Cellular inflammatory response of rainbow trout to PKX
by Elizabeth MacConnell

A thesis submitted in partial fullfillment of the requirements for the degree of Master of Science in Veterinary Science
Montana State University

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Cell identification was based on the literature for peripheral blood leukocytes. In contrast to most Myxosporeans, PKX provoked a severe host response. Initially, the response to PKX was hempoietic hyperplasia followed by proliferation of mononuclear cells. The major lesion was a marked granulomatous nephritis. Resolution of lesions without fibrosis and elimination of PKX was seen by termination of the study. The macrophage was the predominant cell type involved in the inflammatory response to PKX throughout the study. Clay induced a chronic granulomatous response only in the viscera. Intense proliferation of melanomacrophages was the predominant response seen in kidneys, but clay particles that reached the kidney were phagocytosed primarily by macrophages. In this study, PKX and a non-infectious agent, clay, were effectively removed from the kidney by macrophages. Hemtopoietic cell types in fish are still far from being adequately charcterized, in particular the immature forms.

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5/13/88
Date

C A Speer
Chairperson, Graduate Committee

Approved for the Major Deparment

5/13/88
Date

C A Speer
Head, Major Department

Approved for the College of Graduate Studies

May 25, 1988
Date

Henry J Parsons
Graduate Dean

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TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

The cellular inflammatory response of rainbow trout to PKX was investigated. The period studied was from three to twenty weeks post-injection of PKD-infected kidney homogenate. The inflammatory response to PKX was compared to that of a non-infectious agent, bentonite clay. Kidney samples were examined by light and electron microscopy. Cell identification was based on the literature for peripheral blood leukocytes. In contrast to most Myxosporeans, PKX provoked a severe host response. Initially, the response to PKX was hempoietic hyperplasia followed by proliferation of mononuclear cells. The major lesion was a marked granulomatous nephritis. Resolution of lesions without fibrosis and elimination of PKX was seen by termination of the study. The macrophage was the predominant cell type involved in the inflammatory response to PKX throughout the study. Clay induced a chronic granulomatous response only in the viscera. Intense proliferation of melanomacrophages was the predominant response seen in kidneys, but clay particles that reached the kidney were phagocytosed primarily by macrophages. In this study, PKX and a non-infectious agent, clay, were effectively removed from the kidney by macrophages. Hemtopoietic cell types in fish are still far from being adequately charcterized, in particular the immature forms.

INTRODUCTION

Proliferative kidney disease (PKD) is a potentially severe parasitic disease of economic importance in intensively cultured salmonid fishes in Europe and North America. This disease is caused by an unclassified protozoan referred to as PKX. It has been proposed that PKX belongs to the phylum Myxozoa because myxosporean trophozoites and developing spores have been observed in the renal tubules of PKD-infected fish (Hedrick et al., 1984; Kent and Hedrick, 1985). Identification of the infective stage, mode of transmission, intermediate or reservoir hosts and source of the infection are unknown. In addition, the life cycle of this parasite is poorly understood.

Proliferative kidney disease was initially described in fingerling rainbow trout (Salmo gairdneri) by Roberts and Shepherd in 1974. Since then, PKD has been reported in most European countries (Clifton-Hadley et al., 1984). The first reported outbreak of PKD in North America occurred in rainbow trout in 1981, at the Hagerman State Fish Hatchery, Hagerman, Idaho (Smith et al., 1984).

Typically, PKD affects fingerling trout or salmon of the 0+ year age class. The disease has been reported in several trout and salmon species, most often in rainbow trout. Outbreaks of PKD vary markedly in severity; morbidity can be 100% with mortality from 0% to 90% (Clifton-Hadley et al., 1984). Fish with PKD show poor tolerance to stress, increased feed conversion rate and greater susceptibility to secondary infections.

It has been suggested that salmonid species are aberrant hosts for this parasite due to incomplete spore development and the severe inflammation seen in infected fish, which is unusual for most myxosporean infections (Dykova and Lom, 1978). Similar characteristics shared between the family Shaerosporidae and PKX have been described by Hedrick et al.(1984; 1988) and Kent (1985). Shaerospora sp. has been found in tui chub (Gila bicolor) inhabiting the water supply of PKD-infected Hot Creek State Hatchery, California and stickleback (Gasterosteus aculeatus) from Quinault Lake, Washington, water supply of a PKD-infected steelhead hatchery (Hedrick et al., 1988). Although Shaerospora sp. has been implicated as the possible etiologic agent of PKD, cross transmission experiments conducted by Rafferty (1985) with Shaerospora infected roach (Rutilus rutilus) and carp (Cyprinus

carpio), and by Hedrick (pers. comm.) with tui chub and stickleback have been unsuccessful.

The initial cellular response observed by light microscopy to PKX is the proliferation of hemopoietic cells in the interstitium of the kidney. Subsequently, the major kidney tissue response to PKX is interstitial hypercellularity, attributed to the infiltration and proliferation of mononuclear cells. There are variable reports in the literature concerning the cells involved in the immune response to PKX. A variety of morphological and staining characteristics have been used to describe these cells, but the cell types have not been defined. The objective of this study was to describe, by light and electron microscopic examination of rainbow trout kidney, the predominant cell types involved in the inflammatory response to a non-infectious agent and to PKX during initial and later stages of disease.

LITERATURE REVIEW

Historical Background

A disease syndrome in fingerling rainbow trout with gross kidney changes was first named prolferative kidney disease (PKD) by Roberts and Shepherd in 1974. Subsequently, Ferguson and Needham presented the first indepth description of PKD (1978). Possibly, earlier diseases described as "amoebiasis" of trout (Plehn, 1924), infectious kidney swelling and liver degeneration (Schaperclaus, 1954) and infectious anemia (Besse, 1954) were actually PKD. Proliferative kidney disease is now considered one of the most devastating diseases in the fish farming industry in Europe.

Etiological Agent

Taxonomy

Initially, PKD was thought to be caused by an amoeba because the organism formed pseudopodia (Ghittino et al., 1977). Ghittino et al. (1980) subsequently concluded that this amoeba was a contaminant. Ferguson and Adair (1977) suggested that PKX was an amoeba or myxosporean but the

inability to culture the organism or find spores precluded a definite classification. An ultrastructural study of the PKX parasite conducted by Seagrave et al. (1980) showed haplosporean features, namely the occurrence of what they referred to as "haplosporosomes", and multivesicular bodies in the cytoplasm; similar to those seen in the oyster parasite, Martiela sp., currently in the phylum Acetospora. Structures similar to "haplosporosomes" have also been described from members of the phylum Myxozoa (Current, 1979). Recent studies by Kent and Hedrick (1985) indicate that PKX is a myxosporean, as evidenced by the presence of intraluminal trophozoites and developing spores in kidney tubules of infected steelhead trout. The fine structural features of PKX as described by Feist and Bucke (1987) are consistent with early stages of sporogenesis of a myxosporean. However, the precise taxonomic status of PKX has yet to be determined.

Morphology

PKX organisms are 5-20 um in diameter, stain weakly eosinophilic with H&E, have periodic acid-Schiffs (PAS) positive cytoplasmic granules, small pseudopodia, distinct plasmalemma and contain one to several secondary or daughter cells (Ferguson and Needham, 1978; Hedrick et al., 1984; Smith et al., 1984). Ultrastructually, a prominent

feature of PKX is the presence of electron-dense cytoplasmic inclusions, referred to as "haplosporosomes" (Seagrave et al., 1980). They are 0.14-0.20 um in diameter, have an electron-lucent bar, are often associated with the plasmalemma and are found only in the primary cell (Ferguson and Needham, 1978). The nature and function of PKX "haplosporosomes" are not known.

The most consistently described characteristics of the PKX primary cell are a well developed endoplasmic reticulum, numerous multivesicular bodies, lipoid bodies and a prominent plasmalemma (Kent and Hedrick, 1984). Secondary or daughter cells found within primary cells have a distinct Golgi apparatus, double cell membranes and numerous cytoplasmic ribosomes (Feist and Bucke, 1987) typical of myxosporean generative cells (Current 1979). Bundles of microtubules within secondary and tertiary cells have also been observed, an indicative feature of myxosporeans (Feist & Bucke 1987). Two spherical polar capsules with coiled filaments found within capsulogenic cells have been described in spores developing in kidney tubules of steelhead and rainbow trout (Kent and Hedrick, 1985).

Many similarities exist between PKX and early stages of a Shaerospora sp. first described as "Csaba's blood protozoan", in the blood of common carp (Cyprinus carpio)

(Bucsek and Csaba, 1981). Further studies determined that the Csaba's blood protozoan was an early stage of S. renicola and the parasite that causes swimbladder inflammation (Molnar, 1984; Csaba et al., 1984). Shaerospora renicola forms endogenous daughter cells which are released and sporulate in kidney tubules, similar to the pattern of development that has been observed for PKX (Csaba et al., 1984; Kent and Hedrick, 1985).

Development of intraluminal forms of PKX are similar to the sporogenic stages of Shaerospora spp. Both myxosporeans are monosporous and sporoblasts are formed within pseudoplasmodia. The outer enveloping cell of the intraluminal PKX is analogous to the pseudoplasmodia described for Shaerospora (Lom et al., 1983). Although there is no evidence of valve formation, valvogenic cells surrounding PKX capsulogenic cells have been observed and the immature spores of PKX and Shaerospora are very similar in size and shape (Kent and Hedrick 1985).

Shaerospora has been reported in brown trout from PKD enzootic waters (Ferguson, 1984; Fischer-Scherl et al., 1986) and one rainbow trout from a hatchery where PKD is enzootic (Hedrick et al., 1988).

Epizootiology

Host and Geographical Location

Proliferative kidney disease is found principally in salmonid species, most commonly in hatchery-reared rainbow trout. The disease has also been reported in cultured chinook salmon,(*Oncorhynchus tshawytscha*) and coho salmon (*O. kisutch*) (Hedrick et al., 1984); Atlantic salmon,(*Salmo salar*) and brown trout,(*S. trutta*) (Ellis et al., 1982); wild grayling, (*Thymallus thymallus*) (Seagrave et al., 1981) arctic char, (*Salvelinus alpinus*) (Bucke et al., 1985). Northern pike, (*Esox lucius*) and roach, (*Rutilus rutilus*) are the only non-salmonid species in which PKD has been described (Seagrave et al., 1981).

Since proliferative kidney disease was first described in 1974, it has been diagnosed in England, Wales, Scotland, Northern Ireland, Sweden, Republic of Ireland, Italy, France, Germany (Clifton-Hadley et al., 1984) and Denmark (Olesen et al., 1983). Following the discovery of PKD in 1981 in Idaho (Smith et al., 1984), the disease has been reported in hatcheries in California, Washington and British Columbia, Canada (Hedrick et al., 1984; Hoskins, 1986). This may not be indicative of an increased range of the disease but improved recognition of PKD. Review of

histological records at the American River California State Fish Hatchery since 1966 revealed the presence of PKD, previously referred to as "lupus" (Hedrick et al., 1985).

Transmission

Natural transmission of PKX occurs by exposure to enzootic waters. There is no evidence of transmission from fish to fish or by feeding homogenized or trypsinized preparations of infected kidneys to healthy fish (Ferguson and Ball, 1979, D'Silva et al., 1984). Holding disease-free fish in aquaria with feces from infected fish, or with infected fish was also unsuccessful in transmitting PKX (D'Silva et al., 1984). Experimental transmission of PKX has been successful only by intraperitoneal injection of infected kidney homogenates (Clifton-Hadley et al., 1984; D'Silva et al., 1984), or with whole blood or spleen homogenates (Kent et al., 1985).

There is little evidence as to the mode of entry of the PKX parasite into the fish. Access through the gill (Clifton-Hadley et al., 1983) or ingestion (Ghittino et al., 1977) have been suggested as the most likely, because the parasite would then be able to travel via the lymphatic and/or circulatory systems to the target tissues.

Environmental Factors

The severity of PKD can vary markedly and is probably influenced by several environmental factors. Low oxygen levels and fish handling result in increased mortalities due to the respiratory distress caused by the anemia induced in infected fish.

The disease occurs most frequently at water temperatures of 15 C or above (Clifton-Hadley et al., 1984). Spontaneous infections and experimentally-induced disease have occurred at lower water temperatures (Ellis et al., 1982; Hedrick et al., 1984). Several studies suggest that onset and severity of the disease are dependent on water temperature (Ferguson and Ball, 1979; Ferguson, 1981; Clifton-Hadley et al., 1985; Foott et al., 1986). Most likely water temperature affects both the development of the parasite and the host response (Clifton-Hadley et al., 1986). Time of peak infectivity may be seasonal due to the cyclical development of the parasite, which may not be associated with rising water temperature (Hedrick et al., 1985). At one study site in California, the infective stage of PKX was present in the water from April through November with peak prevalence of infection occurring in June (Foott et al., 1986). Data from the Hagerman Idaho State Fish Hatchery also support the seasonality of PKD. At

this facility the water temperature is a constant 15 C throughout the year but PKD only occurs from April through December (Smith, pers. comm.)

Concurrent infections with bacteria, viruses and other protozoans often occur, causing greater losses and making cause of mortality more difficult to determine. Initially, PKD was reported to occur only in soft water conditions (Roberts, 1978; Ferguson and Needham, 1978), but the disease occurs in hard alkaline waters as well (Scott, 1979).

Pathogenesis

Initial diagnosis of PKD is made by microscopic examination of imprint (Klontz and Chacko, 1983; Clifton-Hadley et al., 1984) or wet mounts of kidney prepared from affected fish. Confirmation is obtained by observation of the parasite and associated host inflammatory response by histologic examination of affected kidney tissue (Hedrick et al., 1984). Serological diagnostic tests are not available for the diagnosis of PKD.

Six to eight weeks after infection, clinical signs including anemia, abdominal distention due to ascites, darkened coloration, exophthalmia, renalmegaly and

splenomegaly appear (Ferguson and Needham, 1978). Presumptive diagnosis is often difficult because these signs are often encountered with other fish diseases that affect kidney function. Affected kidneys are usually grey and markedly swollen; in severe cases they appear corrugated. Fish exhibiting clinical signs of PKD are often anemic. The anemia has been classified as a chronic hemolytic anemia, possibly caused by toxins released from the parasite (Hoffman and Lommel, 1984). Leukocytosis has not been reported in fish with PKD.

Gross or microscopic signs of PKD are rarely detected before four weeks following natural or experimental infection to PKX. The characteristic kidney lesions are areas of diffuse granulomatous reaction that often surround one or more PKX parasites (Hedrick et al., 1986). Ferguson and Needham (1978) described a whorling appearance of inflammatory tissue with centrally placed PKX cells, nephron destruction and sclerosis of glomeruli. Severe necrotizing vasculitis and subsequent occlusion of renal and hepatic vessels are observed frequently due to PKX organisms adhering to and destroying vessel walls (Smith et al., 1984). PKX can invoke a similar granulomatous response in the spleen, gills, muscle, pancreas and a mononuclear infiltrate in liver and intestinal mucosa of heavily infected fish (Ferguson and Needham, 1978;

Clifton-Hadley et al., 1984).

The location of the parasite during early infection is unknown. Using antiserum to PKX from rabbits, Rafferty (1986) was able to produce marked kidney tubular fluorescence by the indirect immunofluorescent test (IFT) during the one to three week period post-injection. The parasite was not observed in these samples during this period. Tubular fluorescence did not occur in control fish and to a very small degree during the subsequent course of the disease, but fluorescing PKX cells were observed in the hemopoietic tissue from four to nine weeks post-injection.

Kent and Hedrick (1986) observed an early form of PKX in the kidney interstitium at three weeks post-exposure. They described the organism as small, condensed, eosinophilic, and containing a daughter cell. Clifton-Hadley et al. (1983) found the first evidence of PKX at five weeks post-exposure in the peritubluar capillaries, often with one or more basophilic, crescent-shaped bodies associated with their outer surface. PKX has been detected in tubular epithelium and lumina as early as seven weeks after injection (Kent and Hedrick, 1986). The intraluminal parasites further develop to form multicellular spores with polar capsules, but no valves (Kent and Hedrick, 1986).

The sequential pathologic changes described in the

literature for PKD varies in the time course from initial response and lesion development to resolution. The initial cellular response to PKX, observed by light microscopy five to seven weeks post-injection, is the proliferation of hemopoietic cells in kidney tissue (Clifton-Hadley et al., 1984). Proliferation of melanomacrophage centers as a feature of early lesions has also been reported (Rafferty, 1986). The major kidney tissue response to PKX is interstitial hypercellularity which is attributed to the infiltration and proliferation of mononuclear cells, presumably macrophages, and is prominent between eight to eleven weeks post-injection (Kent and Hedrick, 1986). These cells obliterate much of the normal hemopoietic tissue, renal tubules and glomeruli by nine weeks post-injection (Ellis et al., 1985). The principal cell types in lesions have been described tentatively as macrophages, lymphocytes, fibroblasts and cells that may be transforming into plasma cells (Ferguson and Needham, 1978; Clifton-Hadley et al., 1983). Lymphocytes are also abundant and have been reported closely associated with PKX (Clifton-Hadley et al., 1983; Kent and Hedrick, 1986). Organization and resolution of kidney lesions and necrosis of PKX has been reported by 12 weeks after onset of disease (Ellis et al., 1982). Nodules of chronic inflammation containing intact PKX and fibrous tissue have also been

observed (Clifton-Hadley et al., 1983). Resolution of lesions involves destruction of the parasite and resorption of dead and dying cells. In most fish surviving PKD there is little or no histologic evidence of lesions at 21 weeks post-injection (Clifton-Hadley et al., 1983).

Control

Until recently the use of antibacterial and/or antiprotozoal compounds had been unsuccessful in controlling PKD (Ghittino et al., 1977; Ferguson and Ball, 1979; Bucke et al., 1981; Clifton-Hadley et al., 1984). Most recently, successful treatments of Shaerospora renicola infections of common carp by oral administrations of fumagillin, an antibiotic produced by the fungus Aspergillus fumigatus, led Hedrick et al. (1988) to test the drug against PKD in juvenile chinook salmon. The drug provided protection against PKD but toxicity problems were encountered. Development of PKD was found to be delayed when clinically and subclinically affected fish were treated with repeated doses of high concentrations of malachite green (Clifton-Hadley and Alderman, 1987). However, unacceptable levels of malachite green were found in the fish. Further study on toxic effects of these chemicals on fish will be necessary before they can be used for the

treatment of PKD in hatcheries.

In Scotland, salination of the water supply has been reported to alleviate signs of PKD (O'Hara, 1985). However, an experiment conducted in California in which chinook salmon with PKD were transferred to full strength sea water did not demonstrate the same beneficial effects (Hedrick and Aronstein, 1987). Usually, modifications of husbandry practices such as delayed movements to infected water, reduced handling, lowered densities, increased oxygen levels and decreased water temperatures are employed to reduce the affects of PKD, increasing the economic cost of the disease.

Immunity

Fish have non-specific, natural immunity and specific humoral and cell-mediated immune mechanisms similar to, but not identical with, those of higher vertebrates (Ellis and Munroe, 1976). A characteristic feature of both cell-mediated and antibody-mediated immune response in fish is their dependence on water temperature. Several studies on the relationship between water temperature and the rate of development of the acute and early chronic inflammatory response have shown an approximate 50% reduction in rate of development for a reduction in temperature of 10 C (Finn

and Nielson, 1971; McQueen et al., 1973; Roberts et al., 1973; Anderson and Roberts, 1975).

There are three major lymphoid organs in fish: the thymus, spleen, and kidney. The anterior portion of the kidney is the primary site of hemopoiesis. Posteriorly, much of the extrarenal tissue is also composed of blood-forming tissue (Yasutake and Wales, 1983). Many undifferentiated cells, as well as immature and mature red blood cells and white blood cells are present. Based on mammalian morphological criteria, fish leukocytes have been classified into five main groupings: thrombocytes, lymphocytes, granulocytes, monocytes and hemocytoblasts (Ellis, 1977). There is no clear division of lymphocytes into T- and B-cells, although there is some evidence of functional analogues to T- and B-cells (Lewis et al., 1979). Fish possess only one, Ig-M like, tetrameric immunoglobulin class (Dorson, 1981).

Many features of the fish defense system are poorly understood such as the role of the neutrophil, the function of the complement system, the presence, nature and role of histaminogenic cells and the nature, origin and possible role of the melanin and lipofucsin pigments associated with inflammatory lesions.

Morphologically the cells involved in a granulomatous response are generally similar to their counterpart cells

in higher animals, both at the light microscopic and ultrastructural levels (Timur and Roberts, 1977). However, the inflammatory response in fish is less intense and slower to appear and resolve than in mammals (Finn and Nielson, 1971).

Host Response to PKX

There are limited reports in the literature concerning immunity to PKD. Yearlings not previously exposed to PKD readily contract the disease, whereas survivors show complete resistance to reinfection (Kent and Hedrick, 1985). However, previous exposure alone is not sufficient to produce immunity; recovery from active infection is necessary (Ferguson and Ball, 1979; Kent and Hedrick, 1985). The nature of resistance to infection most likely results from strong humoral (Olesen and Jorgensen, 1985; Klontz et al., 1985) and cellular responses (Ellis et al., 1982). Passive transfer of serum from recovering fish to actively infected fish speeds the recovery and reduces the incidence of parasites and lesions (Hedrick et al., 1985).

Hypoproteinemia is also a characteristic of PKD. Scott (1984) measured changes in serum protein levels during an outbreak of PKD and suggested that the increase of one protein, most likely an acute phase protein, was indicative

of a defense response. Klontz et al. (1986) reported a progressive increase in the serum beta globulins in the IgM range for salmonids and decrease in albumin in clinically ill fish.

Different stocks of Atlantic salmon have exhibited diverse susceptibilities to infection with PKX (Ellis et al., 1982). Reports have also suggested different levels of susceptibility to PKD based on the varied intensities of the host response. For example, Kent and Hedrick (1985) observed a higher incidence of intraluminal parasites and corresponding milder proliferative response in PKD-infected kidneys of brown trout when compared to rainbow trout.

There are variable reports in the literature concerning the cells involved in the immune response to PKX. Giant cells, typical of Type IV delayed hypersensitivity, have occasionally been described in brown trout and Atlantic salmon but never in rainbow trout with PKD (Ellis et al., 1985). Cells which may be transforming into plasma cells have also been described (Ellis et al., 1985), however there is incomplete evidence for plasma cells in fish. Ferguson (1976) proposed that immunoglobulin is produced by stimulated lymphocytes that do not fully differentiate into plasma cells. Lymphocytes found closely associated with PKX suggest an active immune response (Olesen and Jorgenson, 1985).

Host cells associated with PKX are often described by a variety of morphological and staining characteristics such as: large frothy cells; small basophilic cells; epithelioid in form (Ellis et al., 1985); basophilic, crescent-shaped bodies; large sac-like cells (Clifton-Hadley et al., 1984); and aberrant macrophages (Rafferty et al., 1985).

Host Reponse to Non-Infectious Material

Carrageenin, a seaweed extract, has been used successfully to induce granuloma in plaice (Pleuronecctes platessa), maintained in water at 10 C. Within 24 hours of injection, a local inflammatory resonse consisting of neutrophils, macrophages and lymphocytes, had developed (Timur and Roberts, 1977). There was no evidence of intracellular carrageenin at this time. Initial phagocytosis was not observed until day five and was not completed until day 42 (Timur and Roberts, 1977). By the 10th day, the inflammatory cells were almost exclusively macrophages. Granulation tissue was still present at the termination of the experiment on day 80 (Timur and Roberts, 1977). Fish held in 10 C water showed all the features of a marked granulomatous response by day 18, including epithelioid development. However, the first appearance of macrophages with a distinctive foamy cytoplasm and

fibroblastic activity was not observed until day 18 in fish held at 5 C (Timur and Timur, 1985).

Studies involving the intraperitoneal (IP) injection of colloidal carbon in plaice held in 8-10 C water have shown that the carbon gains access to the circulation and is phagocytosed primarily by the ellipsoids of the spleen and the reticuloendothelial cells in the kidney and heart (Ellis and Munroe, 1976). These reticuloendothelial cells became free macrophages and were highly phagocytic. Aggregates of carbon containing macrophages in the kidney and spleen were first observed within or on the periphery of melanomacrophage centers, four days after injection (Ellis and Munroe, 1976). Ferguson (1984) found that bacteria were phagocytosed by macrophages closely associated with endothelium of the renal portal circulation, but not by the endothelial cells.

MATERIALS AND METHODS

Host Response to PKX

This experiment was initiated on April 1, 1987 and terminated twenty weeks later on August 18, 1987, at the Fish Disease Laboratory, University of California, Davis, California.

Animals

Unexposed, age 0+ rainbow trout (10 g) from Hot Creek California State Fish Hatchery were maintained at the Fish Disease Laboratory UC Davis, and used as test animals. Fish were held in 133 liter tanks supplied with 15 C well water in a flow through system, and fed a commercial fish feed at a daily rate of 1.5% body weight.

Fish were given prophylactic treatments of nitrofurazone to prevent infections with Flexibacter columnaris, which is carried by the fish. There was no apparent mortality due to columnaris in either group of fish during the study. However, eleven weeks after initiation of the study, the external parasite Costia (Ichthyobodo sp.) caused a substantial loss (approximately 20%) in the PKD-infected group. Fish were subsequently

treated with formalin at a rate of 100 ppm. The control group did not experience problems with Costia.

Experimental Protocol

PKX was obtained from kidneys of naturally infected rainbow trout from the Hot Creek Hatchery. Infection was confirmed by wet mount followed by histologic examination. Kidney tissue was removed, minced with an equal volume of minimal essential media (MEM), forced through a sieve and mixed 1:1 with phosphate buffered saline. Eighty unexposed rainbow trout were anesthetized with MS-222 (.04mg/ml) and received 0.2 ml PKD-infected kidney homogenate via an intraperitoneal injection just anterior to the pelvic fins. The dose of PKX per fish as determined by examination of ten fields at 40x was 1.45 x 103 parasites. Sixty control fish were each injected with 0.2 ml MEM.

Tissue Collection and Processing

At three, five, seven, ten and twenty weeks post-injection (PI) ten PKD-inoculated trout were randomly collected. Ten control fish were sampled at six, ten and twenty weeks PI. Fish were killed with an overdose of MS-222 and kidney tissue was removed immediately and fixed for light and electron microscopy.

For light microscopy, tissue was collected from posterior kidney, preserved in Davidson's fixative for 24 hours, transferred to 70% ethanol, embedded in paraffin and sectioned at five micrometers. Sections were stained with H&E, Giemsa, hematoxylin-PAS, Masson's trichrome, and Turnbull's blue.

Corresponding kidney tissue was fixed in 2.5% glutaraldehyde for 18 hours at 4 C, buffered to pH 7.2 in .1M cacodylate phosphate buffer; then post-fixed in 1% aqueous osmium tetroxide, dehydrated in graded ethanols, embedded in Spurr's embedding medium, and sectioned on a Sorvall MT 5000 ultramicrotome. Thick sections were stained with toluidine blue to select representative areas and PKX parasites. Thin sections (45 nm thick) were stained with uranyl acetate and Reynold's lead citrate and examined by transmission electron microscopy (TEM) with a JEOL-100CX electron microscope.

Tissue collection and fixation for all sampling periods except week seven were done by Fish Disease Laboratory personnel at UC Davis, and sent to the Fish Technology Center, Bozeman, Montana. I traveled to UC Davis to confer with the staff and personally collect the week seven PI samples.

Host Response to Clay

This experiment was conducted at the U.S. Fish and Wildlife Service, Fish Technology Center, Bozeman, Montana. The study was initiated on September 2, 1987 and terminated 20 weeks later on January 14, 1988.

Animals

Disease free age 1+ rainbow trout (190 g) were maintained similarly at the Bozeman Fish Technology Center but at a water temperature of 10 C. These fish were used as comparative controls to observe the cells involved in a chronic inflammatory response to a non-infectious substance.

Experimental Protocol

Preliminary data using finely ground bentonite clay as an inoculum demonstrated a granulomatous response. Therefore, bentonite clay was used as the test material in the comparative controls. The clay was mixed with sterile 0.85% NaCl (6g clay/40ml saline) and 0.8ml injected IP into each of eighteen anesthetized fish. Six control fish were each injected IP with 0.8ml sterile saline. Previous experiments injecting the inoculum at the same rate of PKD kidney homogenate (0.02 ml/g body weight) produced high

mortality. Studies indicated that bentonite clay administered by IP injection at the rate of 0.004 ml/g body weight was sufficient to induce a granulomatous response.

Tissue Collection and Processing

Sampling began one week PI because studies with colloidal carbon have shown carbon containing macrophages in kidney at four days PI (Ellis and Munroe, 1976). Three comparative controls were sampled at one, three, five, seven, ten and twenty weeks post-injection. Half the saline controls were collected at five weeks and the remainder at the termination of the study. Posterior kidney was dissected and processed in the same manner as mentioned above for light and electron microscopic examination of PKD-infected kidney tissue. Pyloric caeca from these fish were also dissected, processed and examined because of visceral granulomata formed in response to clay.

Cell Classification

Kidney interstitial cells that responded to inoculum were classified as; lymphocytes, polymorphonuclear leukocytes (PMN), macrophages, thrombocytes and hemopoietic blast cells. Cells were classified at the light microscopic level using the following criteria based on the

literature (Ellis, 1976; Cannon et al., 1980; Yasutake and Wales, 1983; Hightower et al., 1984) for hematoxylin-eosin stained sections:

Lymphocyte - small spherical cells, eccentrically located spherical, reddish-purple nucleus with a narrow band of lightly basophilic cytoplasm that frequently contained granules.

PMN - round to ovoid cell with bilobed to multilobulated, eccentric nucleus and blue-gray cytoplasm.

Macrophage - large, reniform, reddish-purple nucleus with moderately basophilic cytoplasm that often contained fine granules and vacuoles.

Melanomacrophage - macrophage containing brownish-black melanin granules.

Thrombocyte - the long form has an elliptical, dense basophilic nucleus often with one or two clefts, very light basophilic cytoplasm that can appear streaked. The spheroid form is indistinguishable from small lymphocytes.

Plasma cell - eccentrically located nucleus, reddish-purple with eosinophilic cytoplasm.

Blast cell - large pale, centrally located nucleus with prominent blue-purple nucleoli and basophilic cytoplasm.

The ultrastructural criteria primarily for plaice (Ferguson, 1976), carp (Blaxhall, 1983; Cenini, 1984) and

channel catfish, Ictalurus punctatus, (Cannon et al., 1980) leukocytes was used as a basis for cellular classification and are as follows:

Lymphocyte - 3-10 um, large sometimes indented nucleus with dense compact chromatin, thin rim of cytoplasm with limited rough endoplasmic reticulum, few elongate mitochondria and small pseudopodia.

PMN - 8-10 um, irregular and sometimes eccentric nucleus with dense, patchy chromatin, no pseudopodia and specific granules (crystalline or fibrillar) in the cytoplasm.

Macrophage - 12-20 um, eccentric nucleus with loosely packed chromatin, well developed rough endoplasmic reticulum, prominent Golgi apparatus, vesicles of varying size and electron-density, few to numerous ovoid mitochondria, pseudopodia. The cytoplasm often contains small, round, dark granules with an electron-lucent rim and phagolysosomes.

Melanomacrophage - smilar to macrophage above, but contains a large number of membrane bound vesicles and electron-dense melanin granules.

Thrombocyte - 5-8 um, indented nucleus with cross hatched heterochromatin and euchromatin, few mitochondria, sparse rough endoplasmic reticulum, small Golgi apparatus, prominent electron-lucent vesicles, centrioles and numerous

microtubules.

Plasma cell - 10-12 um, eccentric nucleus with radially arranged chromatin and distinct nucleolus, and abundant rough endoplasmic reticulum.

Blast cell - large, oval, euchromatic, centrally located nucleus and prominent nucleolus. Cytoplasm contains few mitochondria and numerous ribosomes.

RESULTS

The results presented here are based on interpretations following the examination of paraffin sections and electron micrographs. The severity of lesions associated with PKD and the prevalence of PKX varied from moderate to extensive in kidney tissue collected at each sampling period. PKX was not found in control samples.

PKX

Typical PKX was detected in the kidney vasculature, interstitium, tubular epithelium and lumina of infected fish. By light microscopy, the primary cell was lightly eosinophilic with a prominent, deeply staining nucleolus and PAS positive cytoplasmic granules. Ultrastructurally, primary cells were uninucleate with a euchromatic nucleus and prominent electron-dense nucleolus, electron-dense plasmalemma, granular cytoplasm and characteristic haplosporosome-like bodies. These "haplosporosomes" had an electron-dense matrix with electron-lucent bars one-half to one-third the diameter, especially prominent in those near the plasmalemma. The cytoplasm contained abundant rough

endoplasmic reticulum, spherical to elongate mitochondria with plate-like cristae, lipoid and multivesicular bodies. The lipoid and multivesicular bodies appeared to be more prominent in parasites that contained daughter cells.

Vegetative reproduction of interstitial PKX by binary fission and internal cleavage was observed. Primary cells containing up to four daughter cells were seen in infected kidneys. Daughter cells were encircled by the outer cell membrane and contained prominent, electron-dense nucleoli, few mitochondria, little rough endoplasmic reticulum, abundant free ribosomes and occasionally lipoid bodies (Figure 1). "Haplosporosomes" were never seen in daughter cells but were found adjacent to the primary cell membrane surrounding the daughter cell. The secondary cell often appeared to be separating from the enveloping primary cell (Figure 2).

PKX was observed in kidney tubular epithelium five weeks PI and tubular lumina ten weeks PI. Intraluminal forms varying from small, uninucleate to larger, multinucleate, eosinophilic cells were observed in paraffin sections. Small parasites that resembled daughter cells and multinucleate sporoblasts were found in distal tubular lumina by electron microscopy. Multilaminate bodies were seen in the enveloping cell of the intraluminal sporoblast, but polar capsules were not observed.