Hamilton and Canning, 1988). Such procedures cannot be employed for PK'X' because mature parasite spores have not been described and the harsh treatments employed to isolate spores would harm the integrity of the extrasporogonic parasite. Trophozoite stages of *Ceratomyxa shasta* have been isolated from the ascites associated with this disease (Bartholomew *et al.* 1989). However PK'X' has not been described from the ascites formed in PK'X' infected fish (Ferguson and Needham 1978; Hedrick *et al.* 1993).

Isolation of PK'X' antigens has recently been achieved by using recovered fish serum in affinity chromatography (D. Saulnier, INRA, Jouy en Josas, France. pers. comm.) however this procedure relies on the destruction of the parasite and only enables isolation of those antigens that are involved in the fishes' antibody mediated immune response.

The development of MAbs that bind to PK'X' cells suggests that these cells can be purified utilising a variety of immunological separation techniques. These include panning, fluorescent activated cell sorting (FACS) and immunomagnetic separations. Immunomagnetic separation is relatively inexpensive compared to FACS and enables high cell specificity compared to panning (Secombes 1994). It also permits fast separations and is a relatively gentle separation technique maintaining the extracted cells' viability. Sterility of the technique may easily be achieved allowing the separated cells to be cultured.

Early attempts at magnetic cell separation employed the properties of the cells to aid in separation. Phagocytic cells were purified by allowing them to ingest magnetic particles before separation with a magnet (Kemshead and Gibson 1985). Another method of magnetic separation utilised the iron content of selected cells, such as the haemoglobin in erythrocytes, by chemically reducing the haemoglobin of these cells and placing them within intense electromagnetic fields (Owen and Sykes 1984). Such methods however are limited to the separation of only a few cell types.

The *in vitro* production of monoclonal antibodies by Köhler and Milstein (1975) allowed cell populations to be specifically targeted. The subsequent development of monosized magnetic particles permitted the positive and negative selection of various cell populations, by conjugating the magnetic particles to antibodies specific for the cell population required (Lea, Vartdal, Davies and Ugelstad 1985; Lea, Smeland, Funderud, Vartdal, Davies, Beiske and Ugelstad 1986). The conjugation of antibodies to particles also means that the particles may be coated with an intermediate antibody, such as anti-mouse IgG, allowing separations to be carried out using a range of antibodies. Magnetic separations have now been achieved for many different cell types including protozoan parasites and bacteria (Nese and Enger 1993; Seesod, Lundeberg, Hedrum, Aslund, Holder, Thaithong and Uhlen 1993). There are three main types of magnetic particles available for magnetic cell separations. These include immunocolloids, paramagnetic particles and polystyrene beads coated in iron (Treleaven, Gibson, Ugelstad, Rembaum and Kemshead, 1984; Hancock and Kemshead, 1993). The sizes of these different particles are 50nm, 1μm and 20μm respectively.

The general procedure for recovery of a cell population using immunomagnets is as follows. The mixed cell population is incubated with an antibody specific for the cell type required. The cells are washed to remove excess antibody which has not bound to the required cells. The cells are then incubated with magnetic particles coated with anti-mouse Ig, at a ratio of 10 particles per cell to be extracted. These magnetic particles bind to the antibodies which are coating the required cell population. The cells are then placed

into a strong magnetic field which attracts the particles, thus separating the cells of interest. The remaining cells are aspirated leaving behind the separated cell population.

Problems frequently encountered with magnetic cell separations include, non specific cell-cell interactions, cell-particle interactions and the removal of particles from the cells after selection. To reduce non specific interactions magnetic separations are often conducted in the presence of various detergents, proteases and blocking buffers (Padmanabhan, Corsico, Howard, Holter, Fordis, Willingham and Howard, 1988). Methods for the removal of magnetic particles include competitively removing the particles by incubating with anti-mouse Fab antiserum, treating the cells with trypsin or by growing the cells in culture for 48 hours, whereupon particles are removed from the cell surface by membrane turnover (Lea *et al.* 1985; Funderud, Erikstein, Asheim, Nustad, Stokke, Blomhoff, Holte and Smeland 1990; Rasmussen, Smeland, Erikstein, Caignault and Funderud 1992).

3.2 **Objective**

The objective of this part of the study was to develop a purification procedure for PK'X' using immunomagnetic separation with the MAbs developed in chapter 2.

3.3 **Materials and Methods**

3.3.1 *Preparation of Plastics used in Separation Procedure*

All plastics were incubated overnight at 4°C with Blocking solution (1% low fat milk Marvel, 0.5% Bovine serum albumin, BSA sigma in 0.2M PBS, cooled to 4°C) to reduce non-specific binding. Immediately prior to use the plastics were rinsed twice with distilled water.

3.3.2 *Assessment of PK'X' Infection Within Kidneys*

Infected kidneys from moribund rainbow trout were excised, blotted dry on paper towels, and briefly placed on clean slides to form kidney impression smears. The smears were air dried before staining with Rapi-diff (Arnold lab supplies) and examined at x200 under bright field using an Olympus microscope. Kidneys with high levels of PK'X' (>1 per visual field when viewed at x200 magnification) were selected for immunomagnetic separation.

3.3.3 *Homogenisation of Infected Kidneys*

The excised kidney was incubated at 4°C for 2 hours to reduce macrophage activity (R. Hedrick pers. comm.). It was placed on a fine gauze on top of a 40μm nylon mesh in a 9cm plastic petri dish on ice. 5ml of blocking solution was gently pipetted over the

kidney into the dish. The kidney was pushed through the gauze using the plunger from a 5ml disposable syringe to disassociate its cells. The remaining cells were washed through the gauze into the petri dish using a further 5mls of blocking solution. The gauze and mesh were removed and the kidney homogenate transferred to a 25ml universal on ice. After 5 minutes, to allow the settling of large aggregates, the supernatant was pipetted to a fresh universal. Cell suspensions obtained in this way contained PK'X' which were surrounded by clumps of adherent macrophages (figure 3.1).

3.3.4 *Reduction of Cell Aggregates*

To increase the effectiveness of separations, attempts were made to reduce the level of cell aggregation associated with the PK'X' parasites. These included sonication, trypsinisation and using detergents on the cells.

In order to determine the effectiveness of sonication on cell aggregates, cell suspensions were placed in a sonicating water bath for intervals of 1, 5 and 10 minutes before being poured into petri dishes to be examined. Cell suspensions were also exposed to different concentrations of trypsin, 0.01mM, 0.05mM and 0.1mM. placed into petri dishes and examined over 30 minutes. All petri dishes were examined under a Zeiss Telaval 3 inverted microscope with occasional agitation. Levels of cell aggregation were compared to controls consisting of untreated cell suspensions.

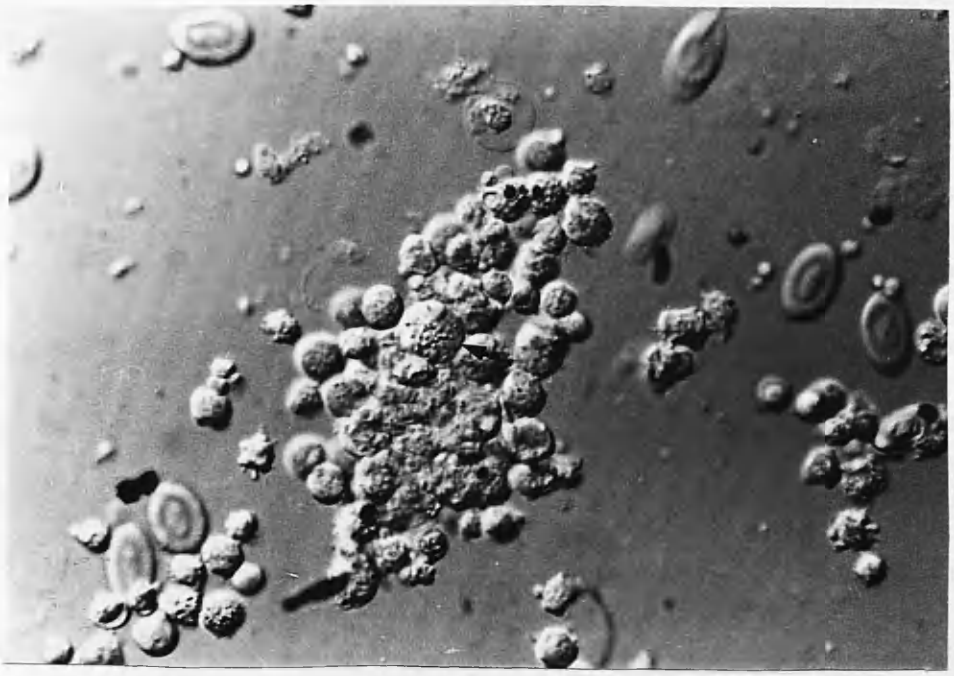


Figure 3.1
PK'X' cell (arrow) surrounded by a large clump of adherent cells, composed primarily of macrophages and lymphocytes.
Mag. X400

A modified method of Padmanabhan *et al.* (1988) was also examined as a possible method of reducing cell-cell interactions when using protein specific antibodies in magnetic separations. Briefly 20mM of EDTA was added to the cell suspension and incubated on ice with occasional agitation for 10 minutes. The cells were centrifuged onto 1ml of FCS at 75g for 10 minutes using a wifug centrifuge. The resultant pellet resuspended in 10mls of Sorting solution (0.2M PBS containing 4mM EGTA, 1mM Mg Cl_2, 100μg chondroitin sulfate, 1mg/ml gelatin, 8mg/ml nonfat dried milk and 10μg/ml BSA, cooled to 4°C), transferred to a petri dish and examined in conjunction with a untreated suspension, under a Zeiss Telaval 3 inverted microscope for cell aggregation.

3.3.5 *Cell Separations*

The procedure for the extraction of PK'X' cells from the kidney suspension was modified from Biomag and Cell Sorting (Advanced magnetics product instruction manual). All of the experiments included negative controls of uninfected kidney homogenate and DMEM replacing the antibody supernatant.

3.3.5.1 *Extraction of Cells Using Carbohydrate Specific Antibodies*

Each of the carbohydrate monoclonal probes, MAbs A3, C5 and D4 were separately used to extract PK'X' cells from kidney homogenate.

5ml of antibody supernatant was added to the kidney suspension (obtained from section 3.3.3) and slowly rotated at 4°C for 1 hour on a varispeed rotamix. Excess antibody was removed by centrifuging the cells at 75g for 10 minutes onto 1ml of FCS using a wifug

centrifuge. The cells were resuspended in 10ml of Blocking solution and the number of PK'X' cells estimated using a haemocytometer. The appropriate amount of Biomag (a suspension of paramagnetic particles, 1μm in diameter, coated with either ~mouse IgM or ~mouse IgG obtained from Advanced magnetics inc, Cambridge), calculated using the formula below (Advanced magnetics product instruction manual), was added to the suspension and rotated for another 30 minutes at 4°C.

$$\frac{\text{Number of targeted cells/ml}}{5\text{x}10^6} = \text{volume (μl) of Biomag suspension needed for cell separation.}$$

Magnetic isolation of labelled cells was achieved by vertically attaching the tube containing the suspension onto the side of a large rare earth magnet (two neodynium iron boron magnets affixed in an iron matrix. Immunocon ltd), using masking tape, and left for 10 minutes at 4°C. During this time cells attached to the paramagnetic particles migrated to the side of the tube nearest the magnet. After separation the remaining cell suspension was aspirated from the tube and the separated cells washed with 3x10 mls of blocking solution followed by one wash of 10mls 0.2M PBS. The tube was removed from the magnet and the separated cells resuspended in 2.0mls of 0.2M PBS.

The success of the separation was assessed by cytospinning 200μl of collected cells and 200μl of the original cell suspension onto slides at 125g for 4 minutes using a Shandon cytospin. The slides were air dried before staining with Rapi-diff^{tm} and viewed at x200 under bright field using an Olympus C-12 microscope.

3.3.5.2 *Concentration of PK'X' Using Negative Selection*

An anti-trout IgM mouse monoclonal, MAb 4C10 (courtesy of Dr A. Thavander, Department of Pathology, Swedish University of Agricultural Sciences, S-75007, Uppsala, Sweden) was used to remove macrophages from the kidney suspension thus increasing the concentration of PK'X'. The number of macrophages was assessed using a haemocytometer and the formula below (Advanced magnetics product instruction manual) used to establish the amount of Biomag to be incubated with the cells.

$$\frac{\text{Number of macrophages/ml}}{1\text{x}10^6} = \text{volume (µl) of Biomag suspension needed for cell separation.}$$

The extraction of macrophages was carried out as in section 3.3.5.2 using MAb 4C10 and the revised volume of Biomag.

3.3.5.3 *Extraction of Cells Using a Protein Specific Antibody*

The monoclonal probe, MAb B4, was used to extract PK'X' cells from kidney homogenate following the above procedure (section 3.3.5.1).

Separations with MAb B4 were also performed on kidney suspensions treated with EDTA and sorting solution as outlined in section 3.3.5. The separation protocol was the same as in section 3.3.5.1 however all blocking solution was replaced with sorting solution.

3.3.5.4 *Separation of Cells Using Three IgM Monoclonal Antibodies*

1.66mls of each of the MAbs supernatants A3, B4 and D4 were combined and this solution used to extract PK'X' cells using the protocol above (section 3.3.5.1). Following separation, the protocol was repeated using the three MAbs on the aspirated cell suspension and the resulting extractions combined.

3.4 **Results**

All of the separation procedures resulted in extraction of macrophages and lymphocytes together with PK'X' cells as a result of cell aggregation and non-specific interactions. Analysis of the extracted cell populations was further confused by the formation of amorphous masses of cells that prohibited the identification of individual cell types.

3.4.1 *Reduction of Cell Aggregation.*

Attempts to reduce the cell aggregations of lymphocytes and macrophages associated with the PK'X' parasites using sonication or trypsinisation were unsuccessful. Both exposure to long periods of sonication (>1 minute) and concentrations of trypsin, greater than 0.05mM trypsin for 10 minutes, resulted in cell lysis. This was determined by a the marked increase in viscosity of the cell suspension. Prior to cell lysis there was no significant reduction in the size or number of cell aggregates associated with the PK'X' parasites.

The application of 20mM of EDTA and sorting solution resulted in the reduction in size of the cell aggregations associated with the PK'X' parasites, however these aggregations of cells were never completely removed from the parasites.

3.4.2 *Cell Separations*

Non-specific extraction of lymphocytes and macrophages was observed in all of the extractions including the controls. Red blood cells however were rarely extracted during the process.

Purification of PK'X' from kidney tissue using the carbohydrate probes was inconclusive. Large amorphous aggregates of cells frequently occurred throughout the separated medium that were highly specific for the magnetic particles. The staining characteristics of these aggregates were consistent with PK'X' however their cellular composition could not be determined using light microscopy (figure 3.2). PK'X' cells surrounded by large aggregations of macrophages and lymphocytes were also extracted. These extractions when cytospun onto slides and stained with rapi diff^{tm} appeared to show many of the parasite primary cells rupturing releasing their secondary cells into their surroundings. However, whether this was due to a result of the extraction or the cytospinning could not be determined with cell viability not being assessed in the study. Comparison of the unseparated and separated cell suspensions revealed that the majority of the PK'X' parasites had not been extracted using this technique. The percentage

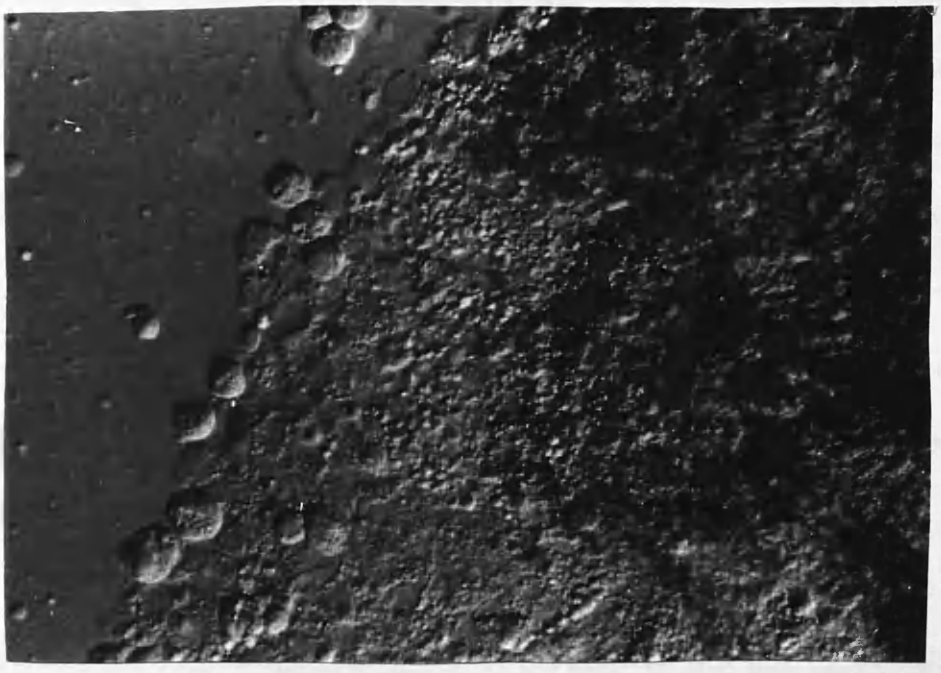


Figure 3.2
Large, amorphous, cellular aggregate resulting from immunomagnetic separation using MAb A3. Presence of immunomagnetic particles within the aggregate indicated with *
Mag X 400

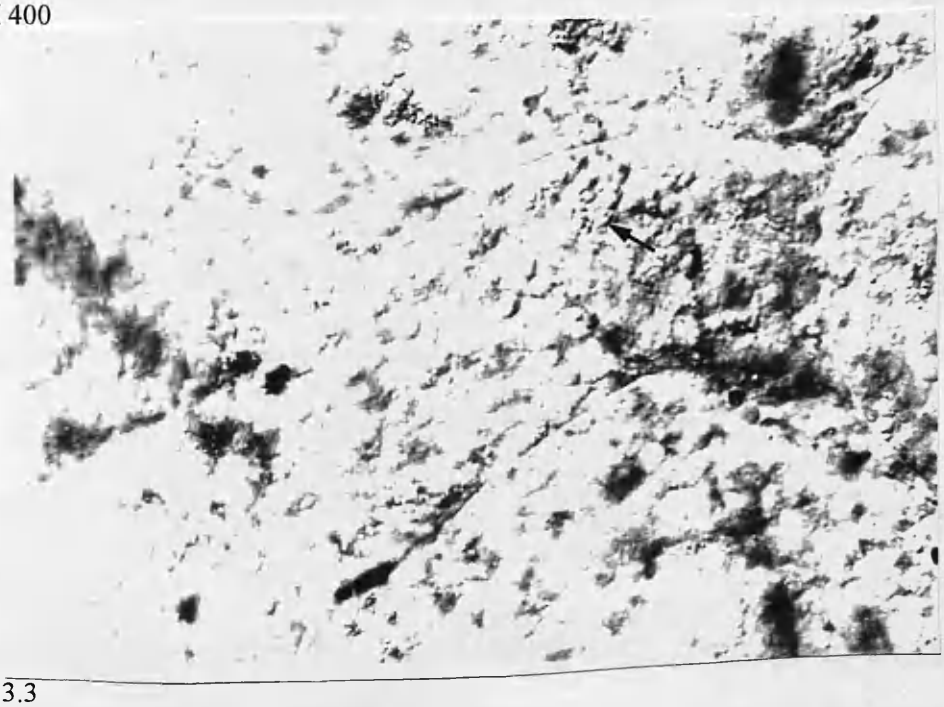


Figure 3.3
Extraction of tubules containing sporogonic stages of PK'X' (arrow) using immunomagnetic separation with MAb B4.
Mag X400

enrichment of PK'X' obtained from the procedure could not be determined due to the presence of large numbers of unidentifiable cells contained within the amorphous aggregates. Negative selection of macrophages using MAb 4C10 resulted in the selective removal of macrophages, lymphocytes and PK'X' from the suspension. The unidentifiable aggregations of cells noted for the other separations were not present within the separation. Comparison of the extracted cell population with the original suspension revealed that only a fraction (about 30%) of cells had been separated using this technique.

Purification of PK'X' from tissue using the protein MAb B4 was also inconclusive with few secondary or primary cells extracted. However kidney tubules containing sporogonic stages of the parasite, as determined by phase contrast microscopy, were targeted and extracted by the magnets (figure 3.3). Comparison of separations using the sorting solution and blocking solution revealed that the sorting solution did result in the reduction of non-specific binding of the magnets to macrophages and lymphocytes. Some indistinct cellular aggregates similar to those noted in extractions using the carbohydrate binding MAbs were also observed within these extractions

Separation of PK'X' using a cocktail of three monoclonals resulted in the extraction of amorphous aggregates of cells and kidney tubules. PK'X' cells surrounded with macrophages and lymphocytes were also extracted although the use of a cocktail of MAbs did not appear to significantly improve separations as determined by comparison of the extracted cells with the controls and original suspension.

3.5 **Discussion**

The aim of this chapter was to implement a novel method for the purification of PK'X' using magnetic cell separation. The separations performed in this study utilised the antibodies obtained in chapter 2 for the positive selection of the parasite. Negative selection of the parasite was also attempted using a monoclonal antibody developed specifically against trout IgM (MAb 4C10).

Separations involving MAbs binding to carbohydrate epitopes resulted in large aggregates of cells being extracted. The cellular constituents of these could not be determined using light microscopy and the origin of these aggregations remains unclear. Immunomagnetic cell separations often result in target cell populations rosetting around magnetic particles resulting in particle-cell aggregates. This is caused by the particles ability to bind onto more than one cell at a time (Padmanabhan *et al.* 1988; Hardingham, Kotasek, Farmer, Butler, Mi, Sage and Dobrovic 1993). At the height of the infection PK'X' primary cells often appear highly vacuolated under light microscopy (Clifton-Hadley *et al.* 1987a). These cells when viewed under electron microscopy are described to be in the process of disintegration, finally releasing their cellular contents into the surrounding interstitium (Kent and Hedrick, 1986; MacConnell *et al.* 1989; chapter 4 this volume). Large aggregates consisting of such disintegrating primary cells when placed within a magnetic field may achieve sufficient momentum to rupture some cells when they reach the side of the tube. Ruptured PK'X' cellular constituents such as DNA and lysosomes may also be targeted by the magnetic particles for extraction from the kidney

suspension. These released cell constituents may result in the non-specific entrapment and lysis of neighbouring cells within the developing rosettes, thus resulting in the formation of apparently amorphous cellular aggregations.

Further study is needed to determine the precise cellular composition of the separated aggregations. These studies may include comparison of the aggregates with infected and uninfected kidneys using SDS gel electrophoresis. Alternatively a DNAase may be used to degrade any released DNA contributing to the amorphous appearance of the aggregations, followed by immunohistochemistry with the lectin GS-I to identify intact PK'X' cells.

The level of parasite separation from the kidney suspension was poor using the carbohydrate-binding MAbs although immunogold localisation determined that the antigens to which the MAbs bound were present on the parasites cell surface (chapter 4 this volume). Poor separations could be attributed to the low affinity of the MAbs to their respective epitopes (Gee, Mansour and Weiler 1991; Myklebust, Pharo and Fodstad 1993). Smith *et al.* (1984) suggested that PK'X' primary cells may possess secretory material around the cell. How this may interfere with immunomagnetic extractions is not known. Cocktails of MAbs have been used to successfully remove cell populations with a corresponding increase in the amount of cells extracted compared to separations relying on one MAb (Vredenburgh and Ball 1990; Myklebust *et al.* 1993). The application of a cocktail of MAbs to remove PK'X' cells did not result in any significant improvement in

the cell separations. The results of this however should be viewed with caution as MAbs A3 and D4 may be specific for the same epitope (chapter 2).

The negative selection of PK'X' using MAb 4C10 resulted in the removal of PK'X' primary cells macrophages and lymphocytes from the cell suspension. The extracted parasites were covered with macrophages and lymphocytes suggesting their incidental extraction with these cells. Ultrastructural studies on PK'X' have demonstrated that the parasite is usually engulfed by one or two macrophages which in turn are closely associated with surrounding lymphocytes (Klontz *et al.* 1986; MacConnell *et al.* 1989). The association of these cells with PK'X' primary cells would inhibit antibody binding and subsequent extraction. To improve purification of PK'X' using immunomagnetic separation it is desirable to remove these cells from the parasites' surface. Attempts to remove the macrophages from the parasite in this study using trypsin and sonication were unsuccessful.

In parasitic infections such as *Leishmania*, where parasites are totally engulfed by macrophages, difficulties have occurred in obtaining pure suspensions, often with the free living parasite stages being targeted for study (Alexander and Russell 1993). Free living forms of PK'X' have been reported in the blood and renal sinuses early in the infection (Klontz *et al.* 1986; MacConnell *et al.* 1989). Targeting these stages with magnetic particles may improve separations by circumventing possible problems associated with adherent macrophages and the disintegration of the trophozoite primary cells. One drawback however would be a low yield due to the relative paucity of

parasites present. Intratubular sporogonic cells of the parasite, as described by Kent and Hedrick (1986), are also free of adherent macrophages allowing them to be more accessible for immunomagnetic separation.

Attempts to extract PK'X' sporogonic stages using MAb B4 did not directly remove the sporogonic parasite, however it did remove tubules containing these stages. Extraction of these tubules may indicate that the magnetic particles are binding onto secondary cells that are migrating through the tubule towards the tubule lumen. However, the magnetic particles do not appear to extract secondary cells or sporogonic cells that are free in the kidney suspension. Immunolocalisation studies on MAb B4 suggest that it is specific for a protein that is released from the secondary and sporogonic cells of the parasite (chapter 4). The extraction of tubules containing sporogonic stages may therefore indicate the presence of immobilised antigen amongst the kidney tubule cells. The ultrastructural studies also indicated the presence of antigen on the surface of the secondary cells, although this may represent residues of fused excretory vacuoles which may not be physically attached to the cell surface.

All of the extraction procedures investigated in this study resulted in the non-specific selection of macrophages from the kidney homogenate by the magnetic particles. These non-specific interactions were only partly alleviated by the blocking and sorting solutions used in the extractions. Fish macrophages have been reported to become inactive at low temperatures (Fryer, Pilcher, Sanders, Rohovec, Zinn, Groberg, and McCoy 1976) suggesting that interactions with particles are due to insufficient blocking

of non-specific binding sites. Magnetic cell separations are often hampered by non-specific binding although these interactions can be significantly reduced by the incubation of cells with the appropriate blocking reagents (Padmanabhan *et al.* 1988).

Comparison of the sorting and blocking solutions used in the MAb B4 extractions demonstrated that the sorting solution improved separations by limiting non specific interactions and reduced the size of the cell aggregates. The sorting solution however is not suitable for use with carbohydrate specific MAbs which bind to the earlier stages of the PK'X' organism, as it reduces carbohydrate epitopes and so further MAbs specific for protein antigens on PK'X' trophozoites need to be developed.

To further improve separations the sorting and blocking solutions need to be optimised. This may include the additions of a DNAase to reduce the effects of disrupted trophozoite primary cells and mouse sera or higher concentrations of goat sera to the solutions.

This study attempted to purify PK'X' cells using immunomagnetic separation and illustrate the potential of this technique for further separations (Adams and Morris 1994). The results obtained indicate that further work is needed to improve the separations. Factors identified as hampering extractions included non-specific binding of macrophages and lymphocytes, the possible extraction of disrupted cellular constituents and release of antigen from the target cell. Future work may avoid many such problems

by targeting extractions on early bloodstream stages and other forms of the parasite unassociated with cellular reactions such as intratubular sporogonic stages.

Chapter 4

Immunogold localisation of Monoclonal Antibodies

4.1 **Introduction**

The ultrastructural development of PK'X' in the kidney has been well documented (Ferguson and Adair 1977; Ferguson and Needham 1978; Seagrave *et al.*1980; Smith *et al.* 1984, Kent and Hedrick 1985a, 1986; Feist and Bucke 1987; MacConnell *et al.* 1989). Under low magnification the parasite appears to be 20μm in diameter with a highly vacuolated cytoplasm which has a granular appearance. Under higher magnification the cytoplasm contains smooth and rough ER, mitochondria, golgi apparatus, nucleus, multivesicular bodies, lysosomes, vacuoles, lipid droplets, free ribosomes, and distinctive electron dense bodies, which characterise the parasites primary cell (Seagrave *et al.* 1980; Lom, Feist, Dykova and Kepr 1989). The electron dense bodies are membrane bound spheroids, uniformly 160nm in diameter, with an electron lucent bar extending into them, lined by an infolding of the bodies delimiting membrane. The bodies are often associated with the plasmalemma to which the electron lucent bar is orientated at 90° (Ferguson and Needham 1978; Seagrave *et al.* 1980). The plasmalemma is electron dense, has an associated glycocalyx and appears highly folded (Smith *et al.* 1984; Ellis *et al.* 1985; MacConnell *et al.* 1989) In some PK'X' cells the cytoplasm immediately under the plasma membrane appears to be free of organelles except for the electron dense bodies (Ferguson and Needham 1978; Smith *et al.* 1984). Rafferty (1986) reported the possible presence of actin filaments within this layer.

The primary cell of the extrasporogonic parasite often contains one or more inclusion bodies referred to as secondary or daughter cells which may in turn contain tertiary cells

within them. These inclusion cells contain mitochondria, few vacuoles, nucleus and are delimited from the primary cell's cytoplasm by a enveloping vacuole (Seagrave *et al.*1980; Kent and Hedrick 1986). Secondary and tertiary cells are usually devoid of the electron dense bodies although their presence has been reported contained in vacuoles within them (Rafferty 1986). The tertiary cells are believed to arise by one secondary cell enveloping another secondary cell within the primary cell. The tertiary cell is speculated to divide to produce the constituent parts of the mature spore (Feist and Bucke 1987). However, complete sporogenesis within rainbow trout has not been observed (Kent and Hedrick 1986; Feist and Bucke 1987; MacConnell *et al.* 1989).

Immunogold localisation using monoclonal antibodies has become a standard immunological technique (Vandell and Polak 1987). Ultrastructural studies using immunogold labelling require that the maximum number of antigenic sites are exposed to the antibodies whilst retaining the clearest possible structural definition. However, fixation and embedding of tissues for routine electron microscopy often results in a marked reduction of antigenicity. This is due to the properties of the antigens, fixatives and resins used. Because of this there is not a standard procedure for the immunogold localisation of antigens. However, there are guidelines for successful localisation studies. These include the omission of osmium tetroxide as a secondary fixative, because it masks antigenic sites, the replacement of gluteraldehyde with paraformaldehyde, and the use of cryosections or acrylic resins instead of epoxy resins, for heat sensitive antigens. Paradoxically, retaining antigenicity often results in poorer fixation and so a compromise

has to be met to retain as much immunoreactivity as possible without sacrificing structural definition.

For further investigations on the parasite using the MAbs decribed in chapter 2 it was desirable to identify the structures within the PK'X' cells to which they were specific.

Previous immunogold investigations of a myxosporean parasite used Spurrs resin to investigate the immunolocalisation of antigen on *Ceratomyxa shasta* (Bartholomew, Yamamoto, Rohovec and Fryer 1990). Spurrs resin however is highly toxic and so the study presented here investigated the use of the acrylic resin LR white (London Resin Company) as a possible alternative for the localisation of MAb binding sites. Because immunohistochemistry (section 2.3.5) required the tissues to be exposed to temperatures exceeding 60°C without apparent destruction of antigenicity, araldite was also investigated as a possible alternative to LR white.

4.2 **Objectives**

The objective of this chapter was to investigate the location of the antigens to which the MAbs bind within the PK'X' cell at a ultrastructural level using immunogold electron microscopy.

4.3 **Materials and Methods**

4.3.1 *Inoculation of Fish with PKD*

Twenty fingerling rainbow trout (~20grams) obtained from a farm with no record of PKD were maintained in two 150 litre plastic tanks supplied with dechlorinated tap water at a constant 18°C. Ten of the fish were injected intraperitoneally with 0.2 ml of PK'X' infected kidney homogenate as described by Clifton-Hadley, Richards and Bucke (1984b). Briefly, kidney homogenate was prepared as described in section 2.3.1 and placed into a 1ml disposable syringe connected to a 21g needle. A saturated solution of benzocaine dissolved in ethanol was added to water to give a final concentration of 25ppm benzocaine (Roberts and Shepherd 1986). Ten fish were individually placed into this solution until they were anaesthetised. After an intraperitoneal injection of 0.2ml of the kidney homogenate the fish were placed into vigorously aerated water until they recovered. The remaining ten fish were injected intraperitoneally with 0.2ml of sterile PBS to act as controls.

Ten weeks post-injection, the fish were killed with a sharp blow to the head, immediately followed by destruction of the brain. The kidneys were excised, blotted dry on a clean paper towel and briefly placed onto a clean slide to form a kidney impression smear. The slide was allowed to air dry before being stained using Rapi-diff™ and examined for PK'X' infection under bright field using an Olympus C-10 microscope

X200 magnification. Kidneys resulting in impression smears that had more than five PK'X' parasites per visual field were selected for embedding.

4.3.2 *Embedding of Infected Kidneys*

The kidneys were fixed for immunogold labelling following the procedure of Dr I. M. Roberts (Invergowerie plant research, Dundee. pers. comm.). The kidneys were cut into $0.5mm^3$ cubes and fixed overnight, at 4°C, in 5% gluteraldehyde/ PIPES buffer (PIPES buffer: 200mM N'N' bis[2- Ethanesulphonic acid], pH 5.5 adjusted with 0.1M NaOH). They were rinsed in PIPES buffer three times followed by 2 washes in distilled water. The tissue was dehydrated through a graded alcohol series and brought to 100% propylene oxide and then infiltrated with 1:1 propylene oxide/ araldite for 24 hours followed by a further 24 hours in araldite, before polymerisation overnight in a 70°C oven. All araldite contained the accelerator BDMA.

Tissues to be embedded in LR white were fixed following the above method whilst infiltration and embedding followed the procedure of LR white for Electron Microscopic Immunocytochemistry (LR white product instruction manual). Briefly, following fixation the kidney tissue was dehydrated to 70% alcohol before a 1 hour infiltration with 70% resin in absolute alcohol followed by two 1 hour rinses in 100% resin. The tissue was embedded in resin in gelatin capsules with overnight polymerisation in a 60°C oven.

All infiltration steps for araldite and LR white were performed on a varispeed rotator. Ultra-thin sections of the blocks obtained for immunogold localisation were cut at 70nm and mounted on nickel grids.

Samples of infected kidney were also processed for routine electron microscopy for comparison with the unosmicated tissues. Briefly 0.5mm^3 cubes of infected kidney were fixed in Karnovskys fixative for 4 hours and rinsed in cacodylate buffer (pH 7.2) overnight. They were then postfixed in 1% Osmium textroxide, dehydrated through an acetone, alcohol series, embedded in araldite, sections cut at 70nm on copper grids and stained using 2% uranyl acetate and lead citrate (Watson 1958; Venable and Coggeshall 1965).

4.3.3 *Immunolabelling of Unosmicated Sections*

Immunolabelling of sections was performed using a modified procedure of Biocell technical information manual (Biocell Research limited). All incubations were performed at 4°C in covered petri dishes containing moist filter paper under a piece of nescofilm for the placement of buffers and grids.

The grids were floated section down on drops of wash buffer (TBS, 1% Tween-20, 1% w/v BSA) containing 10% FCS overnight. They were transferred to drops of hybridoma supernatant and again incubated overnight. The sections were washed by floating the grids on wash buffer contained within 24 well tissue culture plate wells (Corning) with

occasional agitation for 90 minutes followed by a change in buffer and another 90 minute wash. Two gold probe conjugates were used in the study, 5nm goat anti-mouse IgG and 10nm goat anti-mouse IgM (Sigma). The optimal dilution of gold probe was determined by incubating sections with varying dilutions (1/5, 1/10, 1/20, 1/40, 1/80) of the gold conjugates overnight. The optimum dilution for this study was a dilution of 1/40 in wash buffer for both the 5nm and 10nm gold probe. After extensive washing the 5nm probe labelling was enhanced by incubating for 2 minutes with a silver enhancing kit (Biocell). After washing as previously described the grids were held under a stream of distilled water, counterstained using uranyl acetate and lead citrate, and viewed under a Phillips 201 electron microscope at 80kv. Sections to be examined using the protein specific MAb B4 were pre-treated by floating on 20mM periodic acid for 10 minutes, briefly washed under distilled water, and incubated as previously described.

4.4 **Results**

4.4.1 *Parasite Ultrastructure*

Electron microscopy revealed the typical features of the parasite as previously documented (Ferguson & Needham 1978; Seagrave *et al.*1980; Smith *et al.* 1984; Kent and Hedrick 1986; Rafferty 1986). These included the nucleus, nucleolus, multivesicular bodies (MVBs), lysosomes, vacuoles, golgi apparatus, ER and RER, mitochondria, lipid reserves, and electron dense bodies (figure 4.1). The genesis of electron dense bodies on the *trans*-golgi face (figure 4.2) and occasional 'tail' forms were also noted (figure 4.3).

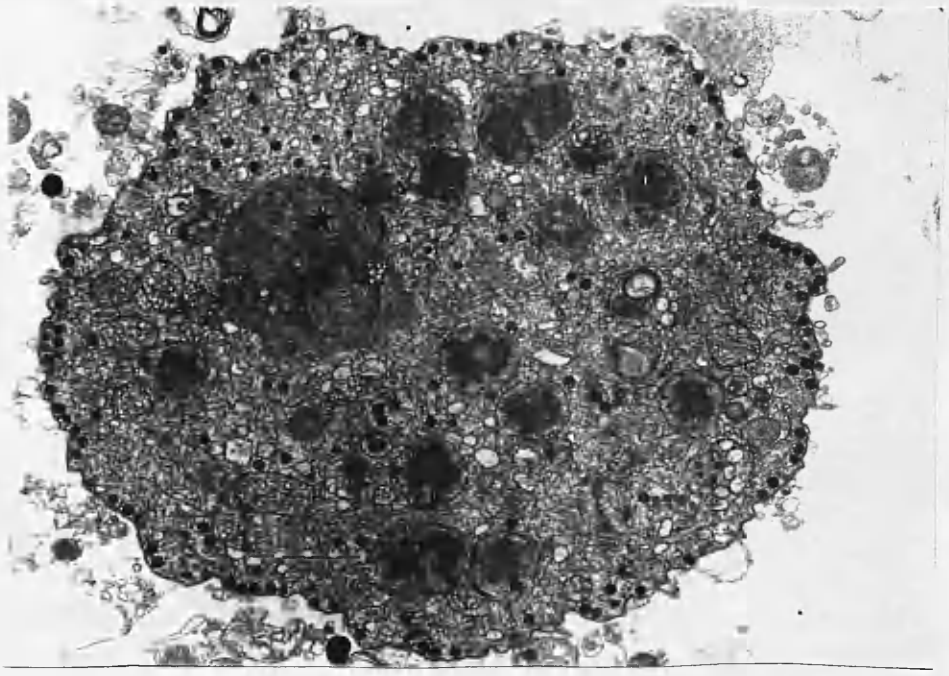


Figure 4.1.
Typical PK'X' parasite within rainbow trout kidney interstitium as viewed with electron microscopy. Identifying features include numerous 'electron dense bodies' associated with the periphery of the primary cell and formation of one or more intracellular secondary cells (indicated with *).
Araldite, post fixed with Osmium tetroxide. Mag X 11,000

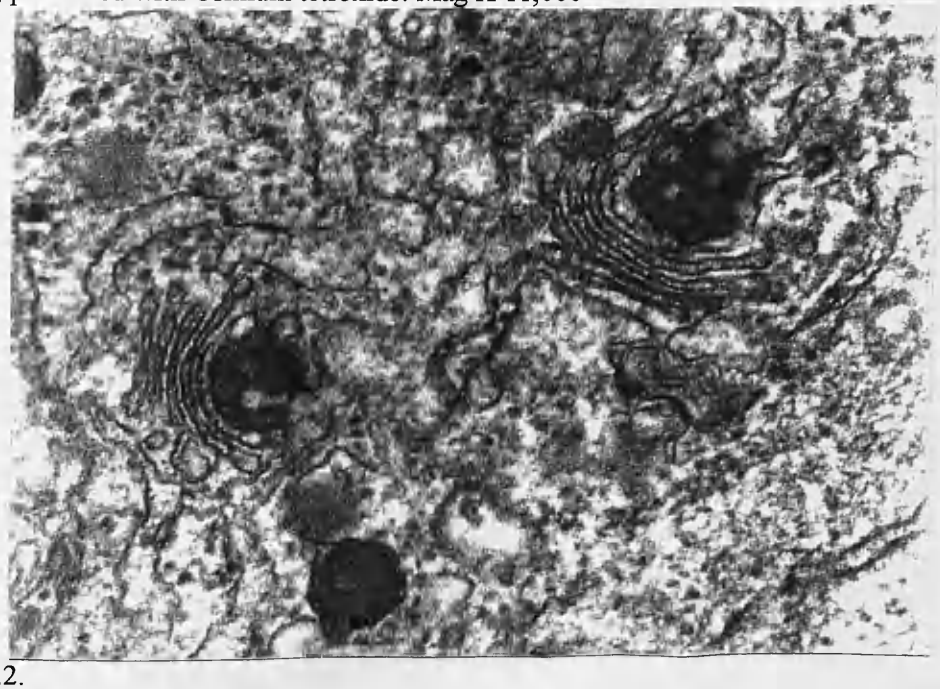


Figure 4.2.
Formation of electron dense bodies on the *trans*-golgi face of the golgi apparatus within the primary cell of PK'X'.
Araldite, postfixed with Osmium tetroxide. Mag X 125,000

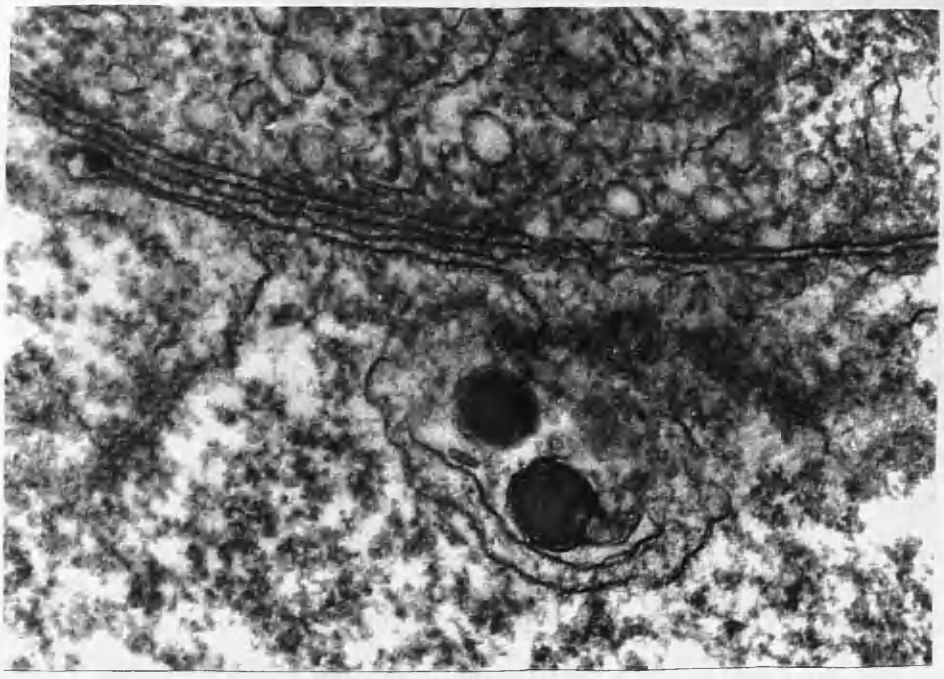


Figure 4.3.
'Tail' form of electron dense body present within the cytoplasm of the PK'X' primary cell. Araldite, post fixed with Osmium tetroxide. Mag X 125,000

Evidence of fusion of electron dense bodies to the plasma membrane was only noted once (figure 4.4). Secondary cells within primary cells were contained within a tightly fitting vacuole. Tertiary cells when present within the secondary cells appeared electron dense and contained a nucleus and tightly packed mitochondria. Fusiform crystals as described by Clifton-Hadley *et al.* (1985) were observed in the interstitium, occasionally engulfed by macrophages.

Features not previously described ultrastructurally included the endocytosis of the primary cell by secondary cells exemplified by the presence of electron dense bodies inside secondary cell phagosomes (figure 4.5). Electron dense bodies were also observed apparently being actively engulfed by MVBs (figure 4.6) within the primary cell. These bodies were also observed within maturing MVBs (figure 4.7). Unosmicated tissues indicated that some electron dense bodies possessed an electron lucent 'T' instead of an electron lucent bar (figure 4.8). There appeared to be two membranes associated with some of the electron dense bodies. These were the surrounding outer membrane which also lined the electron lucent bar whilst a distinct membrane appeared to cap the bar (figure 4.9). A previously undescribed structure was noted that appeared to be closely associated with the electron dense bodies. This resembled a bundle of microtubules, 80nm across and 240nm long (figure 4.10). However this was only observed once and may represent an infolding of the plasma membrane.

Often the primary cell of the PK'X' parasites was totally engulfed within a macrophage. These parasites appeared to be morphologically identical to free parasites within the

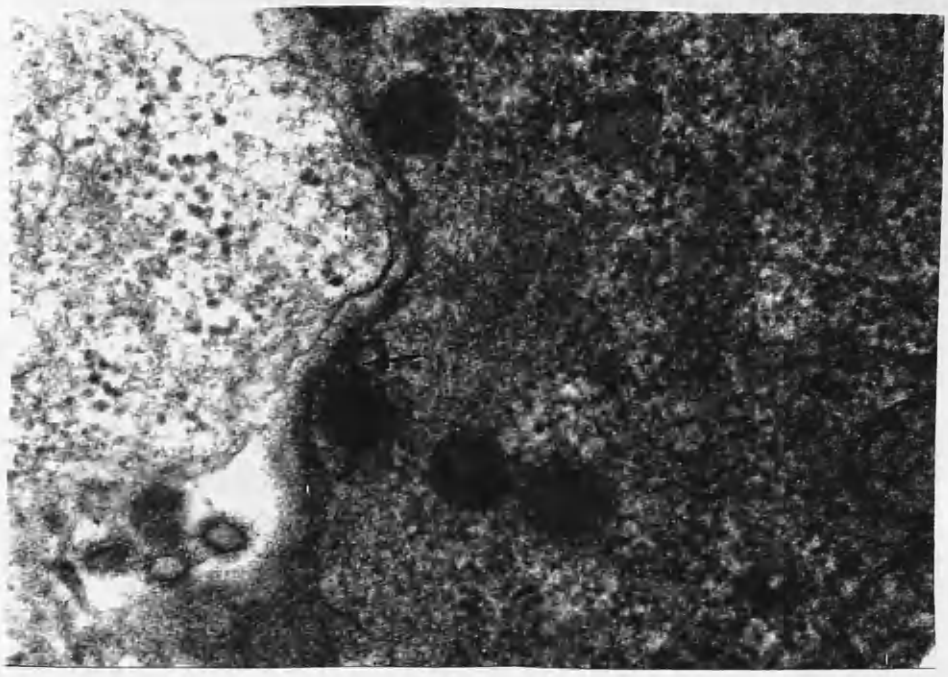


Figure 4.4.
Apparent fusion of electron dense body's electron lucent bar with the plasma membrane of the PK'X' primary cell (arrow).
Araldite post fixed with Osmium tetroxide.Mag X 81,220

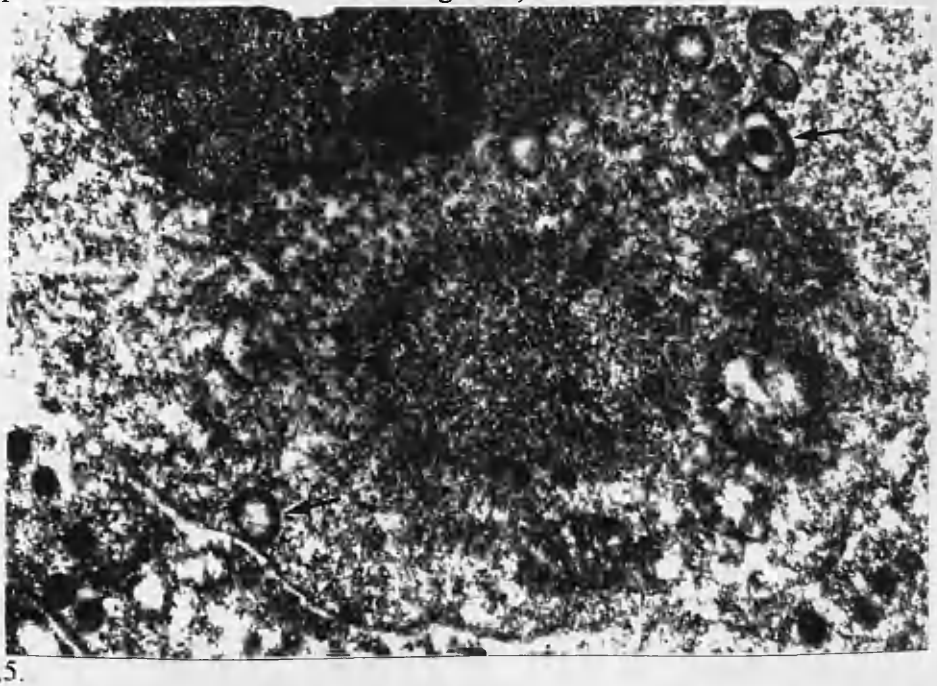


Figure 4.5.
Endocytosis of primary cell by secondary cell. Arrows indicate the formation of a endocytotic vacuole by the secondary cell and the presence of a 'electron dense body' contained within a internalised vacuole within this cell.
LR White, etched with periodic acid and immunostained with MAb B4. Mag X 36,140

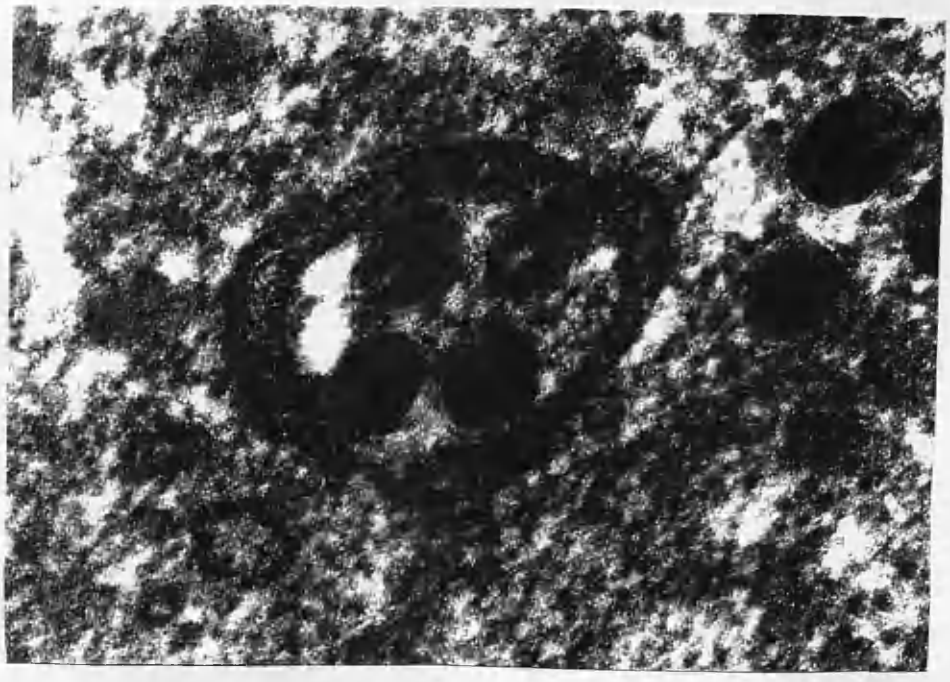


Figure 4.6.
Active engulfment of 'electron dense bodies' by multivesicular body within the primary cell of PK'X'.
Araldite post fixed with Osmium tetroxide. Mag X 125,730

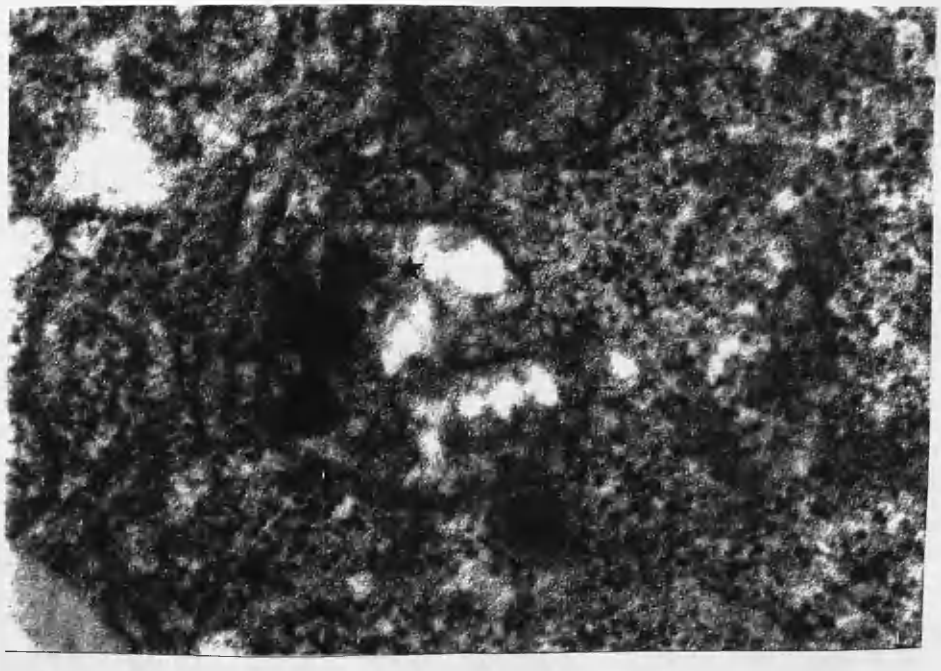


Figure 4.7.
Presence of 'electron dense bodies' contained within maturing multivesicular body (indicated with *).
Araldite post fixed with Osmium tetroxide. Mag X 95,620

Figure 4.8.
Electron lucent 'T' contained within occasional unosmicated 'electron dense bodies'.
LR white, etched with periodic acid, immunostained with MAb B4. X102,830

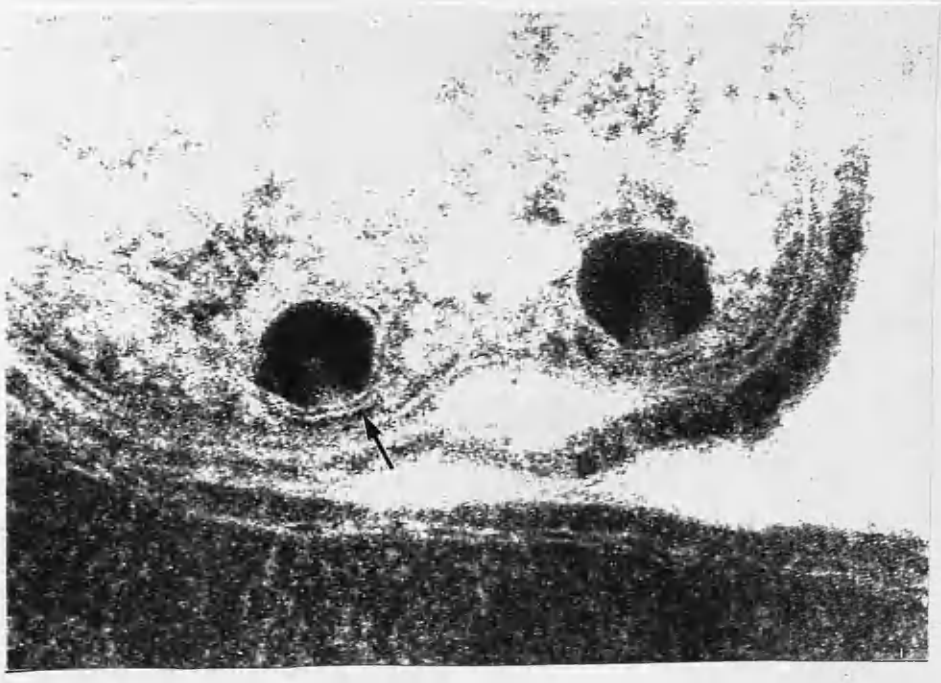


Figure 4.9.
'Capping Membrane' associated with electron dense bodies (arrow).
LR white, immunostained with MAb C5. Mag X 132,750

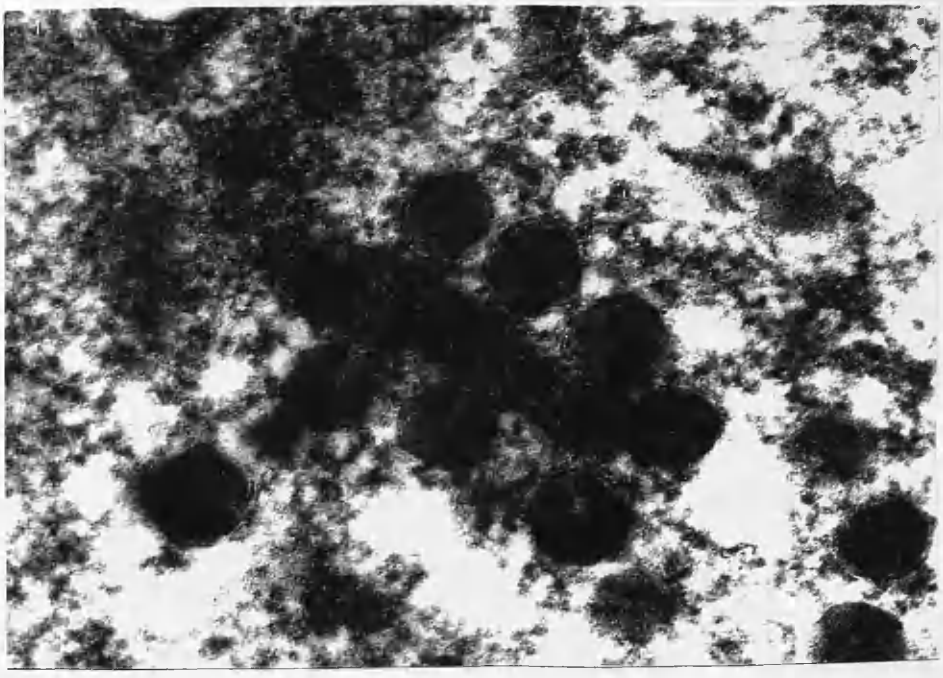


Figure 4.10.
Unidentified structure demonstrating a strong affinity with the 'electron dense bodies' contained within the PK'X' primary cell.
Araldite post fixed with Osmium tetroxide. Mag X 103,840

interstitium. Disintegrating primary cells, releasing secondary cells into the immediate environment apparently resulted in the detachment of the surrounding macrophages (figure 4.11). Ruptured primary cell cellular constituents, free in the kidney interstitium, were observed to be actively phagocytosed by macrophages (figure 4.12).

4.4.2 *Comparison of Araldite and LR white resins*

Both of the resins investigated enabled the immunogold labelling of PK'X' using all of the MAbs produced in chapter 2. Non-specific interactions with the probes and the sections was minimal with occasional probes binding onto the nucleus of host cells. In terms of immunoreactivity, preservation of structural integrity and ease of use LR white was superior to araldite. However araldite did appear to be slightly more beam stable at 80kv.

4.4.3 *Carbohydrate Probe Localisation*

The carbohydrate probe staining determined that all of these MAbs were specific for the same structures in the primary cell. These were primarily the lysosomal and vacuolar membranes, including the membrane enclosing the secondary cell, although labelling also occurred on the lysosomal contents, plasma membrane and sporadically throughout the cytoplasm (figures 4.13, 4.14). Areas of interstitium containing ruptured primary cell contents, exemplified by the presence of free electron dense bodies were also recognised by the probes (figure 4.15). The membranes associated with the electron dense bodies

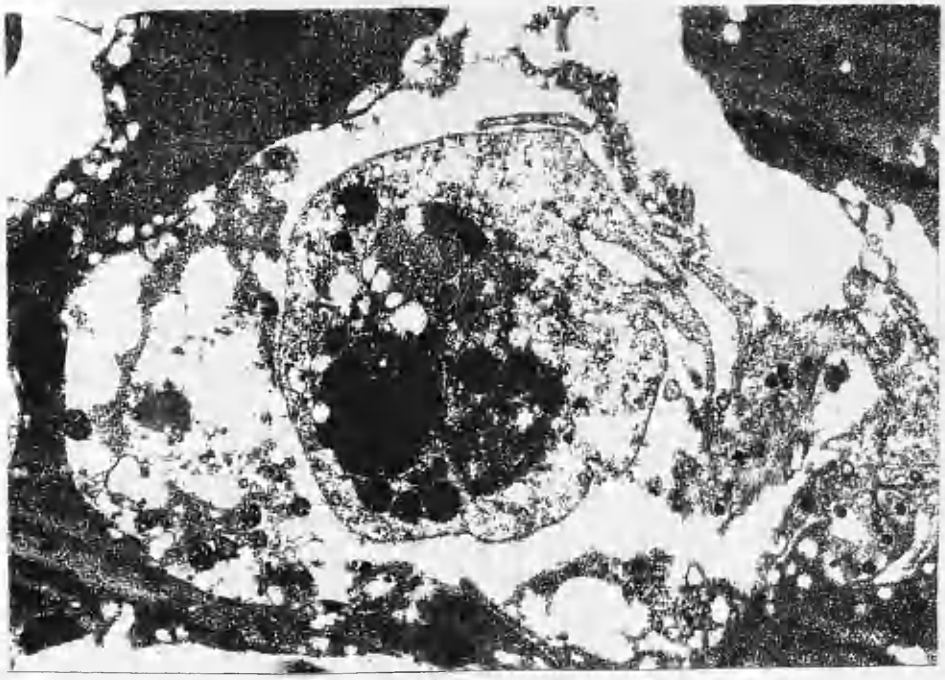


Figure 4.11.
Rupturing primary cell releasing secondary cell into kidney interstitium. This process appears to aid in the detachment of adherent macrophages associated with the parasite (top of picture).
LR white, etched with periodic acid, immunostained with MAb B4. Mag X 18,270

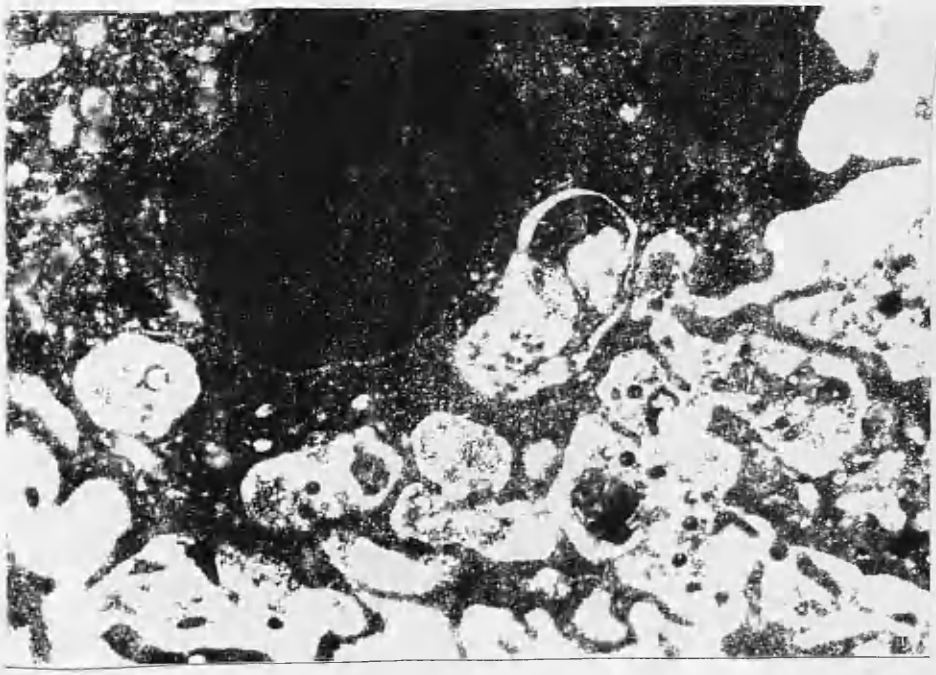


Figure 4.12.
Active phagocytosis of PK'X' primary cell constituents, exemplified by the presence of 'electron dense bodies', free in the kidney interstitium.
LR white, etched with periodic acid, immunostained with MAb B4. Mag X 20,730

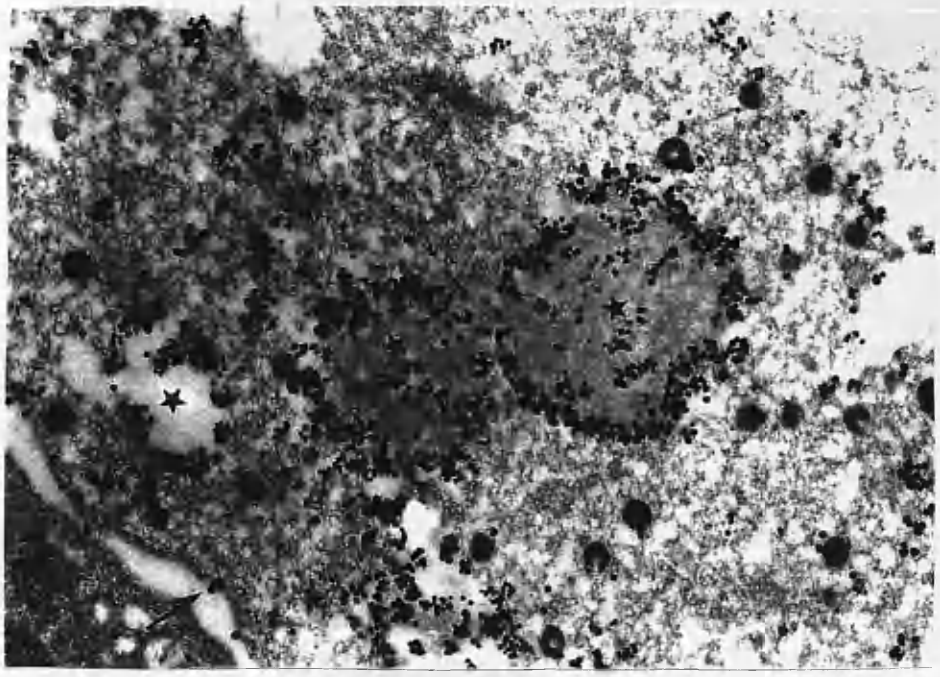


Figure 4.13.
Immunolocalisation of carbohydrate binding MAbs within the PK'X' primary cell. Lysosomal and vacuolar membranes (indicated with *) show strong affinity for the MAbs. Arrow indicates MAb binding with plasma membrane.
LR white, immunostained with MAb C5, silver enhanced. Mag X 37,200

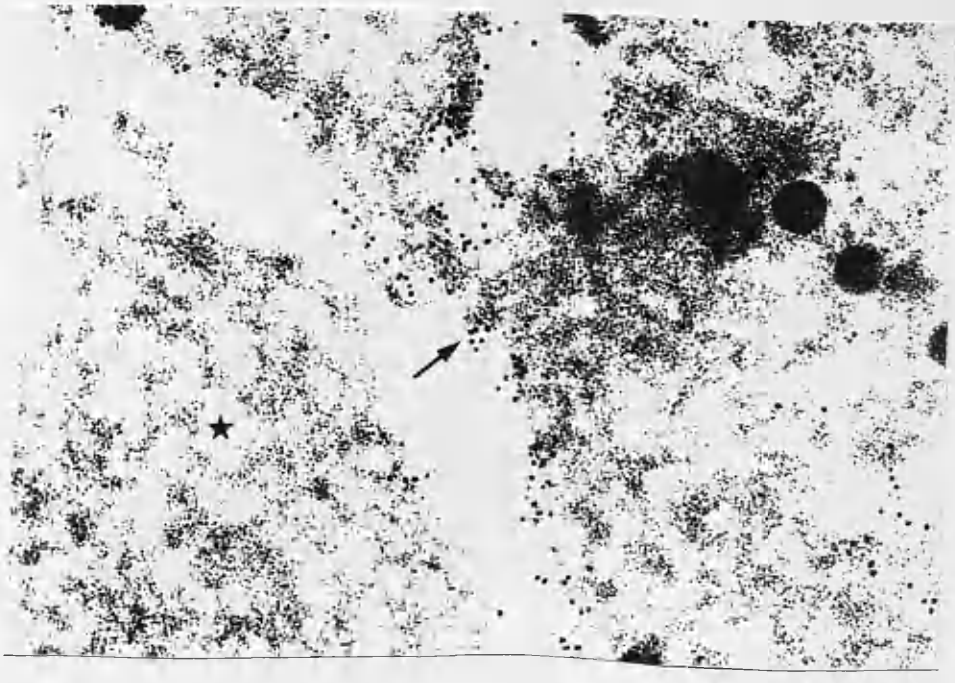


Figure 4.14.
Immunolocalisation of carbohydrate binding MAbs on vacuolar membrane surrounding the secondary cell (arrow indicates immunolabelling, secondary cell indicated with *).
LR white, immunostained with MAb A3. Mag X 57,100

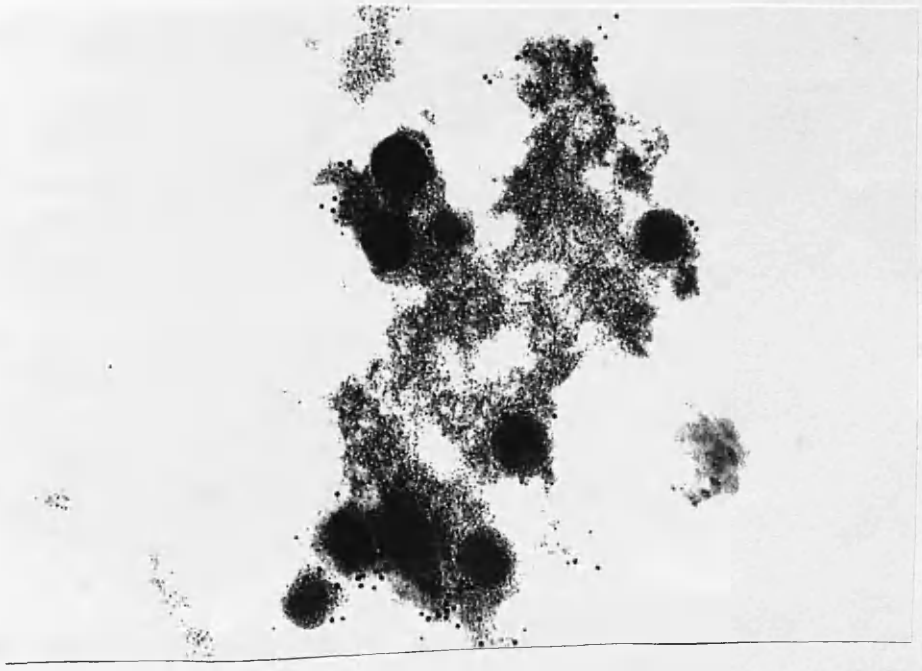


Figure 4.15.
Ruptured primary cell constituents free in kidney interstitium demonstrating immunoreactivity with carbohydrate binding MAbs.
LR white immunostained with MAb D4. Mag X 73,090

were also labelled with the carbohydrate MAbs. The immunogold localisation on the electron dense bodies was generally light although the membrane capping the electron lucent bar of some bodies were heavily targeted by the probes (figure 4.16).

The localisation of carbohydrate MAbs on the secondary cells revealed that there was little binding to the plasma membrane and areas of localised binding within the cell (figure 4.17). These localised areas were thought to represent endocytosed primary cell antigen contained within the secondary cell.

4.4.4 *Protein Probe Localisation*

MAb B4 was specific primarily for the secondary cell's plasma membrane, occasional vacuoles within it and the immediate extracellular environment. Labelled secondary cells were observed free in the kidney interstitium, often surrounded by the remnants of a ruptured primary cell (figure 4.18). On development of tertiary cells within the secondary cell the labelling of MAb B4 became more diffuse throughout the secondary cells with these cells becoming increasingly vacuolated and disrupted (figure 4.19). Tertiary cells did not appear to bind with any of the MAb probes. Primary cells, containing labelled secondary cells, also bound with the probe although this binding was not localised within the cell.Some secondary cells without tertiary cells did not bind with the probe although structurally they appeared identical. Macrophages were occasionally observed, containing engulfed secondary cells which appeared to be disintegrating as determined by probes binding onto these phagocytosed cells (figure 4.20).

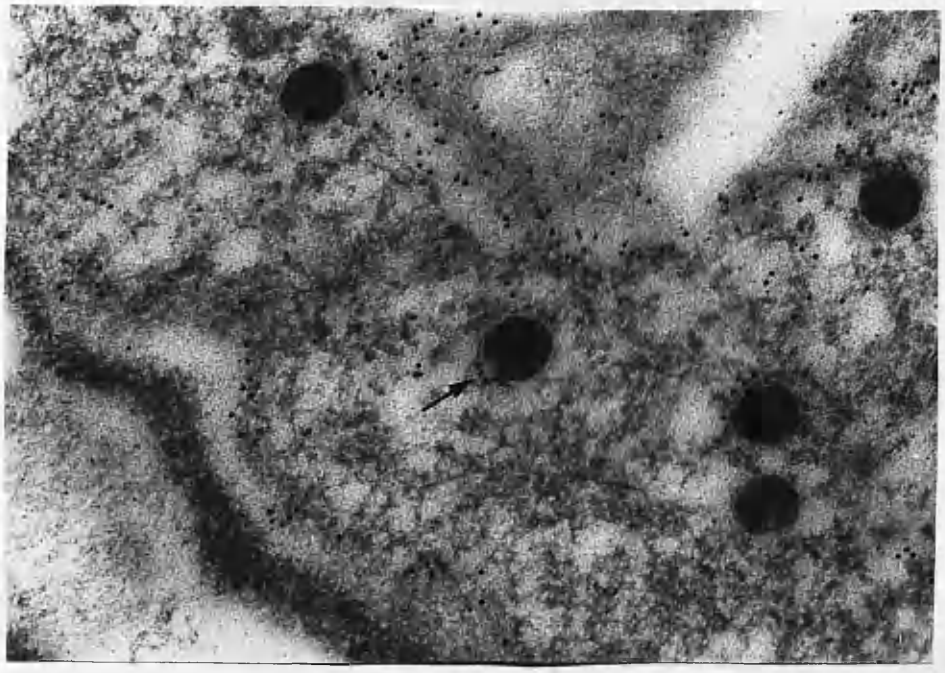


Figure 4.16.
Immunolabelling of capping membrane of electron dense bodies (arrow) in primary cell of parasite.
LR white, immunostained with MAb A3. Mag X 74,700

Figure 4.17
Localisation of primary cell carbohydrate antigen within secondary cell (indicated with *).
LR white, immunostained with MAb A3. Mag X 74,940

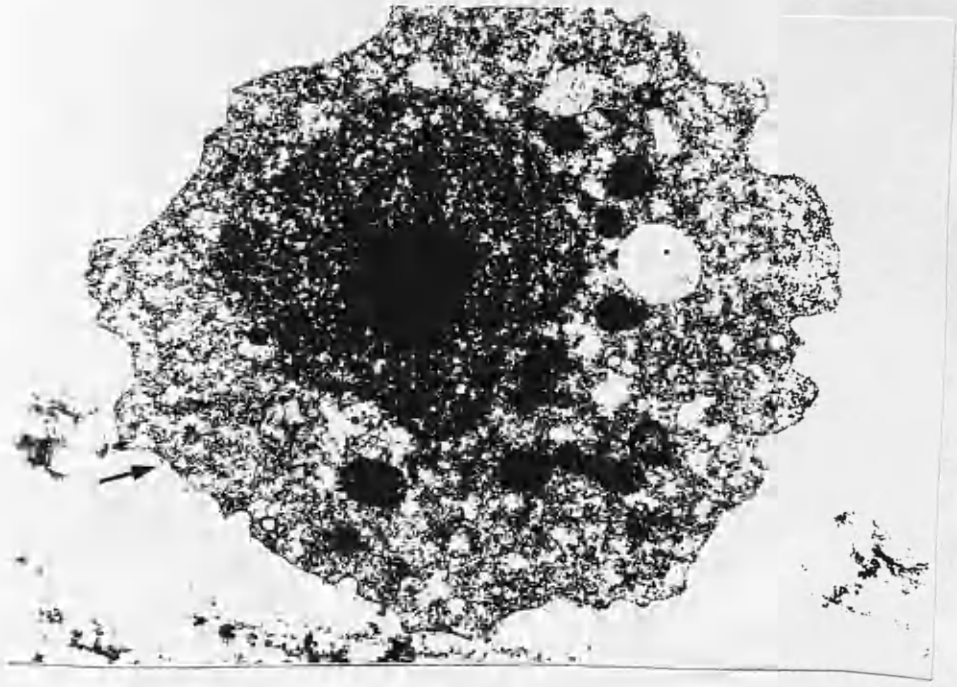


Figure 4.18.
Secondary cell free in kidney interstitium. Arrows indicates immunolocalisation of MAb B4 in vacuoles and associated with the cells surface.
Araldite, etched with periodic acid, immunostained with MAb B4. Mag X 21,875

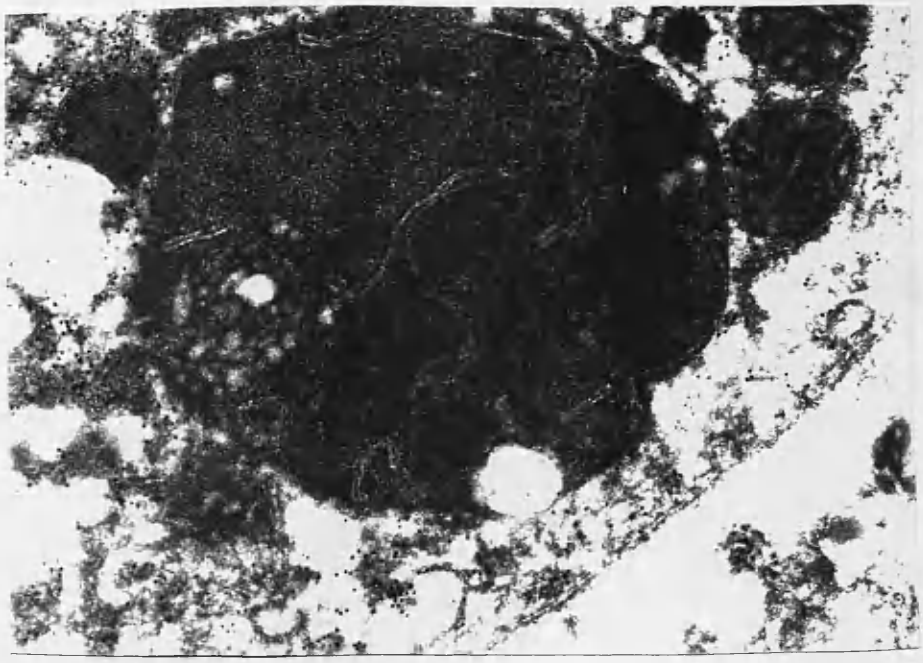


Figure 4.19.
Maturation of secondary cell with condensed tertiary cell containing tightly packed mitochondria showing dispersed immunostaining throughout the secondary cell.
LR white, etched with periodic acid, immunostained with MAb B4. Mag X 57,310

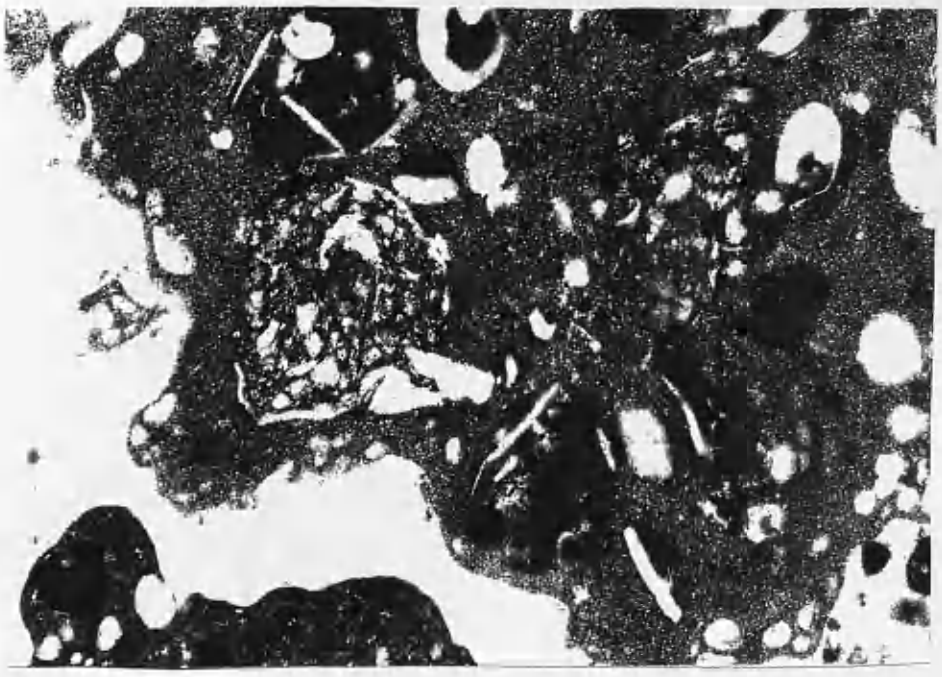


Figure 4.20.
Engulfment of secondary cell by macrophage as determined by immunogold localisation of MAb B4 binding antigen (indicated with *)
LR white, etched with periodic acid, immunostained with MAb B4. Mag X 19,130

Figure 4.21.
Necrotic, electron dense, structure located in tubule lumen. This structure demonstrates a strong affinity for MAb B4 and may represent a necrotic sporogonic form of the parasite.
Araldite, etched with periodic acid, immunostained with MAb B4. Mag X 41,160

Complex electron dense structures specific for MAb B4 were observed in the interstitium and tubule lumens. These were thought to represent necrotic sporogonic stages of the parasite (figure 4.21). No maturing sporogonic stages as described by Kent and Hedrick (1985a) were observed within this study.

4.5 **Discussion**

The results obtained from the immunogold localisation studies of the MAbs indicated that they recognise stage specific antigens produced by the parasite. The carbohydrate specific MAbs A3, C5 and D4 all appeared to bind to the same structures within the primary cell of the parasite whereas MAb B4 was associated with the parasite's secondary cell. The western blotting analysis and double immunolabelling studies described in chapter 2 suggested that MAb A3 and D4 were to the same antigens whereas MAb C5 reacted to only a proportion of these antigens. The results from this study further confirm a relationship between these MAbs.

The carbohydrate probes' localisation within the primary cell of the parasite was principally on the lysosomal and vacuolar membranes. This suggests that the vacuoles are related to lysosomes and could represent compartments connected with degradative events. Lysosomal membranes have been shown to be particularly rich in heavily glycosylated membrane proteins, referred to as LAMPs, across a range of vertebrates (Fukuda 1991; Kornfeld and Mellman 1989). Protozoans, such as trypanosomes, have also been shown to possess heavily glycosylated lysosomal membrane proteins and it is

suggested that these molecules play an important role in the physiology of all eucaryotic cells (Brickman and Balber 1993). The localisation of the carbohydrate probes within this study demonstrates that the lysosomal membranes of PK'X' are rich in carbohydrates and these molecules may be related to the lysosomal glycoproteins of other eucaryotes. The relatively small amount of carbohydrate localisation on the plasma membrane of the parasite is consistent with the distribution of the LAMP proteins within vertebrates and the lysosomal glycoproteins of trypanosomes (Fukuda 1991; Brickman and Balber 1993). The relative abundance of antigen on the vacuolar membrane surrounding the secondary cell suggests that this delimiting membrane may be more related to a modified lysosomal compartment than the plasma membrane. As such, it may serve to provide the developing secondary cell with a distinct physiological environment in which to mature.

The carbohydrate probes were observed to bind to the membranes of the electron dense bodies. Structures related to these bodies occur throughout the Myxozoa (Lom *et al.* 1989). The electron dense bodies have been compared to the haplosporosomes found in members of the Haplosporidea and it has been implied that this phyla be grouped in a common taxon with the Myxosporea (Seagrave *et al.* 1980; Desportes and Ginsburger-Vogel 1977, as cited by Kent and Hedrick 1985). Lom *et al.* (1989) suggested that an important difference between the electron dense bodies and the haplosporosomes may be the negative Théiry reaction elicited by the electron dense bodies within PK'X'. However, the results from this study indicate that glycoconjugates do occur on the membranes surrounding the electron dense bodies and this is consistent with the

localisation of carbohydrates on haplosporosomes (Azevedo and Corral 1985). Seagrave *et al.* (1980) indicated that the electron lucent bar within the electron dense bodies was lined by the membrane surrounding the bodies. Hoffmann and El-Matbouli (1994) suggested that this was a distinction between the electron dense bodies and haplosporosomes. However, the presence of an additional membrane capping the electron lucent bar had not been previously described. Although this capping membrane did not always appear as a discrete membrane on all of the bodies studied, the majority of them did possess it. The presence of this membrane suggests that the electron lucent bar represents a membrane bound vesicle within an electron dense core and as such the relationship between these bodies and haplosporosomes should not be discounted.

The purpose of the electron dense bodies remains unclear. Their genesis within the primary cell was attributed by Smith *et al.* (1984) to the *trans*-Golgi face and a 'vesicular entity' which appeared to be packaging them. Although apparently morphologically dissimilar these authors did not rule out the possibility of these two structures being part of the same production system. Rafferty (1986) also noted the presence of the 'vesicular entities' within the primary cell. Such 'vesicular entities' were not encountered within this study although the genesis of electron dense bodies was often observed on the *trans*-Golgi face of the Golgi apparatus. The vesicular entity may represent part of a *trans*-Golgi network (TGN), viewed face on, and appears to be morphologically identical to such structures described in other cell types (Teichberg and Holzmann 1973). The function of the TGN is believed to be for the production of membrane delimited carrier

structures that transport materials across the cell, such as lysosomal enzymes, secretory substances and material destined for the cell surface (Holzmann 1989).

LAMP proteins in vertebrates are thought to protect lysosomal membranes from enzymatic attack and provide ligand structures for cell-adhesive molecules on the cell surface (Fukuda 1991). The possible localisation of lysosomal carbohydrate conjugates, as determined by immunogold labelling, on the membranes associated with the electron dense bodies, particularly the capping membrane, implies that this membrane is involved in the adhesion and orientation of the bodies to the plasmalemma. Once the body is correctly orientated the capping membrane may also allow for the active transport of materials, such as enzymes, between the electron lucent vesicle and the surface of the parasite. The electron dense bodies have previously been described as fusing with the plasma membrane to release their contents into the extracellular environment (Ferguson and Needham 1978; Smith *et al.* 1984; Rafferty 1986). However, this was not conclusively observed within this study and suggests that if fusions do occur then they are either infrequent or extremely rapid. The presence of 'tail forms' of the electron dense bodies suggests that the electron lucent bar can be everted by the body. Upon removal of the capping membrane the electron lucent bar may evert to fuse with the plasmalemma. This presumably would allow the contents of the electron dense body to be ejected by the parasite into its immediate environment.

The observation of the active engulfment of the electron dense bodies by cup like structures and their appearance within MVBs suggests that they enter the lysosomal

pathway to be degraded by the cell. However, whether this could be attributed to crinophagy, autophagy or for the reduction of materials collected from the cell surface was not determined.

The carbohydrate probes' localisation within secondary cells indicates that these cells do not produce the antigens endogenously but acquire them from the primary cell through endocytosis. This suggests that the composition of PK'X' lysosomal membranes are stage specific to the primary cell of the parasite. The expression of different lysosomal membrane glycoproteins at different developmental stages has been reported for both trypanosomes and vertebrates (Carlsson, Roth, Piller and Fukuda 1988; Lee, Wang and Fukuda 1990; Brickman and Balber 1993). Changes in the antigenic characteristics of a Myxosporean parasite throughout its life cycle are not unique to PK'X' and have been reported for *C. shasta* (Bartholomew *et al.* 1989). Many other parasitic diseases are documented as possessing stage specific antigens and this may be due to the different environments that their alternating life stages encounter (Brickman and Balber 1993).

The immunogold localisation of MAb B4 demonstrates the presence of a protein that is produced and released by the secondary cell, upon its maturation within the primary cell of the parasite, into its immediate environment. The initial localisation within the secondary cell appears to be within secretory vacuoles and as the cell develops a tertiary cell within it, so production of the protein increases. The localisation of the probe in the immediate environment around the secondary cell indicates that the protein may become denatured shortly after its release from the cell. Proteins released by myxosporeans into

the extracellular environment have previously been described for *M. cerebralis* and *Kudoa* spp. Only proteolytic enzymes have been characterised with these enzymes being produced by the maturing spores (Bilinski, Boyce, Jonas and Peters 1984; Stehr and Whitaker 1986; Hamilton and Canning 1988; Whitaker and Kent 1991). The function of the protein in this study remains unclear though a proteolytic function, perhaps to aid the release and migration of the cell to the tubule lumen cannot be ruled out.

Necrotic electron dense structures were found in the kidney tubules and interstitium which were highly specific for MAb B4 and were thought to represent abortive sporogonic stages. Kent and Hedrick (1986) reported the presence of sporoblastic cells and the incomplete development of polar capsules within rainbow trout tubule lumens. No such stages were encountered within this study although this could be attributed to the sections studied. The inflammatory response caused by the PK'X' myxosporean and the absence of viable spores suggest that rainbow trout are not the definitive host for the parasite (Seagrave *et al.* 1980; Kent and Hedrick 1986; Feist and Bucke 1987; MacConnell *et al.* 1989). The presence of necrotic structures specific for MAb B4 within the kidney interstitium and tubules observed in this study supports this theory.

Angelidis *et al.* (1987) suggested that PK'X' cells are able to suppress the immune response elicited by the adherent macrophages against them. Manipulations of enveloping macrophages have been recorded for other parasites such as *Leishmania spp.* (Alexander and Russell 1993). Presumably such manipulations of enveloping macrophages in fish would be elicited by the intact primary cells of the parasite.

MacConnell *et al.* (1989) reported that although macrophages were closely associated with the PK'X' parasite early on in infection, effective elimination of the cell by fish did not appear to occur until the release of secondary cells. Rafferty (1986) noted the presence of free electron dense bodies in the kidney interstitium and postulated that these bodies may be derived from degenerated primary cells. Rupturing primary cells releasing cellular constituents along with secondary cells into the kidney interstitium were noted in this study confirming Rafferty's premise. The immunolocalisation of the carbohydrate probes on PK'X' cell fragments within the kidney interstitium indicate that these cell constituents are antigenic. The observation of secondary cells disintegrating within macrophage phagosomes and the active phagocytosis of ruptured PK'X' cell constituents implies that these structures are highly immunogenic. Kent and Hedrick (1986) described the disintegration of the primary cell as an integral part of the developmental cycle of the parasite allowing for the release and migration of secondary cells to the tubule lumens. Myxosporeans, generally, have been documented as involving the disintegration of primary cells for the release secondary cells as common facets of their life cycles (Lom 1987). It may be that the disintegration of the primary cell in the interstitium occurs prior to the cells' migration to the tubule lumen. This may be caused by the movements of the parasite being restricted by the inflammation associated with the disease (Kent and Hedrick 1985a, 1986; MacConnell *et al.* 1989). Therefore the fish may only provide an efficient immune response for the elimination of the parasite after the disintegration of primary cells and release of the secondary cells into the interstitium.

Further work is needed to precisely characterise the antigens to which the MAbs are specific, thus elucidating their role within the parasite and to determine whether MAbs A3 and D4 are specific for the same epitope. Analysis of the MAb B4 specific protein would provide information on the function of this molecule within the maturation and release of the secondary cell and any immunological consequences. Cytochemical investigations should also be undertaken to study the parasite. These studies would verify the presence of phosphatase activity within the carbohydrate covered vacuoles and aid in determining their lysosomal function and determine the composition of the electron dense bodies.

Chapter 5

Studies On The Life Cycle of PK'X' inside Rainbow Trout using Immunohistochemistry

5.1 **Introduction**

The life cycle of PK'X' in salmonids has been partially documented using conventional light microscopy (Kent and Hedrick 1986; Clifton-Hadley *et al.* 1987a). The parasite first appears in the kidney and the spleen, 2-4 weeks following initial exposure of fingerling trout to infective waters. The location of the parasite in the fish prior to this remains unknown. Once in the kidney interstitium the parasite proliferates evoking a strong inflammatory response in the surrounding tissues causing granuloma formation (Clifton-Hadley *et al.* 1987a). This results in a corresponding degeneration of the glomeruli and tubules. There is also a strong cellular response with the stem cell population of the kidney becoming progressively replaced by macrophages and lymphocytes (MacConnell *et al.* 1989). The tissue shows signs of recovery as early as 12 weeks postexposure to infected waters with full recovery occurring after 20 weeks (Kent and Hedrick 1986; Clifton-Hadley *et al.* 1987a).

The PK'X' parasite has two known developmental stages within the salmonid host, the extrasporogonic and sporogonic stages (Kent and Hedrick 1985a). The extrasporogonic stages are the first to appear in the kidney and are found in the blood and kidney interstitium where they are often the focus for the inflammatory response (Ferguson and Needham 1978; Kent and Hedrick 1985a; Clifton-Hadley *et al.* 1987a). These cells are seen to contain one or more secondary cells, which may in turn contain tertiary cells within them. The primary cells are presumed to migrate to the tubule lumens where they disintegrate, releasing the secondary cells to form the intratubular sporogonic stages of

the parasite (Kent and Hedrick 1986; Marin de Mateo *et al.* 1993). These sporogonic stages occur 2-3 weeks after the initial appearance of the parasite in the kidney although complete sporogenesis has yet to be described (Kent and Hedrick 1987). This makes an exact classification of the parasite difficult as myxosporean taxonomy is dependent on spore morphology (Lom and Arthur 1989).

5.2 **Objectives**

The objectives of this part of the study were to determine whether the MAb probes produced were specific for PK'X' from different geographical regions and fish species. The prevalence of PKD in the United Kingdom also was assessed using MAb C5. The course of the disease in rainbow trout was examined and possible sites for the initial infection explored.

5.3 **Materials and Methods**

5.3.1 *Testing of MAbs with PK'X' From Different Localities and Fish Species*

Paraffin wax embedded kidney samples of PK'X' infected rainbow trout from Denmark, Spain and Italy (courtesy of Dr M. Marin de Mateo, Dipartmeno di Patologia Animale, Facoltá di Medicina Veterinaria, Via Nizza 52, I-10126, Torino, Italy) and paraffin wax embedded kidney sections of brown trout, pike, grayling and atlantic salmon infected with PK'X' (courtesy of Dr S. Feist, MAFF laboratories, Weymouth, and Dr J.

McGeorge, Institute of Aquaculture, Stirling University) were obtained and tested using immunohistochemistry (section 2.3.5) with the MAbs obtained in chapter 2.

5.3.2 *Study to Determine the Prevalence of PKD Throughout Scotland and England*

This work was performed in conjunction with Dr A. Adams and Mrs H. McEwan (Institute of Aquaculture, Stirling University)

PKD infection during July and August of 1994 was examined on 25 trout farms throughout Scotland and England. The kidneys of 25 fingerling rainbow trout from each farm were removed, fixed in formalin and examined using immunohistochemistry with MAb C5 as outlined in section 2.3.5.

5.3.3 *Study of the Course of PK'X' Infection within Rainbow Trout*

5.3.3.1 *Sampling sites*

Two sampling sites were used in this study. Site A was a rainbow trout fish farm on the river Avon, southern England which was enzootic for PKD. Site B was a rainbow trout farm on the river Test, also in southern England. Both farms suffered mortalities attributed to PKD from the beginning of July through to September. Preventative measures taken on the farms to limit mortality included aeration of the pond water and treatment of fish with malachite green. All of the fish used in this experiment were

untreated with malachite green. Site A reported incidence of whirling disease, *Myxobolus cerebralis,* as a concurrent infection to PKD.

5.3.3.2 *Experiment to Examine the Expression of Antigens During the of Maturation of PK'X'*

Ten fish were sampled weekly from Site A. Samples were taken from the 3/5/93, one week after fish were introduced to the farm from bore hole water, until 15/8/93. Samples of kidney, spleen, fin, gill, intestine, skin and liver were fixed in 10% buffered formalin. The tissues were processed in a Histokinette 2000 processor and embedded in paraffin wax, sections cut at 5μm, and tested using immunohistochemistry with all of the MAbs (section 2.3.5). The slides were examined under bright field, x200 magnification using an Olympus 210 microscope.

Blocks representative for each week of the study were recut at 5μm onto clean slides and tested using immunohistochemistry with the lectin GS-I following the method of Castagnaro *et al.* (1991). Briefly, sections were deparaffinized and hydrated as in section 2.3.4. The sections were incubated with 3% hydrogen peroxide for 10 minutes at 40°C, washed three times in PBS followed by a 20 minute incubation with 0.1% trypsin-calcium chloride solution, pH 6.8 at 37°C. The sections were rinsed again in PBS before being delimited using a PAP pen. Non-specific binding was inhibited by incubating the sections for 30 minutes with a filtered solution of acetone dried mouse liver (100μg ml^{-1} in PBS) in a moist chamber. This solution was removed by gently tapping the slides onto a paper towel. The sections were then incubated with 30μg ml^{-1} biotinylated GS-I lectin

for 1 hour in a moist chamber. After washing with PBS they were incubated for 30 minutes with avidin-biotin peroxidase complex ABC (Vector labs) diluted in PBS (sol. A 1μg ml^{-1}, sol. B 2.5μg ml^{-1}). After washing with PBS the sections were stained using DAB for 10 minutes, immersed in tap water followed by counterstaining with haematoxylin and coverslipped (section 2.3.5.3). The slides were examined using an Olympus CH-2 microscope x200 magnification, bright field.

PK'X' infected tissue and control sections of uninfected tissue were routinely tested with all of the MAbs throughout the study. To compensate for possible variations in tissue processing all of the tissues were randomly selected for processing and staining. Blocks representative for each week of the study were recut and tested with immunohistochemistry with each of the MAbs to provide standardisation of the immunohistochemical technique. All photographs were taken using an Orthomat photomicroscope.

5.3.3.3 *Experiment to Determine Entry Site and Early Developmental Stages of PK'X'*

For this part of the study fish obtained from site B were used. Fingerling rainbow trout were introduced into concrete ponds on the farm from bore hole water on the 7/5/94. Sampling started on the 10/5/94 and continued weekly until parasites were detected in the kidney tissue. It has been suggested that actinosporeans, believed to be the infective agents of myxosporeans are released at night (Yokohama, Ogawa and Wakabayashi 1993a; McGeorge 1994). To detect possible infective stages in the epithelium of fish, and to complement the previous study, night time sampling was employed. Five fish

were sampled hourly from 12am to 5am from the same pond every week. The fish were killed by a sharp blow to the head immediately followed by decapitation. Samples were taken of a ventral fin, $1cm^2$ area of skin and underlying muscle from the fishes flank, intestine, eye, gill, kidney, half of the head vertically bisected, and swimbladder. These were all fixed in 10% buffered formalin and processed in a Histokinette 2000 processor. They were embedded in paraffin wax, sections cut at 5μm, floated onto poly-L-lysine coated slides and stained using all of the MAbs and immunohistochemistry (section 2.3.5). Control sections of uninfected and PK'X' infected rainbow trout were tested throughout this experiment.

Kidney impression smears (section 3.3.2) and blood smears were routinely taken on the sampling days to identify any unrecognised blood forms of the parasite. Blood smears were produced by placing a drop of blood from the caudal artery of the fish onto a clean slide. The edge of a large coverslip was then placed at an angle into the drop and used to drag the blood across the slide. The slide was allowed to air dry and stained using Rapi-diff^{tm}. All of the slides were examined at x200 using an Olympus C-12 microscope under bright field.

5.4 **Results**

5.4.1 *Analysis of Antibodies to PK'X' from Different Localities and Fish Species*

All of the MAbs reacted with the PK'X' parasites on the sections studied with no cross reaction with any of the fish tissues. This indicates that the antigens recognised by the MAbs are conserved by the parasite within rainbow trout, brown trout, grayling, atlantic salmon and pike in British waters. The antigens are also conserved in rainbow trout from Denmark, Spain, Italy and England.

5.4.2 *Study to Determine the Prevalence of PKD Throughout Scotland and England*

The results of this study are summarised in figure 5.1. Of the 25 fish farms studied 8 were infected with PKD. Six of these farms were situated in the south of England on four different river systems. The remaining two farms were on different river systems in North Yorkshire. All of the farms studied in Scotland were free from infection.

5.4.3 *Study of the Course of PK'X' Infection within Rainbow Trout*

5.4.3.1 *Expression of Antigens During the Maturation of PK'X'*

The pathology of the disease progressed as previously described (Kent and Hedrick 1986, Clifton-Hadley *et al.* 1987a; MacConnell *et al.* 1989). Briefly, PK'X' cells were first observed primarily in the intrarenal sinuses of all of the fish sampled on 11/6/93.

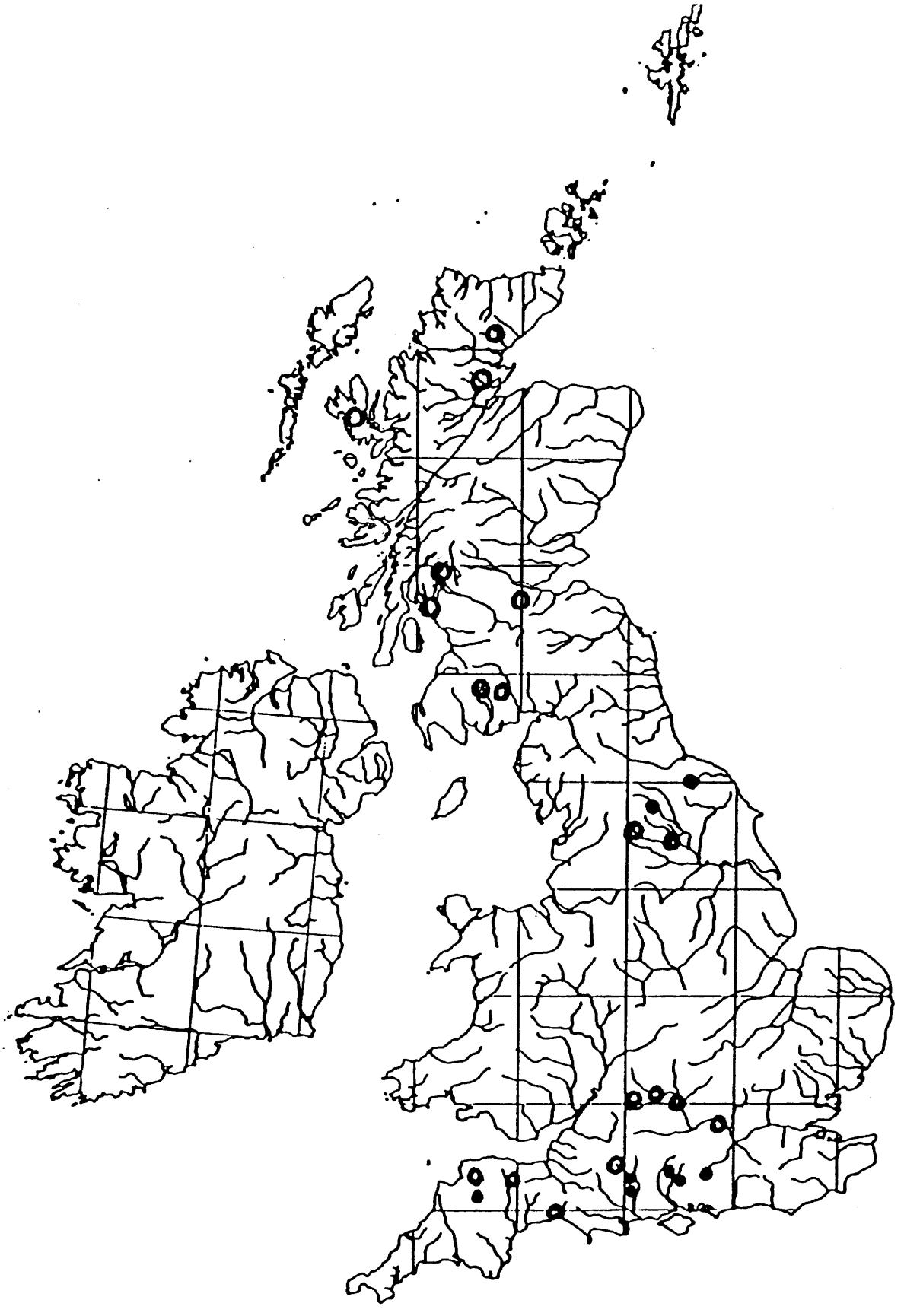


Figure 5.1
Map to show location of fish farms sampled to determine prevalence of PKD throughout Scotland and England. ● indicates fish farms that were positive for PKD while O indicates fish farms that were negative for PKD.

Occasional PK'X' were also detected in the spleen and gill lamellae blood vessels at this time as well as the kidney interstitium. Intratubular sporogonic stages of the parasite were first detected on the 30/6/93. Parasites were detected in the blood vessels of the liver from the 30/6/93. As the infection progressed, reaching a peak mid-July, so the prevalence of the parasite increased in these tissues with the kidney and spleen becoming the most heavily infected tissues. Pathological changes evident in the kidney at this time included haemopoietic hyperplasia, granulomatous nephritis and chronic inflammation. The intestine was only once noted to contain parasites within its vasculature with the skin and pectoral fin remaining unaffected throughout the infection. Slight clubbing of the gill lamellae was noted but not to the extent reported by Clifton-Hadley *et al.* 1987a. At the end of July the level of kidney inflammation was at its most pronounced with some of the kidneys starting to show signs of recovery, characterised by the replacement of granulomatous tissue with haemopoietic tissue. At the end of the study most of the fish were recovering from the disease with few or no visible parasites in their tissues. The kidneys of these fish showed reductions in haemopoietic and granulomatous tissue with evidence of some tubule regeneration. Fusiform crystals as reported by Clifton-Hadley *et al.* (1985) were recorded in only two of the kidneys sampled in August. All of the fish examined after the initial detection of the parasite were infected.

The staining properties of all of the probes tested changed over the course of the infection. The three MAbs specific for carbohydrate epitopes (MAbs A3 C5 and D4) and the lectin GS-I all displayed similar staining properties with the PK'X' parasite whilst the staining of MAb B4 was unique. All of the MAb probes recognised the PK'X'

parasite with no cross reactivity with any of the fish tissues studied. However the lectin GS-I was observed to bind to the epithelial goblet cells of the gill, skin, fin, intestine and the epithelium of some of the tubules in the kidney.

PK'X' parasites were first detected in the fish sampled on the 11/6/93 by all of the carbohydrate binding MAbs and the lectin GS-I. The blood forms of the parasite at this time, in the intrarenal sinuses and gill lamellae, appeared to be lightly staining (figure 5.2) whilst those in the kidney interstitium had the appearance of pronounced circles of staining on them (figure 5.3). Secondary cells were visible within some of the primary cells at this time although they remained small and unstained. As the disease progressed towards its height, during mid-July, so the staining of the carbohydrate binding MAbs and the lectin became more pronounced with the replacement of circles of staining with strong brown staining over the whole of the primary cell of the parasite (figure 5.4). The primary cells developed large vacuoles during the remainder of the infection, finally appearing to break up. Corresponding to this increase in vacuolation was a weakening of the staining on these cells with very heavily vacuolated cells appearing to be pale yellow in colour (figure 5.5).

The carbohydrate binding MAbs and the lectin GS-I stained maturing secondary cells within primary cells a week after the parasites' first appearance in the kidney. As these cells developed, evidenced by an increase in size from ~5μm to 10μm, so their staining intensified principally around their edges. The cells usually remained distinct from the

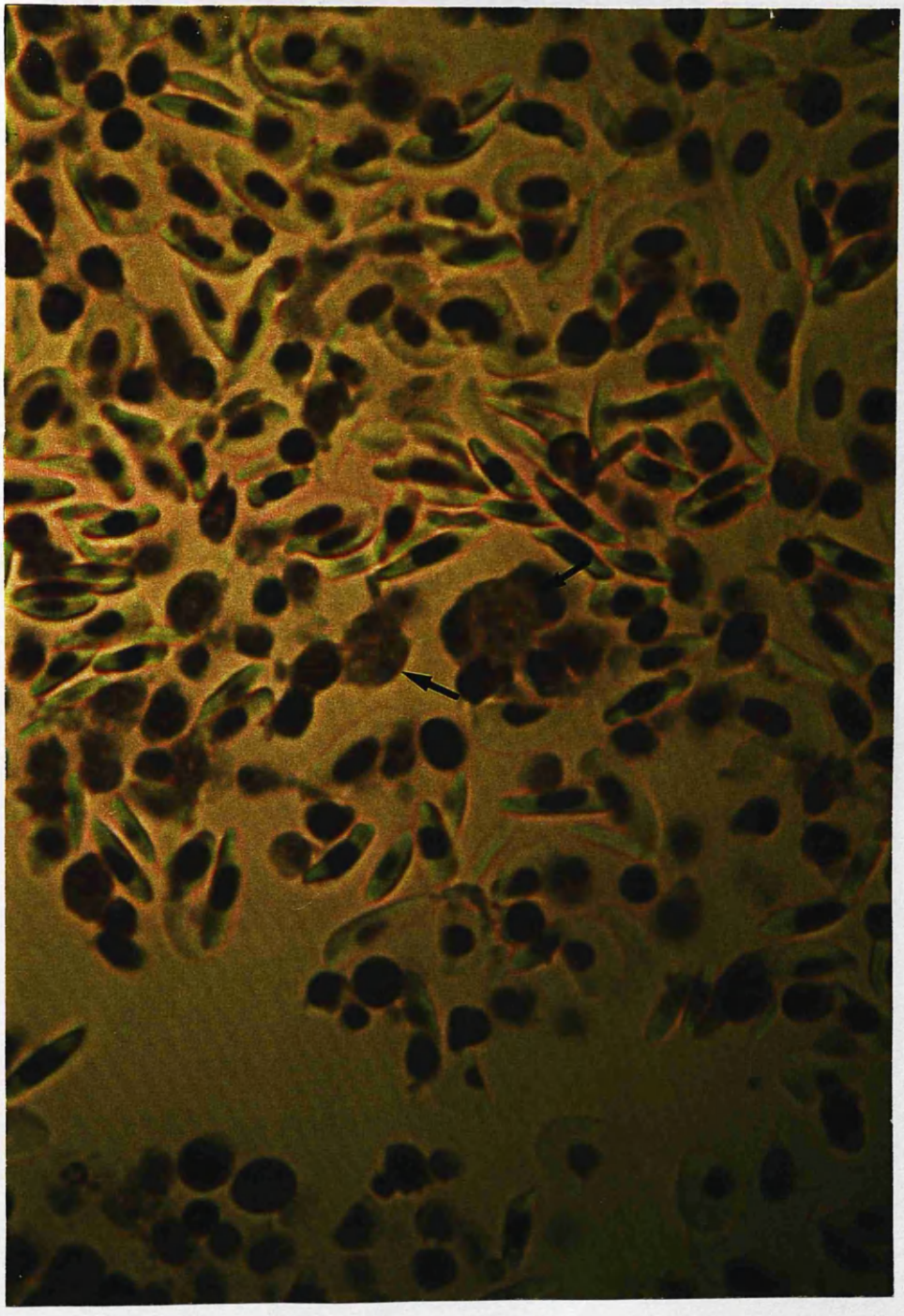


Figure 5.2.
Early forms of the PK'X' parasite (arrows) detected within the intrarenal sinuses of the kidney. Immunostained with MAb C5. Mag X 1,000

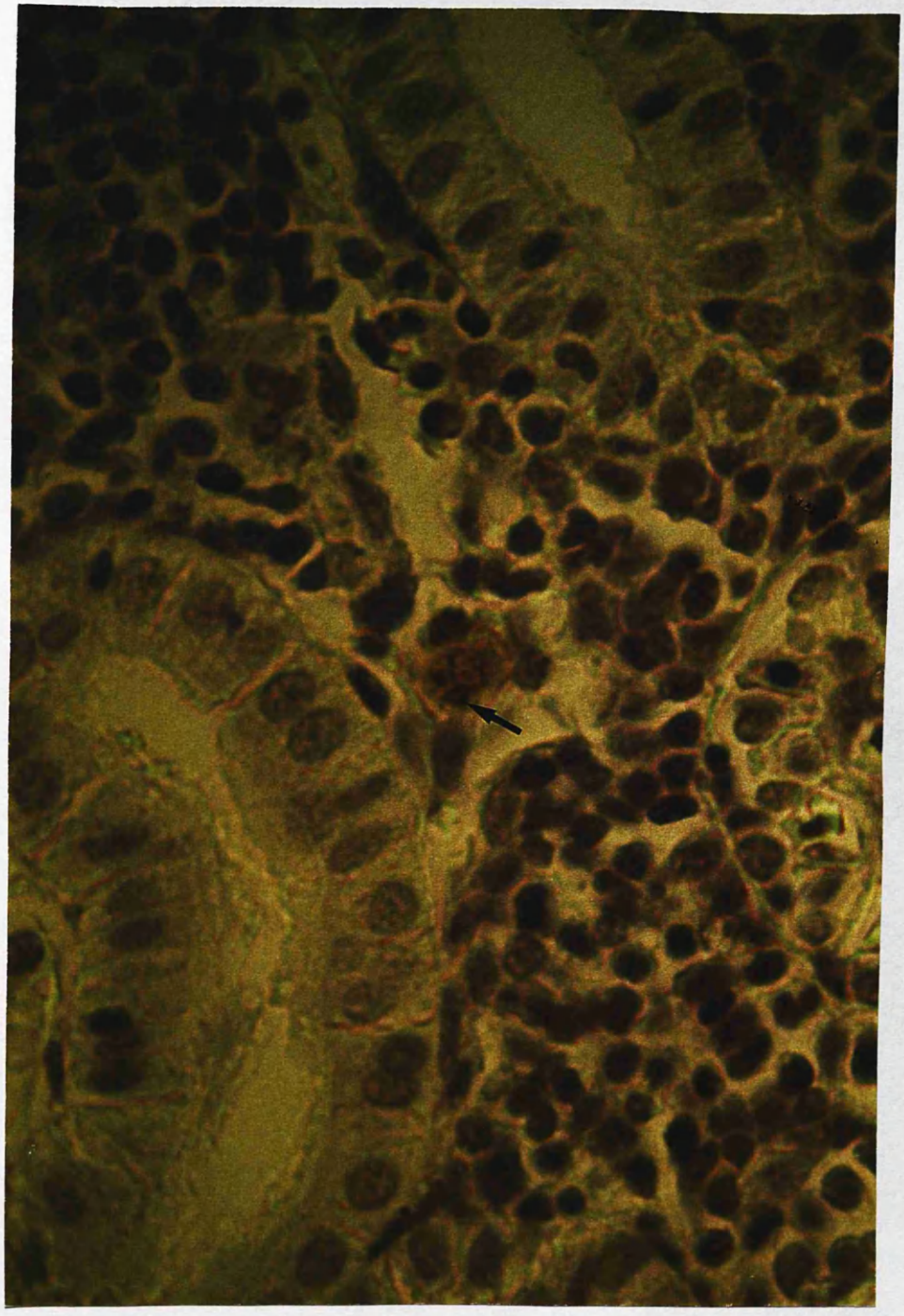


Figure 5.3.
Early form of the PK'X' parasite detected within the kidney interstitium. Arrow indicates pronounced circles of antibody staining within the parasite.
Immunostained with MAb C5. Mag X 1,000

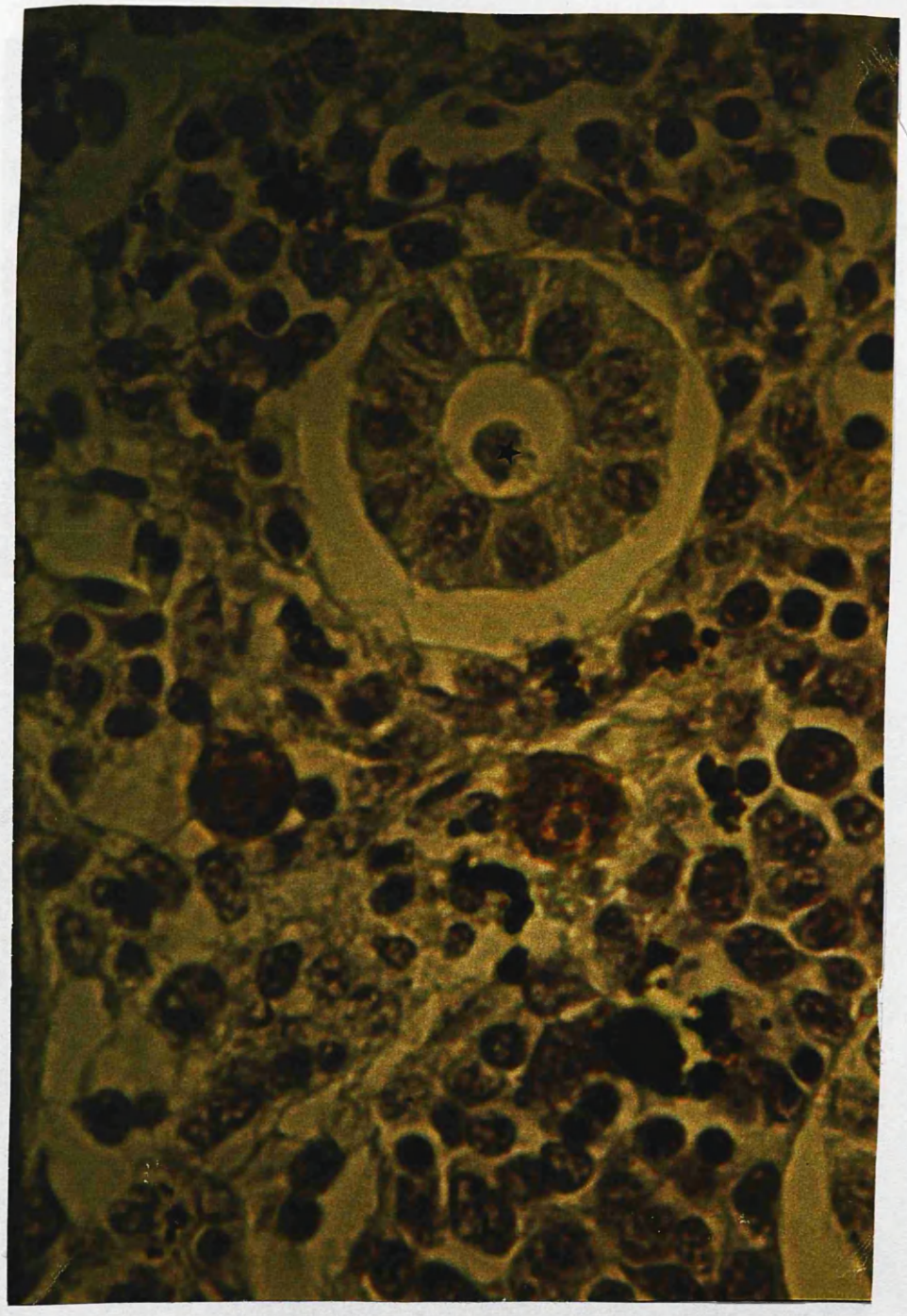


Figure 5.4.
Increasing intensity of staining on the primary cell of the parasite with the carbohydrate binding MAbs at the height of the infection (July). Intratubular sporogonic form of the parasite remains unstained (indicated with *).
Immunostained with MAb C5. Mag X 1,000.

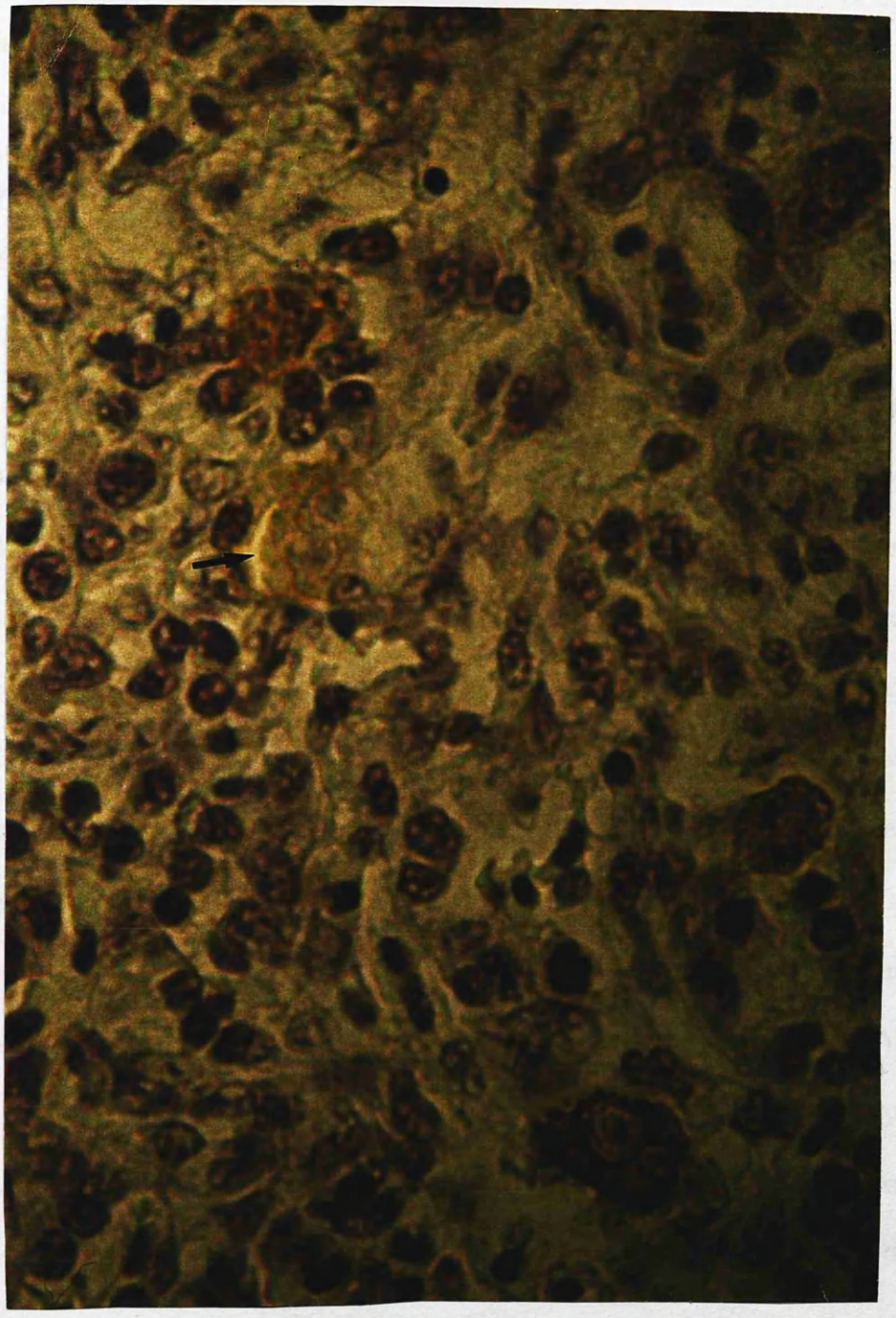


Figure 5.5.
Disintegration of PK'X' cell (arrowed). Other PK'X' cells on this section demonstrate different intensities of immunostaining with MAb suggesting that they are at different stages of development.
Immunostained with MAb C5. Mag X 1,000.

primary cell by the presence of a clear 'halo' around them. Intratubular sporogonic stages of the parasite were detected within tubule lumens on the 30/6/94.

As previously noted the staining characteristics of MAb B4 were unique amongst the probes used. Staining only occurred with developing secondary cells and intratubular sporogonic stages (figure 5.6). Immature secondary cells remained unstained. Staining with MAb B4 on the secondary cells started on the 30/6/93 and coincided with the appearance of the intratubular sporogonic stages of the parasite. Occasional small cells in the interstitium also stained with MAb B4 and these were believed to be secondary cells devoid of a primary cell coating (figure 5.7). Secondary cells stained with MAb B4 were seen to be migrating though the tubule walls at this time to form the intratubular sporogonic forms (figures 5.8, 5.9). Initially only one developing secondary cell was observed per primary cell. However as the disease progressed some primary cells contained up to three secondary cells simultaneously (figure 5.10). Recovering fish displaying no interstitial parasites did not appear to possess intraluminal sporogonic stages. Direct comparison between sections stained with the carbohydrate specific MAbs and MAb B4 indicated that substantially more intratubular sporogonic stages were detected with MAb B4 (around a fourfold increase). None of the small cells recognised by MAb B4 in the interstitium were detected by the carbohydrate binding MAbs or the lectin GS-I.

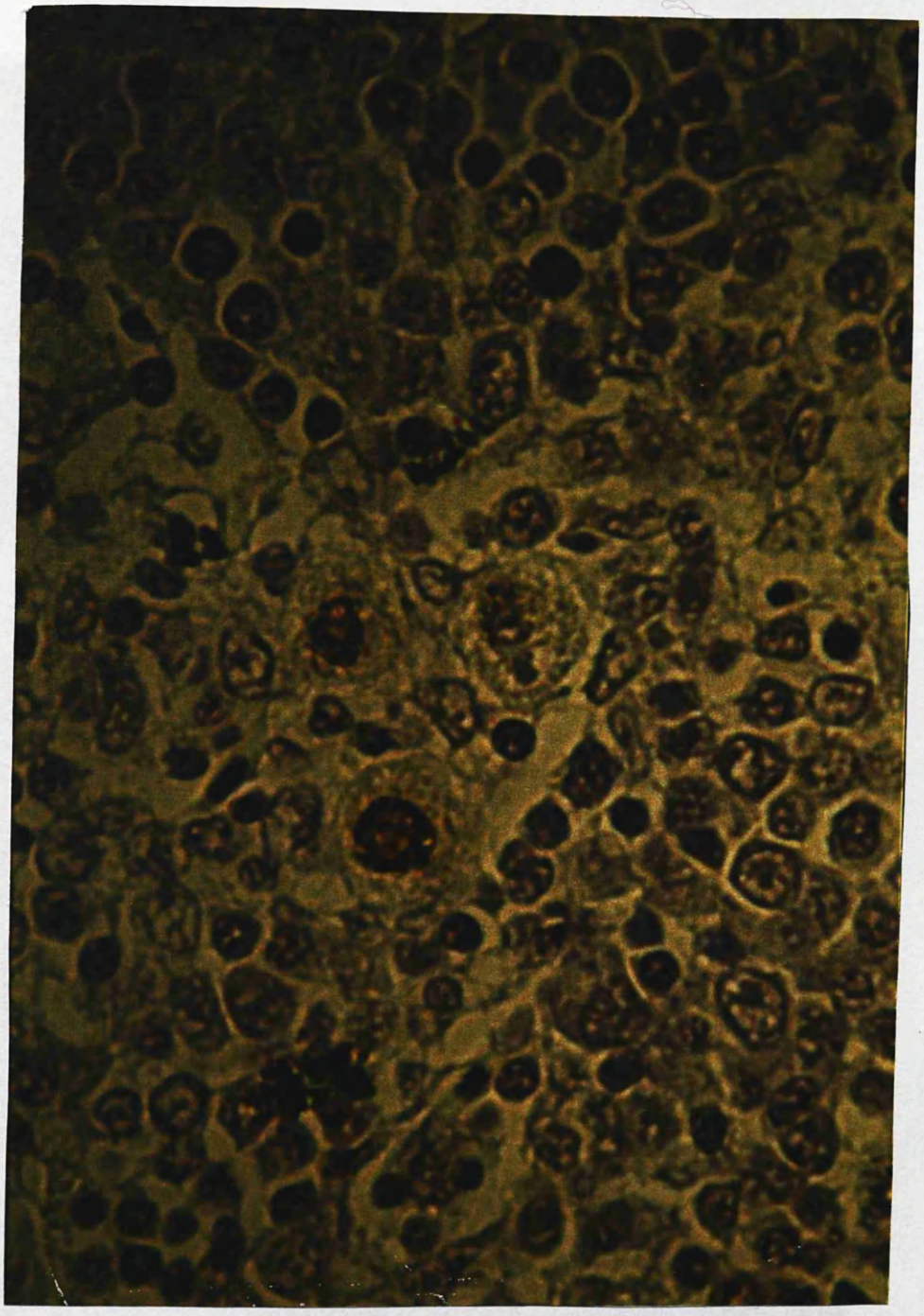


Figure 5.6.
Development of secondary cells within PK'X' primary cells with a corresponding increase in the intensity of the immunostaining with MAb B4.
Immunostained with MAb B4. Mag X 1,000.

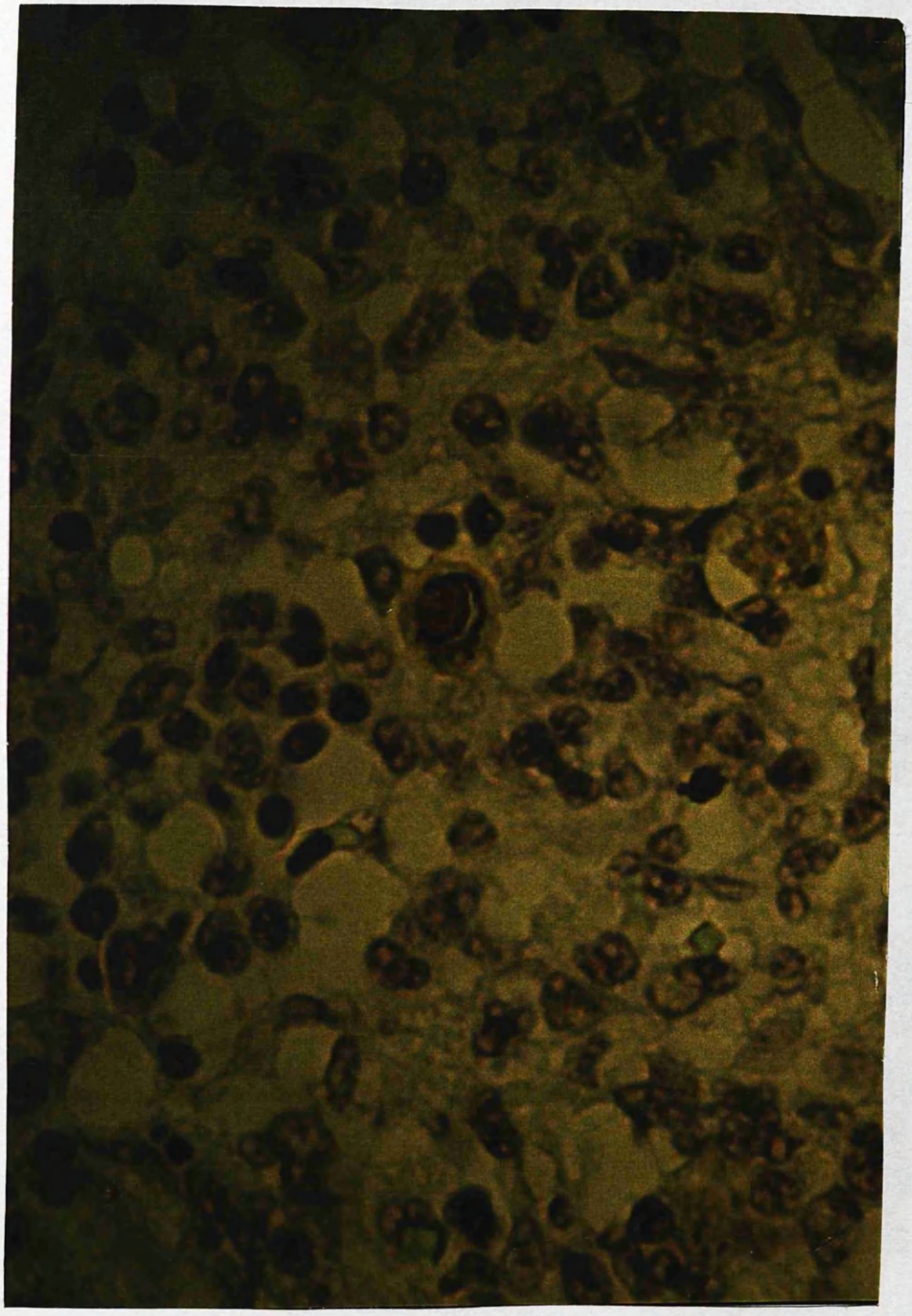


Figure 5.7.
Secondary cell, devoid of primary cell, in the kidney interstitium.
Immunostained with MAb B4. Mag X 1,000.

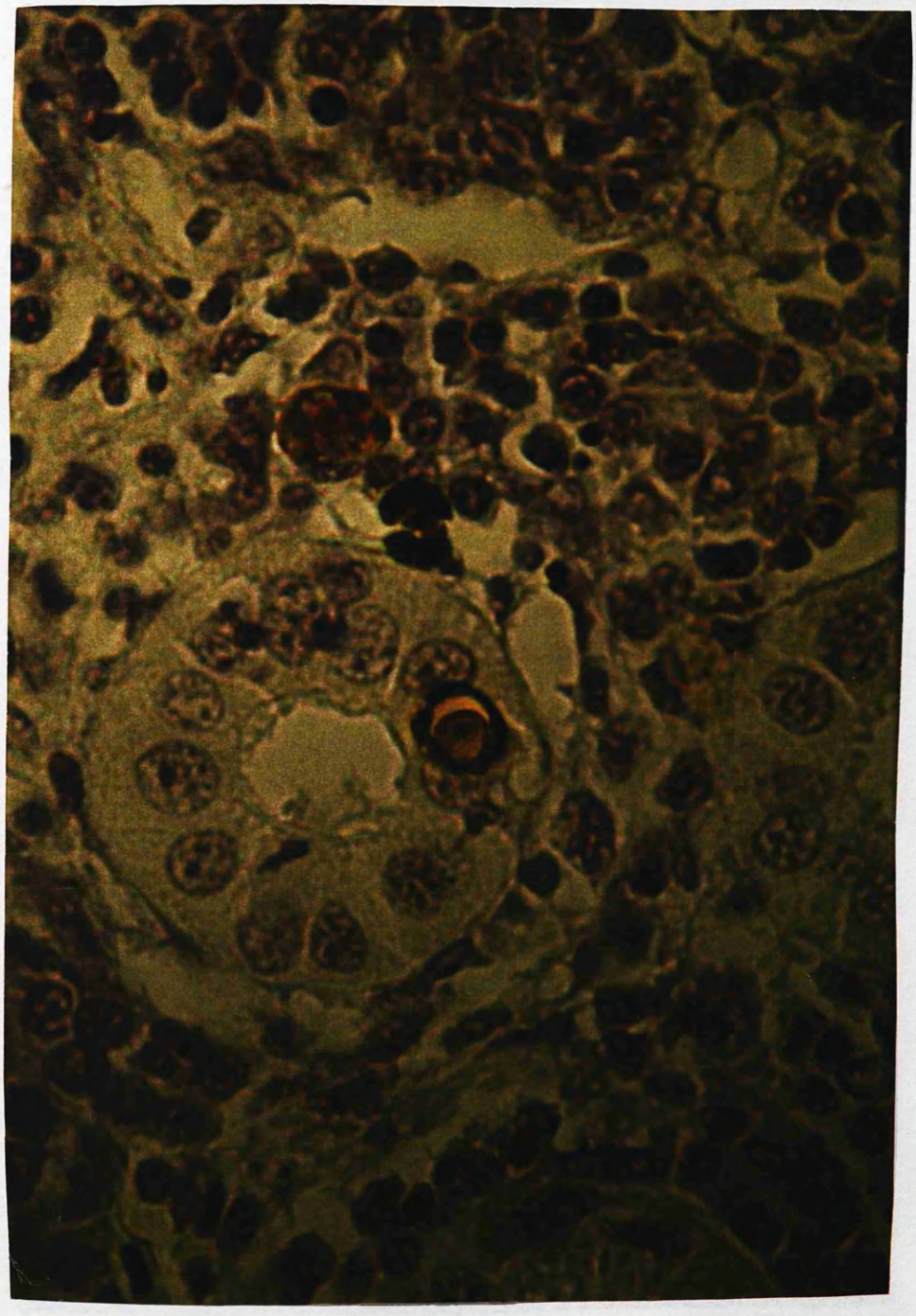


Figure 5.8.
Migration of secondary cell through the wall of a kidney tubule.
Immunostained with MAb B4. Mag X 1,000.

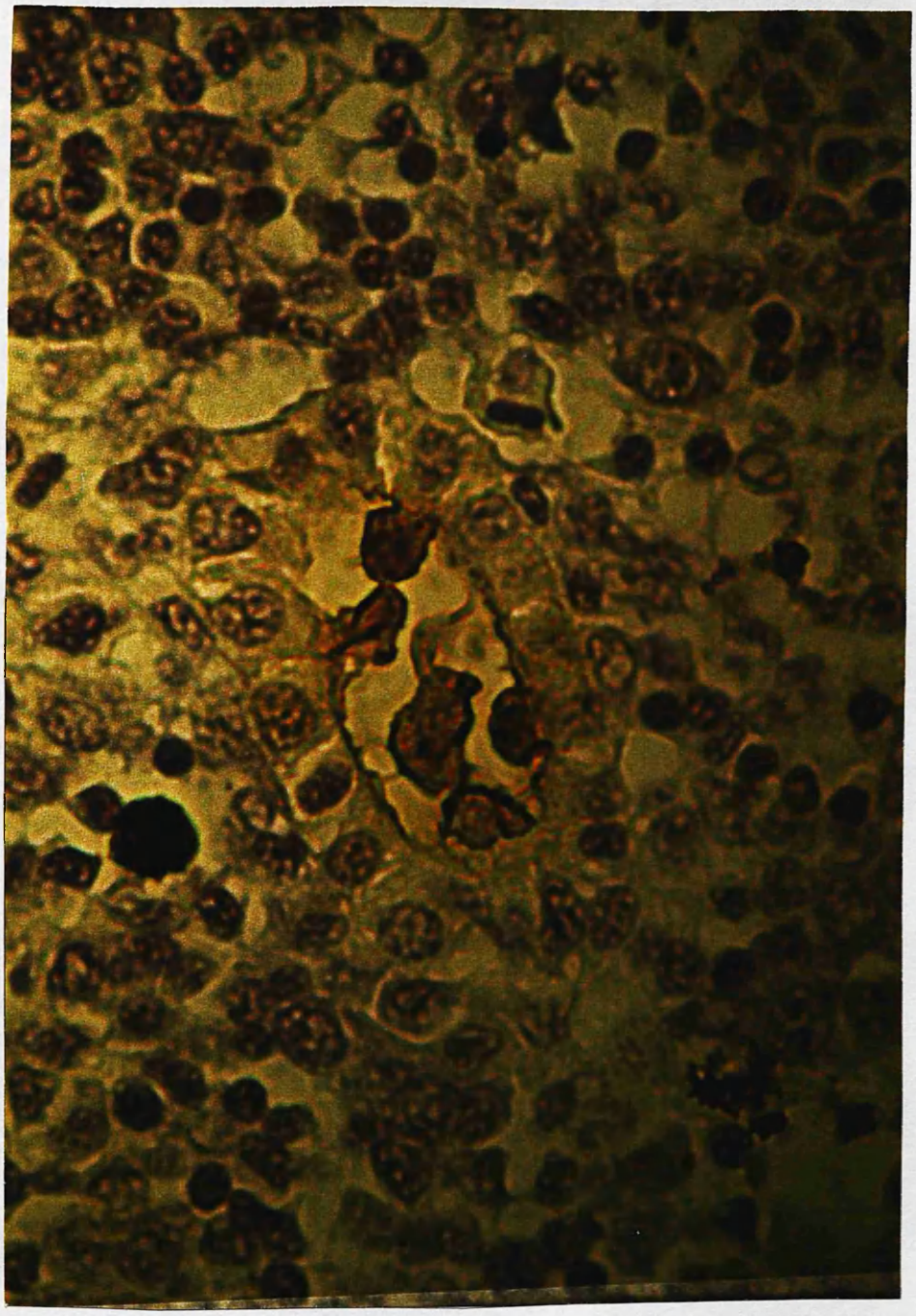


Figure 5.9.
Intratubular sporogonic forms of PK'X'.
Immunostained with MAb B4. Mag X 1,000.

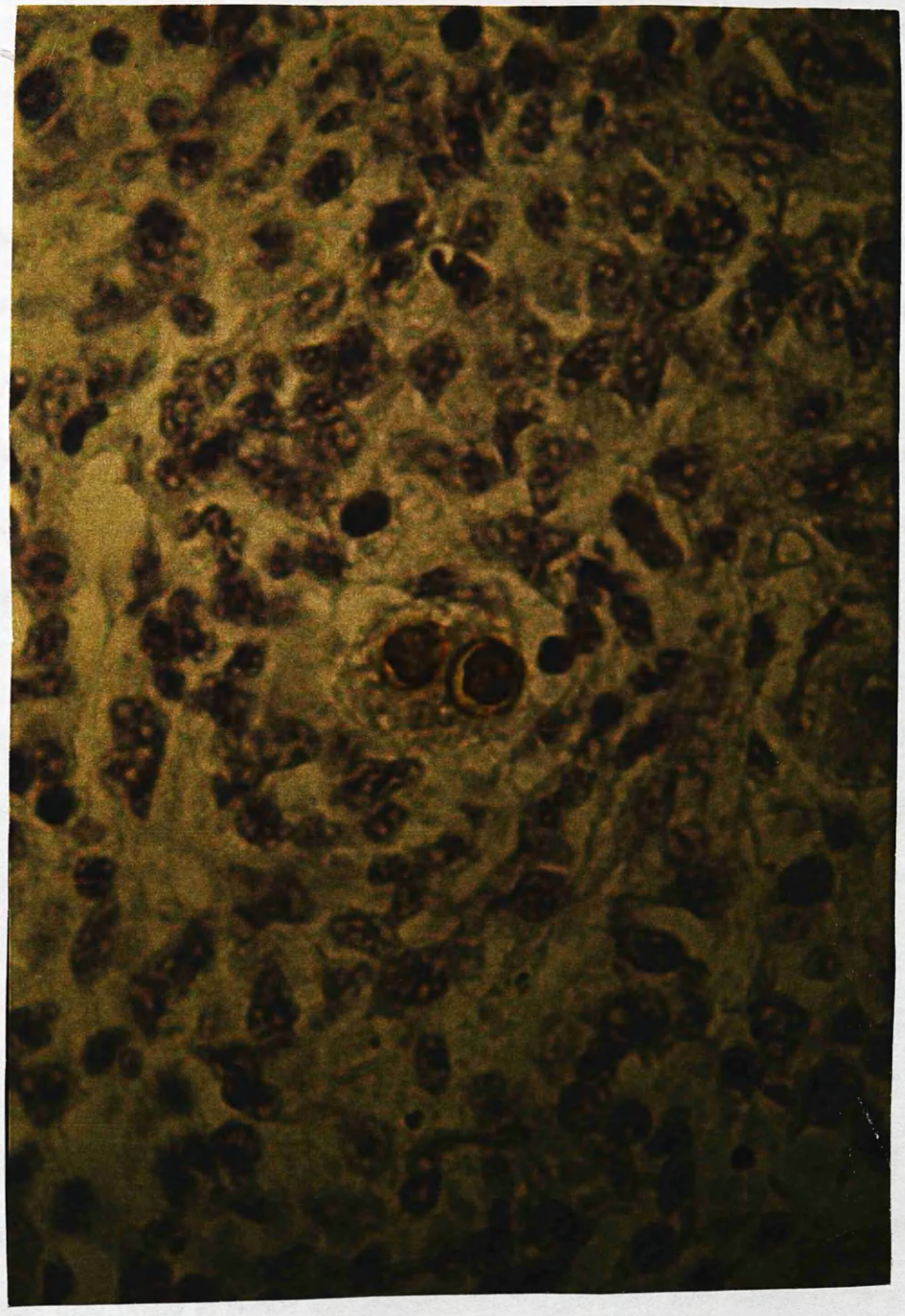


Figure 5.10.
Simultaneous development of two secondary cells within one PK'X' primary cell.
Immunostained with MAb B4. Mag X 1,000.

During the infection it was noted that PK'X' at different stages of development were present in the same section. This was accentuated by the different staining characteristics of the MAbs and lectin on these stages (figures 5.4, 5.5).

Infection of rainbow trout by *M. cerebralis* was evident in 10% of the fish examined after 6/6/93. This was demonstrated by the presence of mature spores within the cartilage of the gill arches. The areas of cartilage destruction associated with the spores were recognised by MAb B4. *Trichodina* spp. and *Ichthyopthirius* spp. were also noted to infect some of the gills throughout the study although cross reaction with the MAbs was not observed.

A structure presumptively identified as a Haplosporidian sporocyst (Barrow 1961) was noted between the gill filaments of one fish sampled on the 6/6/93. MAbs B4, C5 and D4 all appeared to strongly bind with this structure (figure 5.11). The sporocyst was not present on the section immunostained with MAb A3.

5.4.3.2 *Initial Entry Site and Early Developmental Stages of the Parasite.*

From the blood and kidney impression smears trophozoite PK'X' parasites were first detected in the fishes' blood and kidney from the 13/6/94. All of the tissues examined before this date failed to reveal the presence of any parasites. Occasional binding was observed in the epithelium of the head and in the brain of some fish. However this appeared to be for host tissue and was thought to represent non-specific binding as a result of insufficient blocking or washing steps.

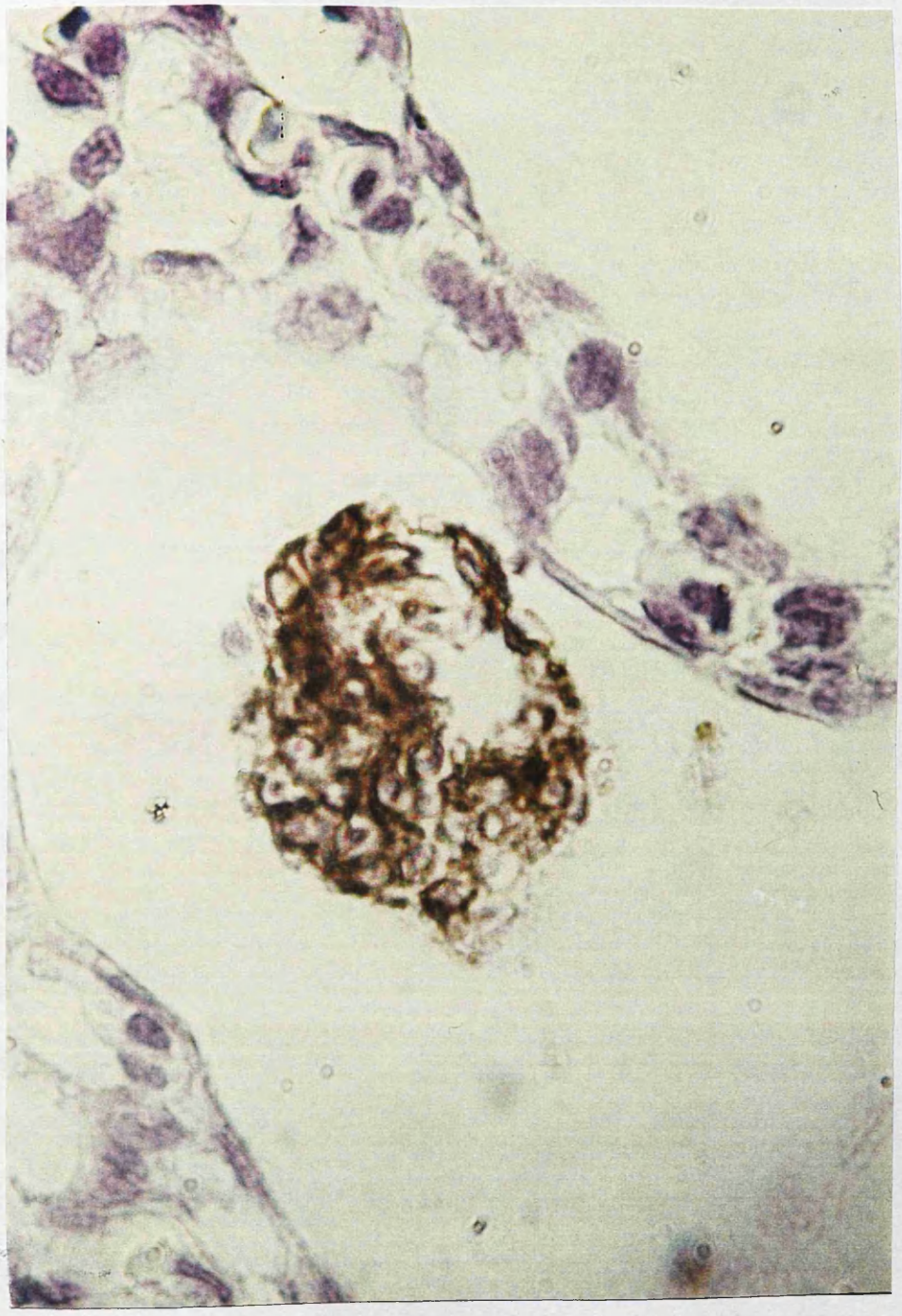


Figure 5.11.
Haplosporidian sporocyst present between the gill filaments of rainbow trout.
Immunostained with MAb A3. Mag X 1,000.

5.5 **Discussion**

The results obtained from the testing of rainbow trout from different localities and other fish species demonstrate that the antigens recognised by the MAbs are conserved by the parasite and do not indicate strain/species differences between them. Homology of PK'X' antigens from different species and localities has also been recorded for the lectin GS-I and MAb 12 (Marin de Mateo *et al.* 1993).

Seagrave *et al.* (1981) surveyed 45 farms in England and Wales and determined that 48% of the farms in England were infected with PKD. The results from this survey indicate that the incidence of PKD in England has remained reasonably constant with around 44% of farms tested infected with the disease. The slight decrease may be attributed to the difference in sample size. PKD has previously been reported in Scotland although it was not noted within this study (Ferguson and Needham 1978; Ellis *et al.* 1985). It has been suggested that incidence of the disease is related to summer temperatures which may account for the variations in distribution (Ferguson 1981). An increase in the incidence of the disease has been reported during the summer of 1995 (O. Robinson, British Trout Association pers comm.). This may reflect the unusually hot summer of that year (1995 was one of hottest summers on record). The production of monoclonal antibodies will allow farms to be accurately diagnosed for the disease in the future and may help to determine a relationship between environmental conditions and the distribution of the disease.

The experiment to examine the expression of antigens during the maturation of PK'X' within rainbow trout determined that some of the antigens of PK'X' are stage specific with the antigenic profile of the parasite changing throughout its life history.

All of the carbohydrate binding MAbs and the lectin GS-I displayed similar staining properties with the parasite although the lectin cross reacted with fish tissue. This had been noted by Marin de Mateo *et al.* (1993) in relation to the kidney tubules. The presence of this antigen within epithelial goblet cells suggests that PK'X' shares antigens with fish mucus. Antigenic homology between the fish and parasite has also been reported by Adams *et al.* 1992 between PK'X' and tubule cells using MAb 18. Antigen mimicry by parasites is thought to help with parasite evasion from the host's immune response (Pauly 1974). Complete mimicry, however, is not usually achieved as some antigens are essential for the parasites' life cycle (Hall 1994).

The results obtained from chapter 4 indicated that the antigens recognised by the carbohydrate binding MAbs are found principally within lysosomes and may be related to the LAMP molecules of other eucaryotes. The increase in the staining of the parasite by the carbohydrate binding MAbs as the disease progresses suggests that there is either an increase in the amount of antigen being expressed within existing lysosomes or there is an increase in lysosomal structures within the PK'X' primary cell. Changes in the glycosylation of LAMPs have been detected in macrophages responding to inflammatory stimuli within vertebrates (Rabinowitz and Gordon 1991). However such environmentally induced changes within PK'X' appear unlikely as parasites in the same

location within the kidney show different staining characteristics. It has been documented that the PK'X' primary cell becomes more degenerated as the disease progresses, with a corresponding enlargement of 'vacuoles' within its cytoplasm, finally resulting in the cell's disintegration (Seagrave *et al.* 1980; Kent and Hedrick 1986; MacConnell *et al.* 1989). The results from this study suggest that these vacuoles are lysosomal in origin with the increase in staining by immunohistochemistry representing an increase in the number of lysosomes within the PK'X' primary cell. The subsequent fusion of these lysosomes may result in the large vacuoles as visualised with immunohistochemistry and light microscopy. The degeneration of the cytosol corresponding to this increase in lysosomes can be explained by the cell autophagocytosing with the development and release of secondary cells. Myxosporean parasites commonly have primary cells that disintegrate to release secondary cells (Lom 1987). This autophagocytotic degeneration may not only aid in the release of the secondary cells but may also provide a source of nutrition for them.

The staining characteristics of MAb B4 demonstrate that this antigen is produced by maturing secondary cells within the primary cell of the parasite and its presence is concurrent with the appearance of intratubular sporogonic forms of the parasite. The observed developmental sequence of the secondary cells strongly suggests that these cells migrate to the tubule lumens to form the sporogonic stages of the parasite. Migrating secondary cells are often surrounded by a primary cell that appeared to be recognised by MAb B4. Small cells were also observed in the interstitium that bound with MAb B4 and it is believed that these represented secondary cells that were devoid of a primary cell

coat. The results obtained from chapter 4 indicate that the antigen recognised by MAb B4 is released by the secondary cell into its environment, thus explaining its presence in the primary cell of some PK'X'. The absence of staining around the PK'X' primary cells and released secondary cells suggests that the antigen is either rapidly degraded once outside the cell, or is leached from the tissue sections during processing. The presence of the antigen in the cartilage associated with *M. cerebralis* spores suggests that the latter is the case. The association between the antigen and areas of cartilage destruction in *M. cerebralis* also suggests a proteolytic function for this protein.

The parasite was not seen to produce spores and recovering kidneys did not appear to possess the intratubular sporogonic forms as reported by Kent and Hedrick (1985a,1986). Such sporogonic forms have not been reported by other workers studying PKD (Clifton-Hadley *et al.* 1987a; MacConnell *et al.* 1989). Recently Kent *et al.* (1993a, 1994, 1995) have suggested that sporogonic forms of the parasite may persist in kidney tubules until the maturation of the fish where upon complete sporogenesis occurs. The results from this study indicate that this does not happen within rainbow trout in Britain with recovering fish displaying no sporogonic stages. Whether this is due to rainbow trout being of triploidy stock and therefore infertile is open to conjecture. Brown trout and Atlantic salmon, however, both appear to possess more advanced stages of the disease and perhaps represent the definitive hosts for this disease (Clifton Hadley and Feist 1989; Bucke *et al.* 1991; Marin de Mateo *et al.* 1993).

Experiments to determine hitherto undescribed stages of the parasite were not conclusive in this study. El-Matbouli *et al.* (1995) have recently described the complete life cycle of *M. cerebralis* in rainbow trout and demonstrate an intracellular stage of development, followed by migration to the brain before sporogenesis in the gill filaments of the host. This suggests that different presporogonic developmental sites may occur before the final site of sporogenesis is reached. This has also been proposed for *S. renicola* and may be a common facet of myxosporean infections (Lom and Dykova 1986). Previous studies on the course of PK'X' infection indicate that exposure to the infective stages of the parasite occurs at least 10 days before the parasites' appearance in the kidney (Chilmonczyk pers com. to Hedrick *et al.* 1993). The initial appearance of PK'X' in the renal sinuses, spleen and vasculature of the gills in this study shows that the parasite reaches the kidney from the blood. This suggests that early stages of the parasite would be closely associated with the vascular system of the fish. Early developmental blood stream forms of the parasite have not been described for PK'X', although they have been reported for other species of *Sphaerospora* such as *S. renicola* (Lom, Dykova and Pavlaskova 1983a,b; Lom, Pavlaskova and Dykova 1985; Molnar 1988).

The failure to elucidate the site of entry and subsequent development of PK'X' before its appearance in the kidney may be due to a number of reasons. These may include the antibodies not recognising early stages of the parasite, these stages being too small to identify using light microscopy, infection occurring at a different time from that when the samples were taken, the wrong organs being taken for analysis or that the parasites, although present in the organs, not being in the sections examined. To determine the

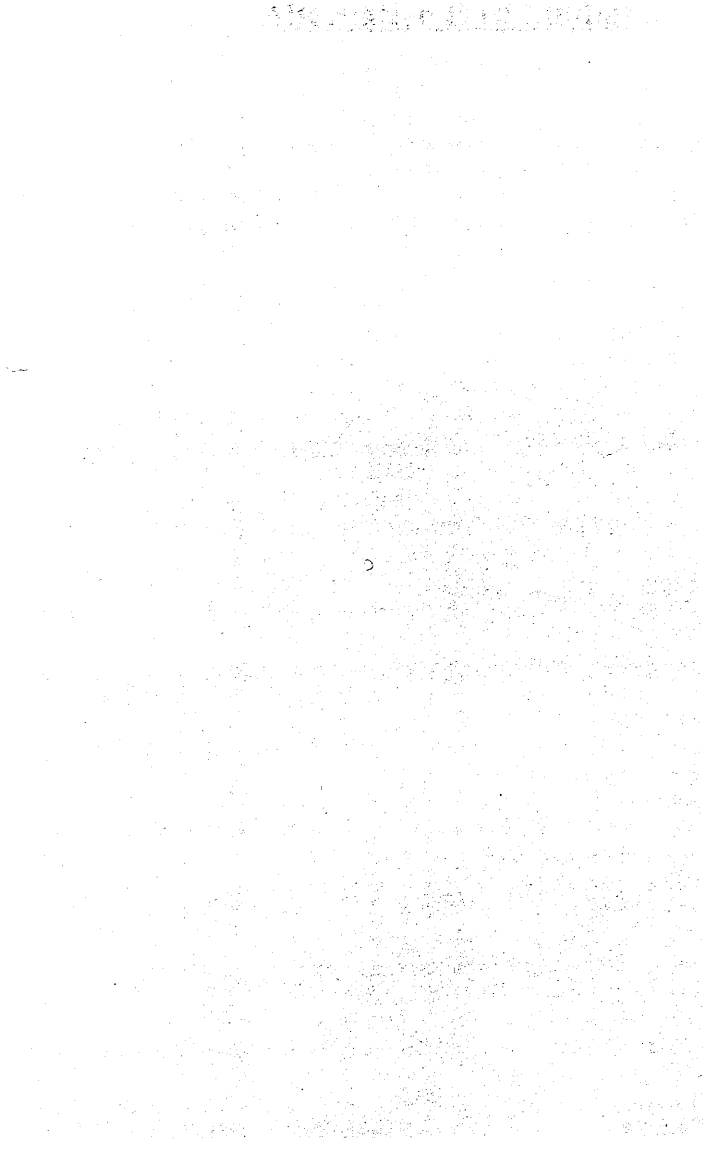
course of *M. cerebralis* infection, El-Matbouli *et al.* (1995) exposed the fish to thousands of infective stages. The infective stage of PK'X' have not yet been identified and so such high infections would be difficult to achieve experimentally.

The observation of a Haplosporidian sporocyst between the gill filaments of a pre-clinical fish that appeared to bind to three of the MAb probes is intriguing. Comparisons between the PK'X' organism and members of the Haplosporidia have been made (Seagrave *et al.* 1980; chapter 3). Future studies on the transmission of the disease should address the possibility of a member of the Haplosporidea being the infective agent of PKD.

Examination of the life cycle of PKD could be clarified with the development of DNA probes against the disease. This would allow for specific organs to be screened for early infection using the polymerase chain reaction (PCR) thus circumventing possible problems encountered using monoclonal antibodies as outlined above. Such DNA probes have been developed for *C. shasta* (Bartholomew, Rodriguez and Arakawa 1994), and are presently being developed for PK'X' (Saulnier, Brémont, Delaunney, Thierry and de Kinkelin 1995).

The results from this chapter confirm that the PK'X' organism possesses stage specific antigens. The intensification of staining of the carbohydrate binding MAbs using immunohistochemistry on the primary cell of the parasite indicates that this cell undergoes autophagy with the development and release of the secondary cell. The

staining of MAb B4 demonstrates the relationship between the intratubular sporogonic stages of the parasite and the secondary cells. This antigen is released by the maturing secondary cells and may have a proteolytic function.



Chapter 6

Alternative Host Studies

6.1 **Introduction**

The infective agent for PKD remains unknown. Studies to transmit PK'X' to rainbow trout directly from moribund fish have been achieved by the intraperitoneal, intradermal and intravenous injection of homogenised PK'X' infected tissue into naive fish (Clifton-Hadley *et al.* 1984b; D'Silva, Mulcahy and de Kinkelin 1984; Kent and Hedrick 1985b; Rafferty 1986). Experimental infections involving the feeding of fish with infected tissue and holding in the same tanks as both live and dead fish infected with PK'X' have all failed to produce the disease (Clifton-Hadley 1986; Rafferty 1986). Such results strongly indicate that an alternative host is needed in the life cycle of PKD.

Studies on *M. cerebralis* by Markiw and Wolf (1983) first demonstrated that an aquatic oligochaete was needed to complete a myxosporean life cycle. This was initially disputed by Hamilton and Canning (1987) who could not replicate their results although further studies conducted by El-Matbouli and Hoffmann (1989) confirmed that an oligochaete was required. Oligochaetes have now been implicated in the life cycle of other myxosporeans including *Hoferellus cyprini*, Proliferative Gill Disease, *Hoferellus carassius*, *Myxidium giardi*, *Myxobolus articus*, *Myxobolus cotti* and Kidney Enlargment Disease (El-Matbouli and Hoffmann 1989; Styer, Harrison and Burtle 1991; El-Matbouli, Fischer-Scherl and Hoffmann 1992; Großheider and Körting 1992; Benajiba and Marques 1993; Kent, Whitaker and Margolis 1993b; Yokoyama, Ogawa and Wakabayashi 1993b). Spores released from myxosporean infected fish are believed to settle onto the river bed where they are ingested by the oligochaete population. The spore

develops in the epithelium of the worms' intestine where it transforms into an Actinosporean sp. (Wolf, Markiw and Hiltunen 1986; El-Matbouli and Hoffmann 1989; Odening 1991; Lom and Dykova 1992). This stage is released by the worm where it travels to the surface of the water column with the aid of large float cells (Marques and Ormieres 1982). When the actinosporean comes in contact with fish mucus it extends polar filaments and stimulates the release of its sporoplasm (Yokohama *et al.* 1993a; 1995). The infective sporoplasm contained within the actinosporean penetrates the epithelium of the fish to continue the life cycle of the myxosporean in the fish host (Markiw 1989a; El-Matbouli *et al.* 1995).

Epizootialogical studies indicate that the infective stage of PKD is water borne and is present in river water from April through to October (Ferguson and Ball 1979; Foott and Hedrick 1987). Its presence in the water at this time is thought to be related to the higher temperatures during the spring and summer (Ferguson 1981; Schlotfeldt 1985). Experiments to filter river water from farms suffering PKD have demonstrated that the infective agent is under 60μm in diameter (Alderman and Rodgers 1984). Chilmonczyk *et al.* (1989) studying an indoor recirculating unit enzootic for PKD determined that rainbow trout exposed to sediments taken from the unit became infected with the disease. Filtration of these sediments through graded screen meshes demonstrated that the infective agent of the disease was collected on a 500μm screen and it was proposed that an oligochaete, possibly *Stylaria lacustris*, was the intermediate host of PKD (Hedrick *et al.* 1992b). Infectivity was also noted in the water associated with the sediments that

passed through a 50μm mesh screen and this was believed to contain a PKD infective actinosporean, of the genus *Aurantiactinomyxon*, that was released from infected worms.

One problem in the study of the life cycle of PKD is the absence of mature spores in rainbow trout. This and the severe inflammatory response in the trout has led to speculation that this may be the wrong host for PK'X' (Seagrave *et al.* 1980; Kent and Hedrick 1986). Studies on other fish species inhabiting PKD enzootic rivers have yet to determine the presence of a definitive or reservoir host for the parasite although myxosporean infections have been reported from these fish (Hedrick *et al.* 1986; Hedrick, Kent, Toth and Morrison 1988; MacConnel and Smith 1990). Species of *Sphaerospora* have also been found as concurrent infections to PK'X' (Fischer-Scherl, El-Matbouli and Hoffmann 1986; Odening *et al.* 1988). Studies on *Sphaerospora oncorhynchi* in sockeye salmon (*Oncorhynchus nerka*) and kokanee salmon (non-anadromous sockeye salmon) have suggested that this is the PK'X' myxosporean, the complete sporulation of the parasite being initiated by the sexual maturation of the fish (Kent *et al.* 1993a, 1994). However, *O. nerka* is not present in European waters and so a mature salmonid of a different species may contain fully developed spores of PK'X'.

6.2 **Objectives**

The objectives of this study were to investigate potential aquatic oligochaete hosts to PK'X' using immunohistochemistry, electron microscopy and transmission studies. Wild fish kidneys from PKD infective waters were also examined using

immunohistochemistry to ascertain antigenically similar myxosporeans to PK'X' which may indicate the definitive host for the disease.

6.3 **Materials and Methods**

6.3.1 *Study to Determine the Infective Agent of PK'X'*

6.3.1.1 *Sampling Sites*

Worms were sampled from four rainbow trout farms in southern England which suffered annually from PKD. The sampling sites A and B have been previously described in chapter 5 (this volume), site C was a fish farm 6 miles upstream from site B whereas site D was a fish farm 4 miles downstream from site B, both on the river Test. The sampling of sites A, B and C was performed during May when epidemiological studies indicated that this was the time when the infective stage might be released. The remaining samples were taken during January, from sites A and D. These were brought up to temperature in an 18°C incubator for 2 weeks prior to study to stimulate the release of infective stages.

6.3.1.2 *Collection of Mud Samples*

Mud samples containing oligochaetes were collected from the inlets and outlets of infected farms as well as from any sediment tanks present. The samples were obtained by throwing a closed Peterson grab into the river/tank and dragging it across the sediment. This material was then placed into large plastic bags containing a small amount of river water and transferred to the lab for examination.

6.3.1.3 *Collection of Worms from Mud Samples*

Oligochaetes were sorted from the sediments by washing the sediments through graded sieves, placing each sieve in a shallow dish containing water and placing under a bright light. These were then left for 1 hour for worms to migrate through the sieve mesh. The sieves were then emptied into a fresh dish containing water. Oligochaetes were removed from the dishes to a container containing filter sterilised, aerated river water using a plastic pipette.

6.3.1.4 *Examination for Actinosporean Releasing Worms*

All worms were studied using a modification of the cell-well technique of Yokoyama *et al.* (1991). Groups of 5 worms were transferred to individual cell wells on 24 well plates each containing 2ml of dechlorinated tap water. The water was replaced twice a week with fresh declorinated tap water. The plates were stored outside away from direct sunlight and examined daily for released actinosporean stages. The wells were scanned using a Zeiss Treval 3 inverted microscope through the water column of each well. Samples of surface water were also taken, placed on a coverslipped slide and observed at x 400 under phase contrast microscopy using an Olympus C-12 microscope. Worms within wells containing released actinosporeans were placed separately into fresh wells and left overnight. They were subsequently scanned as previously described to identify the infected worms.

6.3.1.5 *Identification of Released Actinosporeans*

Released actinosporeans were photographed under both phase contrast and bright field. Measurements were taken from the enlarged photomicrographs of a known magnification. Spores were identified using the keys of Janiszewska (1955, 1957), Marques (1984) and compared to published reports.

6.3.1.6 *Identification of Actinosporean Infected Worm Species*

The anterior third of infected worms was removed and fixed with 10% buffered formalin. They were washed twice with distilled water before being placed individually onto slides in a few drops of 50:50 glycerin in 70% alcohol, coverslipped and left overnight to clear. Immediately before examination the mount was flattened by slight pressure exerted on the coverslip. The worms were then examined under the appropriate magnification and identified using the key of Brinkhurst (1963).

6.3.1.7 *Immunohistochemical Studies on Actinosporean Infected Worms*

Small pieces of worm were fixed in 10% buffered formalin, processed using a histokinette processor, embedded in paraffin wax, sections cut at 5μm and placed on poly-l-lysine coated slides (BDH). Immunohistochemistry was performed on the sections as previously described (section 2.3.5) using all of the MAbs.

6.3.1.8 *Immunohistochemical Studies of Released Actinosporeans*

200μl samples of well water containing actinosporean stages were collected and spun onto poly-l-lysine coated slides using a Shandon cytospin for 4 minutes at 125g. The

slides were fixed in methanol and air dried before immunohistochemical examination. Immunohistochemistry was performed as previously described (section 2.3.5) on the actinosporeans using all of the MAbs and the lectin GS-I (section 5.3.3.2).

6.3.1.9 *Ultrastructural Studies on Infected Worms*

Small pieces of infected worms were fixed in Karnovsky's fixative for 4-6 hours at 4°C. They were rinsed twice in cacodylate buffer for 30 minutes, post fixed in 0.5% osmium tetroxide in cacodylate buffer for 1 hour, dehydrated through a graded acetone series and embedded in araldite epoxy resin (section 4.3.2). Worms were also embedded in LR white. These blocks were prepared by rinsing the tissue in Karnovsky's fixative containing 1% phosphotungstic acid overnight at 4°C. They were rinsed and post fixed as above before dehydrating through a graded acetone/ ethanol series to 70% ethanol. They were then rotated for 1 hour in 70% ethanol/LR white before transferring to 100% LR white. They were rinsed three times in LR white for 1 hour before embedding in LR white, in gelatin capsules overnight in a 70°C oven. Semi-thin sections of the resin blocks were cut at 0.5μm and stained in methylene blue. Preparations containing actinosporean parasites were selected and ultra thin sections cut at 70nm, placed on 400 mesh copper grids and stained with 2% uranyl acetate in methanol/ lead citrate for the araldite blocks and with 2% uranyl acetate in water/ lead citrate for 15 minutes for the LR white blocks and examined using a Philips 301 transmission electron microscope at 80kv.

6.3.1.10 *Experimental Infection of Rainbow Trout with Actinosporeans*

Five naive fingerling rainbow trout (mean weight 30g) were placed in a small plastic fish tank, containing a small amount of water. The fish were immobilised against the side of the tank by gently pushing the lid of the tank against them. The contents of a actinosporean infected well were then pipetted onto the head of each fish and immediately in front of the mouth. The volume of water in the tank was then increased, aerated and the fish left for 15 minutes before transferring to stock tanks maintained at 18°C. The fish were subsequently netted and exposed to fresh actinosporeans released by the worms using this method for a further 4 times over ten days. Eight weeks following initial exposure to actinosporeans the fish were killed and kidney impression smears taken and stained using Rapi-diff^{tm} for the presence of PK'X'.

6.3.2 *Analysis of MAb Binding to Other Myxosporea Present in PK'X' Infective Waters*

Paraffin embedded kidney samples of *Coregonus lavaretus*, *Salmo trutta*, *Thymallus thymallus*, *Pungitius pungitius*, *Esox lucius*, *Leuciscus leuciscus*, *Salmo salar* containing various myxosporean infections were obtained (courtesy of Dr S. Feist, MAFF, Weymouth and Dr J. McGeorge, Institute of Aquaculture, University of Stirling) and tested using immunohistochemistry.

The antibodies were also tested using immunohistochemistry on paraffin embedded kidneys infected with *S. onchorhynchi*, *S. renicola* and *Ceratomyxa shasta* by Dr M. Marin de Mateo at Stirling University.

6.4 **Results**

6.4.1 *Study to Determine the Infective Agent of PK'X'*

The results of experiments conducted in section 6.3.1 are summarized in table 6.1.

Over 1500 worms were obtained from the collected sediments. The majority of these worms were identified as species of *Tubifex* although, *Nais* and species of *Limnodrilus* were also noted.

Very few of the oligochaetes studied were infected with actinosporean stages so biological studies often involved one worm. This limited the number of studies that were possible on the worms and so incomplete studies were performed.

Four species from different genera of actinosporean were identified. These were identified as species of *Triactinomyxon*, *Aurantiactinomyxon*, *Neo-aurantiactinomyxon* and *Echinactinomyxon* respectively (figures 6.1-6.5). The spore dimensions are outlined in tables 6.2 and 6.3 and with the dimensions of comparable actinosporean species. From this it is suggested that the *Echinactinomyxon sp.* is of the same dimensions as *Echinactinomyxon radiatum* with a protoplasmic strip noted along the epispore float cells as described by Janiszewska (1957). Observations on the release of this actinosporean indicated that they were released from the worm as a group of spores

Table 6.1: Summary of results of experiments 6.3.1 conducted on actinosporeans and actinosporean infected worms

Actinosporean species	Oligochaete host genus	Number of oligochaetes infected	MAb Immuno-histochemistry	Lectin Immuno-histochemistry	Infected worm Immuno-histochemistry	Ultrastructral studies	Experimental transmission studies
Triactinomyxon	*Tubifex*	5	no reaction	no reaction	no reaction	no sporoplasmasomes	no transmission
Echinactinomyxon	*Tubifex*	3	no reaction	no reaction	-	no sporoplasmasomes	no transmission
Aurantiactinomyxon	-	1?	no reaction	-	-	-	-
Neo-aurantiacinomyxon	*Tubifex*	1	no reaction	binding to sporoplasm	no reaction	-	-

Table 2: Measurements of *Triactinomyxon* and *Echinactinomyxon* species compared to known species

	Triactinomyxon		*Echinactinomyxon*	
	present study	*Triactinomyxon ignotum* (Marques 1984)	present study	*Echinactinomyxon radiatum* (Janiszewska 1957)
epispore cavity length	25-42µm	30-50µm	29(28-31)µm	25-30µm
style (inc polar capsules)	211(205-218)µm	140-170µm	-	-
epispore float	245(205-275)µm	175-220µm	117(101-125)µm	100-125µm
sporozoites	8	8	-	-

Table 3: Measurements of *Neo-actinomyxon* and *Aurantiactinomyxon* species

	Neo-actinomyxon sp.	*Aurantiactinomyxon sp.*
endospore cavity diameter	16(15-17)µm	31(30-32)µm
whole spore diameter	39(36-41)µm	-
epispore float length	13(12-15)µm	25(21-28)µm
epispore float width (widest point)	23(22-24)µm	19(17-22)µm

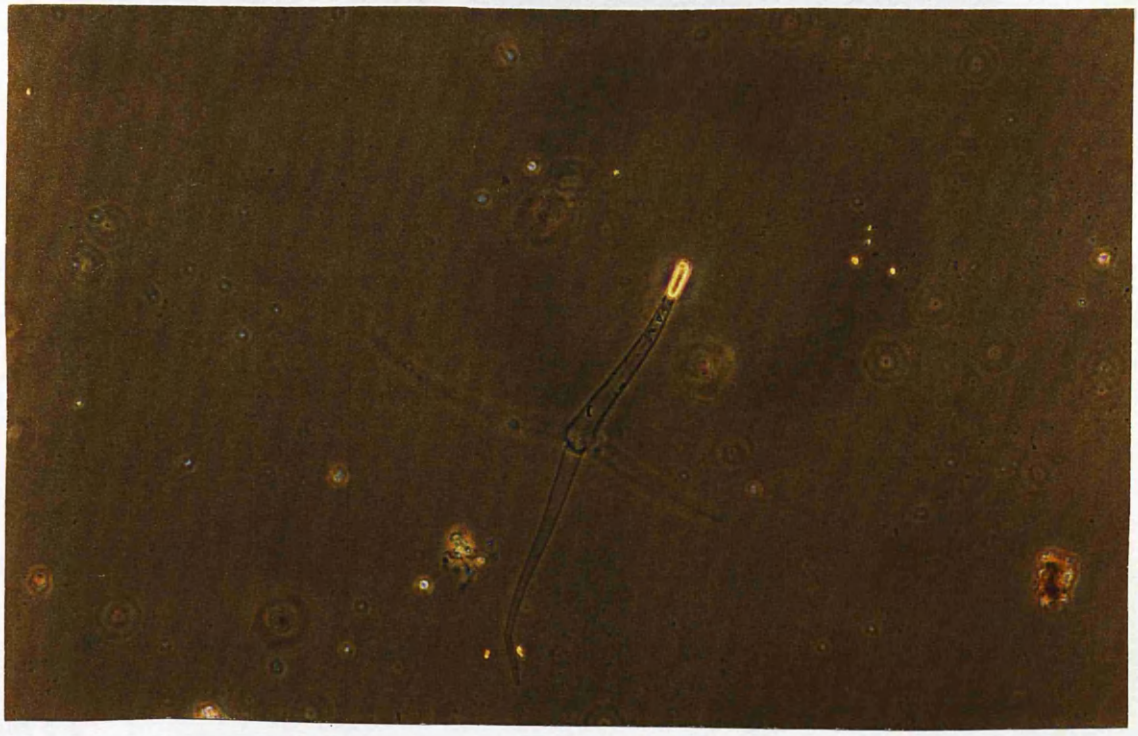


Figure 6.1
Triactinomyxon species found in worms present in PKD enzootic waters.
Phase contrast illumination. Mag X 100

Figure 6.2
Detail of the sporoplasm of *Triactinomyxon* species containing 8 sporozoites.
Phase contrast illumination. Mag X 1,000

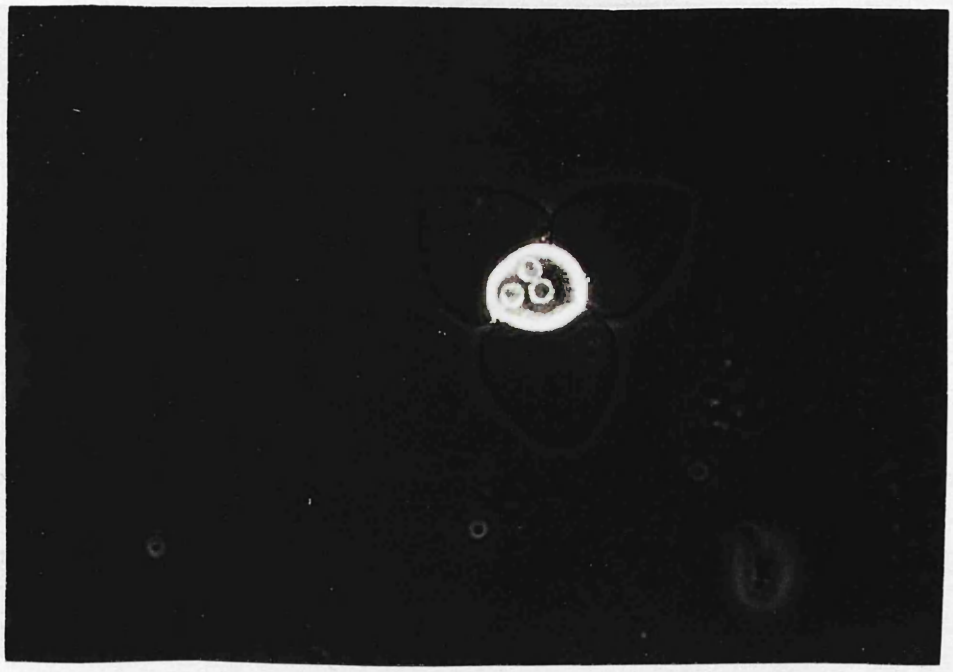


Figure 6.3
Aurantiactinomyxon species found in a worm present in PKD enzootic waters.
Phase contrast. Mag. X 800

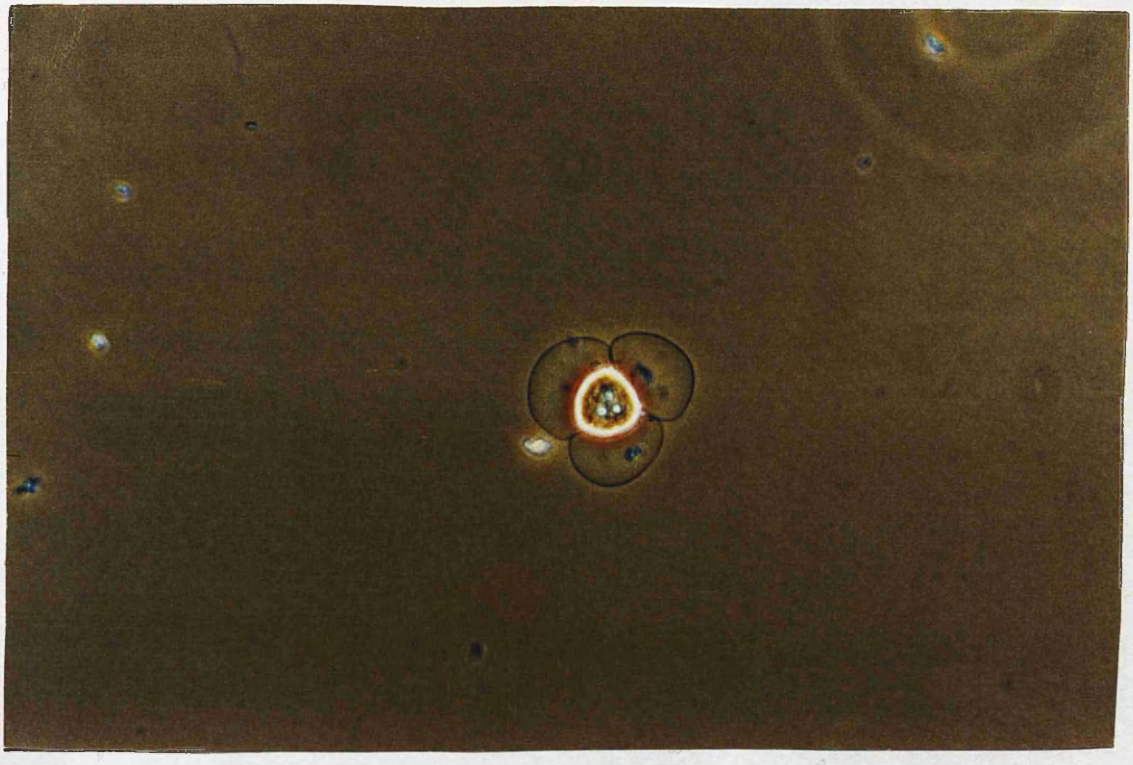


Figure 6.4
Neo-aurantiactinomyxon species found in a worm present in PKD enzootic waters.
Phase contrast illumination. Mag X 400

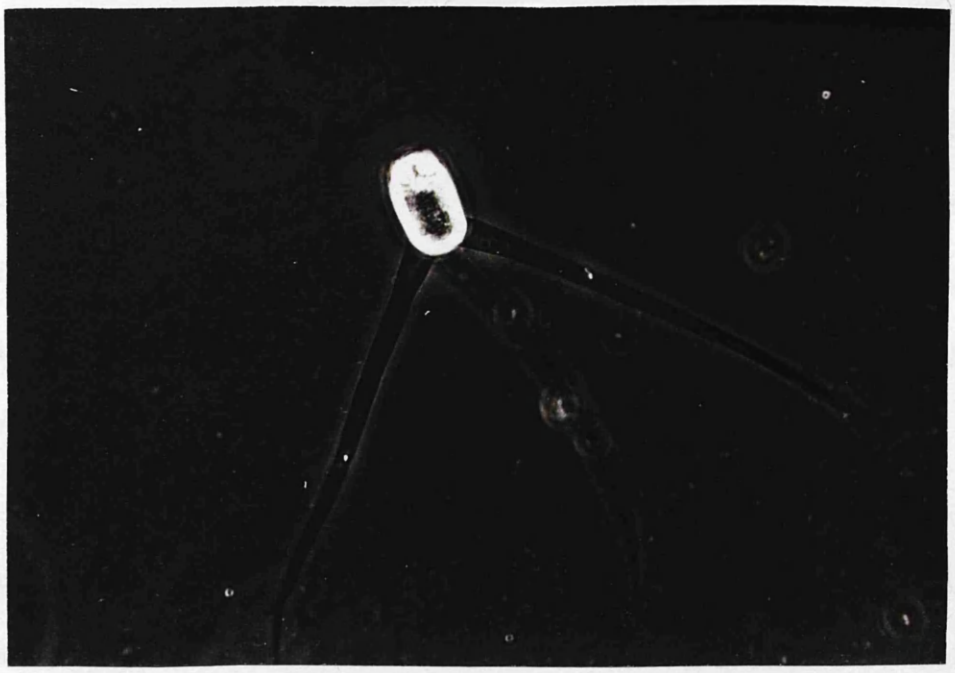


Figure 6.5
Echinactinomyxon species found in worms present in PKD enzootic waters.
Phase contrast illumination. Mag. X 400

finally to separate and settle on the bottom of the cell well, with their polar capsules oriented uppermost, the float tips appearing to possess adhesive qualities. The *Triactinomyxon sp.* was similar to *Triactinomyxon legeri* although the style length was consistently shorter. No comparable actinosporeans could be identified for the *Aurantiactinomyxon sp.* or the *Neo-aurantiactinomyxon sp.*.

The distribution determined that the *Triactinomyxon sp.* was present at sites A and B and was found in 5 worms. The *Echinactinomyxon sp.* was present at sites B and C and was found in 3 worms. The *Neo-aurantiactinomyxon sp.* was identified at site A in only one worm. The *Aurantiactinomyxon sp.* was located at site C although the releasing worm could not be identified.

6.4.1.1 *Identification of Releasing Worms*

All of the worms identified, belonged to the genus *Tubifex.* The oligochaete host releasing the *Aurantiactinomyxon sp.* could not be identified as it died before it could be isolated.

6.4.1.2 *Immunohistochemistry of Released Actinosporeans.*

None of the MAbs reacted with any of the released actinosporeans although the lectin GS-I appeared to selectively bind to the sporoplasm of the *Neo-aurantiactinomyxon sp.* (figures 6.6, 6.7).

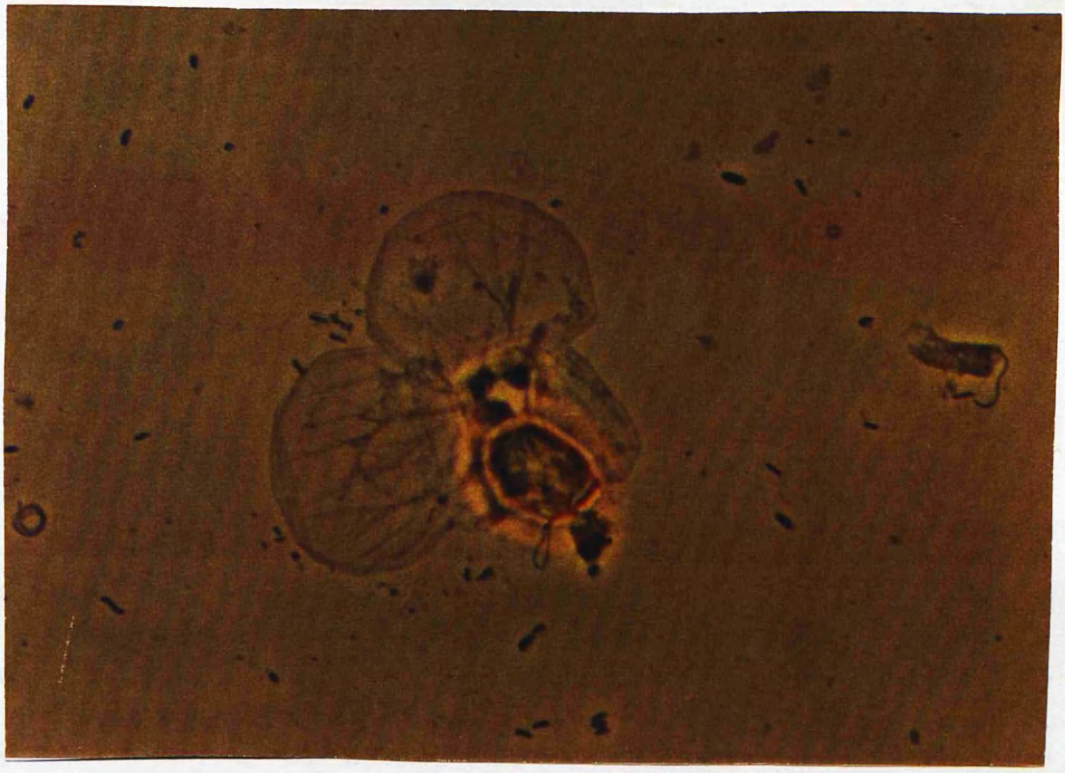


Figure 6.6
Pattern of staining with the lectin GS-I on emerging sporoplasm of the *Neo-aurantiactinomyxon* species
Phase contrast illumination. Mag X 1,000

Figure 6.7
Pattern of staining with the lectin GS-I on the emerging sporoplasm of the *Neo-aurantiactinomyxon* species.
Bright field illumination. Mag X 1,000

6.4.1.3 *Immunohistochemistry on Releasing Worms*

The worms tested with the monoclonal probes using immunohistochemistry did not result in a reaction.

6.4.1.4 *Ultrastructural Examination of Infected Worms*

Ultrastructural examination was carried out for the worms containing *Triactinomyxon* and *Echinactinomyxon* parasites. Developing *Triactinomyxons* were rarely detected within infected worms. Polar capsule formation could not be identified although valvogenic cells and developing float membranes were observed (figure 6.8). No electron dense bodies, indicative of PK'X' were observed in the cytoplasm of the *Triactinomyxon*. In the *Echinactinomyxon* infected worms, the intestinal epithelium appeared heavily infected with the parasite, with pansporoblasts observed in the intestinal epithelium and mature spores occurring within the intestines lumen. Polar capsules were identified, and these were seen to contain six turns of the polar filament (figures 6.9 and 6.10). No electron dense bodies indicative of PK'X' were identified in the sporoplasms of either species of actinosporean.

A coincident infection of an unidentified ciliate inhabiting the coelom of the oligochaete infected with the *Triactinosporean sp.* was also noted (figure 6.11).

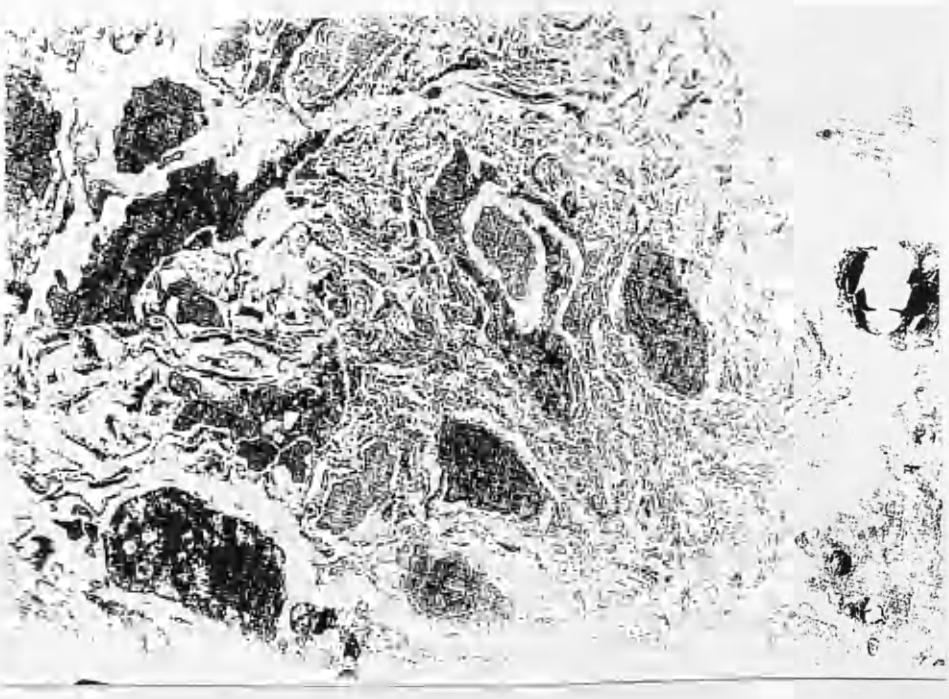


Figure 6.8
Triactinomyxon species within the coelom of a infected tubifex worm. Valvogenic cells are present (indicated with *) forming thin outgrowths of membrane which produce the future stylus and/ or anchor-like extensions of the actinosporean.
LR white. Mag X 5,400

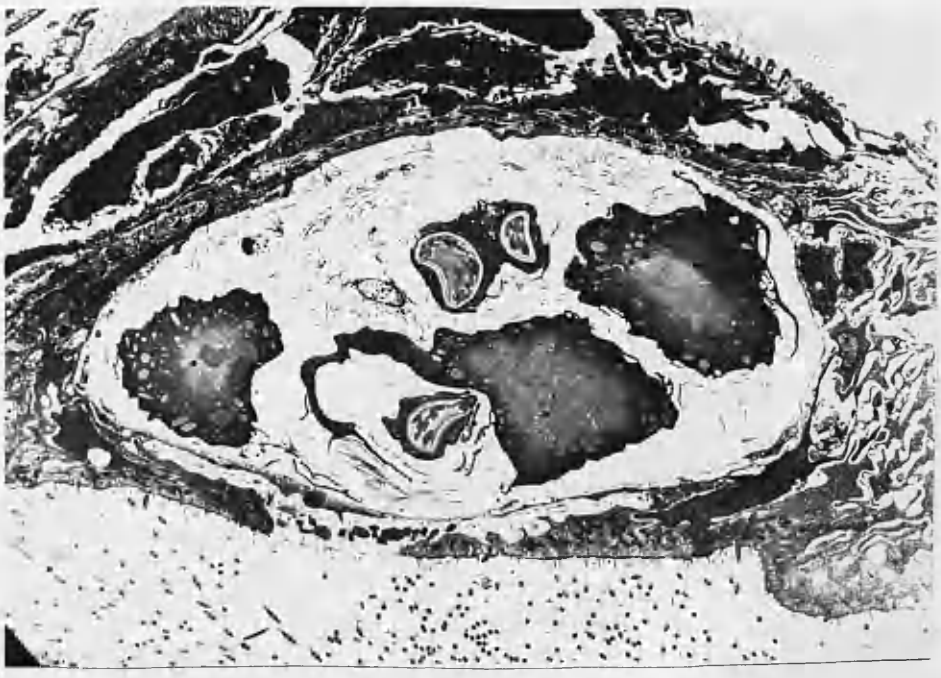


Figure 6.9
Echinoactinosporean species pansporoblast formation within the intestinal wall of a infected tubifex worm.
LR white. Mag.X 4,400

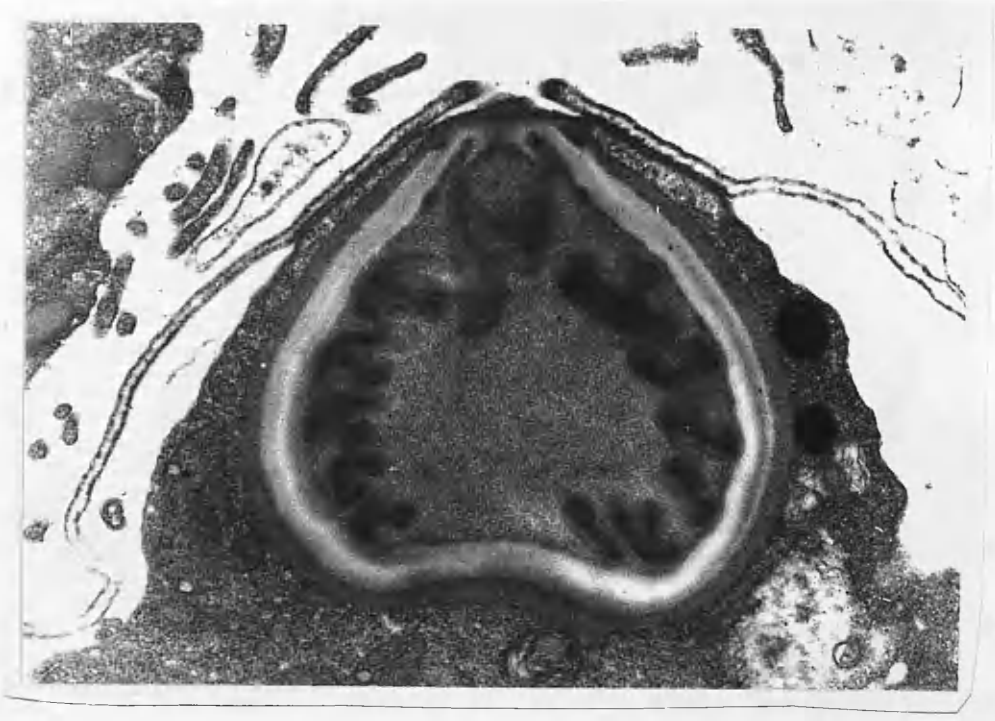


Figure 6.10
Polar capsule formation of *Echinactinomyxon* species displaying six turns of the polar filament.
LR white. Mag.X 22,000

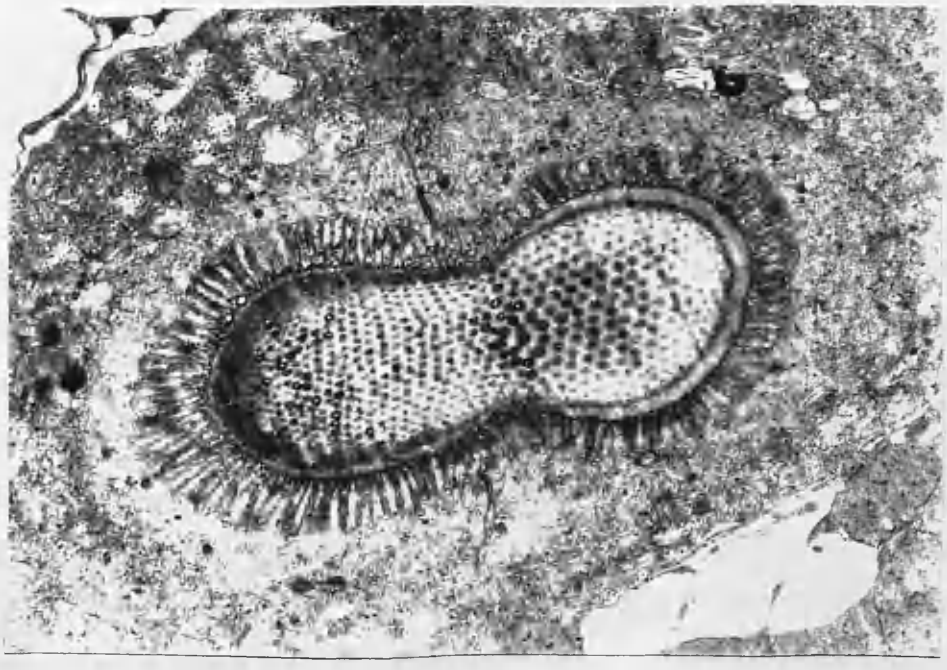


Figure 6.11
Unidentified ciliate, apparently dividing, occurring within the coelom of tubifex infected with *Triactinomyxon* species.
LR white. Mag X 13,000

6.4.1.5 *Experimental Infection of Fish*

Because of their relatively high prevalence the *Triactinomyxon sp.* and the *Echinactinomyxon sp.* were used in the infection trials. All of the fish remained PK'X' negative after experimental infection with these actinosporeans.

6.4.2 *Analysis of Antibody Binding to Other Myxosporea*

The binding characteristics of the MAbs on different myxosporean parasites is detailed in table 4. MAb B4 was found to react with several species of *Sphaerospora* and *M. cerebralis*. All of the MAbs tested reacted with an unidentified intratubular species of *Sphaerospora* infecting *S. salar*. This was the only species of Myxosporean that MAbs A3, C5 and D4 recognised apart from PK'X'.

6.5 **Discussion**

The analysis of oligochaetes from PKD infective waters could not determine the alternative host to the parasite. Although several actinosporean species were detected in the study, no single species was found common to all of the sites investigated. Actinosporean infections of oligochaetes are considered to be rare with only 0.1-4.1% of worms infected (Marques 1984; Yokoyama *et al.* 1991; McGeorge 1994). Furthermore, actinosporeans appear to remain viable in the water column from three days up to twenty-five days after release from the oligochaete, allowing them the opportunity to travel several miles before reaching a fish farm (Markiw 1992; Yokoyama *et al.* 1993a).

Table 4: Results of immunohistochemistry on myxosporeans inhabiting wild fish

Fish Species	Location/ date sampled	Parasite species	MAb binding			
			A3	B4	C5	D4
Salmo trutta	Haweswater. /7/87	*Chloromyxum spp.*	-	-	-	-
S. salar	R. Usk. /11/86	*Sphaerospora spp.*	-	+	-	-
S. salar	R. Test. /1/87	*Sphaerospora spp.*	+	+	+	+
C. lavaretus	Haweswater. /6/87	*Sphaerospora spp.*	-	-	-	-
L. leuciscus	R. Avon /8/86	*Sphaerospora spp.*	-	-	-	-
E. lucius	R. Avon /9/86	*M. lieberkuehni*	-	-	-	-
L. leuciscus	R. Avon /6/87	*Chloromyxum spp.*	-	-	-	-
L. leuciscus	R. Avon /6/87	*Myxobolus muelleri*	-	-	-	-
L. leuciscus	R. Avon /6/87	*Myxobolus spp.*	-	-	-	-
L. leuciscus	R. Avon /6/87	*Hoferellus spp.*	-	-	-	-
Pungtius pungitius	R. Avon /9/87	*Sphaerospora elegans*	-	+	-	-
Phoxius phoxius	R. Avon /5/87	*Sphaerospora spp.*	-	-	-	-
O. mykiss	R. Avon /7/93	*M. cerebralis*	-	+	-	-
O. mykiss	USA	*Ceratomyxa shasta*	-	-	-	-
Cyprinus carpio	Scotland	*S. renicola*	-	+	-	-
O. nerka	Canada	*S. onchorhynchi*	-	+	-	-

Thus, it is conceivable that possible actinosporean stages of the PK'X' life cycle were not identified in this study through constraints on sampling.

Actinosporean infected oligochaetes and released actinosporeans tested using immunohistochemistry and MAbs A3, B4, C5 and D4 did not show any reaction with the MAbs. The failure of the MAbs to recognise any of the actinosporeans does not exclude them as the infective agent of PK'X' as myxosporeans, including PK'X', have been shown to change their antigenic characteristics throughout their life cycles (Bartholomew *et al.* 1989; chapter 5). Immunohistochemistry with the lectin GS-I did however show a reaction with the sporoplasm of the *Neo-aurantiactinomyxon sp.* This demonstrates an antigenic relationship between this actinosporean and the PK'X' parasite. Sharing of antigens between an actinosporean and the corresponding myxosporean phase of the myxosporean life cycle has been previously documented for *M. cerebralis* and *H. exilis* (Markiw 1989b; Belem and Pote 1994). However, the lectin GS-I is not specific to the PK'X' parasite within the fish reacting with both tubule epithelium, and goblet cells (Marin de Mateo *et al.* 1993; chapter 5). As the lectin GS-I was only tested against two species of actinosporean in this study further work is needed to ascertain whether this *Neo-aurantiactinomyxon sp.* is the only species of actinosporean the lectin recognises. Recent studies on the infective agent have indicated that an unidentified *Aurantiactinomyxon sp.* prevalent in enzootic waters also displays antigens common to PK'X' (O'Flynn, Hanjavanit and Mulcahy 1995; D. Saulnier, pers. comm). Therefore results of lectin immunohistochemistry against the *Neo-aurantiactinomyxon sp.* although

interesting should be viewed with caution until experimental transmissions are carried out with this actinosporean species.

Ultrastructural studies of the *Echinactinomyxon sp.* and *Triactinomyxon sp.*, the most prevalent actinosporeans detected within the study did not suggest that they were part of the life cycle of PK'X'. The ultrastructural data of the sporoplasms of these actinosporeans failed to demonstrate the presence of the electron dense bodies that indicate a PK'X' cell. However, the electron dense bodies appear to be stage specific within the extrasporogonic parasite of PK'X' and therefore may not be expressed within other phases of its life cycle (Kent and Hedrick 1986).

The experimental transmission studies conducted with the *Echinactinomyxon sp.* and *Triactinomyxon sp.* on naive rainbow trout failed to elicit PKD within the fish. Previous attempts to experimentally transmit myxosporean diseases using actinosporeans suggest that large numbers of actinosporeans are needed to evoke a clinical infection and it is possible that the fish were not exposed to a sufficient number of stages (Markiw 1992). However, the measurements taken of the two actinosporeans in this study indicate that they would be too large to pass through a 50μm mesh filter, determined by Hedrick *et al.* (1992b) as allowing passage of the infective agent of PKD. Deformation of actinosporean floats under pressure could conceivably allow the spores to pass through such filters although studies of *T. gyrosalmo* suggests that this would not occur (Markiw 1986). These results from the studies in this chapter suggest that the *Echinactinomyxon sp.* and the *Triactinomyxon sp.* encountered are not the infective agents of PKD.

Studies to identify the definitive host for the PK'X' myxosporean using immunohistochemistry with MAbs A3, B4, C5 and D4 indicated the presence of an unidentified *Sphaerospora sp.* infecting *S. salar* during January which bound to all of the MAbs. Binding for this parasite only occurred with intratubular sporogonic stages, the extrasporogonic stages not appearing to be present in the kidney. Although the study demonstrated that MAb B4 was not specific for PK'X' MAbs A3, C5 and D4 appeared to bind only to PK'X' and the unidentified salmon *Sphaerospora sp.* It is possible that this *Sphaerospora sp.* is PK'X' and is analogous to the sporogonic stages of PK'X' reported in the USA that remain in the kidney tubule lumens months after the fish have recovered from the clinical infection (Kent and Hedrick 1986, 1987). Kent *et al.* (1993a, 1995) studying *Onchorhynchus nerka*, suggested that these stages remain in the kidney lumens until the maturation of the fish where they finally sporulate to form spores of *Sphaerospora onchorhynci*. Sections of *S. onchorhynchi* infected tissue tested with the MAbs in this study only reacted with MAb B4. Marin de Mateo, McGeorge, Morris and Kent (1996) using immunohistochemistry did not observe binding of the lectin GS-I to *S. onchorhynchi* sections, contrary to the observations of Kent *et al.* (1993a). MAbs A3, C5 and D4 are all specific for the extrasporogonic cell of PK'X' and the staining pattern of lectin GS-I is consistent with this (chapter 5). Discrepancies in the staining characteristics of the probes may be explained by antigens being lost from the developing spores as their pseudoplasmodium disintegrates finally to release the spores. To circumvent this problem, analysis of antibody binding should aim to examine the extrasporogonic and sporogonic stages of myxosporeans.

In view of the difficulties experienced in collecting and detecting actinosporeans future work on the life cycle of PK'X' should concentrate on the elucidation of the definitive host for the disease. Such studies in this chapter indicate that a *Sphaerospora sp.* found in *S. salar* may represent an advanced stage of the disease and also a possible definitive host. In light of this and the findings of Kent *et al.* (1993a, 1995) studies for the final host should focus on salmonids endemic for PK'X' infected river systems such as *S. salar* and *S. truttae*. Only when the definitive host for the disease is determined can large scale infections of oligochaetes and other marine invertebrates with mature spores be performed in controlled conditions, thus allowing the complete study of the life cycle.

Chapter 7

Summary and Conclusions

Four hybridomas which synthesised PK'X' reactive MAbs were successfully produced by a polyethylene glycol mediated fusion between spleen cells from an inoculated mouse and a mouse myeloma cell line. These MAbs were termed MAbs A3, B4, C5 and D4 respectively. Immunological characteristics of the MAbs were assessed. MAbs A3 and D4 were of the same immunological class and appeared to bind to the same antigens whereas the characteristics of MAbs B4 and C5 were unique. MAbs A3, C5 and D4 all reacted with the extrasporogonic parasite whereas MAb B4 bound to intratubular sporogonic stages and developing secondary cells within the extrasporogonic cell. The antigenic homology expressed between these stages indicates that secondary cells are released from the extrasporogonic cell to form the intratubular sporogonic stages.

One of the problems encountered with the manufacture of mouse anti-PK'X' MAbs was that a pure suspension of the parasite could not be obtained. Immunomagnetic separation using the MAbs was examined as a possible method for the parasites' future purification. Separations targeted against the extrasporogonic parasite using MAbs A3, C5 and D4 could not be achieved as a result of aggregates of macrophages attached to the parasites' surface. Ultrastructural observations indicated that many of the parasites were often totally engulfed by one or more macrophages. Attempts to reduce these aggregates, by sonication, trypsination and exposure to detergents resulted in limited success.

Immunomagnetic experiments to separate sporogonic stages using MAb B4 resulted in only tubules containing sporogonic stages being targeted. This was thought to be a result

of the MAb B4 binding antigen being secreted by the cell into its immediate environment.

The results from all of the separations were confused by the magnetic particles forming large amorphous aggregates with extracted cells. These aggregates were deemed as a result of extracting cells at the peak of the clinical disease when the extrasporogonic parasites cellular structure was disintegrating. Thus the observed aggregates would be composed of ruptured PK'X' cells and constituents such as DNA and lysosomes that would entrap and lyse host cells.

Further work is needed to improve the separations obtained by immunomagnetism. Possible modifications to the procedure may include targeting the parasite in its pre-clinical phase, thus circumventing many of the problems associated with adherent macrophages and the disintegration of the extrasporogonic parasite. This would be assisted by the development of further MAbs that selectively bind to the pre-clinical parasite. Tubules containing sporogonic stages were successfully extracted by immunomagnetism and MAb B4. Purification of sporogonic stages by this procedure may be achieved in future extractions with the development of MAbs that selectively bind to these stages.

Immunogold electron microscopy was employed to determine the location of the MAbs binding sites within PK'X' cells. Comparison of tissues embedded in LR white and araldite determined that LR white was superior for immunogold electron microscopy.

The localisation of the antigens binding to MAbs A3, C5 and D4 determined that they all recognised the same structures within the extrasporogonic parasite. These structures were primarily the lysosomal and vacuolar membranes although binding was detected on the plasma membrane, electron dense bodies and throughout the cytosol. This suggests that the vacuoles, often observed within the parasite, represent compartments involved in the lysosomal pathway of the cell and the antigens recognised by the MAbs may be related to the LAMP glycoproteins found within the lysosomal membranes of other eucaryotes. Localisation of the antigen on the vacuole enclosing secondary cells also suggests that this structure has a lysosomal origin.

Intensification of the staining of MAbs A3, C5 and D4 by immunohistochemistry on kidney sections taken over the course of the infection suggest that there is a significant increase in the number of lyosomes present within the primary cell of the extrasporogonic parasite. This can be compared to the increase in vacuolation noted by other authors over the course of the disease (Seagrave *et al.* 1980; MacConnell *et al.* 1989). During this vacuolation a decrease in cell organelles and cytoplasmic density has also been noted. This suggests that the primary cell of the extrasporogonic parasite undergoes autophagic digestion with the progression of the disease. The nature of these vacuoles needs to be examined further by cytochemical analysis to detect the presence of lysosomal enzymes such as acid phosphatase so as to precisely define their function within the cell.

Immunogold electron microscopy using MAb B4 demonstrated that the protein antigen recognised by this MAb was synthesised by maturing secondary cells and released into their environment. The purpose of this antigen remains unclear although it is expressed by the sporogonic stages of several myxosporea spp. including *M. cerebralis*. The association between the antigen and the areas of cartilage destruction surrounding the spores in this disease suggests that this antigen may have a proteolytic function.

Immunohistochemical studies on the course of the disease through rainbow trout using MAb B4 demonstrates that the antigen to which it binds is produced by maturing secondary cells within the extrasporogonic cell and the intratubular sporogonic stages of the parasite. As the initial production of the protein within the secondary cells coincides with the appearance of the intratubular sporogonic stages it can be speculated that this protein is associated with the development of the sporogonic parasite. Ultrastuctural studies with immunogold labelling demonstrated secondary cells synthesising the protein antigen being discharged by rupturing primary cells and migrating through the kidney interstitium. Therefore the purpose of the antigen may be to aid the release and migration of the secondary cell to the tubule lumen.

The autophagic digestion of the primary cell appears to be a pre-determined facet of the parasites life cycle and not a response to nutrient deprivation of the host's immune response. Whether this digestion is regulated by the developing secondary cell is unknown. Nutrients obtained from the autophagy of the primary cell would presumably aid in the nutrition of the secondary cell and ultrastructural observations have confirmed

that the secondary cell obtains nutrients by endocytosing the surrounding cell. The autophagic disintegration of the primary cell may also eventually result in this cell's lysis therefore releasing the secondary cell into the kidney's interstitium.

The appearance of intratubular sporogonic stages and the rapid proliferation of the parasite in the kidney was associated with a corresponding increase in the inflammatory response of the kidney. The immune response of fish to PK'X' is poorly understood, however the inflammation coincides with the release of secondary cells from the disrupted primary cells. This may be due to the disrupted primary cell liberating cellular constituents with the release of the secondary cell. This cellular material has been seen to be actively processed by macrophages. Thus the fish may only start to elicit an effective immune response against the parasite after the release of secondary cells. The increase in the inflammation of the kidney may also prevent parasite migration to tubules thus resulting in more parasites releasing secondary cells directly into the interstitium rather than into the tubule lumens. This would result in positive feedback with the kidney becoming more inflamed until all of the parasites have been eliminated.

Further work is needed to elucidate the structure and function of all of the antigens that bind to the MAbs in this study. In particular work should focus on MAb B4 which demonstrates an antigen that is associated with the sporogonic development of the parasite, a process which may help to explain the pathogenic nature of this organism.

Sporogenesis was not observed to occur within the rainbow trout, with tissues appearing to totally eliminate the parasite during mid August. The presence of complex electron dense structures within tubules and the kidney interstitium suggests that the sporogenesis of the parasite is interrupted. Therefore sporulation of PK'X' as suggested by Kent *et al.* (1993a) does not occur within the rainbow trout from European waters and this is not the natural host for the disease. However immunohistochemical studies using the MAbs on other fish species inhabiting PK'X' enzootic waters did indicate the presence of a *Sphaerospora spp.* infecting Atlantic salmon that bound with all of the probes. Although MAb B4 bound to several species of Myxosporean, MAbs A3, C5 and D4 all appeared to be specific for PK'X' and the salmon *Sphaerospora sp.* demonstrating antigenic homology between these parasites. Future studies should investigate the relationship between the *Sphaerospora sp.* and PK'X' in Atlantic salmon to determine whether this is the definitive host for the disease.

Studies to locate the site of the initial infection of the parasite before its appearance in the kidney were inconclusive. The parasite was first detected in the blood vessels of the gill, kidney and spleen indicating that it reaches these tissues via the vascular system of the fish. Analysis of the gill, fin, swimbladder, skin, muscle, eye, vertically bisected head (including the brain) and intestine prior to the detection of the extrasporogonic parasite in the kidney all failed to elucidate the presence of the parasite. Investigations on the maturation of the parasite in the kidney with immunohistochemistry demonstrated that the antigens to which the MAbs bind are stage specific to the parasite's life cycle and therefore early stages may not be recognised by the probes. Alternatively the wrong

organs may have been screened or the parasite was not in high enough numbers to detect. Future studies to determine the initial site of the parasite's infection should utilise PK'X' specific DNA probes that are now becoming available for the diagnosis of the disease (Saulnier *et al.* 1995). These would circumvent many of the problems associated with using MAb probes by permitting the detection of single parasites within a whole organ and not relying on the changing antigenic characteristics of the organism. One possible problem with using DNA probes may be associated with the systemic nature of PK'X'. This may result in tissues and organs harbouring very few parasites being detected in PCR studies, effectively providing false positives for tissues which are not specifically targeted by the parasite for its development.

Experiments to determine the alternative host to PK'X' by studying the aquatic oligocheate population of fish farms were unsuccessful. Although over 1,500 worms were examined only four species of actinosporean were described. None of these species reacted with the MAbs produced in the study. However, the lectin GS-I was found to react with the sporoplasm of a *Neo-aurantiactinomyxon sp.* This actinosporean was also small enough for passage through the 50μm precision mesh screen determined by Hedrick *et al.* (1992b) to pass the infective agent of PK'X'. Further study is needed to determine the role of this actinosporean in the life cycle of the parasite.

The observation of a haplosporidian sporocyst, that reacted strongly with three of the MAbs, associated with the gill of a rainbow trout exposed to PK'X' waters deserves further examination. Comparisons between PK'X' and the Haplosporidia have

previously been noted (Seagrave *et al.* 1980). In this study, ultrastructural examination of the electron dense bodies indicate that they appear to be very similar to the haplosporosomes described in species of the Haplosporidia. Myxosporeans are known to occur in species of amphibians as well as fish, and taking into account the difference between the number of actinosporean spp. described and the number of myxosporean spp. it is not unlikely that other aquatic invertebrates may be involved in the life cycle (Desser, Lom and Dykova 1986). Therefore examination of the alternative host for myxosporean infections should not be exclusively limited to oligocheates.

Examination of PKD enzootic rivers for the alternative host although feasible is both time consuming and laborious with large numbers of worms having to be sampled and screened to determine a relatively small actinosporean population. Unless actinosporean spp. already collected from PKD infected rivers can be screened using PK'X' specific DNA probes it may be more profitable to concentrate studies on locating the definitive fish host for the disease. Not only would this permit the precise taxonomic classification for the disease it would also allow for uninfected invertebrates to be exposed to large numbers of mature PK'X' spores, thus inducing a substantial population of the alternative host to be infected with the PK'X' organism which can be used for experimental transmissions and other biological studies.

PKD remains one of the most commercially important diseases of Salmonid culture in the northern hemisphere. Recent developments in the *in vitro* culture of the parasite and successful passive immunisation experiments suggest that an effective vaccine can be

produced against the disease (McGeorge, Adams, Feist and Richards 1995; Adams, Morris and Richards 1995). With the withdrawal of malachite green in the USA and similar proposed legislation in Europe, further research is undoubtedly needed on this disease to provide a vaccine and comprehensive control strategy.

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