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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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.

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Elizabeth MacConnell

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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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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.

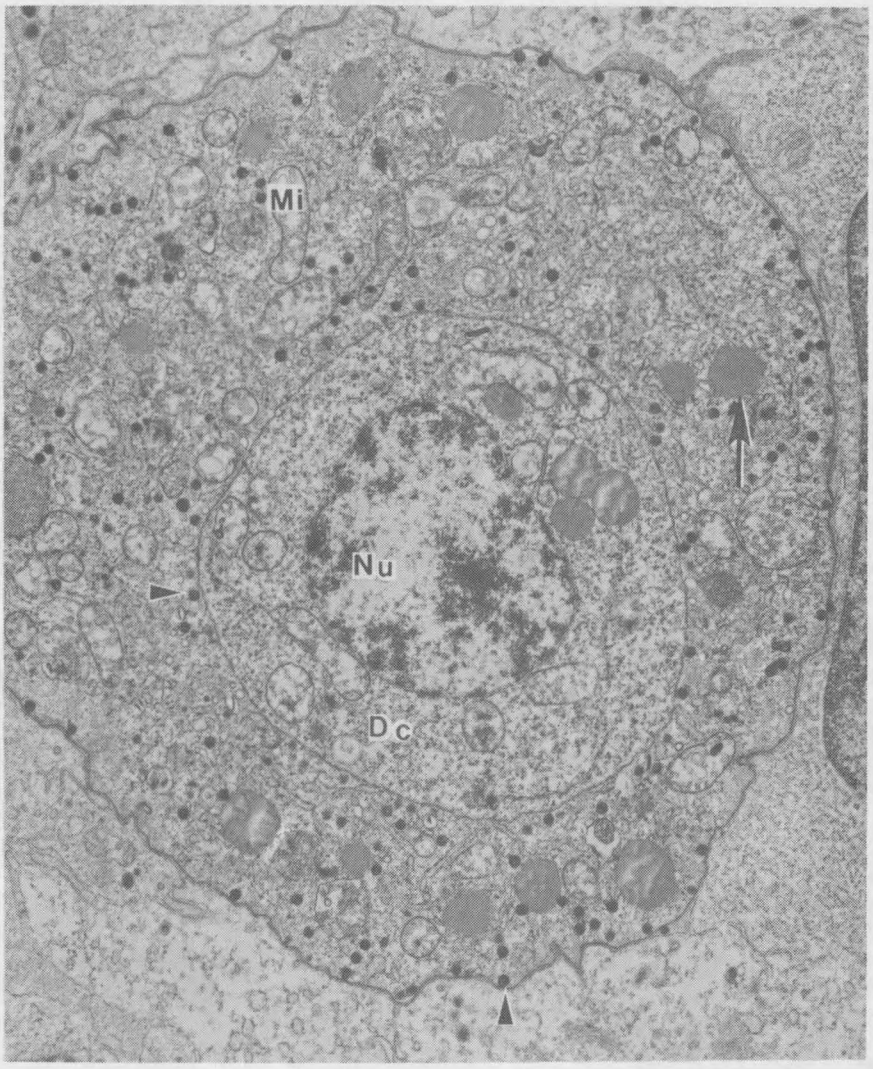


Figure 1. PKX containing internal daughter cell (Dc); haplosporosomes (arrowheads), nucleus (Nu), lipoid body (arrow), mitochondria (Mi)(x9000).

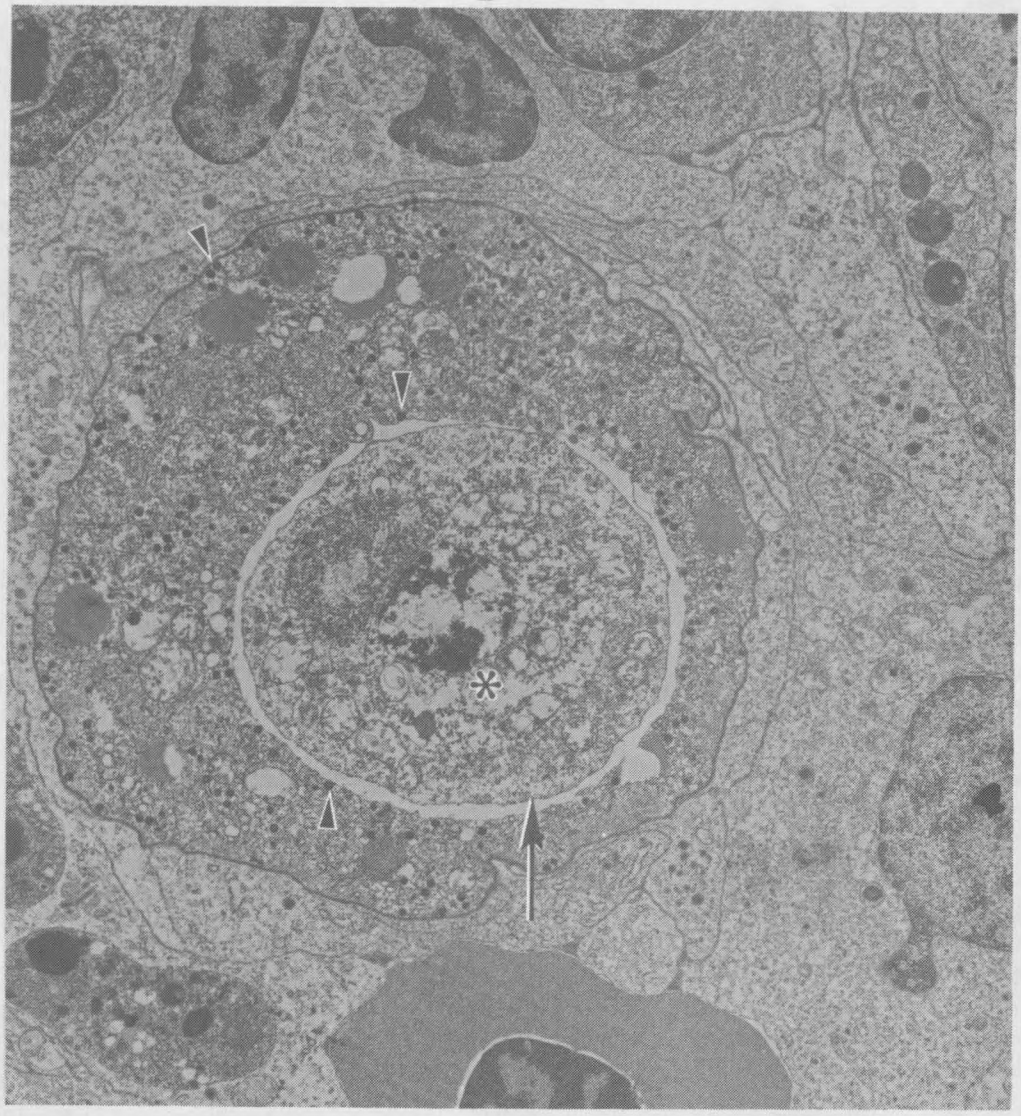


Figure 2. PKX containing daughter cells (*) that appear to be separating (arrow) from enveloping primary cell; haplosporosomes (arrowheads) (x7800).

Host Response to PKX

Week Three

PKX were observed infrequently by light microscopy in five of ten kidneys collected three weeks PI. They were observed in the hemopoietic tissue, sinusoids and peritubular capillaries; most appeared to have macrophages closely adhered to the cell surface of the parasite (Figure 3a). Several parasites were found free in the circulation. Several stages of development were observed; small condensed eosinophilic cells resembling daughter cells, primary cells and primary cells containing daughter cells (Figure 3b). In one sample, small cells, possibly degenerate PKX, were observed in the tubular epithelium and lumina (Figure 4a).

In several samples, focal areas of hemopoietic tissue appeared intensely basophilic, apparently due to increased cellularity. Numerous mitotic figures were evident in these areas. Melanomacrophages were found scattered throughout the hemopoietic tissue, except for one sample in which there was a marked increase in numbers and concentration.

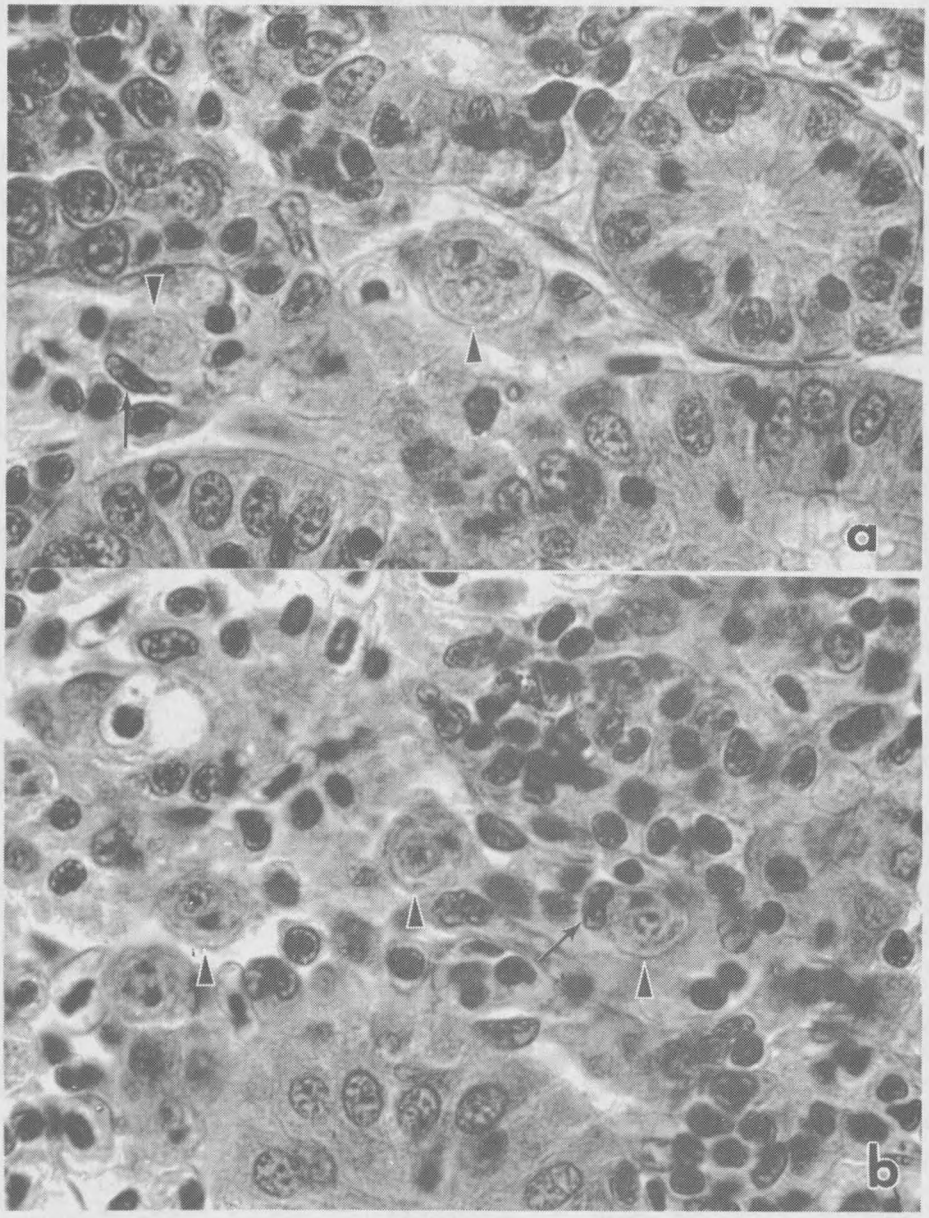


Figure 3. a) PKX (arrowhead) in sinusoid with macrophage (arrow) closely adhered to cell surface, three weeks PI (x400). b) PKX (arrowhead) containing daughter cell in kidney interstitium; macrophage (arrow) (x400).

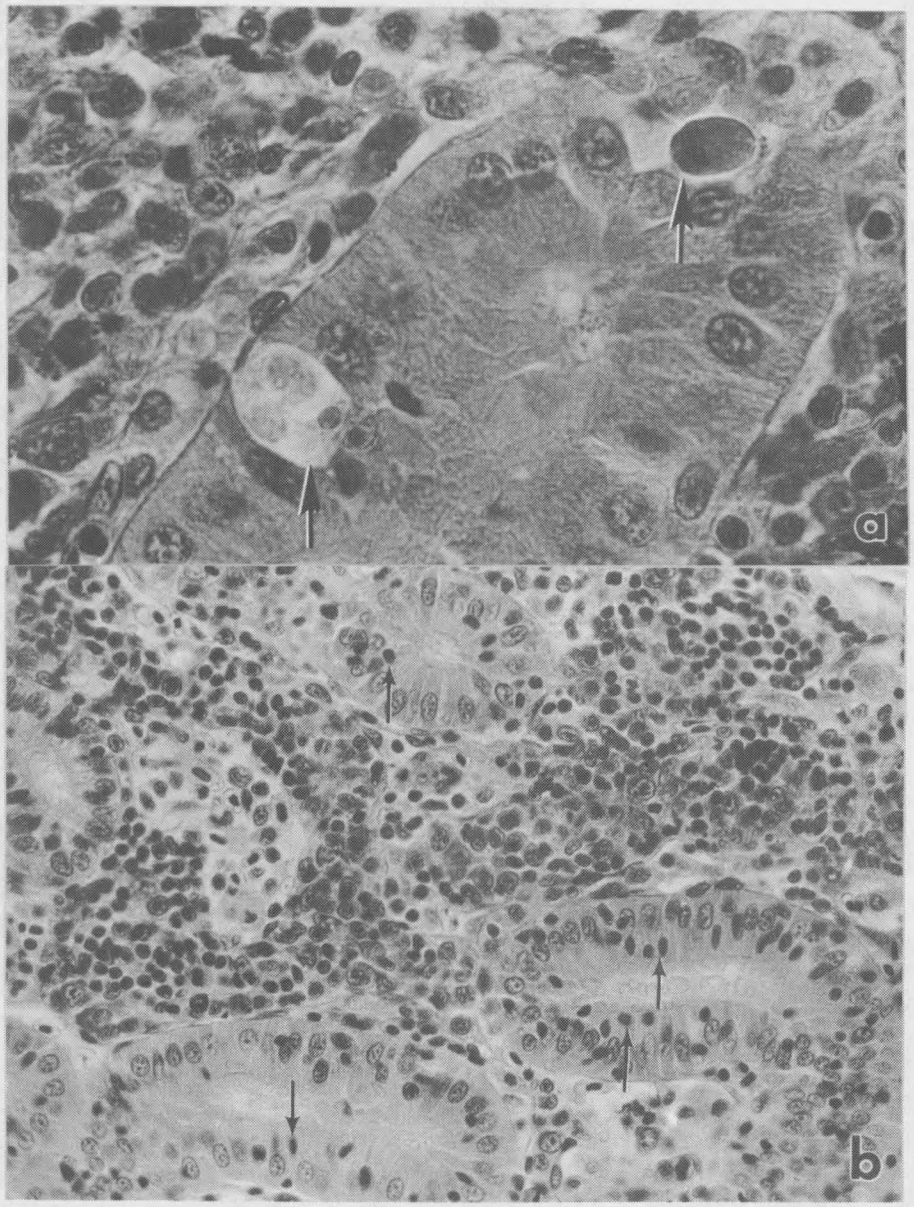


Figure 4. a) Vacuoles (arrows) containing cellular material in tubular epithelium (x400). b) Lymphocytes (arrows) migrating through tubular epithelium (x160).

Numerous lymphocytes were seen migrating through tubular epithelium (Figure 4b). The major tubular change observed three weeks PI was vacuolation of proximal tubular epithelium with hyaline droplet degeneration and accumulations of an eosinophilic granular material in most tubular lumina.

Examination by electron microscopy of kidneys collected at three weeks PI confirmed findings at the light microscopic level, i.e. large cytoplasmic droplets, vacuolation of tubular epithelium, migrating lymphocytes, mitoses of hemopoietic cells and finely granular material in tubular lumina (Figure 5). However, the presence of any developmental stages of PKX were not confirmed by ultrastructural examination of tissue samples.

Endothelial cells lining kidney sinusoids and peritubular capillaries appeared activated as evidenced by the abundance of pinocytotic vesicles adjacent to the luminal plasmalemma and cytoplasmic protrusions. Numerous lymphocytes were seen in the peritubular capillaries. Circulating macrophages were observed attached to the inner surface of vessel walls (Figure 6). Macrophages were seen migrating through tubular basal lamina and into tubular epithelium. Occasionally, macrophages containing

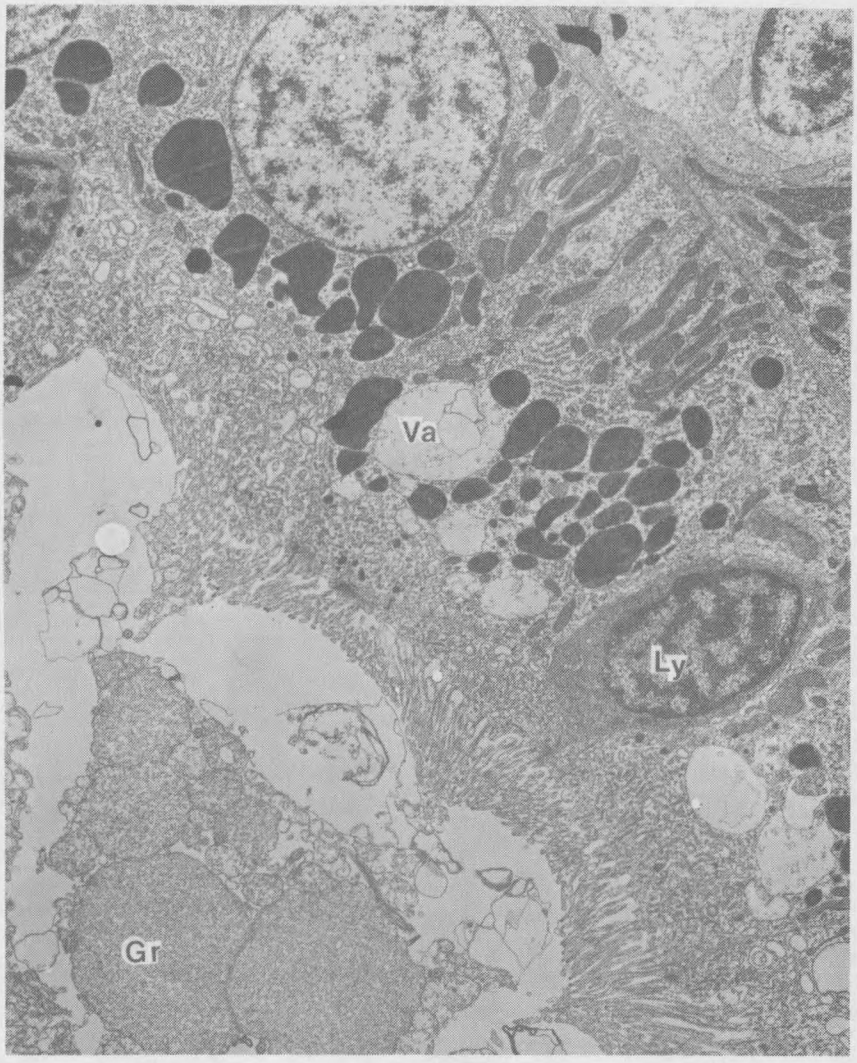


Figure 5. Migrating lymphocyte (Ly) and vacuoles (Va) in tubular epithelium and granular material (Gr) in tubular lumina, three weeks PI (x4680).

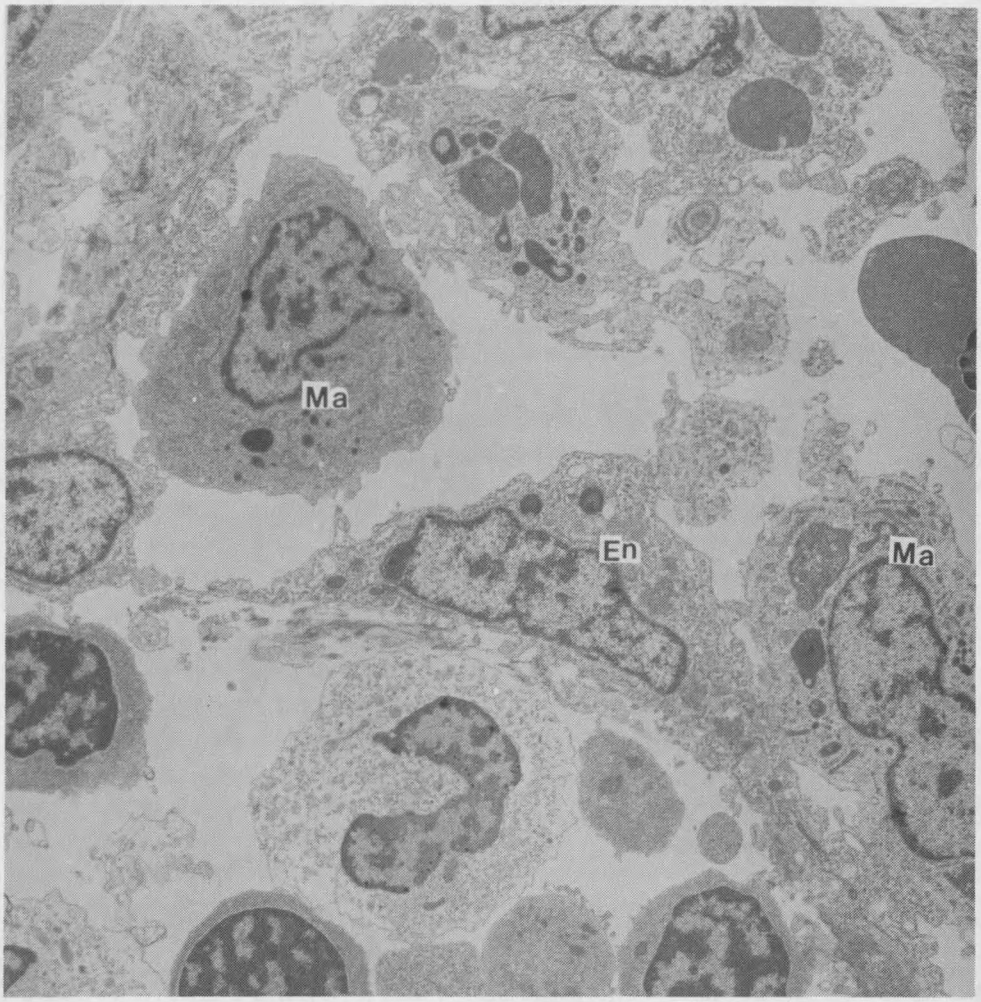


Figure 6. Circulating macrophages (Ma) attached to endothelium (En) of vessel wall, three weeks PI (x5200).

degenerate cellular material in phagocytic vacuoles were overlying endothelial cells.

Large, electron-dense, nucleolar-like material, was seen in macrophages in several tissue samples collected at three weeks PI. An unidentifiable cell observed with relative frequency in the kidney interstitium (Figure 7) at three weeks PI was not found in subsequent samples or controls. The cell was small, contained electron-dense cytoplasm, numerous vacuoles, few mitochondria, numerous long pseudopodia and often a deeply indented nucleus with surrounding microtubules (Figure 8). Disruption of nuclear membranes, swollen mitochondria or condensation of organelles suggestive of degeneration was not observed in these cells.

Week Five

At five weeks PI interstitial inflammation was evident in kidney samples from all ten fish. PKX was identified in seven samples; one heavily, one moderately and the rest lightly infected. Samples collected at this time exhibited a wide range of severity of lesions, from small localized areas of hemopoietic proliferation, vacuolation of proximal tubular epithelium and no identifiable PKX (Figure 9a) to advanced lesions with massive hemopoietic proliferation,

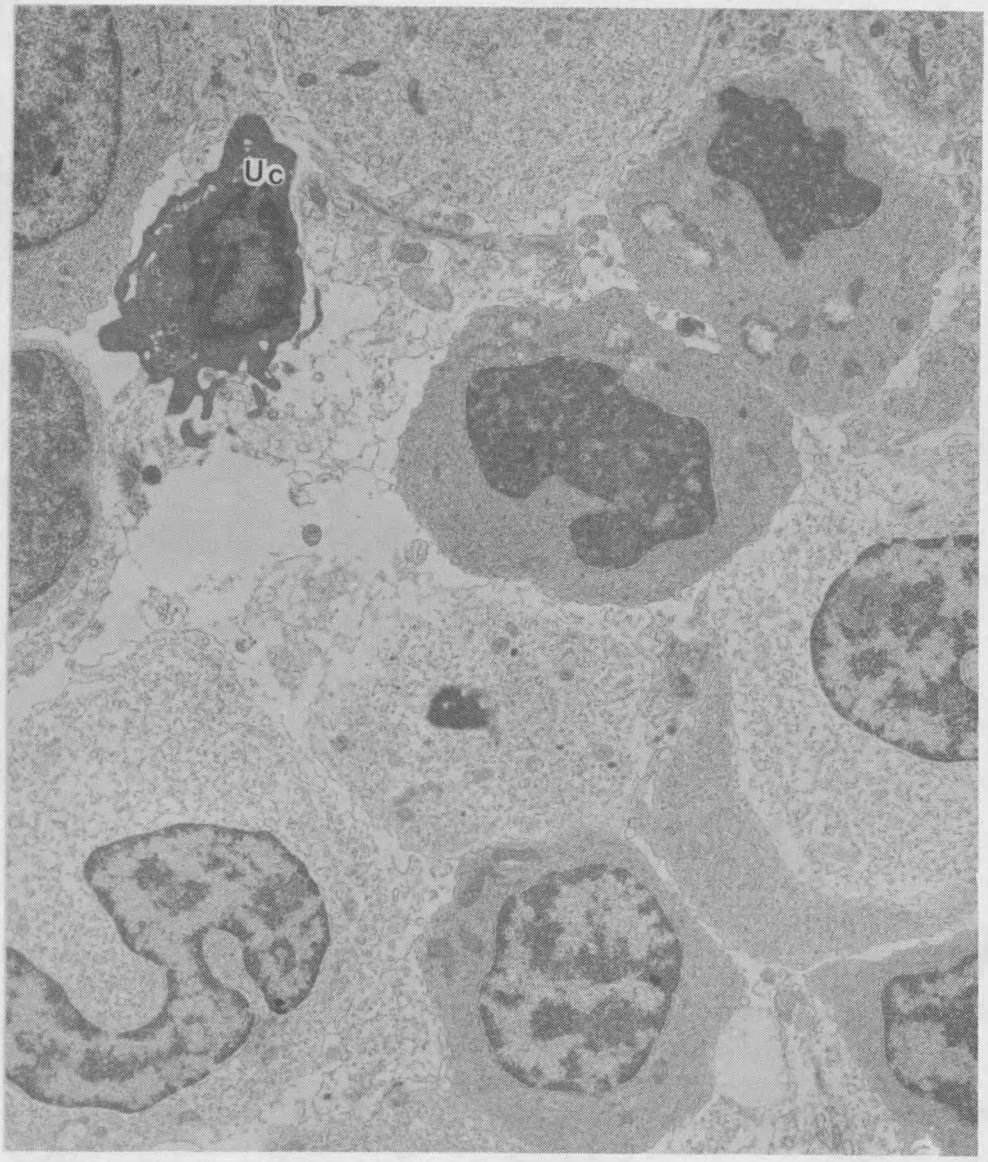


Figure 7. Unidentified cell (Uc) found in kidney interstitium, three weeks PI (x5200).

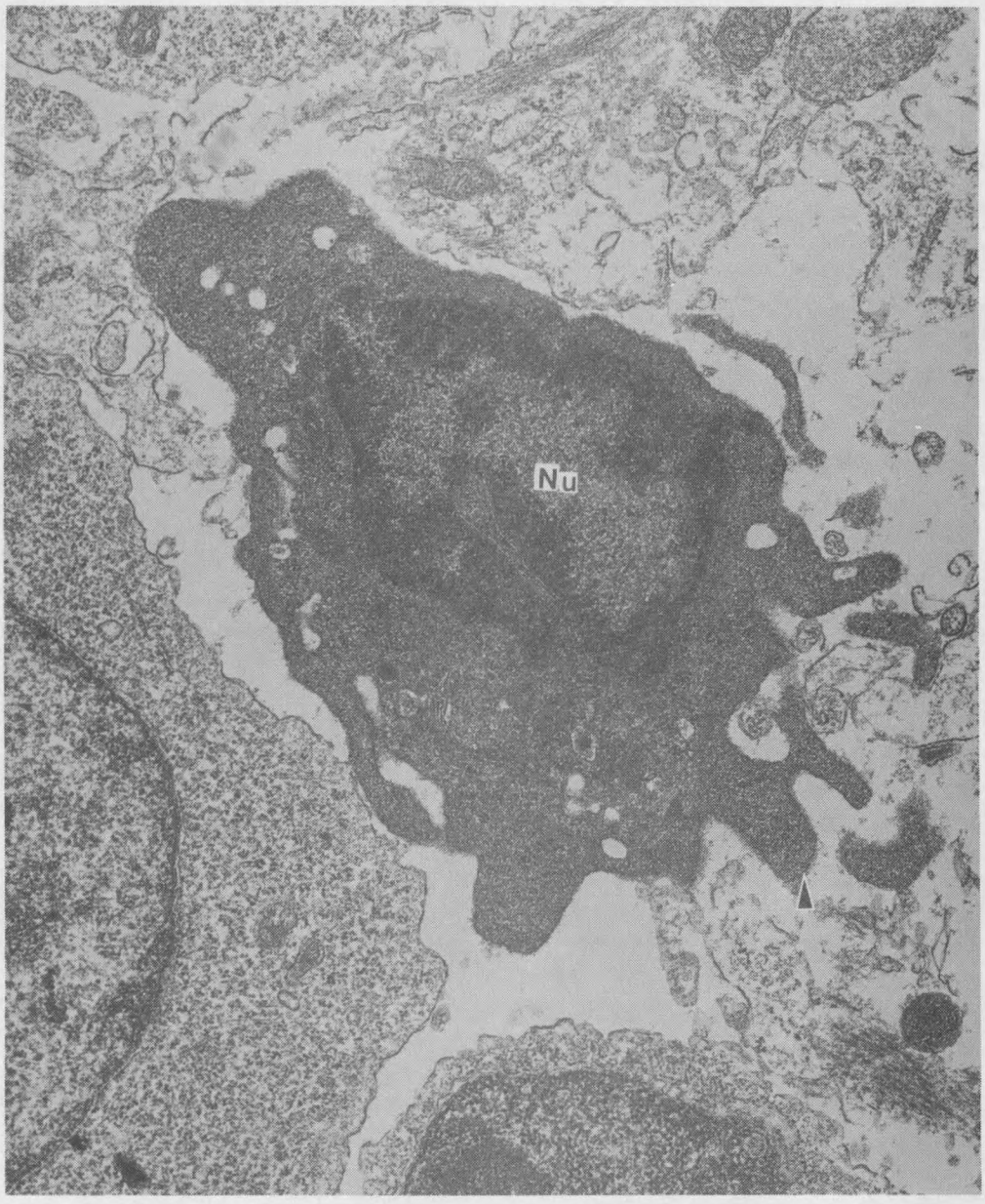


Figure 8. Unidentified cell with electron-dense cytoplasm, vacuoles, indented nucleus (Nu) and pseudopodia (arrowhead) (x15,000).

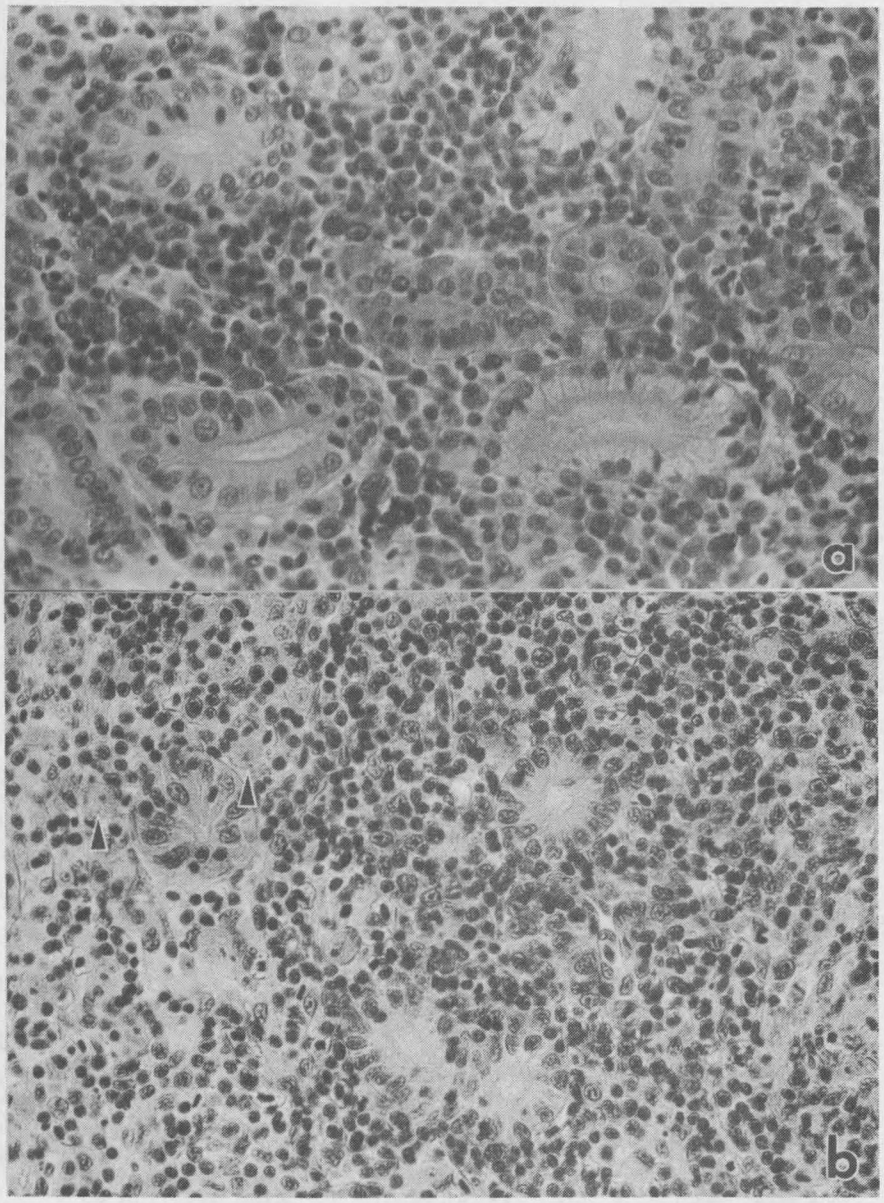


Figure 9. a) Mild PKD (x160). b) Severe PKD, five weeks PI; PKX (arrowheads) (x160).

few intact tubules and numerous PKX cells (Figures 9b). Although inflammation was evident in all ten fish, PKX was not identified in three samples that exhibited mild hemopoietic proliferation.

Prominent features observed by light microscopy were intense basophilia of hemopoietic tissue, numerous mitotic figures, and displacement of renal elements by increased hemopoietic cells. There was a marked decrease in tubular density in two samples. Cells infiltrating the interstitium consisted largely of lymphocytes and macrophages.

One small granulomatous foci with a single PKX located in the center was seen in the hemopoietic tissue of one kidney sample. PKX was observed in tubular epithelium of only one fish that was heavily infected with PKX and exhibited advanced lesions (Figure 10). Most PKX were surrounded by macrophages and occasionally attached to vessel walls.

Severe vasculitis with areas of occlusion was seen in the renal vein of one sample (Figure 11a). Macrophages appeared to be adhering to the surface of PKX cells that were free within the vessel, on the edge of and within the lesion. The lesion was comprised of parasites, macrophages, lymphocytes, and erythrocytes (Figure 11b).

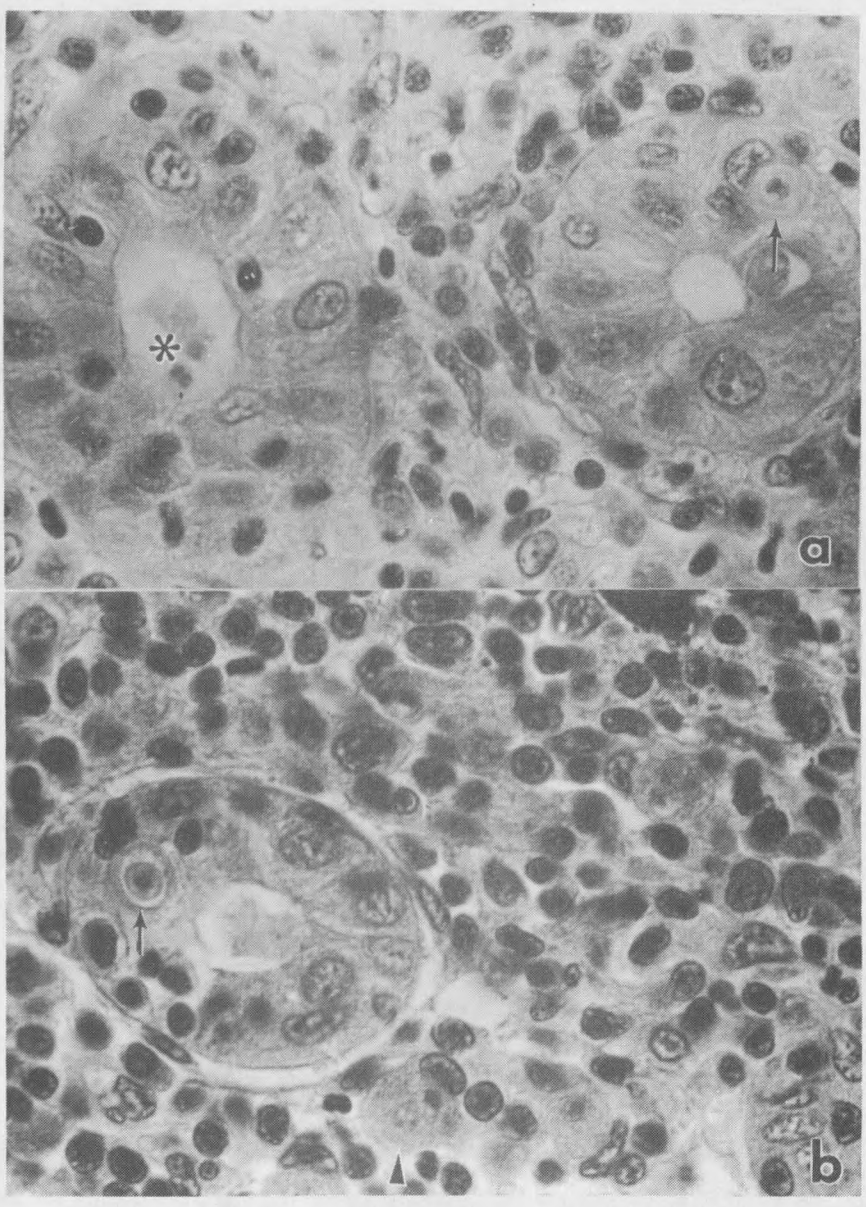


Figure 10. a) PKX in tubular epithelium (arrow) and lumina (*) (x400). b) PKX in tubular epithelium (arrow) and hemopoietic tissue (arrowhead), five weeks PI (x400).

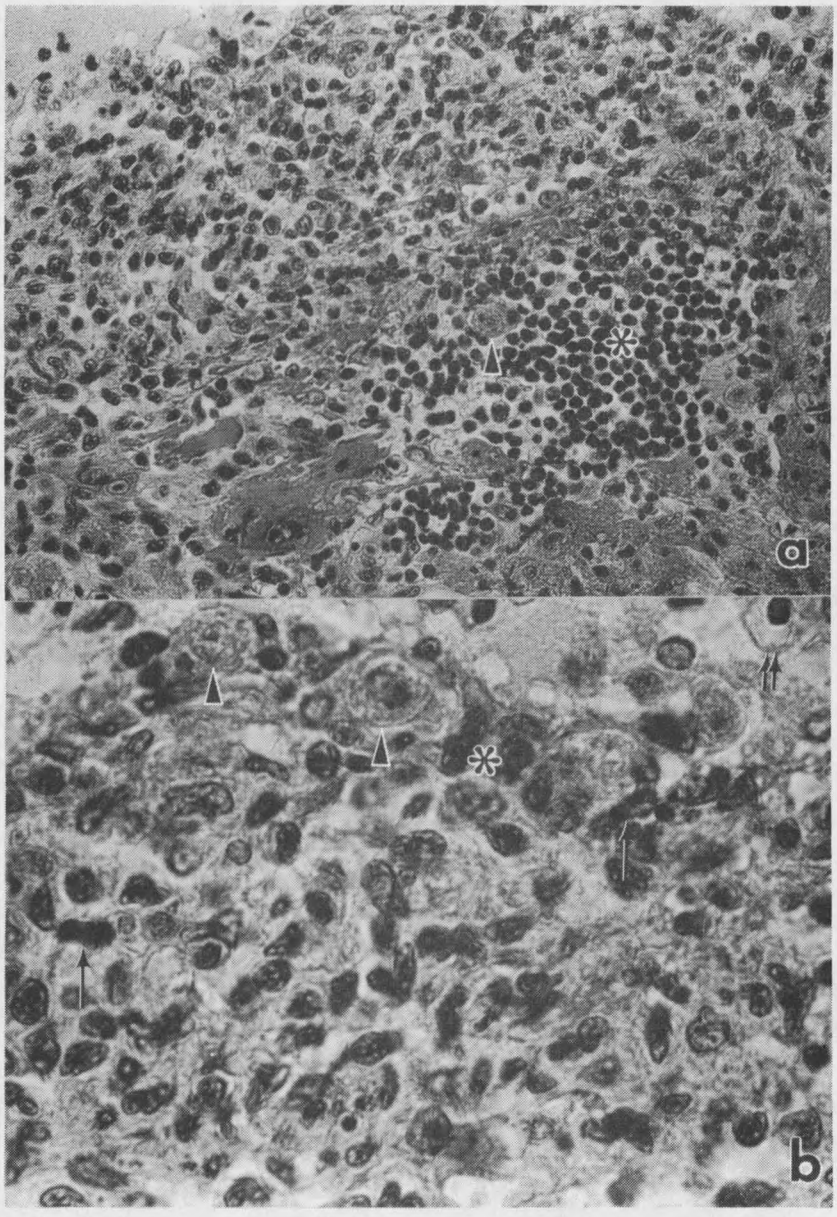


Figure 11. a) Vascular lesion in renal vein, five weeks PI; PKX (arrowhead), lymphocytes (*) (x160). b) Vascular lesion containing PKX (arrowheads), lymphocytes (*), macrophages (arrows) and erythrocytes (double arrow) (x400).

The endothelium and muscularis were often disrupted where the inflammatory lesion was attached, possibly allowing macrophages, lymphocytes and PKX to migrate through the vessel wall into the hemopoietic tissue. A brownish-green pigment which stained negative for hemosiderin, was observed scattered throughout the renal vein, hemopoietic tissue and occasionally lining tubular lumina.

Numerous lymphocytes were seen migrating through the epithelium of collecting tubules, however, this was also observed in controls and may represent a normal condition. A marked increase in melanomacrophages was noted in five weeks PI in experimentally-infected fish and among samples from the control group at six weeks. Apparently this was in response to the infectious agent, F. columnaris, present in both groups of fish.

Electron microscopy showed that PKX found in the interstitium had at least one macrophage, usually two or more closely adhered to their surface (Figure 12). Lymphocytes were often seen in close proximity but not directly adjacent to the parasite. PKX observed in the circulation appeared to be in the process of being engulfed by macrophages (Figure 13). Macrophages containing degenerate cellular components, possibly PKX were observed in tubular epithelium of several tubules

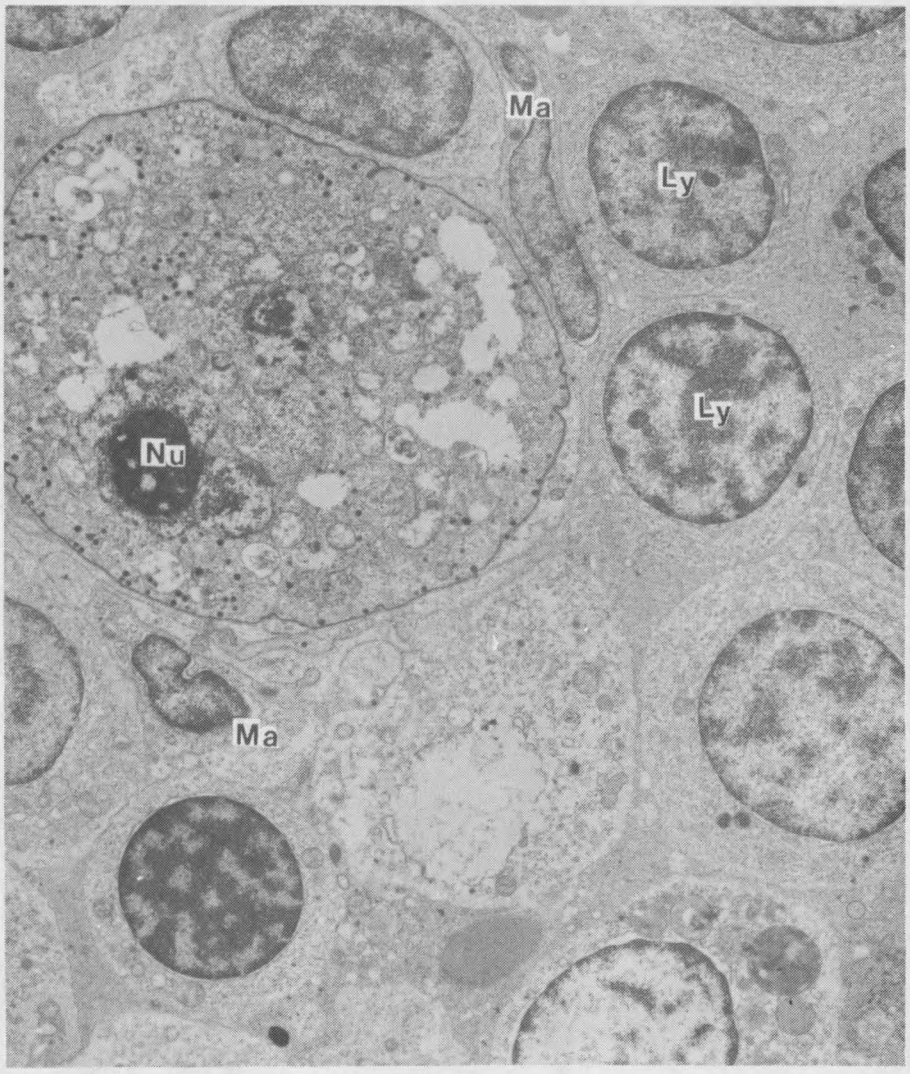


Figure 12. Macrophages (Ma) closely adhered to the cell surface of PKX; lymphocytes (Ly), nucleus (Nu) (x5000).

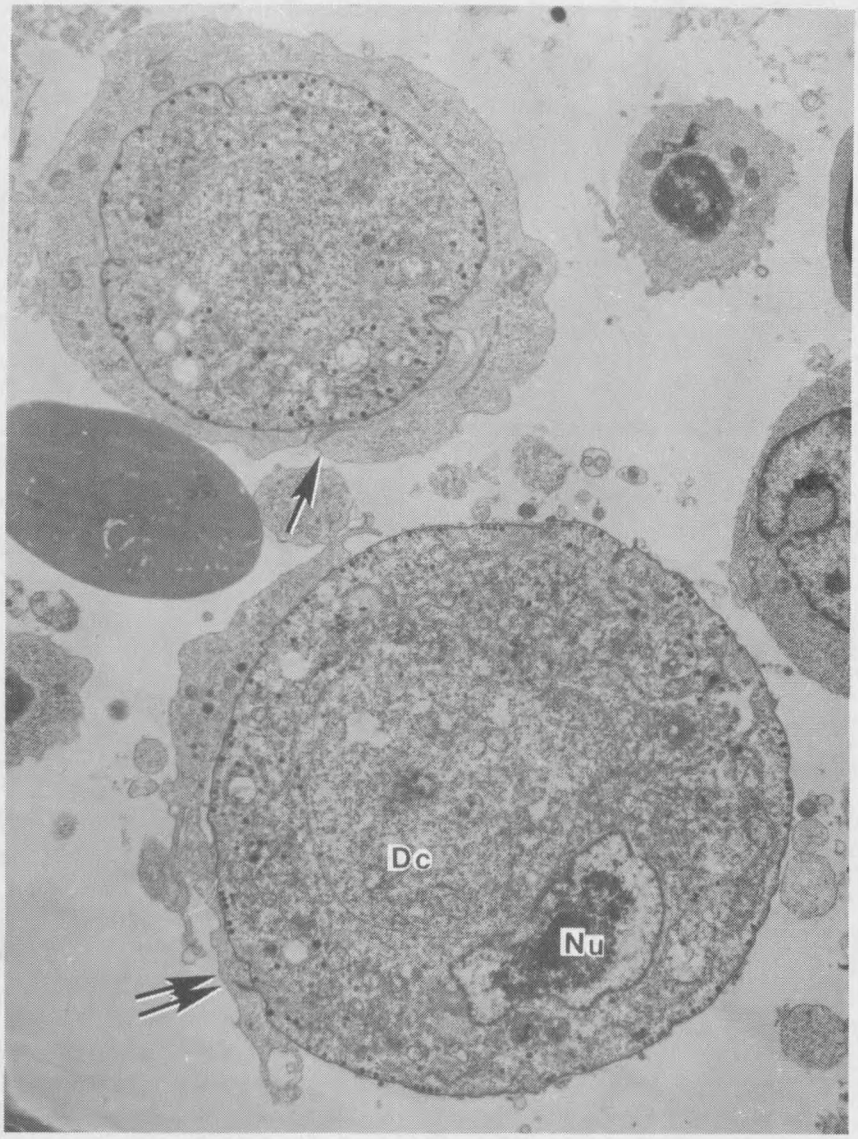


Figure 13. PKX in kidney circulation partially (double arrows) and completely (arrow) engulfed by macrophages; daughter cell (Dc), nucleus (Nu) (x5000).

(Figure 14). In contrast to three week samples, peritubular capillary lumina appeared collapsed from increased cellularity or engorged with blood cells.

Week Seven

Eight of the ten fish sampled at seven weeks PI had grossly swollen kidneys. Histologically, PKX was found in all ten kidney samples; four were heavily, three moderately and three lightly infected with the parasite. There was a marked decrease in tubular density in all ten samples.

Intact as well as degenerate PKX, often with intact daughter cells, were observed in kidney tissue. The intact daughter cells appeared as small condensed eosinophilic cells, similar to the early stage of PKX seen at three weeks PI. Cells, predominantly macrophages, were found adhered to all the parasites observed at this time.

The characteristic feature of samples taken at seven weeks PI was an epithelioid-like granulomatous response to PKX. Granulomatous lesions replaced hemopoietic tissue to varying degrees throughout the kidney interstitium. PKX was usually found in the center of a "whorl" of macrophages (Figure 15). Epithelioid-like macrophages were the

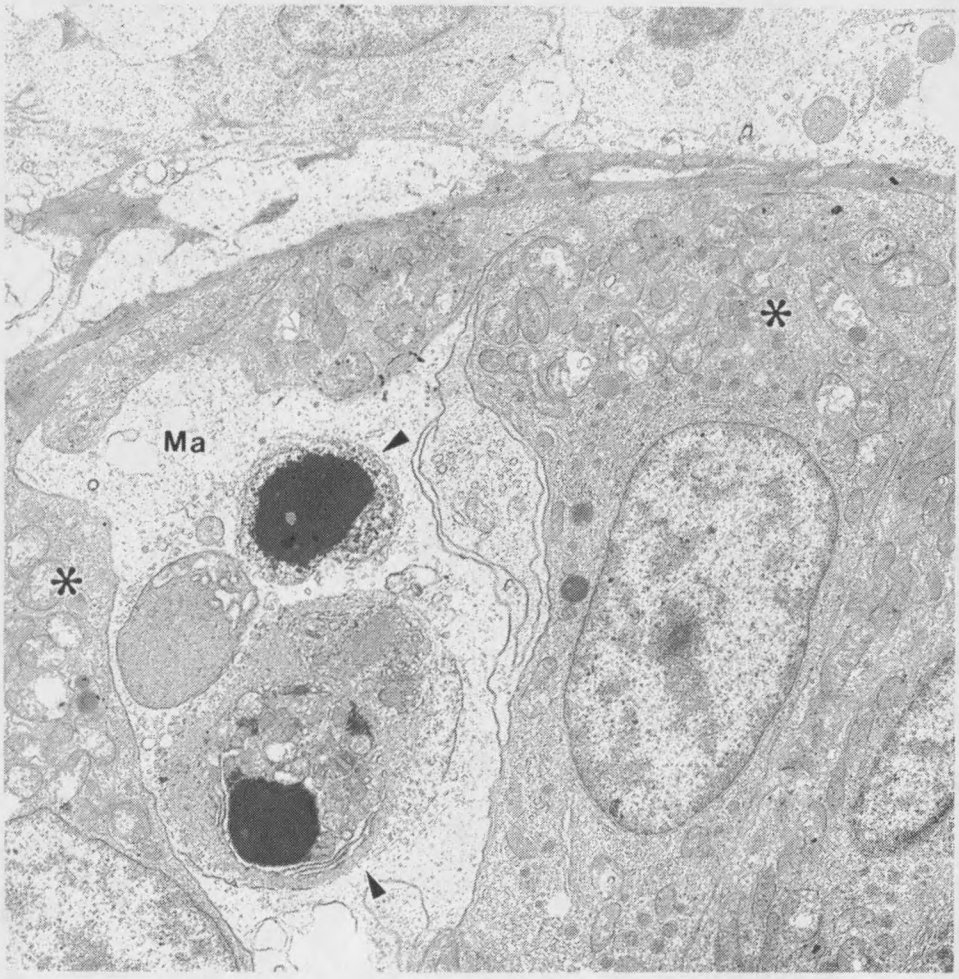


Figure 14. Macrophage (Ma) containing degenerate cellular material (arrowhead), possibly PKX, in tubular epithelium (*), five weeks PI (x5200).

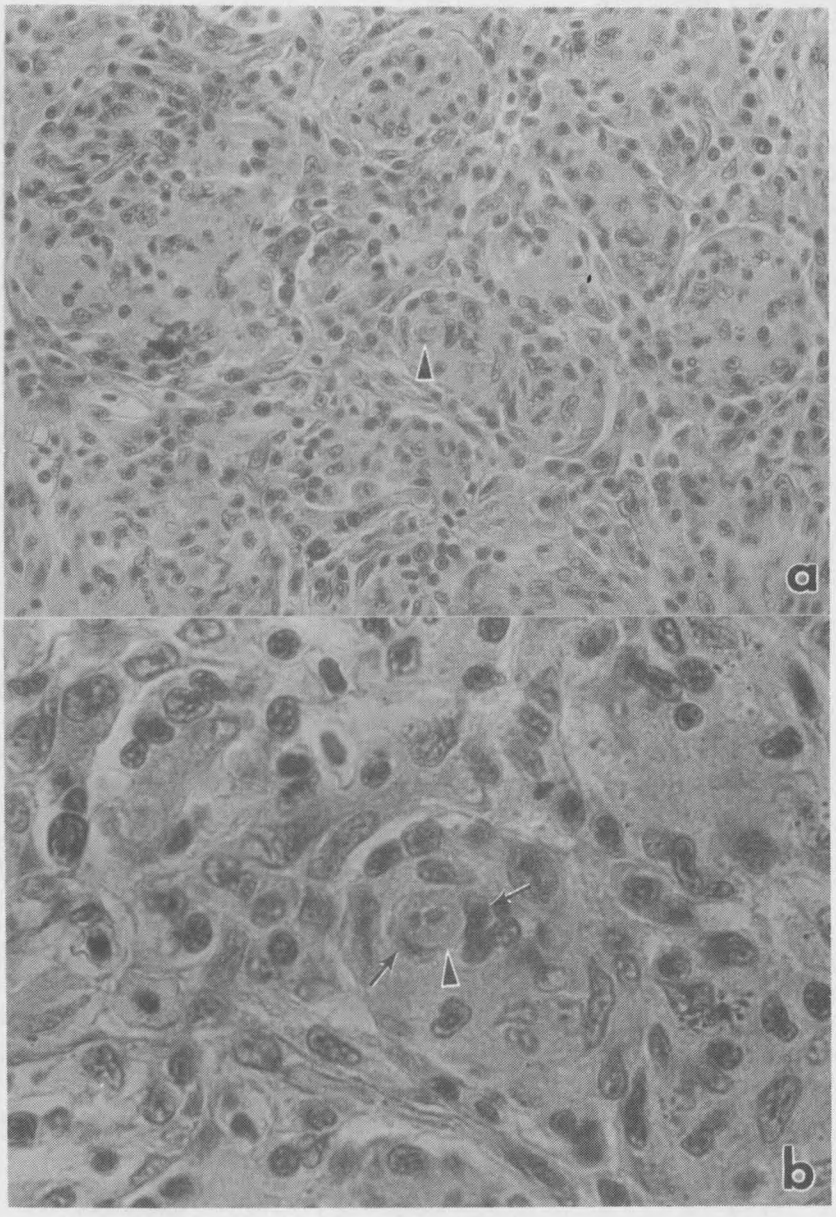


Figure 15. a) Granulomatous lesions seven weeks PI; PKX (arrowhead) (x160). b) PKX (arrowhead) in the center of a whorl of macrophages (arrows) (x400).

predominant cells found in week seven samples. Fibroblasts were found only widely scattered throughout the inflammatory tissue.

The most interesting feature observed at the ultrastructural level was the abundance of plasma cells, found only occasionally at previous sample times. Plasma cells were not found attached to or in close proximity to parasites but were abundant throughout the kidney interstitium. Epithelioid cells were the predominant cell type found in kidney samples (Figure 16). Epithelioid cells were morphologically similar to macrophages but they were usually larger, elongate and did not contain phagolysosomes. The lack of collagen fibrils in the extracellular space close to the cell distinguished these cells from fibroblasts. Macrophages were seen migrating through the tubular basal lamina and into tubular epithelium.

Peritubular and sinusoidal spaces were often collapsed. Tubular basal lamina appeared thickened. Autophagic vacuoles, myelin figures and clumped chromatin, all of which are indicative of degeneration, were often noted in tubular epithelium.

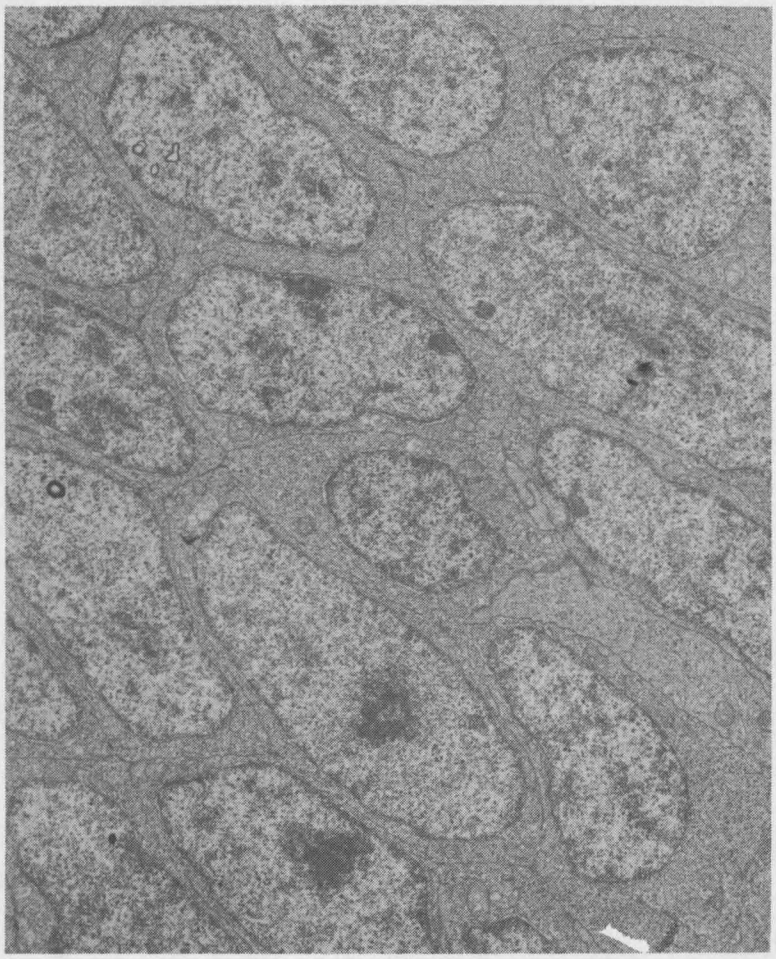


Figure 16. Epithelioid tissue seven weeks PI (x5200).

Week 10

All ten fish sampled at ten weeks PI were positive for PKX. Six fish were heavily, three moderately, and one lightly infected. Intraluminal forms of PKX were observed in kidneys from three fish. Macrophages were attached to most of the parasites in the kidney interstitium (Figure 17a) and blood vessels, but host cells were not found associated with the intraluminal forms of PKX (Figure 17b). PKX cells that had macrophages adhered to their surface appeared to be undergoing degeneration, but daughter cells, of which there were often two or more, were usually intact.

Epithelioid tissue was markedly reduced in ten weeks PI samples as compared to seven weeks PI samples. Proliferative hemopoietic tissue was prominent with diffuse epithelioid tissue scattered throughout the interstitium. PKX cells were mostly found within epithelioid tissue. Few intact tubular elements remained at this time. Numerous lymphocytes were seen in remaining tubules.

An interesting feature of kidney tissue from ten weeks PI was the presence of brownish-black, melanin-like granules, mostly lining tubular lumina (Figure 17b).

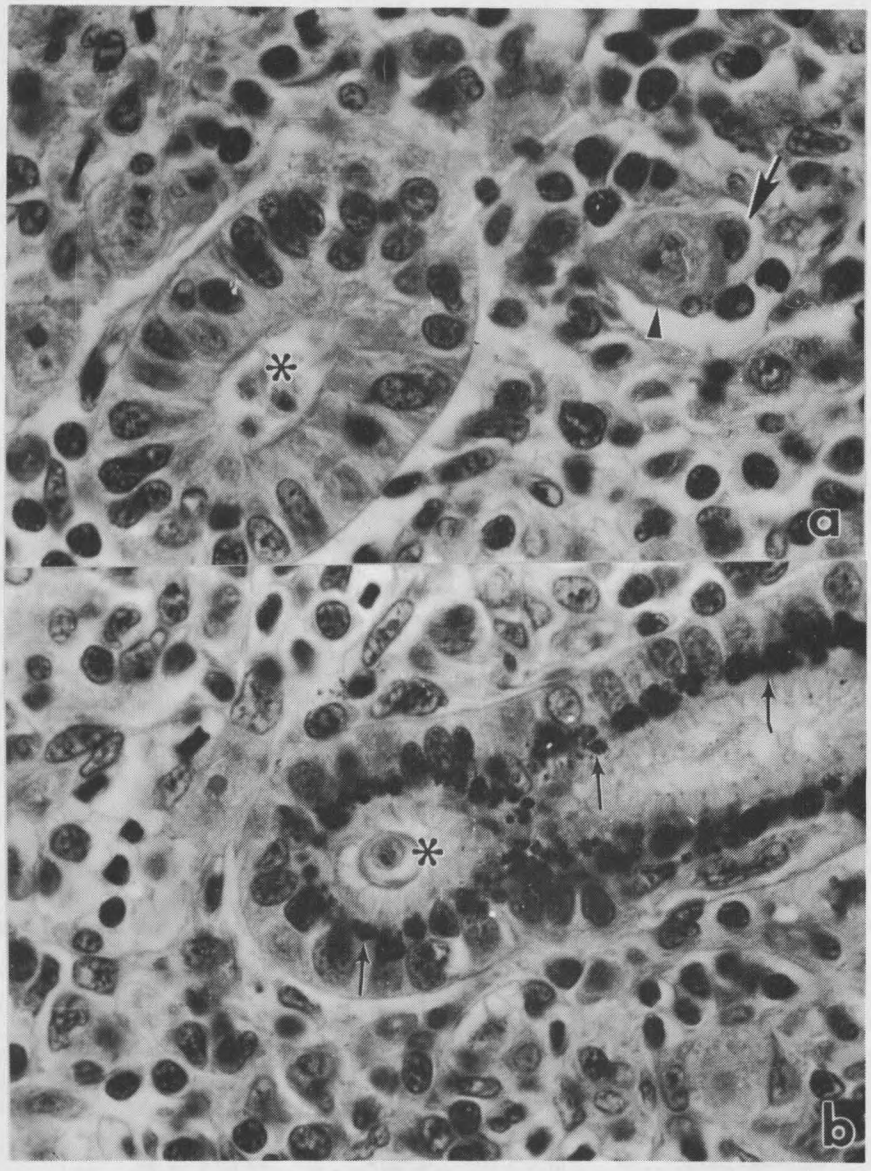


Figure 17. a) Macrophage (arrow) adhered to surface of PKX (arrowhead) in kidney interstitium; intraluminal PKX (*) (x400). b) Melanin-like material (arrow) lining tubule lumina ten weeks PI; intraluminal PKX (*) (x400).

Staining for hemosiderin was negative and identification of this material by ultrastructural examination was unsuccessful.

Most commonly, PKX was seen by electron microscopy with at least one macrophage, usually two or more, adhered to the surface completely surrounding the parasite (Figure 18). Macrophages containing electron-dense granules with an electron-lucent halo, presumably lysosomes, were often observed in close proximity to the plasmalemma bordering PKX (Figure 19). Lymphocytes were often, but not always found in close association with the outermost macrophage plasmalemma but rarely adjacent to PKX (Figure 20).

At ten weeks PI, the hemopoietic tissue was comprised primarily of leukocytes; predominantly macrophages, immature and mature lymphocytes. Few erythrocytic precursors were observed in the hemopoietic tissue. In comparing tissue samples to week three, hypercellularity was evident by the lack of intercellular spaces and the compressed appearance of peritubular capillaries and sinusoids.

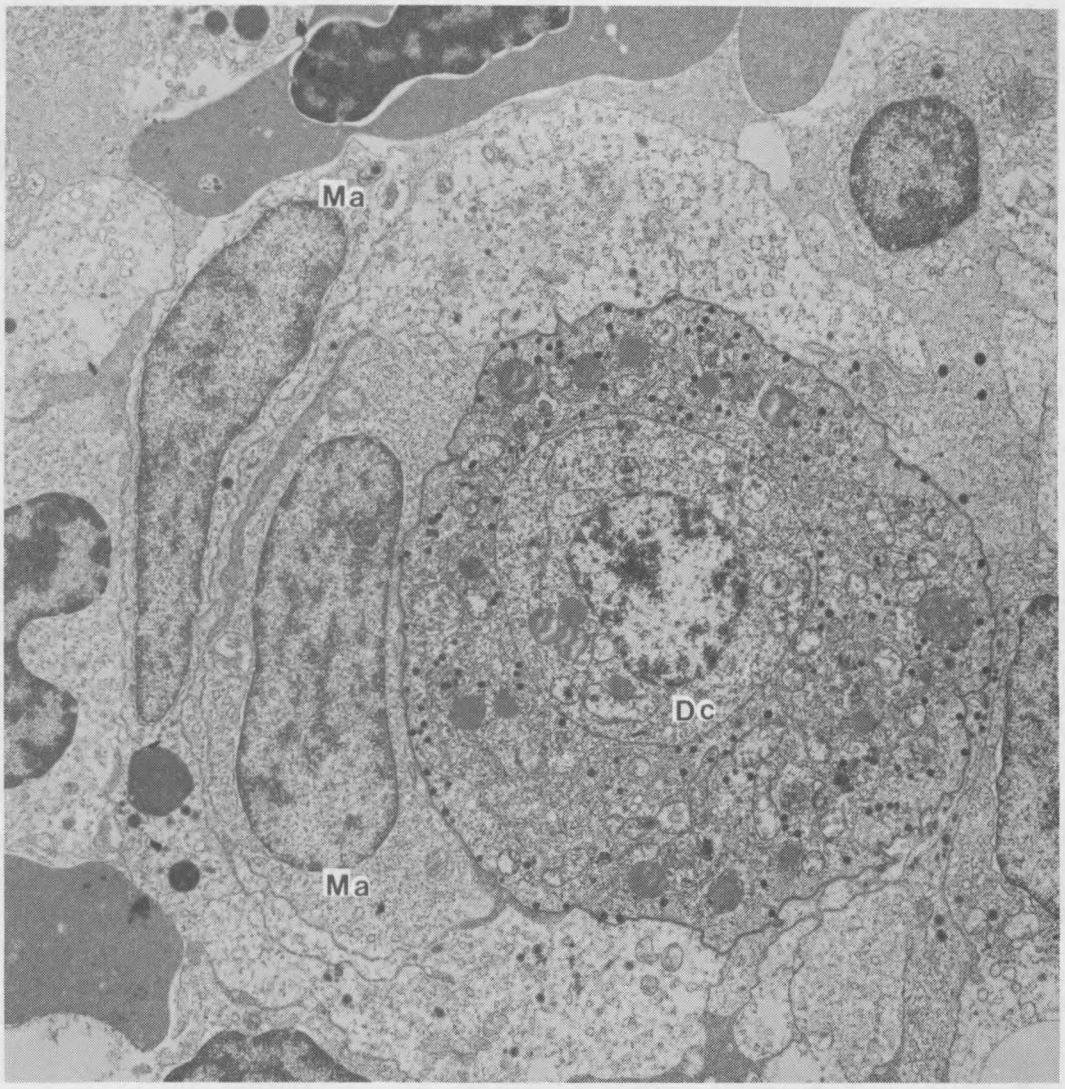


Figure 18. Macrophages (Ma) closely adhered to the surface of PKX; daughter cell (Dc) (x5720).

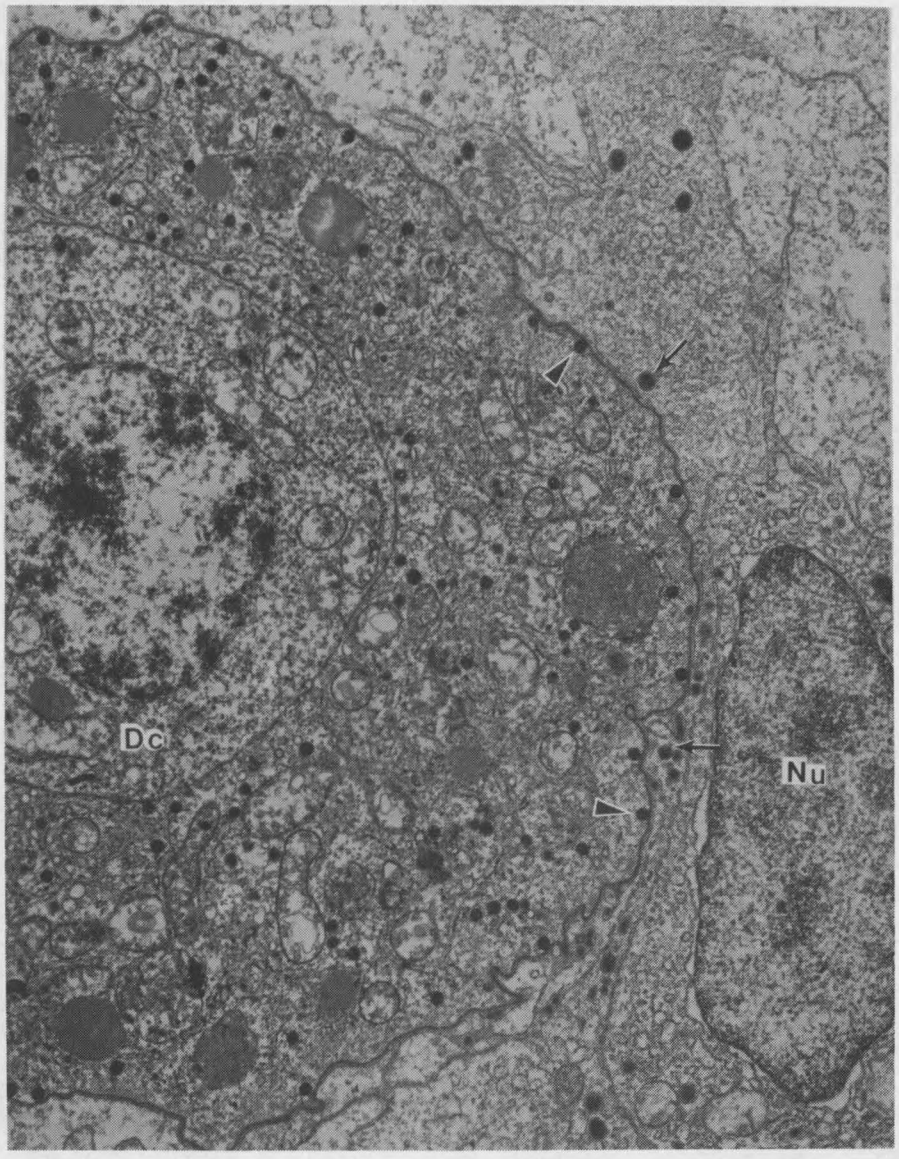


Figure 19. Electron-dense granules (arrows) in close proximity to plasmalemma adjacent to PKX; haplosporosomes (arrowheads), daughter cell (Dc), nucleus (Nu) (x10,000).

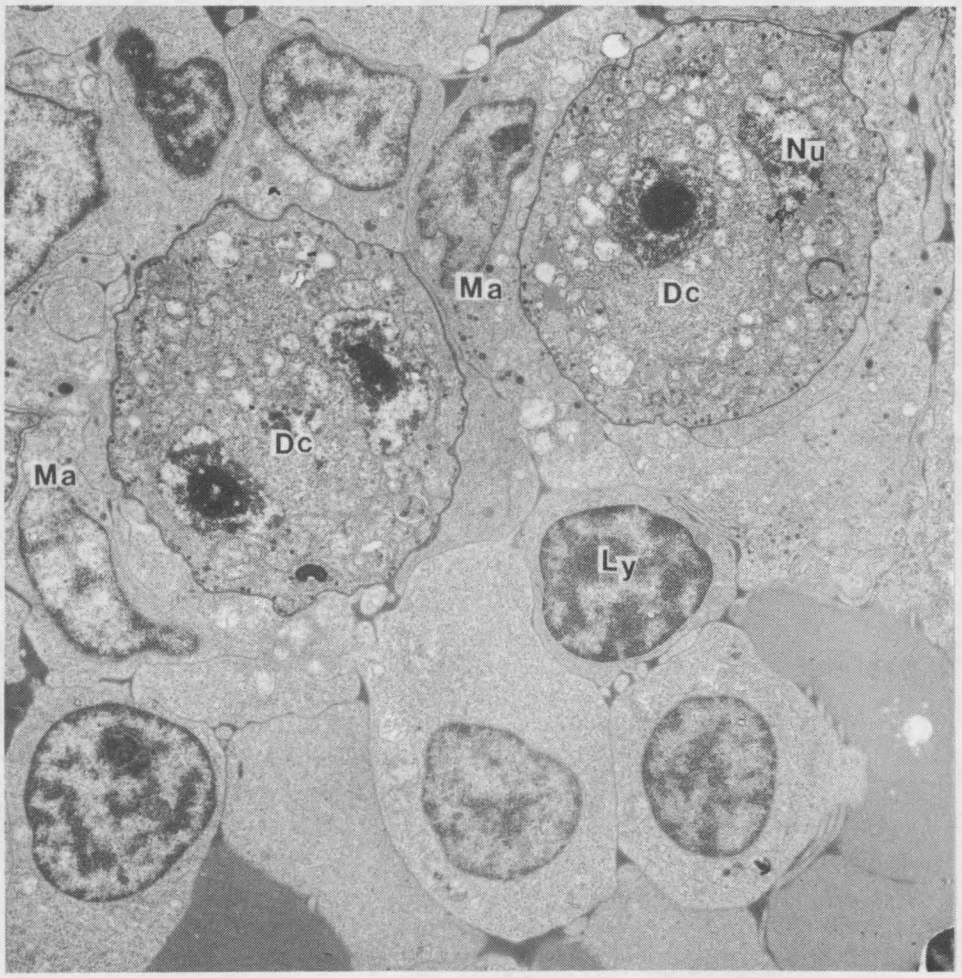


Figure 20. PKX with macrophages (Ma) closely adhered to cell surface and lymphocyte (Ly) closely associated with macrophages; daughter cell (Dc), nucleus (Nu) (x4200).

Macrophages were again found migrating through the basal lamina and into distal tubular epithelium (Figure 21). Tubular epithelium often showed signs of degeneration, such as swollen mitochondria, myelin figures, cytoplasmic vacuolation, and a thickened basal lamina, fibroblasts and epithelioid cells. Like plasma cells seen in week seven, thrombocytes were more numerous in these tissue samples than previously observed (Figure 22).

Week Twenty

The study was terminated at twenty weeks PI. PKX was not identified in the kidney interstitium or tubular lumina in any of these tissue samples. Small granulomatous foci, vacuolated tubules and regenerating tubules were the most prominent features observed by light microscopy. All kidneys appeared to be recovered or recovering from PKD as evidenced by the reduction in hemopoietic and granulomatous tissue, increase in renal elements, and numerous regenerating tubules (Figure 23). Cells in regenerating tubules were distinguished by their deeply basophilic cytoplasm.



Figure 21. Macrophage (Ma) migrating through basal lamina (arrow) into tubular epithelium, ten weeks PI; PKX (x4200).

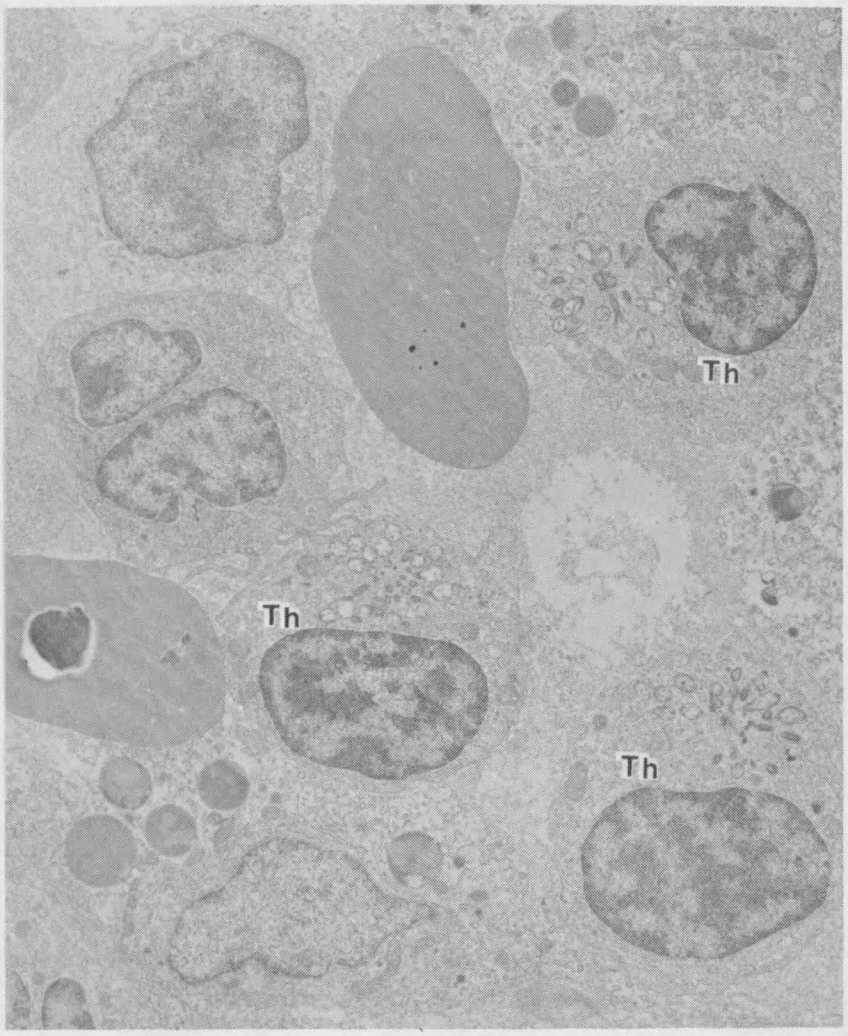


Figure 22. Thrombocytes (Th) found at ten weeks PI (x5460).

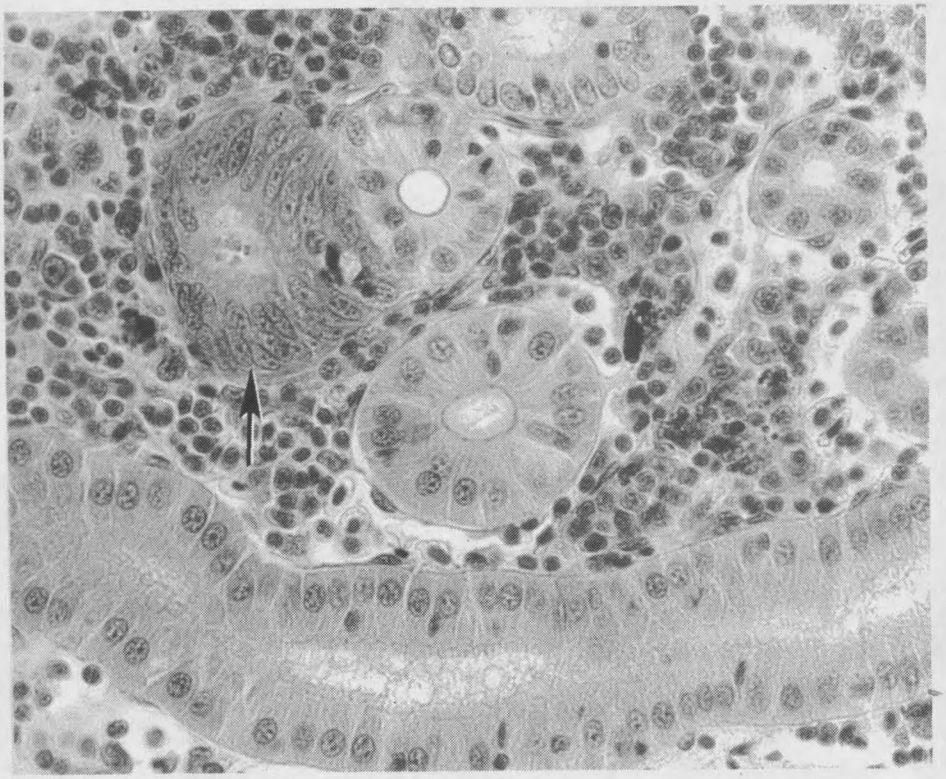


Figure 23. Regenerating tubule (arrow), twenty weeks PI (x400).

Thickened basal lamina, macrophages in tubules, regenerating tubular epithelium and scattered fibroblasts were the most common ultrastructural features of these kidney samples (Figure 24). Cells of regenerating tubular epithelium were squamous to cuboidal in shape, had large nuclei with diffuse chromatin, prominent nucleoli and few or no microvilli.

Host Response to Clay

During the twenty week experiment, there was no mortality of fish injected with bentonite clay and saline. The control fish injected only with saline did not show any changes in the pyloric caeca or kidney tissue samples.

Week One

Histologically, clay particles were visible in tissue surrounding pyloric caeca. Macrophages appeared to be the only cell type responding to the presence of clay in the visceral cavity. The most characteristic feature in the kidney was the increase in melanomacrophages found in the

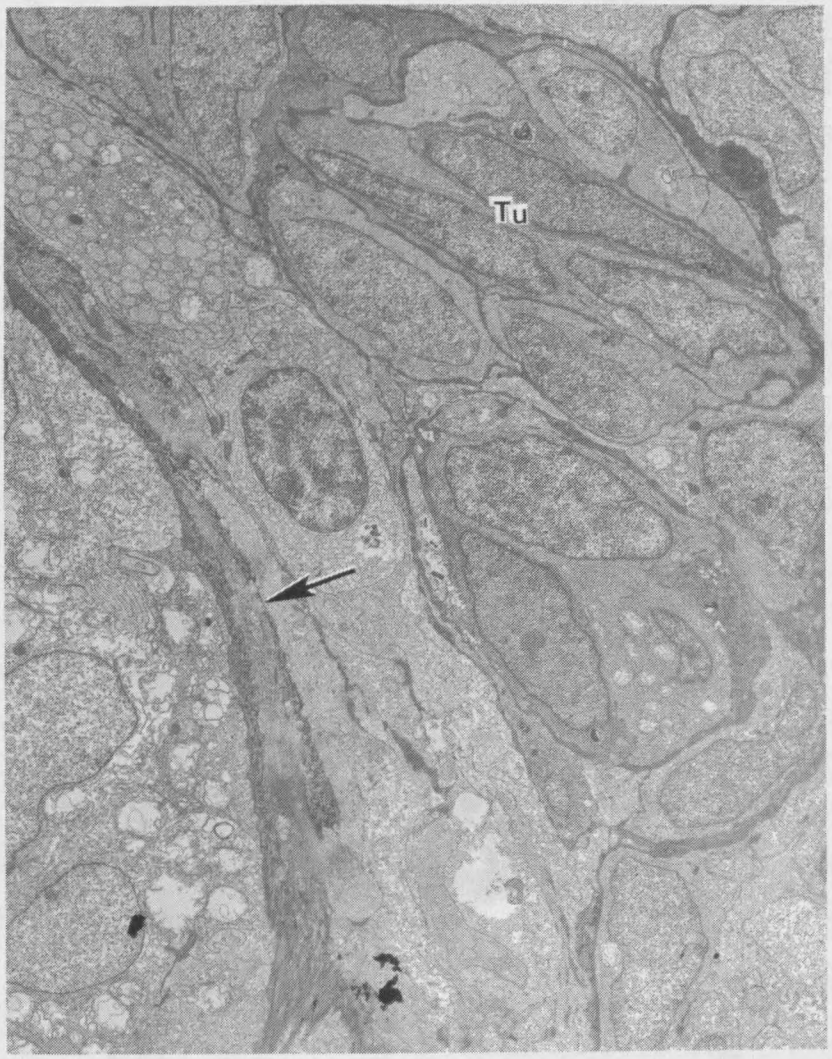


Figure 24. Thickened basal lamina (arrow) and regenerating tubular epithelium (Tu), twenty weeks PI (x4000).

interstitium. Some melanomacrophages contained clay particles as evidenced by the blue color of clay in sections stained with Giemsa. Most proximal tubules appeared heavily vacuolated.

Electron microscopic examination of the kidney samples revealed the presence of clay particles in melanomacrophages and macrophages that appeared to be overlying endothelial cells (Figure 25). Endothelial cells, especially those lining the peritubular capillaries were swollen and markedly vacuolated. In contrast to fish with PKD, PMNs were observed more frequently in the hemopoietic tissue.

Week Three

Grossly, visceral adhesions were first noted in fish collected at week three. An inflammatory response to the clay was evident in tissues surrounding pyloric caeca samples. Bentonite clay induced distinct visceral granulomas that were scattered throughout the fatty tissue. The granulomas were mainly comprised of macrophages containing clay particles and a few multinucleate giant cells.

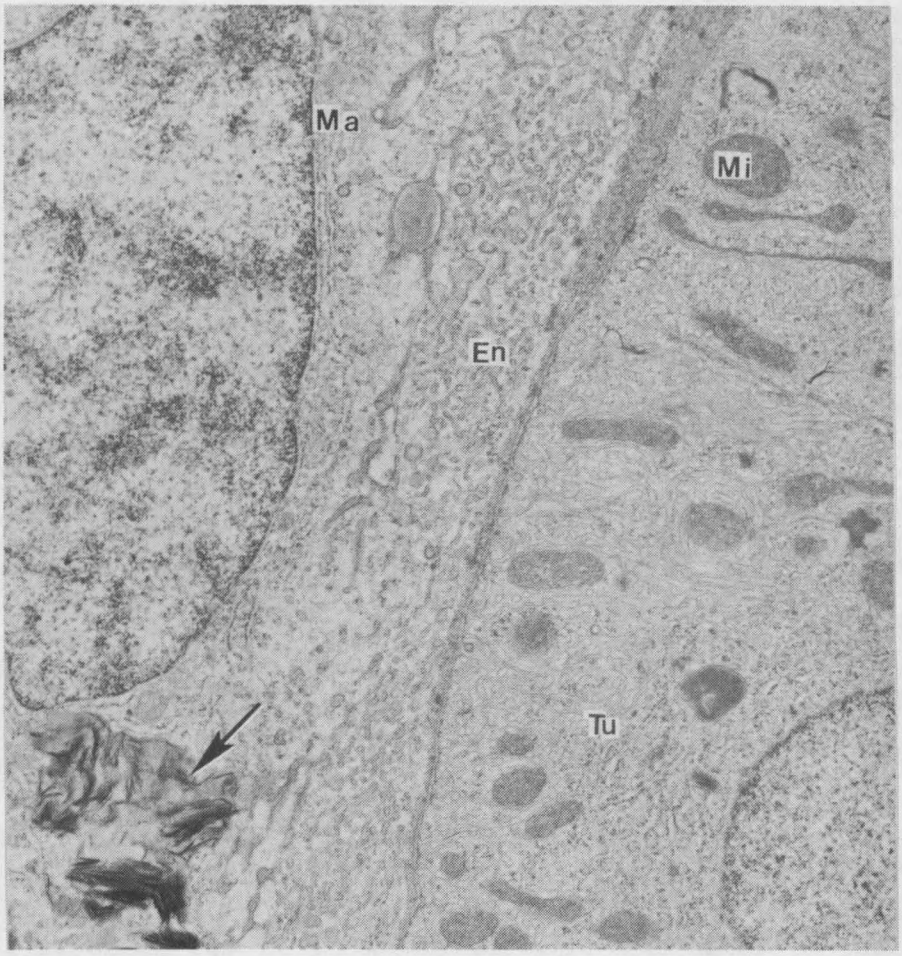


Figure 25. Clay particle (arrow) contained in macrophage (Ma) which is overlying endothelial cell (En); tubular epithelium (Tu), mitochondria (Mi) (x11,500).

Melanomacrophage proliferation in hemopoietic tissue was more intense in the three weeks PI than one week PI samples. Again, sections stained with Giemsa revealed the presence of small clay particles in these cells. Clay stained intensely blue with Giemsa.

Ultrastructurally, activated endothelial cells were observed with clay particles in their cytoplasm. Clay particles were also found again in macrophages and melanomacrophages. Tubular lumina, usually distal segments, were almost occluded with a finely granular material similar to that seen in PKD-infected fish at three weeks PI. Many large, electron-dense droplets, similar to those seen in hyaline droplet degeneration, were found in proximal tubular epithelium.

Week Five

Visceral granulomas were not seen in any of the samples collected five weeks PI. However, kidney tissue from two of three fish sampled revealed an intense proliferation of melanomacrophages in the hemopoietic tissue.

Melanomacrophages were the predominant cell type found in kidney interstitium by electron microscopy (Figure 26). However, clay particles had been phagocytosed mostly by macrophages.

Week Seven

Massive visceral granulomas containing clay particles of various sizes were observed at seven weeks PI. Macrophages, epithelioid cells, and foreign-body giant cells were the predominant cell types found in the lesion (Figure 27a). A lymphocytic infiltrate was not seen in these granulomas. Marked hypercellularity and a decrease in tubular density in the hemopoietic tissue was noted in kidney samples. Melanomacrophages almost filled the entire kidney interstitium (Figure 27b). Clay particles were difficult to discern in these areas of the concentration of darkly staining melanomacrophages.

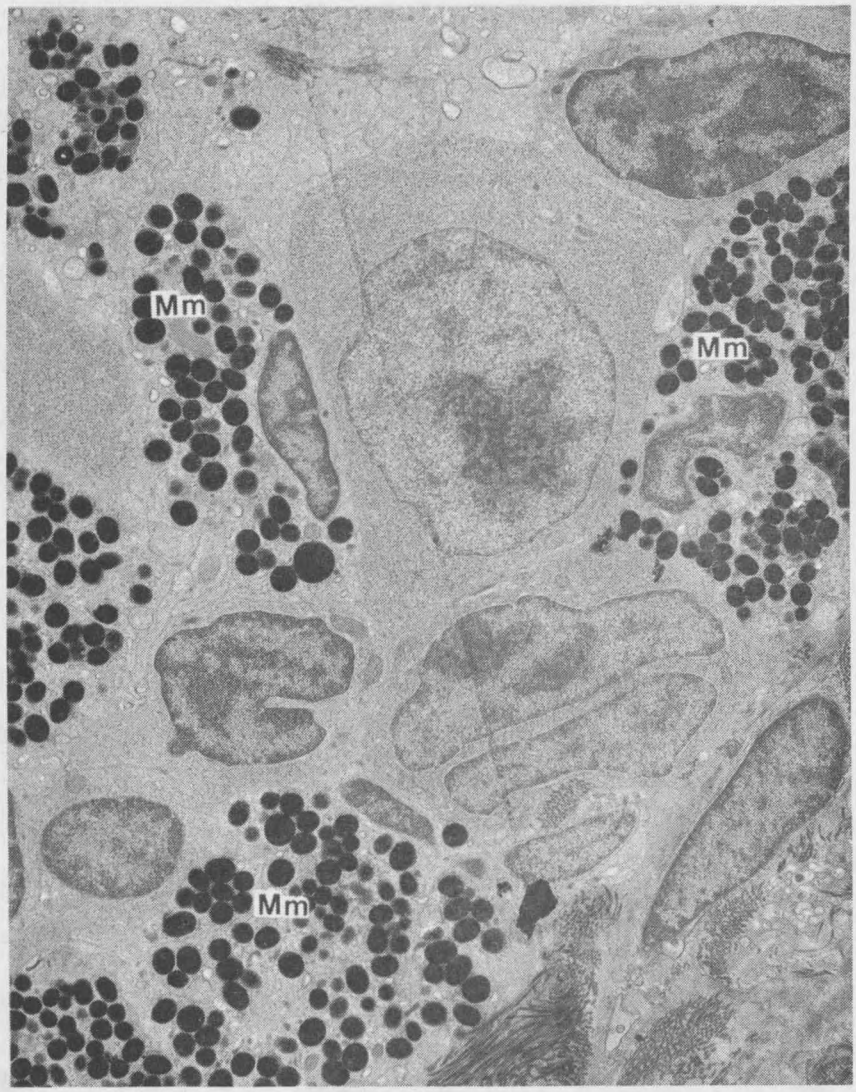


Figure 26. Melanomacrophages (Mm) found in kidney interstitium, five weeks PI (x5200).

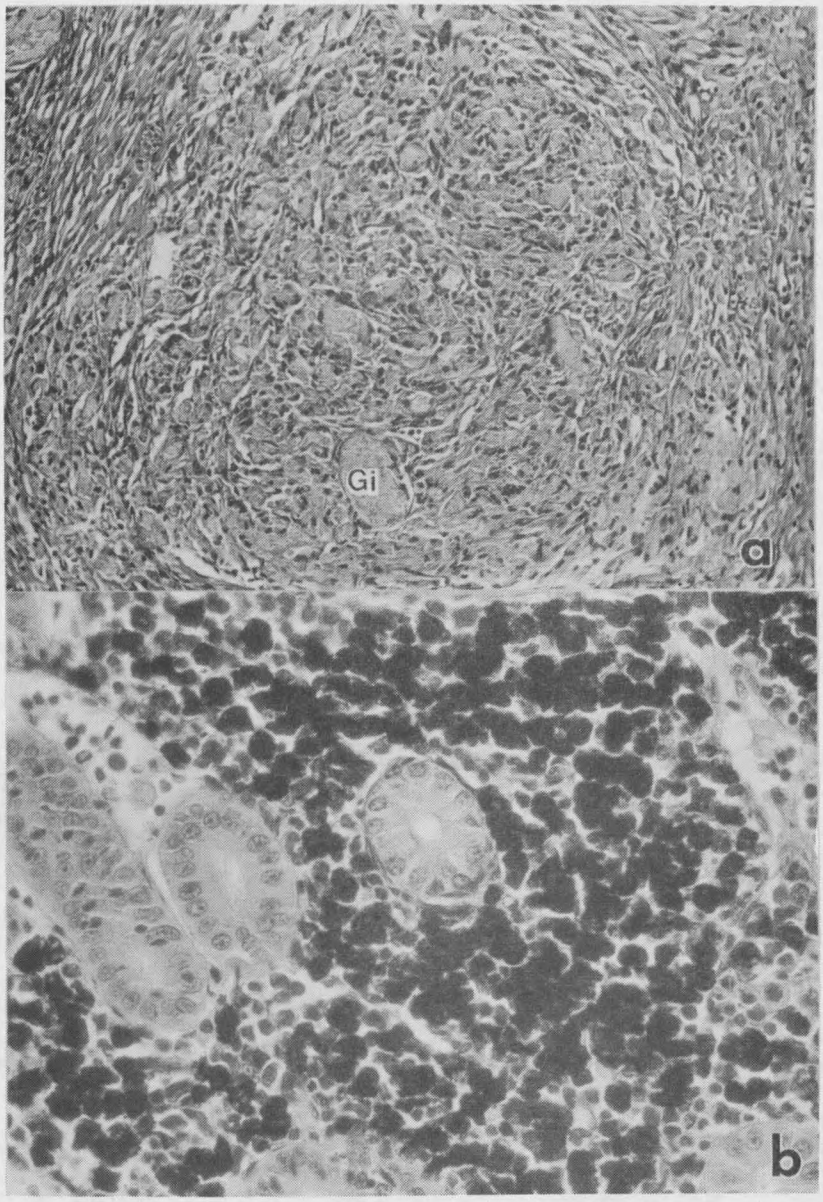


Figure 27. a) Visceral granuloma seven weeks PI; giant cell (Gi) (x160). b) Melanomacrophages dominate the kidney interstitium seven weeks PI (x400).

Electron microscopic examination of kidney tissue showed clay particles mostly in macrophages (Figure 28), occasionally endothelial cells, and very seldom in melanomacrophages although this was again the predominant cell type. An accumulation of large, electron-dense absorption granules was noted in proximal tubular epithelium (Figure 29).

Week 10

Visceral granulomas ranged from small, well circumscribed lesions scattered throughout adipose tissue to massive lesions invading the intestinal muscularis and obliterating most of the fatty tissue. Necrosis was noted in several areas within large granulomas. Lymphocytes and numerous long spindle-shaped fibroblasts were seen in the large, more advanced lesions (Figure 30a).

A moderate decrease in tubular density and hypercellularity in the kidney interstitium were the characteristic features of kidney tissue sampled. Melanomacrophages and mononuclear cells were the

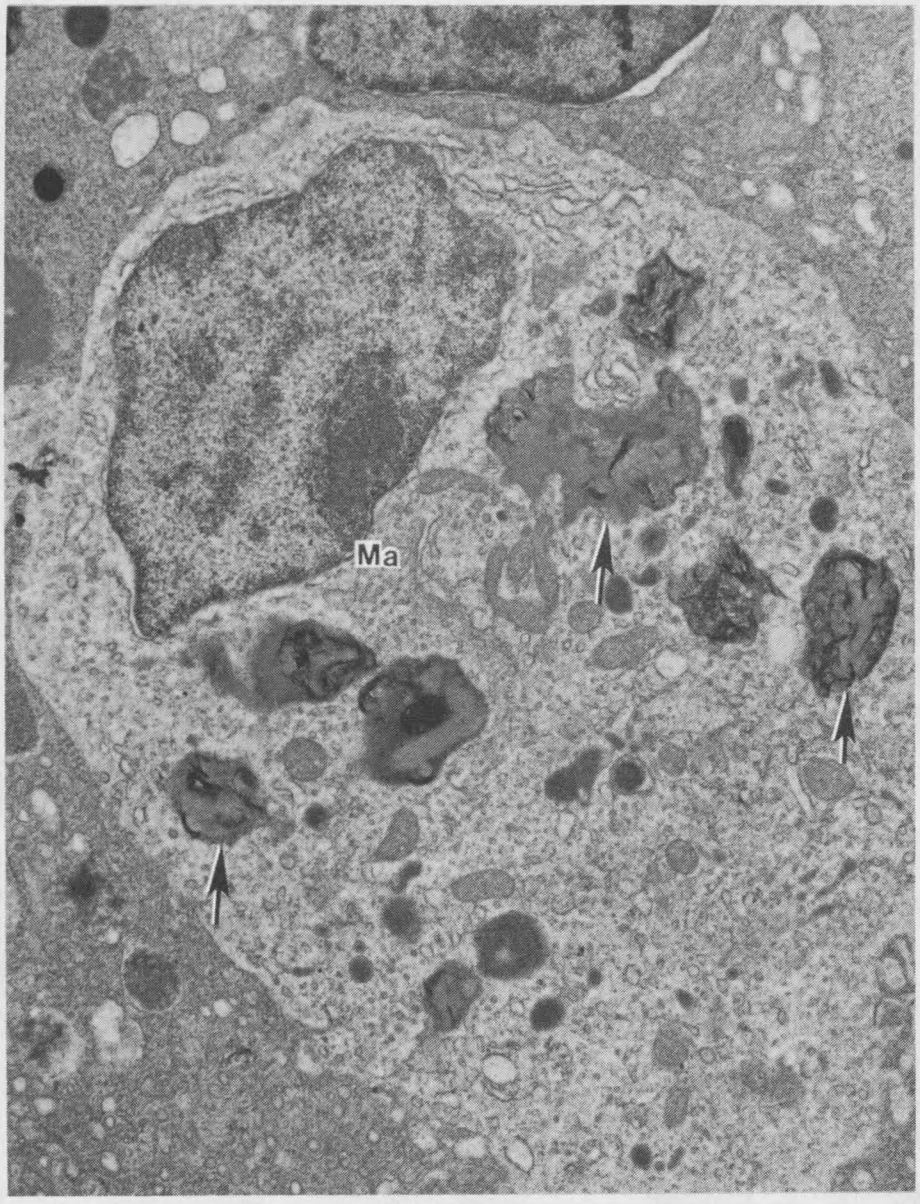


Figure 28. Macrophage (Ma) with phagocytosed clay particles (arrows) seven weeks PI (x10,000).

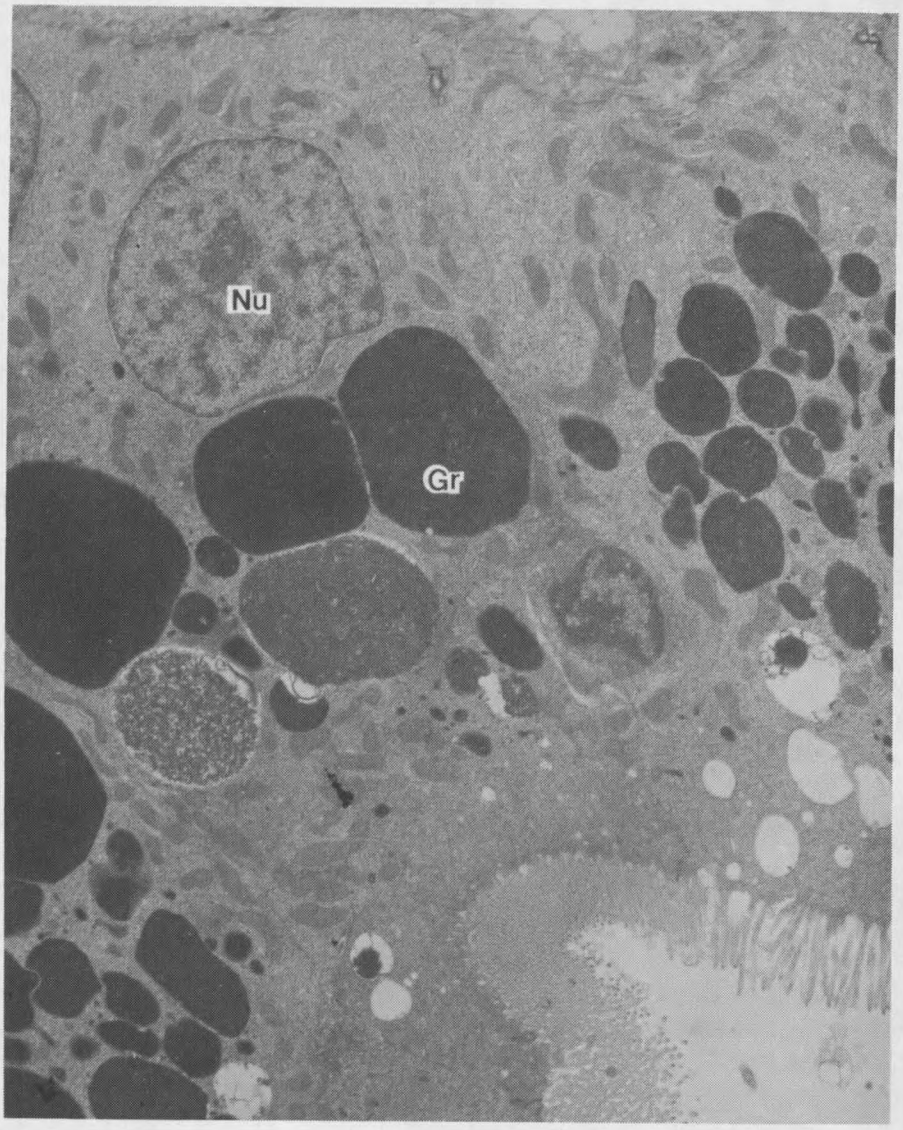


Figure 29. Large, electron-dense absorption granules (Gr) in proximal tubular epithelium seven weeks PI; nucleus (Nu) (x5460).

predominant cell types found in the interstitium. Proximal tubular epithelium appeared to have extremely vacuolated cytoplasm and hyaline droplet degeneration was noted in many of these tubules. Cellular debris was evident in lumina of several collecting tubules. Regenerating tubules were observed throughout the kidney tissue. Melanomacrophages were seen in the kidney circulation (Figure 30b). In sections stained with Giemsa, clay particles were difficult to distinguish in areas of hemopoietic tissue containing large concentrations of melanomacrophages, but easily identifiable in macrophages not located near these areas.

Ultrastructural examination of kidneys revealed results similar to seven weeks PI. Plasma cells were first noted in these samples, often adjacent to kidney tubules (Figure 31). Small, atypical barbell shaped mitochondria suggestive of sublethal injury were often seen in tubular epithelium (Figure 31).

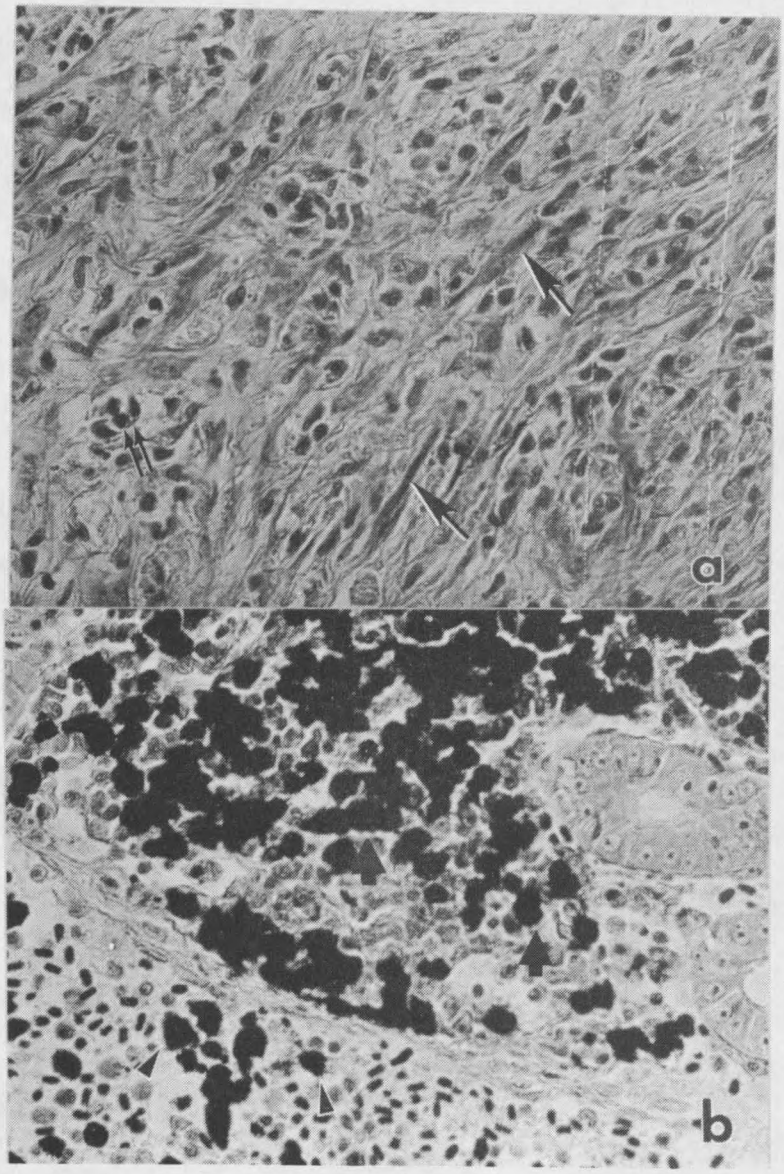


Figure 30. a) Lymphocytes (double arrow) and fibroblasts (arrows) in visceral granuloma (x160). b) Melanomacrophages (arrowheads) in the kidney circulation ten weeks PI (x160).

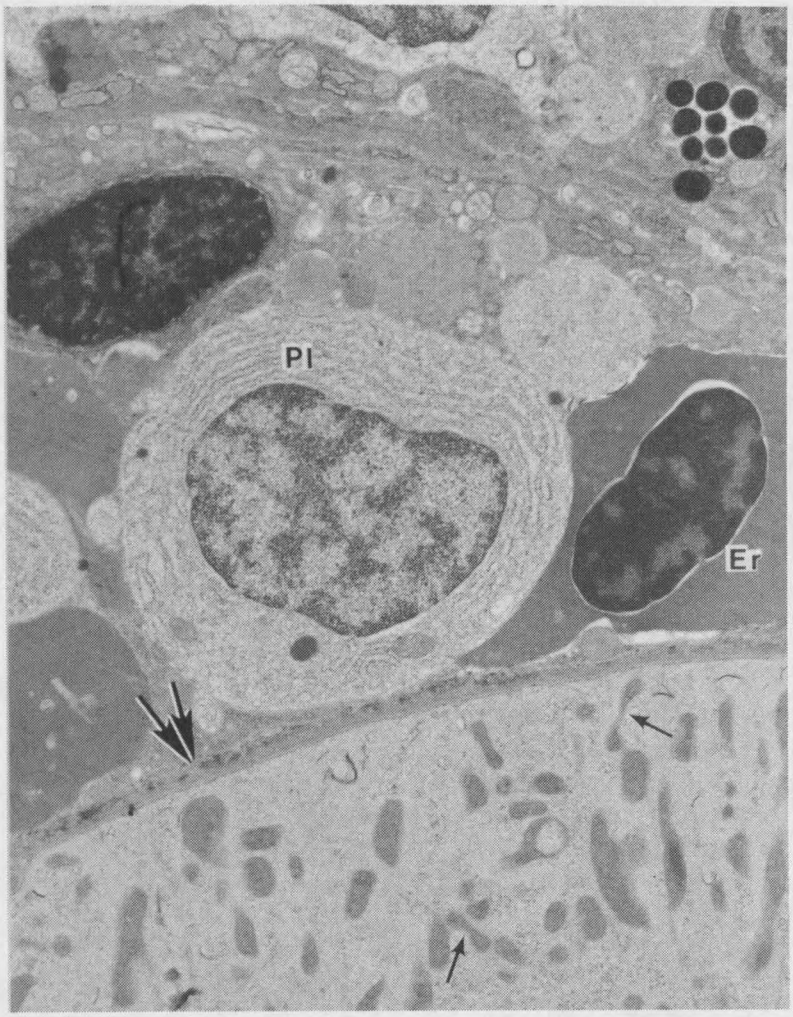


Figure 31. Plasma cell (Pl) adjacent to kidney tubular epithelium with small barbell-shaped mitochondria (small arrows) ten weeks PI; basal lamina (large arrow), erythrocyte (Er) (x7600).

Week 20

Grossly, samples collected at the termination of the study showed visceral adhesions along the intestine and black streaks in the visceral fat and pyloric caeca. Histologically, small to large granulomas, were found scattered throughout the visceral fat, attached to and invading spleen capsules and intestinal muscularis. The characteristic feature of these samples was numerous melanocytes, not seen previously, found primarily in the center of the granulomas (Figure 32).

Kidney tissue more closely resembled saline injected controls at this time. Tubular density appeared normal. Hypercellularity, extremely vacuolated tubular epithelium, and protein material in tubular lumina were markedly reduced. Melanomacrophages were found primarily around blood vessels and lightly scattered throughout the interstitium. Blast cells were evident and clay particles were difficult to find in the hemopoietic tissue. Electron microscopic examination of samples confirmed the above results.

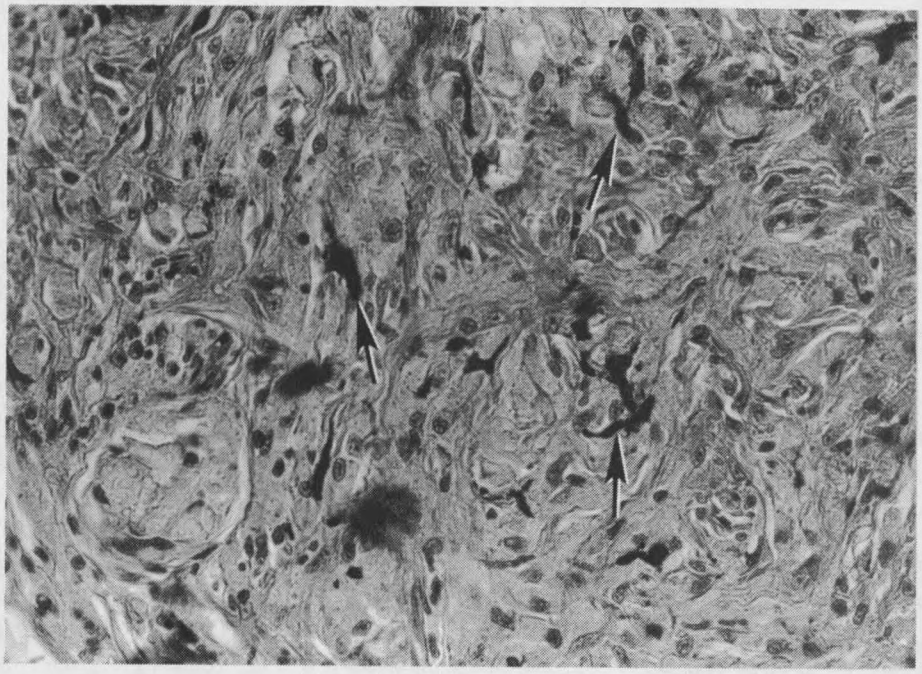


Figure 32. Melanocytes (arrows) in visceral granuloma twenty weeks PI (x160).

DISCUSSION

Developmental stages of PKX previously described by Kent (1986), Rafferty (1986) and Clifton-Hadley et al. (1987) were observed in this study. The small condensed eosinophilic form of PKX at three weeks PI and migration of the parasite through tubular epithelium reported by Kent (1986) were observed by light microscopy but not confirmed by electron microscopy. Experimental fish infected with PKD appeared to mount an effective local cellular response against PKX, interrupting maturation of the parasite. Typical myxosporeans, have primary cells that eventually disintegrate and release daughter cells, which may begin the cycle again, go into the sporogenic phase or be destroyed by the host tissue reaction (Lom, 1987). Intraluminal sporogenic forms were observed infrequently, and were not found in any fish collected twenty weeks PI. Complete sporulation did not take place as evidenced by the lack of polar capsule formation.

Vegetative reproduction by binary fission of PKX internal cells (Kent, 1986) and internal cleavage (Seagrave et al., 1980) typical of the phylum Myxozoa (Lom et al., 1983) was observed in this study. The secondary cell or generative cell has characteristic bundles of microtubules

close to the nucleus, numerous free ribosomes in the cytoplasm, pseudopodia-like extensions and lack a centriole. It is this type of cell that is produced by the released sporoplasm and is the initial stage of the extrasporogenic cycle (Lom, 1987). Unidentified cells seen early in the infection could represent the infective stage of PKX, but may alternately represent foreign fish cells from the kidney homogenate inoculum.

PKX provoked a severe host reaction characterized by interstitial hyperplasia and granulomatous interstitial nephritis. This is in contrast to most myxosporeans which invoke practically no tissue reaction (Lom, 1987). The host inflammatory response seemed to inhibit both migration of PKX to the tubular lumina and subsequent sporulation. Infection rate and intensity of disease did not appear to be dose related. Prevalence of PKX and degree of associated inflammation are dependent on the immunocompetence of the host which is affected by several factors, especially in parasitic infections (Woo, 1987).

Interstitial hyperplasia characteristic of PKD (Kent and Hedrick, 1986; Clifton-Hadley et al., 1987) was observed in fish injected with PKX beginning three weeks PI and extending through ten weeks PI. This response can be compared to bone marrow hyperplasia seen in many infectious diseases of man. Fish sampled at five weeks PI exhibited a

marked cellular response and by seven weeks PI chronic inflammation was evident. The increase in plasma cells observed seven weeks PI was indicative of a humoral response to PKX. Replacement of epithelioid tissue with normal hemopoietic tissue at ten weeks PI may be indicative of recovery not inflammation, since PKX was found mostly in granulomatous lesions. Thrombocytes, believed to be the equivalent of mammalian platelets are probably part of the healing process (Ellis 1981). Fibrosis, typical of resolution of an inflammatory lesion, was seen only to a minor degree during later stages of the disease.

The sequential pathologic changes of PKD observed in this study were similar to that reported by Clifton-Hadley (1987). However, fusiform hemosiderin crystals associated with intravascular lesions and nodules of chronic inflammation were not observed during the study. Glomerular sclerosis as described by Ferguson and Needham (1978) was not observed to any degree during the study.

Rafferty (1986) reported marked melanomacrophage proliferation during early stages of experimentally induced PKD and suggested melanomacrophages were involved in processing PKX antigens to stimulate the immune response. This phenomenon was not observed during this study. Clifton-Hadley (1987) found granules of melanin throughout reactive tissue but few melanomacrophages, similar to the

findings in this study. Melanin is a pigment known for its ability to absorb free radicals and cations and render them inactive. However, melanin also has bactericidal properties and has been associated with lipid peroxidative tissue damage (Edelstein, 1971; Roberts, 1975). Possibly, melanin is mobilized to neutralize free radical and cation activity and explains why the pigment has been commonly observed in sites of infection (Agius, 1985).

Evidence from this study indicates that macrophages are the predominant host cell type associated with PKX throughout the disease. This is not surprising since the macrophage is the most important cell type in host defense against invasion by infectious or noninfectious foreign material (Corbel, 1975). Fish macrophages remove debris, may destroy intracellular microorganisms, may uptake and process antigen, may kill antigen deviant cells, and may actively secrete various substances (Agius, 1985). Little is known about the factors that affect the phagocytic response of macrophages in fish. It has been suggested that their role is more primitive and restricted than that seen in mammals (Ellis, 1981). In this study, PKX was not eliminated until twenty weeks PI even though macrophages were found closely adhered to the cell surface of most parasites at five weeks PI. PKX may delay destruction by prolonging the killing mechanisms of the macrophages,

possibly by producing a substance that prevents fusion of lysosmes or inhibits the production of a sufficient number of lysosomes.

Melanomacrophages were the predominant cell type found in kidney samples from fish injected with clay. However, the clay particles that gained access to the circulation were phagocytosed primarily by macrophages in the hemopoietic tissue in kidney. Perhaps these macrophages later became melanomacrophages. Melanogenesis is poorly understood in melanomacrophages. It is not known if melanin is phagocytosed or manufactured in these cells.

Normally, melanomacrophages accumulate steadily with age and kidneys of older fish may contain large numbers, but this process is often accelerated in the course of some diseases (Agius, 1985). Melanonmacrophages contain melanin that is different than that found in melanocytes of the dermis and has been associated with various pathological conditions (Roberts, 1975). Melanomacrophage centers are thought to represent primitive analogues of germinal centers found in birds and man (Roberts, 1975). All teleosts, except salmonids, have distinct melanomacrophage centers which are considered to be an integral part of the reticuloendothelial system (Roberts, 1975). Inert particles such as carbon are known to be phagocytosed by macrophages that aggregate within melanomacrophage centers

(Agius, 1985). It has been suggested that melanomacrophages are sensitive indicators of stressful conditions and are useful as health monitors (Wolke et al., 1985).

The PMN response to infection is not as important in fish as it is in mammals (Corbel 1975). PMNs were scattered throughout kidney interstitium during the study, but never constituted a major portion of the lesion. Many authors (Sakai, 1984; Suzuki,1986) have reported that fish neutrophils are phagocytic. Clifton-Hadley (1987) found what he believed to be neutrophils surrounding PKX. In this study neutrophils were not phagocytic nor were they found associated with PKX. The precise role of the neutrophil and melanin pigment are poorly understood in fish diseases (Ellis, 1981).

This study has demonstrated an effective cellular response, dominated by macrophages, against infectious and non-infectious foreign material. The inflammatory response in fish is considerably slower than that seen in higher vertebrates, but regenerative capacity is high in fish (Ellis, 1981). Resolution of interstitial inflammation was observed without fibrosis in both experimental groups by twenty weeks PI. Small localized areas of chronic inflammation were still evident but tubular and hemopoietic density were normal.

There are presently many gaps in understanding the fish immune system (Woo, 1987). There is general confusion over the cellular components of the defense system in fish because mammalian hematological terms have been applied without evidence that functional similarities exist. In similarity to mammals there is production of specific immunoglobulin, and phagocytic and cytotoxic cells. Distinctly different, however, are reduced and structural differences in immunoglobulin classes and the absence of lymph nodes (Ellis 1981). The teleost kidney executes functions that are attributable to mammalian lymph nodes and bone marrow.

Interspecies discrepancies of ultrastructural features in leukocytes were encountered in this study. Rainbow trout cells most often resembled those described in channel catfish. However, neutrophils seen in this study had segmented or multilobed nuclei. Hemopoietic cell types in fish are still far from being adequately charactertized, particularly the immature forms. Differences in hemopoietic cells among species, especially those of different water temperature regimes and environmental conditions, need to be investigated.

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