Studies on the Pathology of Bacterial Kidney Disease (*Renibacterium salmoninarum*) in Coho (*Oncorhynchus kisutch*) and Atlantic salmon (*Salmo salar*)

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Abstract

With the intensification of the aquaculture industry along the west coast of Canada, there has been a concomitant increase in the prevalence of bacterial kidney disease (BKD) (Renibacterium salmoninarum) (Rs) in production and government hatchery facilities. Due to the paucity of information on the pathogenesis and epizootiology of Rs, treatment and control measures have been confounded and emergence of BKD exacerbated.

Initially, to enhance specificity over conventional histochemistry for demonstration of Rs in histological sections an avidin biotin conjugated immunoperoxidase technique was developed for use to monitor the histopathological manifestation of BKD.

To ascertain the temporal and spatial histogenesis of BKD, coho (Oncorhynchus kisutch) and Atlantic (Salmo salar) salmon were challenged experimentally by intraperitoneal injection and naturally by cohabitation with Rs-innoculated fish then serially sampled.

Histopathology revealed lesions consistent with past case reports and investigations, as well as previously undescribed manifestations including, inflammatory cell kinetics in the renal perivascular compartment, cresentic glomerulonephritis, extrarenal dissemination (via septic emboli or direct extension), and ovarian follicular cell accumulation of Rs. Pseudocyst formation, renal interstitial hyperplasia and meningoencephalitis were also characterized.

The granulomatous response in coho salmon was predominantly histiocytic; whereas, in Atlantic salmon a tuberculoid response was more apparent. In either species a profound cell mediated immunity was adduced.

To further resolve the nature of the inflammatory response, coho and Atlantic salmon were immunosuppressed by administration of supratherapeutic doses of glucocorticosteroids. On challenge with Rs both species incurred an earlier onset and greater rate of mortality than immunocompetent cohorts. The granulomatous response appeared irregular and expansive with exuberant intra- and extracellular Rs growth. These observations may be attributed to inhibition of prostaglandin and leukotrienne synthesis, as well as an inability to immunologically prime phagocytes.

To evaluate the inflammogenic potential of somatic, cell-wall associated, and soluble fractions of Rs, coho and Atlantic salmon were injected and serially sampled for histopathology. Past studies on Rs virulence determinants and pathogenic mechanisms have focused almost exclusively on a soluble protein, designated p57. In this investigation no histological alterations were appreciated in fish challenged with cell-wall associated or soluble (p57) fractions; however, mild, multifocal pyogranulomata were noted in the renal interstitium of coho and Atlantic salmon challenged with the somatic or peptidoglycan fraction of Rs. Peptidoglycans of a number of mammalian pathogens are strongly inflammogenic, poorly biodegradable, and persist in host tissue for protracted periods. Chemical resolution and in vivo evaluation of subcellular components of Rs is warranted to further resolve the pathogenesis of BKD in salmonid species.

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Introduction

With the secular intensification of aquaculture on the West Coast of Canada, Bacterial Kidney Disease (BKD) has emerged as a major contributor of farmed fish morbidity and mortality (Evelyn, 1988).

The etiological agent of BKD, Renibacterium salmoninarum is a small (0.5 x 1.0 um), Gram positive, PAS positive, non-acid fast, fastidious, asporogenous, nonmotile, diplobacillus with varying degrees of endemicity in farmed, feral and wild Pacific salmon (Fryer and Saunders, 1981).

Infection with R. salmoninarum results in low grade, persistent mortality or sporadic epizootics, either of which causes significant economic loss to producers. Due to the chronicity of infection fish seldom succumb before 6-18 months of age, therefore, considerable financial investment is incurred by the time mortalities arise (Evelyn, 1988).

The predilection sites for the bacteria are the anterior and posterior kidney. Due to the inherent functional reserves of renal tissue in fish, coupled with nitrogenous waste and monovalent ion excretion predominantly via the gills, clinical manifestation of BKD occurs only in the terminal stages of infection and reflects osmoregulatory, coupled with acid-bases imbalances. Moribund fish commonly feature clinical signs of lethargy, anaemia, ascites, exophthalmus, swollen vent, inappetance, as well as cutaneous ecchymosis, petechiae and subdermal cavitations (Fryer and Saunders, 1981; Ferguson, 1989; Klontz, 1983).

Necropsy of fish has revealed species-specific pathology. In Pacific salmon (Oncorhynchus spp) lesions include diffuse, subacute to chronically active fibrinoperitonitis, nephritis,

splenitis, and pericarditis; whereas, in the Atlantic salmon (Salmo spp) fish featured multifocal, chronic, pyogranulomata predominantly in the reticuloendothelial tissue with cicatrization and resolution of lesions reported (Snieszko and Griffin, 1955; Wood and Yasutake, 1956; Bruno, 1986b).

Differentials for these gross lesions include protozoal infections, such as Myxobolus sp, Myxidium spp, Ceratomyxa shasta, or PKX (the etiological agent of proliferative kidney disease, afflicting predominantly rainbow trout (Oncorhynchus mykiss)), viral infections, such as infectious haematopoietic necrosis virus (IHN), or viral hemorrhagic septicemia (VHS), systemic bacterial infections such as, Mycobacterium sp, Nocardia sp, Streptomyces spp, Aerococcus spp, Rhodococcus spp, Lactobacillus spp, or rickettsial infections, mycotic infections, such as Exophiala spp, or Ichthyophonus spp, metazoan parasitic infections, environmental or nutritional diseases, such as nephrocalcinosis or visceral granuloma, and neoplasia (Fryer and Saunders, 1981; Roberts, 1989; Brocklebank, Speare, Armstrong and Evelyn, 1992; Speare, Brocklebank, MacNair et al, 1992).

Diagnosis of BKD is confounded due to prolonged periods (up to 40 days) required for culture, lack of serological sensitivity or specificity, as well as hypoendemnicity in suspect populations. It is contingent on a combination of Gram stains and indirect or direct immunofluorescent antibody techniques of selected tissue smears, histopathology of representative lesions (Bullock, Griffins, and Stuckey, 1980), serodiagnosis by immunodiffusion (Chen, Bullock, and Bullock, 1974), coagulation test (Kimura and Yoshimizu, 1981), counter immunoelectrophoresis test (Cipriano, Starliper, Schachte, 1985), enzyme linked immunosorbent assays (Sakai, Loyama, Atsiata and Kobayashi, 1987; Pascho and Mulcahy, 1987; Elliott and Barila, 1987) and culture

on enhanced nutrient agar, such as KDM-2 (Evelyn, 1977) or SKDM (Austin, Embley and Goodfellow, 1983).

Polyclonal antibody preparations demonstrated that the etiological agent of BKD was antigenically homogeneous (Bullock, Stuckey and Chen, 1974; Getchell, Rohovec and Fryer, 1985; Austin and Austin, 1987); however, more recent work with monoclonal preparations suggests that strain variation exists among isolates (Arakawa, Saunders, and Fryer, 1987; Wiens and Kaattari, 1991). The principle monoclonal epitope for R salmoninarum is a heat stable, water soluble (Daly and Stevenson, 1987) 57 kd surface protein, which is consistently localized on all screened R. salmoninarum cohorts. The protein is a virulence factor with a dose dependent in vivo anaemia-induction factor, in vitro lymphocyte antibody suppression (Turaga, Weins and Kaattari, 1987), and bacterial autoaggregation, a noted characteristic of the bacterium.

Epizootiological determinants of BKD infection and pathogenesis include water temperature, hardness, salinity, as well as stocking density, genetics, carrier incidence, and husbandry practises (Austin and Austin, 1987).

Survival experiments indicate that the bacteria can persist in association with faecal or organic material in fresh and salt water for 4 and 21 days, respectively (Austin and Rayment, 1985). This phenomenon, coupled with repeated failure to isolate the organism from water samples from aquaculture production facilities, suggests that the organism persists for only short periods outside of the host and is, therefore, considered an obligate pathogen. Infected or carrier salmonids are the putative reservoir of infection (Fryer and Saunders, 1981) and the role of paratenic hosts (such as non-salmonid and molluscan species), remains speculative.

Although the prime mode of transmission is considered to be vertical (Wolf and Dunbar, 1959; MacLean and Yoder, 1970; Mitchum, Sherman and Baxter, 1979) horizontal transmission within production facilities (inter-alia), as well as from farmed to feral fish stocks have been adduced (Mitchum and Sherman, 1981; Paterson, Lall, Airdrie et al, 1985). Horizontal transmission has been reported in both freshwater and sea water, as well as between different species of salmonids (Bell, Higgs and Traxler, 1984; Mitchum and Sherman, 1981).

Epizootiological investigations of BKD outbreaks have implicated ostensibly healthy eggs with the introduction of R. salmoninarum into novel geographical locations (Allison, 1958). On hatching, progeny shed bacteria which are capable of persisting outside of the host for a sufficient duration of time to invade and infect other susceptible individuals to establish an enzootic state.

Preliminary investigations by Bullock, Stuckey, and Mulcahy (1978) demonstrated that the pathogen is transmitted to progeny of eggs even after surface disinfection with povidine iodine (to which R. salmoninarum has been shown to be susceptible, Amend and Pietsch, 1972). This observation, coupled by data obtained from Evelyn et al (1984; 1986b) and Bullock and Leek (1986) suggested that intra-ovum infections of yolk material occurred.

Despite recent advancements in chemotherapy and chemoprophylaxis, control and prevention of R. salmoninarum has been hindered by the paucity of information on the epizootiology and pathogenesis of BKD (Elliot, Pascho and Bullock, 1989).

The focus of this work is initially to develop a specific immunohistochemical technique for the demonstration of Rs in histological preparations. Thereafter, this technique will be employed to demonstrate the bacterium in tissue sections of

naturally and experimentally challenged fish. To further resolve the nature of the inflammatory response to Rs, by challenged immunosuppressed fish with Rs and to evaluate subcellular components of Rs for inflammogenic potential.

Chapter I

Immunohistochemical Detection of *Renibacterium salmoninarum* in Salmonid Fish

INTRODUCTION

With the intensification of the aquaculture industry on the west coast of Canada, bacterial kidney disease (BKD) has emerged as the prime contributor to farmed fish morbidity and mortality (Evelyn, 1988).

The etiological agent of BKD, Renibacterium salmoninarum (Rs) is a small (0.5 x 1.0 um), Gram positive, periodic acid Schiff (PAS) positive, non-acid fast, fastidious, asporogenous, nonmotile, coccobacillus reported in farmed, feral and wild Pacific salmon (Fryer and Sanders, 1981).

Detection of Rs is made difficult because of the prolonged periods (up to 40 days) required for culture and the lack of serological sensitivity or specificity, as well as its enzootic status in susceptible populations (Klontz, 1983). An etiological diagnosis is often contingent on a combination of the Gram stain with direct (DFAT)(Bullock, Griffiins and Stuckey, 1980) or indirect (IFAT)(Bullock and Stuckey, 1975; Mitchum, Sherman and Baxter, 1979; Paterson, Gallant, Desautels, and Marshall, 1979; Laidler, 1980) fluorescent antibody techniques, as well as serology or histopathology.

Historically, histochemical demonstration with either PAS, Gram's, or Lillie's allochrome of bacteria morphologically consistent with Rs was sufficient for diagnosis. Histochemistry, however, has inherent limitations (Austin and Austin, 1987). As BKD progresses from subacute to chronic stages of infection the organism sometimes tends to stain Gram negative rather than Gram positive. Also morphological distinction between Rs and pigment granules in the tissues' endogenous pigments or other Gram or PAS positive organisms may be difficult. For example, certain species of Mycobacterium and Rhodococcus sp, prime candidates in the differential diagnoses of granulomatous lesions in fish, also

stain positive with PAS and Gram's stain, respectively (Chandler, 1987; Speare, Brocklebank, MacNair et al, 1992). Thus, despite available cytochemical stains for the detection of Rs in tissue smears, imprints, or histological sections, immunochemical tests, such as DFAT or IFAT, have been developed and are employed for improved specificity (Elliott, Pascho and Bullock, 1989).

Unfortunately, immunofluorescent tests also have intrinsic limitations, such as nonspecific autofluorescence, relatively low sensitivity, impermanence of the stained tissue preparations, and the prerequisite of dark field microscopy (Pinkus, 1982). Conjugation of primary or secondary antibodies with horseradish peroxidase avoids these drawbacks and facilitates detection of cell and tissue-associated antigens which may not be readily apparent using cyto- or histochemical stains (Falini and Taylor, 1983).

Immunoperoxidase staining techniques have been developed for the detection of a variety of fish pathogens, cells and tissue antigens (Anderson, Swanson, Waxman et al, 1984; Peleteiro and Richards, 1985; Hoffman, Bell, Pfeil-Putzien and Ogawa, 1989; Noga, Dyskra, and Wright, 1989; Bartholomew, Yamamoto, Rohovec, and Fryer, 1990; Evensen, Espelid, and Hastein, 1991; Jansson, Hongsi, Lingderb et al, 1991). The application of an avidin biotin conjugated immunoperoxidase for detection of Rs and comparison to conventional stains may prove a valuable adjunct to the histopathology of BKD.

MATERIALS AND METHODS

Representative chinook salmon (Oncorhynchus tshawytscha) renal tissue infected with Rs, as determined by immunofluorescence (IFAT), and histochemistry (Gram stain and

Periodic Acid Schiff), as well as apparently normal renal tissue were obtained from the fish health diagnostic laboratory (Nanaimo, BC) for development of an avidin-biotin conjugated immunoperoxidase system (ABC).

To assess the effects of different fixatives on immunoreactivity, tissues were fixed in either 10% neutral buffered formalin, Bouin's, Davidson's, or Smith's formal dichromate fixatives. A commercially available rabbit anti-Rs polyclonal antiserum (Grand Island, NY, New York) and 4 murine anti-Rs monoclonal (Micrologics, Victoria, British Columbia) antibodies, designated RSO1 (IgM), RSO2 (IgG1), RSO3 (IgM), and RSO4 (IgG1) were procured for evaluation as primary immunoreactants. Monoclonal antibody to the F antigen of Rs were prepared as described by Wiens and Kaattarri (1991) and assayed for specificity by radioimmunoassay (Micrologics, data sheet).

Tissues were processed by an automatic tissue processor (Tissue tek II) and embedded by conventional techniques. Serial 5 um thick sections of normal and Rs infected renal tissue were prepared and affixed to glass slides using poly-D-lysine (Sigma). Tissues were deparaffinized in xylene and rehydrated through a graded series of alcohols to water. Endogenous peroxidase activity was inactivated by incubation of the sections with 0.5% hydrogen peroxide in methanol at room temperature for 30 minutes. Slides were then pretreated in a 0.05% protease XIV (Sigma) solution for 15 minutes at 37C. After two 10 minute washes in 0.1 M phosphate buffered saline (PBS) pH 7.2, nonspecific antibody binding sites were blocked by incubation for 30 minutes at room temperature with either PBS-5% normal goat serum (Sigma) or PBS-5% normal horse serum for the polyclonal and monoclonal primary antibodies, respectively. Serial doubling dilutions of

primary antibody from 1:200 to 1:51200 were applied and slides were then placed a humid chamber. Each dilution was applied to duplicate slides.

Slides were incubated overnight at 4C, and after two 10 minute washes in PBS, dilutions of the secondary (biotinylated) antibody was applied to the slides (checker board titration from 1:100 to 1:1600 dilutions were used to ascertain optimal levels) for 30 minutes in a humid chamber at room temperature. The avidin and biotin complex (ABC) (Elite Kit, Vector Laboratories) was prepared concurrently by incubating the avidin and biotin together for 30 minutes.

After two washes in PBS, the ABC was applied to the slides and incubated for 60 minutes in a humid chamber at room temperature.

The chromogen was prepared by adding 10 ul of 50% hydrogen peroxide to 100 ul 0.1% 3,3-diaminobenzidine-4 HCl (DAB)(Sigma) immediately before its application to the slides. Slides were incubated with DAB and monitored for staining reaction for up to 10 minutes. Slides were washed twice in PBS, counterstained with hematoxylin, dehydrated, and mounted.

Controls included omission of the primary antibody, the primary antibody replaced with either normal rabbit antiserum or homologous serum absorbed against Rs. Normal mouse ascites fluid was substituted for monoclonal antibodies. To assess specificity, formalin fixed sections of histologically and clinically similar Aerococcus viridans and Lactobacillus spp were evaluated with the monoclonal and polyclonal antibodies for cross-reactivity. In addition, the protocol was conducted with omission of the biotinylated secondary antibody, as well as with omission of the ABC complex (DeLillis, Sternberger, Mann et al, 1979; Petrusz, 1983). Treatment of the slides with the

proteolytic enzymes trypsin, pronase, and pepsin (Kapland and Kraft, 1969; Reid, Hall, Smith and Baer, 1983) was evaluated for possible improved staining.

To compare the ability of conventional histochemistry and immunohistochemistry to demonstrate the bacterium, 20 blocks of Rs-infected formalin fixed ovarian tissues were selected. Each block was sectioned and stained with either hematoxylin and eosin, Gram's stain, Schiff's Periodic Acid, direct immunofluorescence (Bullock, Griffins and Stuckey, 1980), or the avidin biotin conjugated immunoperoxidase technique, then examined and graded according to the degree of specific, nonspecific, and background staining (value ranged from 0=negative, 1+=mild, 2+=moderate, to 3+=intense).

RESULTS

Serial dilutions of rabbit anti-Rs polyclonal antiserum and murine anti-Rs monoclonal antibodies were evaluated for optimal detection of Rs antigen by the avidin-biotin conjugated immunoperoxidase technique. The intra- and extracellular Rs antigen was optimally detected with a dilution as high as 1:1600 for the polyclonal antiserum and with dilutions of up to 1:3200 for RSO3, 1:6400 for RSO1 and RSO2, and 1:25600 for RSO4 for the monoclonal antibodies (Figures 1-3). Higher dilutions of the primary antibodies resulted in a concomitant reduction in staining intensity (Table 1).

Immunohistochemical staining for Rs was specific with no positive results noted with the control measures. Optimal dilutions for the biotinylated secondary antibodies were 1:200 for the goat-anti-rabbit polyclonal antibodies and 1:800 for the horse anti-mouse monoclonal antibodies.

Differences in stain intensity were not apparent with any of the enzyme digestion protocols, and tissues fixed with formalin versus Davidson's or Bouin's fixatives yielded equally satisfactory results. However, Smith's formal dichromate fixed tissues consistently resulted in increased nonspecific background staining.

Comparison of the 3 histochemical and 2 immunohistochemical techniques revealed a greater specificity and lesser nonspecificity for the ABCIP and PAS (+2 - +3) on comparison to the H & E (0 - +1), Gram's (+1 - +3) and DFAT (+1 - +3) (Table 2). Control slides for the ABCIP and DFAT were 0 - +1. Background staining for both the PAS and ABCIP ranged from 0 - +1; H & E, Gram's, and DFAT values ranged from +1 to +3.

Table 1. Relative efficiency of rabbit polyclonal and mouse monoclonal antibodies at detecting R. salmoninarum (=Rs) antigen.

Dilution of Ab	G.I.	RSO1(IgM)	RSO2(IgG)	RSO3(IgM)	RSO4(IgG)
1:200	++	++	++	++	++
1:400	++	++	++	++	++
1:800	++	++	++	++	++
1:1600	+-	++	++	++	++
1:3200	-	++	++	+	++
1:6400	-	+	+	+-	++
1:12800	-	+-	+-	-	+
1:25600	-	-	-	-	+-
1:51200	-	-	-	-	-

G.I. = rabbit anti-Rs polyclonal antibody

RSO1-RSO4= mouse monoclonal antibodies

++ = strongly positive

+ = positive

+- = weakly positive

- = negative

Note. Secondary dilutions used were 1:200 for the goat anti-rabbit polyclonal antibodies and 1:800 for the horse anti-mouse monoclonal antibodies.

Figure 1. Immunohistochemical detection of Rs in the renal tissue of a coho (Oncorhynchus kisutch) salmon using the immunoperoxidase stain (ABC). Note florid Rs infection, marked histiocytic infiltration, and lack of distinct granuloma formation. Counterstained with hematoxylin. x300

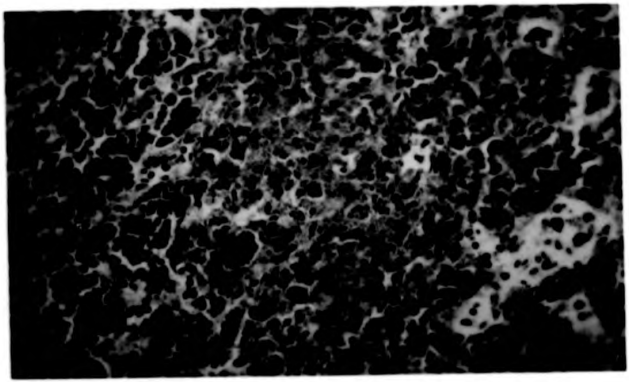

Figure 2. ABC control slide with substituted homologous antibody absorbed against Rs substituted for the primary antiserum. Counterstained with hematoxylin. x300

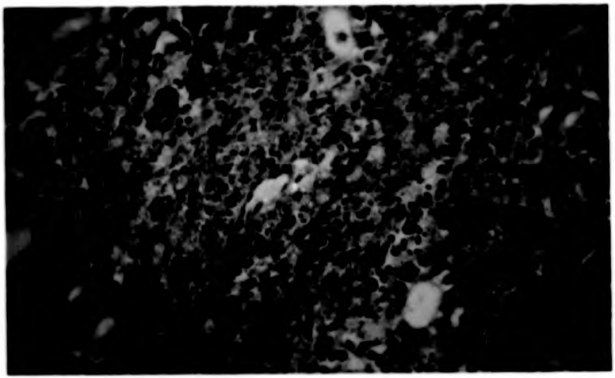

Figure 3. Detection of Rs antigen in a large tuberculoid granulomatous lesions of a naturally infected Atlantic salmon (Salmo salar) using the immunoperoxidase technique. Immunoperoxidase, counterstained with hematoxylin. 75x

Table 2. Comparison of histochemical and immunohistochemical techniques for the demonstration of Rs in histological sections. (a value of 0=negative, 1+=mild, 2+=moderate, 3+=intense). Values represent number of sections with feature out of twenty slides examined.

	H & E				Gram's				PAS			
	0	+1	+2	+3	0	+1	+2	+3	0	+1	+2	+3
detection	12	6	2	0	0	8	7	5	0	0	3	17
nonspecific	0	0	6	14	3	12	4	1	17	3	0	0
background	0	6	9	5	3	8	9	0	17	3	0	0

	DFAT				ABCIP			
	0	+1	+2	+3	0	+1	+2	+3
detection	4	4	10	2	0	0	2	18
nonspecific	4	9	3	4	18	2	0	0
background	3	6	8	2	16	4	0	0

H & E = Hematoxylin and Eosin

PAS = Schiff's Periodic Acid

DFAT = Direct Immunofluorescence (primary 1:60 Rabbit anti-Rs)

ABCIP = Avidin Biotin Conjugated Immunoperoxidase (1:800 primary Rabbit anti-Rs, 1:200 secondary Goat anti-Rabbit)

DISCUSSION

This avidin-biotin conjugated immunoperoxidase (ABCIP) technique extends the diagnostic and research capabilities of conventional BKD histopathology through improved *in situ* detection of Rs antigen.

The use of immunofluorescent techniques for demonstration of Rs infection in salmonids is well established (Bullock et al, 1980; Elliot et al, 1989). Fluorescence, however, is ephemeral and often accompanied by nonspecific autofluorescence, as well as a paucity of tissue (cellular) detail. The ABCIP technique overcomes these factors as permanent, high resolution (even at the subcellular level) preparations result which can be evaluated by conventional light microscopy (Taylor, 1978).

Three immunoperoxidase techniques are presently available for the detection of Rs: an indirect method (IDP)(Hoffman et al, 1989), a peroxidase anti-peroxidase procedure (PAP)(Jansson et al, 1991) and the present avidin biotin conjugated immunoperoxidase technique.

In the IDP method five to seven conjugated secondary antibodies are capable of binding to a primary antibody, increasing the sensitivity of this protocol by a factor of 5-7 fold in comparison to the direct procedures (Haines and Clark, 1991). With the ABC approach the enzyme-conjugated-avidin binds, essentially irreversibly, with the biotinylated secondary antibody. An avidin-biotin lattice configuration results and accounts for up to a 1000 fold increase in sensitivity in comparison to the indirect immunoperoxidase technique (Hsu, Raine and Fanger, 1981).

Comparable magnitudes of sensitivity have been demonstrated with the PAP and ABC techniques (Hsu et al, 1981), however, due

to the versatility of the ABC system for electron microscopy and biotin-lectin conjugation, and because of reagent availability, the ABC technique should prove to be a more valuable procedure for routine diagnostic and research work (Lee and DeLillis, 1987).

Sensitivity refers to the minimal amount of antigen detectable by a detection procedure, whereas antibody efficiency denotes the minimal amount of antibody required for the detection of an antigen (Petrusz, 1983). Several factors influence antigen sensitivity, including, the nature of the antigens (multiple versus single epitopes), the epitope stability in fixatives, the degree of antigen accessibility, detection system employed, and the use of enhancement systems (such as osmium tetroxide or heavy metals with the chromogen) (True, 1990). The antibody efficiency of the ABC technique is three to 25 times as great as that reported for the IDP method. Greater dilutions of primary and secondary antibodies for immunohistochemistry should decrease the amount of antisera required and thereby minimize operational costs. In addition, nonspecific background staining is reduced with the ABC technique (Bains and Miller, 1988).

Immunological nonspecificity may be attributed to endogenous pigments, such as lipofuscin and melanin, and to peroxidase activity, particularly within elements of the myelopoietic and hematopoietic tissue. As the chromogen (DAB) employed in this immunoperoxidase protocol results in a dark brown precipitate, differentiation from melanin granules, particularly in melanomacrophage centers associated with chronic inflammatory processes in fish (Roberts, 1989) may prove difficult.

In our experience, prolonged tissue bleaching, as suggested by Jansson et al (1991), to remove endogenous melanin or

peroxidase activity, resulted in a marked and undesirable depletion of Rs antigen immunoreactivity. Use of alternative substrates, such a 4-chloro-1-naphthol, which yields a blue product, or 3-amino-9-ethylcarbazole (ACE), which results in a red precipitate, and use of appropriated counterstains should resolve the problem of distinguishing between melanin or lipofuscin and Rs antigen and facilitate detection of Rs antigen-antibody complexes.

The recently developed and commercially available immunoperoxidase technique with avidin conjugated to alkaline phosphatase (Vector Laboratories), results in a blue precipitate on addition of the substrate. Nevertheless, with DAB incorporation and comparison of control slides it should facilitate interpretation and localization of antigen-antibody complexes.

As multiple epitopes are recognized with polyclonal antisera, improved sensitivity of antigen detection in comparison to monoclonal antibodies results; however, as monoclonal antibodies are specific for a single epitope, specificity is enhanced with respect to polyclonals. A pool of monoclonals against a number of epitopes may be utilized for improved sensitivity and specificity.

Immunohistochemistry with the monoclonal RS02 revealed predominantly cell associated immunoreactivity, consistent with immunogold electron microscopic localization of the soluble F antigen of Rs in aggregates on and contiguous to bacterial cell walls (Kusser et al, unpublished data).

Antigen immunoreactivity was maintained with all fixatives studied, with increased background staining noted only with Smith's formal dichromate, a fixative employed principally for egg and egg sac fry histology. This phenomenon may be attributed

to solubilization and redistribution of antigen with fixation (Dixon and Eng, 1982). Enhanced detection of Rs in serial sections was consistently noted when compared with Gram's and DFAT and H & E stains (Table 2) and is consistent with observations by Hoffman et al (1989). However, a quantitative estimate of the relative sensitivity of immuno- and histochemical methods would only be possible if stained smears containing increased numbers of Rs cells were examined. This was not performed.

Chapter II

Sequential Histopathology of Bacterial Kidney Disease in Coho (*Oncorhynchus kisutch*) and Atlantic Salmon (*Salmo salar*) Experimentally Challenged with *Renibacterium salmoninarum*

INTRODUCTION

Bacterial kidney disease (BKD) is an insidious, systemic condition afflicting predominantly salmonid species (Fryer and Sanders, 1981). The etiological agent of BKD, Renibacterium salmoninarum (Rs), is a Gram positive, nonencapsulated, obligate intracellular pathogen. Infection with R. salmoninarum results in low grade, persistent mortality or sporadic epizootics, either of which causes significant economic loss to producers. Due to the chronicity of infection fish seldom succumb before 6-18 months of age, often, after considerable financial investment is expended by producers (Evelyn, 1988).

The predilection sites for the bacterium are the anterior and posterior kidney. Due to the inherent functional reserves of renal tissue in fish, coupled with nitrogenous waste and monovalent ion excretion predominantly via the gills, clinical manifestation of BKD occurs primarily in the terminal stages of infection and reflects osmoregulatory, coupled with acid-bases imbalances (Hunn, 1964; Bruno, 1986a). Moribund fish commonly feature clinical signs of lethargy, anaemia, ascites, exophthalmus, swollen vent, inappetance, as well as cutaneous ecchymosis, petechiae, and subdermal cavitations (Fryer and Sanders, 1981; Klontz, 1983).

Necropsy of fish tends to reveal species-specific pathology. In Pacific salmon (Oncorhynchus spp), lesions include diffuse, subacute to chronically active fibrinoperitonitis, nephritis, splenitis, and pericarditis (Wood and Yasutake, 1956); whereas, in the Atlantic salmon (Salmo salar), fish feature multifocal to disseminate, chronic, granulomata to pyogranulomata predominantly in the mononuclear phagocytic system with cicatrization and resolution of lesions reported (Bruno, 1986b).

Preliminary investigations on BKD histopathology characterized renal interstitial lesions in brook trout as tuberculoid, similar to mammalian lesions associated with tuberculosis (Mycobacterium tuberculosis) or mycoses (Snieszko and Griffin, 1955). This work, however, was later discounted by Wood and Yasutake (1956) who adduced a histiocytic infiltration with indiscrete margins, a paucity of fibroplasia, and infrequent giant cells. Histiocytosis was apparent in virtually all tissues examined including the kidney, spleen, liver, gastrointestinal tract, brain, skeletal musculature, and gills (Wood and Yasutake, 1956).

Renal histopathology of BKD may be arbitrarily resolved into excretory and interstitial components.

Recent light and electron microscopy of renal glomeruli in rainbow (Oncorhynchus mykiss) and brook trout (Salvelinus fontinalis) challenged with Rs revealed lesions homologous to mammalian glomerulonephritis (Young and Chapman, 1978; Sami, Fishcer-Scherl, Hoffman and Pfeil-Putzien, 1992). Periglomerular fibrosis (with attendant focal or global synechia), hypercellular glomeruli, as well as peritubular fibrosis and hyaline droplet tubulonephritis were apparent with light microscopy. Thickened glomerular basement membranes with a spike-like appearance was evident ultrastructurally and there was immunofluorescence with rabbit anti-trout IgM detected glomerular immune complex deposition.

Histological changes within the renal interstitium include hemoblast hyperplasia with concomitant lymphopenia within the pronephric interstitium 7 days post Rs challenge (Yasutake, 1955, as cited by Bendele and Klontz, 1975). The inflammatory infiltrate featured a large (20 um) cell with an eccentric vesicular nucleus and prominent eosinophilic cytoplasmic

granules. Cells of this type were localized not only within the kidney, but also within the liver, spleen, and lamina propria of the gastrointestinal tract.

Interstitial macrophages replete with Rs were evident by 35 days post per os challenge with contaminated viscera. Erythrophagocytosis within the renal vascular sinusoids has also been reported (Bendele and Klontz, 1975).

Wolke (1975) has reported coagulation necrosis of renal interstitial and tubular epithelial tissue.

In addition to the cited renal histopathology, there is a plethora of published and anecdotal, tissue or organ-specific, case reports on the histopathological expression of BKD. For example, exophthalmia, initially attributed to extraocular effusion (Bell, 1961; Wolf, 1966; MacLean and Yoder, 1970) has since been ascribed to a retrobulbar granulomatous infiltrate with involvement of, and degenerative changes to, the extrinsic ocular muscles (Hendricks and Leek, 1975). Interestingly, these lesions may occur in the absence of renal interstitial (systemic) involvement (Hoffman, Popp, van de Graaff et al, 1984).

Granulomatous to pyogranulomatous lesions within the central nervous system (CNS) have been noted particularly associated with the tela choroidea (contiguous to the fourth ventricle), saccus dorsalis, and epiphysis of the epithalamus, and optic chiasma (Woods and Yasutake, 1956; Speare, Ostland and Ferguson, 1991).

Within the abdominal cavity, reported lesions have ranged from initial granulocytic to extensive fibrinoperitonitis (pseudodiphtheritic) or granulomatous peritonitis (Smith, 1964). Focal to diffuse subcapsular and parenchymal granulomata have been observed in the liver, spleen, and heart (Ellis et al, 1978). Petechia and ecchymoses throughout the peritoneum

(subserosal haemorrhages) have also been noted.

Manifestation of BKD in skeletal muscle has ranged from systemic involvement with anasarca, to coagulation necrosis, cavitation, and cicatrization (Klontz, 1983).

Of prime importance for vertical transmission of Rs (Evelyn et al, 1984) is infection of the ovaries (oophoritis), and to a lesser extent, infection of the testes (orchitis). Documented ovarian lesions vary from microthrombi and septic emboli within the ovarian (thecal) microvasculature to accumulation of macrophages bearing numerous intracellular Rs within the ovarian stroma (Armstrong, 1989; Bruno and Munro, 1986a). Intra-ovum infection in preovulatory ovaries has been noted and attributed to haematogenous dissemination, ovarian localization, and recruitment from contiguous stroma.

Interpretation of past histopathological data is often confounded due to apparent interspecific variations in response, as well as route and quantity of challenge inoculum. In addition, diet, fish age, and water temperature and hardness appear to influence the clinical expression of BKD (Klontz, 1983). The focus of this study was initially to characterize and then quantify the distribution of Rs-induced lesions in coho (Oncorhynchus kisutch) and Atlantic salmon (Salmo salar) and to ascertain relevant interspecific differences.

MATERIALS AND METHODS

To assess the interspecific variation in the temporal and spatial distribution of Rs lesions in coho (Oncorhynchus kisutch) and Atlantic (Salmo salar) salmon, samples of these species were obtained from Rosewall Creek, Vancouver Island (Big Qualicum Stock) and from United Hatcheries, Vancouver Island,

respectively. The test fish were derived from brood stock that had been screened for Rs by culture and direct immunofluorescence (DFAT) (Bullock et al, 1980). Prior to Rs challenge, the test fish had been maintained on sites supplied by well water and with no previous history of BKD. A subsample of the test fish was evaluated for Rs prevalence.

For logistical reasons coho salmon were retained at Rosewall Creek and Atlantic salmon were maintained at the Pacific Biological Station (PBS), Nanaimo, BC.

Isolate 384 of Rs (Bell, Higgs and Traxler, 1984) was cultured on KDM-2 medium (Evelyn, 1977), harvested at day 18 of growth (in the log phase), and suspended in sterile peptone-saline (0.1% peptone-0.9%NaCl). The optical density was adjusted to 1.25 OD at 540 *nm* and serially diluted to yield doses of 10^7, 10^5, 10^3 Rs cells/fish. The growth curve for Rs is presented in Appendix I. Bacteria were enumerated by the drop plate technique.

Coho salmon between 4-6 gm in size were anaesthetized with 100 mg/l tricaine methane sulphonate (MS222)(Sigma), divided into 3 cohorts of 200 each, and challenged by intra peritoneal(ip) injection with 0.1 ml of peptone-saline containing the appropriate dose of Rs cells. Control fish received peptone saline, ip. Individual groups were maintained in 250 L flow-through tanks supplied with well water between 8-9 C.

After a week of acclimation at the PBS, Atlantic salmon 4-6 gm in size were similarly treated. Fish were randomly divided into lots of 50, and challenged by ip inoculation with 0.1 ml of of peptone-saline containing 10^7, 10^5, and 10^3 Rs cells/ml and maintained in 50 L pot tanks supplied with dechlorinated municipal water between 5-7 C. Control fish received peptone-saline.

All fish were fed a commercially available diet at approximately 2.0% body weight. Mortalities were removed and recorded on a daily basis and frozen for subsequent microbiological evaluation.

Ten coho salmon from each challenge group were sampled on days 1, 3, 7, 14, 28, and every 28 days thereafter (until all of the fish were used up or had succumbed to BKD). Similarly, 3-5 Atlantic salmon were sampled on days 1, 7, 14, 28, 42, 56, and 84. Sampled fish were killed by immersion in a concentrated solution of MS222, their abdomens incised, and then fixed intact in Davidson's solution.

To maintain topographic orientation of the viscera, individual blocks were prepared for histopathology by initial transection of the torso 1-2 cm caudal to the posterior limit of the cranium, then by sagittal sectioning of the cranium and then by parasagittal sectioning of the abdomen. This protocol facilitated histopathological evaluation of virtually all organs.

Tissues were processed through a graded series of alcohols and xylene, embedded in paraffin, sectioned to a thickness of 5 um, and stained histochemically with hematoxylin and eosin. Representative slides were also stained with Schiff's Periodic Acid, Brown and Hopps' Gram stains, Masson's Trichrome Stain, Martius's Scarlet Blue, and immunohistochemically with the avidin biotin conjugated immunoperoxidase for Rs (polyclonal)(chapter 1) and cytokeratins (AE1 and AE3)(Noga, Dykstra and Wright, 1989). The monoclonals against cytokeratins were used at a working dilution of 1:100 in PBS to detect epithelioid cell formation.

Each section was examined, the lesions identified and tabulated according to tissue distribution and location.

RESULTS

Cumulative mortalities

Cumulative mortalities of coho and Atlantic salmon are depicted in figures 4 and 5. There were calculated as total mortality in 7 day intervals (excluding sampled fish) relative to surviving numbers. With the coho salmon, 100% mortality was attained by day 42 with 10^7, day 75 with 10^5, and day 93 for 10^3 Rs; whereas, with the Atlantic salmon 100% mortality occurred by day 30 with 10^7, day 57 with 10^5, and day 79 for 10^3 Rs.

Histopathology of Rs-challenged coho and Atlantic salmon revealed multisystemic involvement with lesion severity and distribution contingent on time post infection, dose administered, and species evaluated (Tables 3 and 4).

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Histopathology of Rs-challenged coho and Atlantic salmon revealed multisystemic involvement with lesion severity and distribution contingent on time post infection, dose administered, and species evaluated (Tables 3 and 4).

Figure 4. Cumulative mortalities of coho salmon held in fresh water at 8.0 C and challenged by intraperitoneal injection of 10^7, 10^5, and 10^3 Rs.

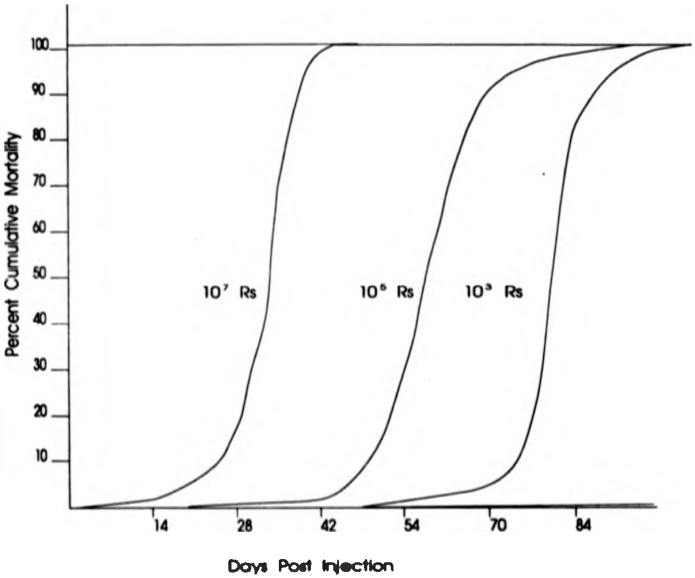


Figure 5. Cumulative mortalities of Atlantic salmon held in fresh water between 5-7 C and challenged by intraperitoneal injection of 10^7, 10^5, and 10^3 Rs.

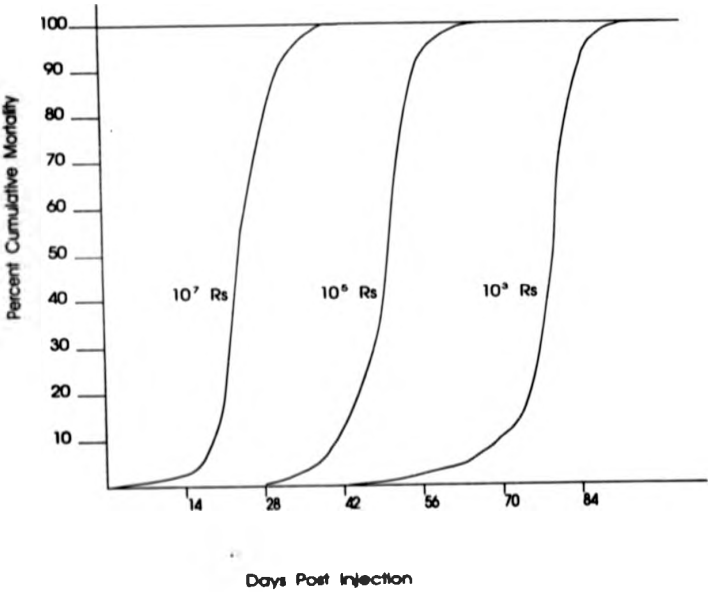


Table 3. Multisystemic lesions in coho salmon challenged with 10^7, 10^5, 10^3 Rs. Fractional numbers= number of fish with feature/number examined. NA= not available

	1	3	7	14	28	56	84
	days post infection						
1) Renal							
perivascular							
neutrophils							
10^7	0/10	5/10	3/15	9/13	0/10	NA	NA
10^5	0/10	10/18	8/17	0/12	0/12	0/16	NA
10^3	0/10	5/10	6/10	6/10	1/10	0/11	2/10
lymphocytes							
10^7	0/10	0/10	12/15	8/10	0/10	NA	NA
10^5	0/10	0/18	5/9	3/12	0/12	2/16	NA
10^3	0/10	0/10	0/10	0/10	6/9	0/10	0/9
interstitium							
granuloma							
focal							
10^7	0/10	0/10	2/15	4/20	1/20	NA	NA
10^5	0/10	0/10	7/12	7/18	0/16	0/15	NA
10^3	0/10	0/10	0/10	3/10	2/9	0/10	0/10
multifocal							
10^7	0/10	0/10	12/15	12/20	15/20	NA	NA
10^5	0/10	0/10	0/12	0/18	4/16	15/15	NA
10^3	0/10	0/10	3/12	10/18	8/16	0/15	NA
free Rs							
10^7	0/10	0/10	0/15	0/20	18/20	NA	NA
10^5	0/10	0/10	0/12	0/18	6/16	14/15	NA
10^3	0/10	0/10	0/10	0/10	0/9	0/10	0/10
glomerular							
10^7	0/10	0/10	5/7	5/9	6/10	NA	NA
10^5	0/10	0/10	0/9	3/9	2/10	5/9	NA
10^3	0/10	0/10	0/10	3/10	2/10	0/10	1/10
tubular							
10^7	0/10	0/10	0/8	6/9	7/10	NA	NA
10^5	0/10	0/10	0/9	3/9	7/8	9/9	NA
10^3	0/10	0/10	0/10	0/10	0/10	0/10	4/10
intravascular							
thrombosis							
10^7	0/10	0/10	0/10	0/10	7/10	NA	NA
10^5	0/10	0/10	0/9	0/18	0/16	5/15	NA
10^3	0/10	0/10	0/10	0/10	0/10	0/10	8/10

	1	3	7	14	28	56	84
			days post infection				
2) Splenic							
ellipsoids							
10^7	0/4	2/4	4/6	2/5	3/6	NA	NA
10^5	0/6	1/5	3/6	4/7	3/7	6/6	NA
10^3	0/4	0/4	2/6	3/6	4/5	3/3	5/5
capsule							
10^7	0/4	0/4	3/6	3/5	3/6	NA	NA
10^5	0/6	0/5	0/6	4/7	6/7	6/6	NA
10^3	0/4	0/4	0/6	4/6	3/5	3/3	5/5
parenchyma							
10^7	0/4	0/4	0/6	4/5	4/6	NA	NA
10^5	0/6	0/5	0/6	1/7	1/7	4/6	NA
10^3	0/4	0/4	0/6	0/6	0/5	2/3	5/5
3) Hepatic							
capsule fibrin							
10^7	0/10	0/9	7/10	8/10	6/10	NA	NA
10^5	0/10	0/10	0/10	1/10	7/10	10/10	NA
10^3	0/10	0/10	0/10	0/10	6/10	8/8	10/10
granulomatous							
10^7	0/10	0/9	0/10	2/10	9/10	NA	NA
10^5	0/10	0/10	0/10	6/10	8/10	10/10	NA
10^3	0/10	0/10	0/10	0/10	0/10	6/8	10/10
parenchyma							
10^7	0/10	0/9	0/10	0/10	4/10	NA	NA
10^5	0/10	0/10	0/10	0/10	1/10	0/10	NA
10^3	0/10	0/10	0/10	0/10	0/10	2/8	2/10

	1	3	7	14	28	56	84
			days post infection				
4) Gastroenteric							
peritonitis							
focal							
10^7	0/10	6/9	5/14	3/17	4/19	NA	NA
10^5	0/10	7/10	2/18	0/10	1/10	0/10	NA
10^3	0/10	0/10	0/10	5/9	0/10	1/10	0/10
multifocal							
10^7	0/10	0/9	7/14	12/17	13/19	NA	NA
10^5	0/10	0/10	13/18	7/10	8/10	9/10	NA
10^3	0/10	0/10	0/10	0/9	9/10	10/10	8/9
free Rs							
10^7	0/10	0/9	0/14	0/17	19/19	NA	NA
10^5	0/10	0/10	0/18	0/10	0/10	0/10	NA
10^3	0/10	0/10	0/10	0/9	7/10	10/10	9/9
esophagitis							
10^7	0/10	0/9	0/10	6/10	9/9	NA	NA
10^5	0/10	0/10	2/10	5/10	9/10	10/10	NA
10^3	0/10	0/10	4/10	9/9	9/9	9/9	8/8
free Rs							
10^7	0/10	0/9	0/10	0/10	9/9	NA	NA
10^5	0/10	0/10	0/10	0/10	0/10	0/10	NA
10^3	0/10	0/10	0/10	0/9	7/9	9/9	8/8
swim bladder							
10^7	0/8	0/9	0/10	0/9	3/9	NA	NA
10^5	0/8	0/10	0/9	0/9	0/7	2/9	NA
10^3	0/10	0/10	0/10	0/10	0/9	4/10	6/8

	1	3	7	14	28	56	84
			days post infection				
5) Cardiovascular							
endocarditis							
10^7	0/7	0/9	3/9	1/10	2/10	NA	NA
10^5	0/10	1/8	1/10	0/10	6/10	4/9	NA
10^3	0/10	2/10	0/10	0/10	6/10	8/8	5/10
pericarditis							
10^7	0/7	0/9	0/9	0/10	3/10	NA	NA
10^5	0/10	0/8	0/10	0/10	0/10	0/9	NA
10^3	0/10	0/10	0/10	0/10	0/10	3/8	0/10
peripheral vasculature							
10^7	2/15	9/16	7/18	0/10	0/10	NA	NA
10^5	6/8	4/11	4/9	4/10	3/9	2/8	NA
10^3	0/10	3/10	5/10	1/10	5/11	0/10	0/10
6) Reproductive							
10^7	0/9	0/9	5/8	7/10	9/10	NA	NA
10^5	0/10	0/10	0/10	7/9	9/10	10/10	NA
10^3	0/10	0/10	0/10	0/10	9/10	8/8	9/9
7) Neural							
10^7	0/10	0/10	0/10	0/10	3/10	NA	NA
10^5	0/10	0/10	0/10	0/10	0/10	2/10	NA
10^3	0/10	0/10	0/10	0/10	0/10	0/8	6/8
8) Ocular							
10^7	0/4	0/4	0/6	0/6	0/4	NA	NA
10^5	0/4	0/5	0/4	0/4	3/3	2/2	NA
10^3	0/4	0/5	0/5	0/5	0/3	0/3	3/3
9) Branchial							
10^7	0/10	0/9	0/10	0/9	3/10	NA	NA
10^5	0/10	0/10	0/10	0/10	2/10	4/10	NA
10^3	0/10	0/10	0/10	0/10	0/10	0/10	2/10
10) Skeletal Musculature							
10^7	0/10	0/10	0/10	0/10	0/10	NA	NA
10^5	0/10	0/10	0/10	0/10	0/10	0/10	NA
10^3	0/10	0/10	0/10	0/10	0/10	0/10	0/10

Table 4. Polysystemic lesions in Atlantic salmon challenged with 10^7, 10^5, 10^3 Rs Fractional numbers= number of fish with feature/number examined. NA= not available

	7	14	28	42	56	84
	days post infection					
1) Renal						
perivascular neutrophils						
10^7	2/6	0/10	0/7	NA	NA	NA
10^5	2/7	0/9	0/7	0/8	0/7	NA
10^3	0/5	0/5	3/9	0/9	0/6	0/5
lymphocytes						
10^7	0/6	0/10	0/7	NA	NA	NA
10^5	NA	NA	NA	NA	NA	NA
10^3	0/5	0/5	4/9	0/9	2/6	0/5
interstitium granuloma focal						
10^7	0/6	0/10	0/7	NA	NA	NA
10^5	0/7	3/9	0/7	0/8	0/8	NA
10^3	0/5	2/8	2/9	0/9	0/8	0/9
multifocal						
10^7	0/6	6/10	7/7	NA	NA	NA
10^5	0/7	5/9	7/7	8/8	8/8	NA
10^3	0/5	0/8	3/9	9/9	8/8	9/9
free Rs						
10^7	0/6	6/10	7/7	NA	NA	NA
10^5	0/7	0/9	0/7	0/8	2/8	NA
10^3	0/5	0/8	0/9	0/9	0/8	2/9
glomerular						
10^7	0/6	0/10	7/7	NA	NA	NA
10^5	0/6	0/7	0/7	4/7	5/7	NA
10^3	0/5	0/6	1/5	4/6	5/7	3/6
tubular						
10^7	0/6	0/10	7/7	NA	NA	NA
10^5	0/6	0/7	0/7	6/6	6/6	NA
10^3	0/5	0/6	0/5	3/6	4/7	6/6
intravascular thrombosis						
10^7	0/6	0/10	0/7	NA	NA	NA
10^5	0/7	0/9	0/7	0/6	0/6	NA
10^3	0/5	0/6	0/5	0/6	0/7	0/6

	7	14	28	42	56	84
		days post infection				
2) Splenic						
ellipsoids						
10^7	2/5	3/4	3/3	NA	NA	NA
10^5	3/3	4/5	1/3	3/3	2/4	NA
10^3	0/4	2/4	3/3	2/3	3/3	4/4
capsule						
10^7	0/5	3/4	3/3	NA	NA	NA
10^5	0/3	0/5	1/3	3/3	3/4	NA
10^3	0/4	0/4	0/3	1/3	2/3	4/4
parenchyma						
10^7	0/5	1/4	3/3	NA	NA	NA
10^5	0/3	0/5	2/3	3/3	0/4	NA
10^3	0/4	0/4	0/3	0/3	0/3	3/4
3) Hepatic						
capsule						
fibrin						
10^7	0/5	3/8	5/8	NA	NA	NA
10^5	0/7	0/6	3/4	5/5	3/6	NA
10^3	0/5	0/5	1/6	5/6	2/8	3/6
granulomatous						
10^7	0/5	2/8	5/8	NA	NA	NA
10^5	0/7	0/6	2/4	5/5	6/6	NA
10^3	0/5	0/5	1/6	5/6	4/8	5/6
parenchyma						
10^7	0/5	0/8	1/8	NA	NA	NA
10^5	0/7	0/6	1/4	0/5	2/6	NA
10^3	0/5	0/5	1/6	0/6	2/8	4/6

	7	14	28	42	56	84
	days post infection					
4) Gastroenteric						
peritonitis						
focal						
10^7	2/7	0/10	0/10	NA	NA	NA
10^5	4/7	7/9	0/8	0/8	0/6	NA
10^3	3/5	0/8	0/9	0/5	0/6	0/5
multifocal						
10^7	2/7	7/10	9/10	NA	NA	NA
10^5	3/7	2/9	8/8	8/8	3/6	NA
10^3	1/5	8/8	8/9	5/5	4/6	5/5
free Rs						
10^7	0/7	0/10	8/10	NA	NA	NA
10^5	0/7	0/9	0/8	6/8	5/6	NA
10^3	0/5	0/8	0/9	2/5	3/6	2/5
esophagitis						
10^7	0/7	4/6	7/7	NA	NA	NA
10^5	2/7	7/9	8/8	5/5	4/6	NA
10^3	2/5	0/5	5/5	5/5	5/6	5/5
free Rs						
10^7	0/7	5/6	7/7	NA	NA	NA
10^5	0/7	0/9	6/8	5/5	4/6	NA
10^3	0/5	0/5	0/5	5/5	5/6	2/5
swim bladder						
10^7	0/7	2/8	3/7	NA	NA	NA
10^5	0/7	0/7	0/6	3/6	4/7	NA
10^3	2/5	2/4	3/4	3/6	3/4	4/5
5) Cardiovascular						
endocarditis						
10^7	0/7	0/8	2/7	NA	NA	NA
10^5	0/5	0/4	2/5	1/4	3/4	NA
10^3	0/5	0/4	1/5	5/5	4/4	5/5
pericarditis						
10^7	0/7	0/8	4/7	NA	NA	NA
10^5	0/5	0/4	3/5	2/4	3/4	NA
10^3	0/5	0/4	0/5	0/5	2/4	1/5
peripheral vasculature						
10^7	0/5	0/7	0/7	NA	NA	NA
10^5	0/7	0/9	0/7	0/6	0/6	NA
10^3	0/5	0/4	0/5	0/7	0/7	0/9

	7	14	28	42	56	84
	days post infection					
6) Reproductive						
10^7	0/7	3/8	3/6	NA	NA	NA
10^5	0/7	0/7	0/6	3/6	3/4	NA
10^3	0/5	0/4	0/5	2/3	3/6	2/3
7) Neural						
10^7	0/7	0/6	6/6	NA	NA	NA
10^5	0/4	0/5	4/6	6/6	3/4	NA
10^3	0/5	0/5	0/5	3/4	1/4	2/5
8) Ocular						
10^7	0/3	0/4	3/3	NA	NA	NA
10^5	0/2	0/3	3/3	2/2	0/3	NA
10^3	0/2	0/3	0/3	1/2	0/3	2/2
9) Branchial						
10^7	0/5	0/7	0/8	NA	NA	NA
10^5	0/3	0/5	0/6	0/5	0/4	NA
10^3	0/5	0/4	0/5	0/5	0/6	0/5
10) Skeletal Musculature						
10^7	0/7	0/8	0/8	NA	NA	NA
10^5	0/7	0/8	0/7	0/8	0/6	NA
10^3	0/6	0/8	0/6	0/6	0/7	0/8

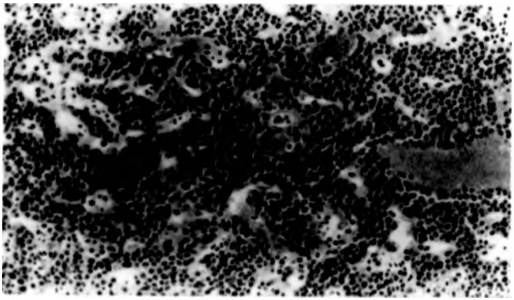
Renal Histopathology in Rs-infected coho salmon

Sequential histopathology of coho salmon challenged with Rs by intra-peritoneal injection revealed morphometric alterations initially involving the perivascular, then the interstitial and excretory, and terminally, the intravascular, compartments of the kidney (Table 3, section 1).

The anterior kidney (pronephros) consists of anastomosing vascular channels lined by attenuated endothelia. Islands or cords of putative stem, progenitor, and mature cells of the lymphomyeloid and erythropoietic lineages are evident.

In Rs infected coho 3-7 days post challenge, focal areas of granulopoiesis were evident with marked expansion of the granulocytic pool in relation to other cell lineages (Figure 6 and 7). This phenomenon was most apparent in fish challenged with 10^5 and 10^7 Rs, particularly in the perivascular areas of the caudal vena cava and pronephric sinusoids; this phenomenon appeared to subside by day 14. Cells extending through vascular adventitia and endothelium were occasionally observed (Figure 8). The endothelium of the caudal vena cava was punctuated and continuous with the vascular sinusoids (Figure 9). In addition, cells with bizarre nuclei and homogeneous finely granular cytoplasm,

Figures 6a and 6b. Histological section of pronephros from a coho salmon on day 7 after challenged with 10^3 Rs cells. Note the homogeneous cell composition of the renal interstitium (a). (H & E, 75x and 300x)



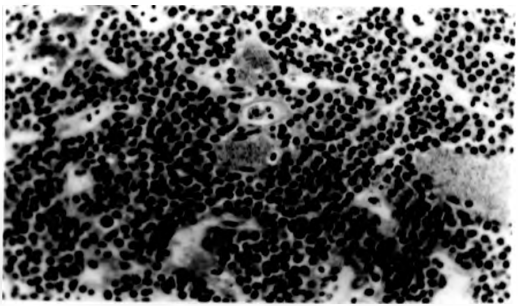


Figure 7. Higher magnification of figure 6. Note the prominent immature and mature neutrophils within the interstitial cords of the pronephros (A). (H & E, 750x)

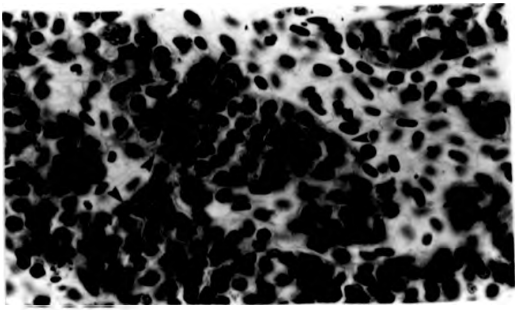


Figure 8. Perivascular area of the caudal vena cava of a coho salmon 14 days post-challenge with 10^4 Rs. The interstitium is more activated than in Figure 1 and is composed of predominantly small lymphocytes and neutrophils at different stages of development. Note the individual cell extending across the vascular adventitia (a). (H & E, 750x)

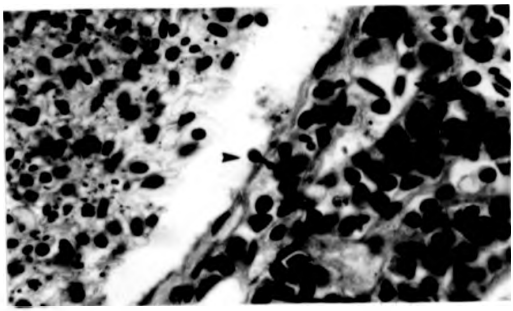
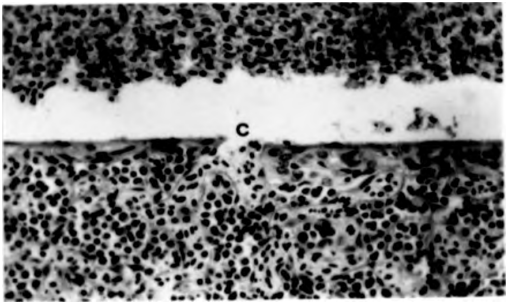


Figure 9. Longitudinal section of the caudal vena cava demonstrating the punctated microvasculature (c) which facilitates communication of the systemic circulatory system with the renal interstitium. (H & E, x300)



possibly effete neutrophils, were noted interspersed within the granulocytic pool of fish challenged with 10^1 Rs on day 14.

An accumulation of histiocytes was observed in the interstitium between days 14-28 and appeared more marked in those fish challenged with 10^1 and 10^7 Rs. Eventually, by day 28 and day 56 for the 10^7 and 10^5 Rs-challenged cohorts, florid extracellular Rs within the interstitium extended to and involved the perivascular compartment. In fish challenged with 10^3 the perivascular compartment by day 56 was composed predominantly of a homogeneous population of monocyte-like cells 15-25 um in size, each with a prominent vesicular circular to ovoid nucleus. Small infiltrating lymphocytes were also noted.

Interstitium

By day 7, the neutrophils which were initially apparent in the perivascular compartment appeared to either extend and diffusely involve the anterior and posterior renal interstitium, or formed microabscesses frequently adjacent to vascular sinuses. In addition to granulocytes, small and large monocyte-like cells were interspersed within the renal interstitium. The small cells were approximately 5-10 um in size and featured a prominent, intensely and homogeneously basophilic circular nucleus and scant amounts of cytoplasm; these cells were histologically consistent with lymphocytes. The larger cells were between 10-15 um in size, featured a conspicuous eccentric to centrally situated ovoid to reniform nucleus with 1-2 prominent nucleoli, and contained abundant peripheral heterochromatin. A moderate amount of cytoplasm was present, which on hematoxylin and eosin staining appeared eosinophilic with a basophilic hue. The cells appeared to belong to either the myeloid or the lymphocytic lineage

(Figure 10).

A gradual increase in the large monocyte-like cells and lymphocytes relative to erythro- and granulocytes was apparent by day 14, and by day 28 the interstitium was mildly hyperplastic and composed predominantly of these 2 cell types in approximately equal numbers. At this stage, on low magnification the interstitium appeared to consist of a relatively homogeneous basophilic cell population. Microabscess formation was recognizable as faintly eosinophilic ovoid to spherical nidi with a slight increase in extracellular ground substance. Frequent mitotic figures, granulopoiesis, and lymphocytic infiltration contiguous to the microabscess was also evident.

At this stage of infection, early granulomatous lesions were composed primarily of neutrophils with occasional histiocytes and lymphocytes (Figure 11). Neutrophils at various stages of development were detected adjacent to the nidus (the cells resemble immature mammalian band cells with an unsegmented, elongated, coiled nucleus with faintly homogeneous basophilic cytoplasm).

Fish challenged with 10^7 and 10^5 Rs featured multifocal to disseminate pyogranulomata; whereas fish challenged with 10^3 featured a more focal distribution of lesions. Lymphocytes aggregated and either formed a mantle around (Figure 11) or infiltrated into the lesion. The hypercellular interstitium eventually compressed and compromised the lumen of the vascular channels. Intravascular accumulation and margination of leucocytes contiguous to the nidi was noted. Fish challenged with 10^1 Rs featured a marked lack of abscess formation.

Moderate interstitial hyperplasia with an increase of large monocyte-like cells relative to lymphocytes was typical in the interstitium by day 28 (Figure 12). Mitotic figures within the

large monocyte-like cells were evident throughout renal sections.

A transition from a predominantly mixed granulocytic-lymphohistiocytic to pyogranuloma and eventually, a histiocytic granuloma was apparent in the interstitial lesions. Large cells, 15-20 um in size with a prominent eccentric reniform nucleus infiltrated the lesion. Frequently, in fish challenged with 10^4 Rs the nuclei appeared displaced to the margin of the plasmalemma and was somewhat attenuated due to cytoplasmic accumulation of Rs (Figure 13).

In fish challenged with 10^7 by day 28, however, massive accumulations of intracellular and extracellular Rs were apparent (Figure 14). In 5 of 10 renal sections examined only scant cellular remnants persisted; karyorrhectic debris was evident throughout the pronephros and intertubular spaces. Remaining fish in this treatment group succumbed to BKD within 4 days of this stage.

By day 28, in fish challenged with 10^3, there was a marked absence of microabscess formation. The interstitium consisted of an approximately equal ratio of lymphocytes to the large monocyte-like cells. From day 28 to 56 there was a marked increase in the number of histiocytes, with granulomata occasionally expanding and becoming confluent. Focal areas of necrosis, phagocytosis of nuclear debris, and numerous mitotic figures were evident within the interstitium of fish challenged with 10^5 Rs. Rs were primarily intracellular (within macrophages); however, focal areas of necrosis and extracellular release and dispersal of bacteria was noted in 6 of 10 fish.

In fish inoculated with 10^3 Rs, there was by day 56 a moderate hyperplasia with a preponderance of the large monocyte-like cells with focal to multifocal accumulations of lymphocytes. At this stage of renal hyperplasia the granulomata were composed

almost exclusively of histiocytes which were surrounded by cells of myeloid lineage. An increased amount of eosinophilic extracellular ground substance was also apparent within pyogranulomata. In 3 of 10 fish, a hyperplastic interstitium was noted with no evidence of pyogranuloma, histiocytosis, or necrosis.

Between days 56 and 84 there was a marked increase in histiocytosis with extension along the lymphomyelopoietic cords. Marked compression of the portal vascular sinuses and displacement of renal tubules was apparent as the granuloma increased in size.

With the exception of mild nephrocalcinosis, no renal lesions were apparent in control fish.

Figure 10. Photomicrograph of renal interstitium of coho 28 days post-challenge with 10^3 Rs. Note the accumulation of the large monocyte-like cells (B) within the interstitial cords. Numerous mitotic figures are evident. H & E, 750x

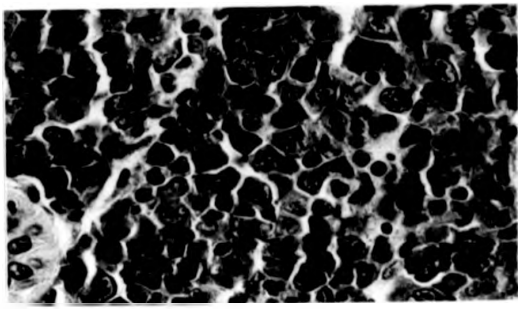


Figure 11. Early granuloma within the renal interstitium of a coho salmon 28 days post-infection with 10^3 Rs. The nidus is composed of a central area of histiocytes and neutrophils with a perilesional infiltration of lymphocytes (a). Note the infiltration of cells within the interstitial cords, as well as in the lumen of the sinusoids (b). (H & E, 300x)

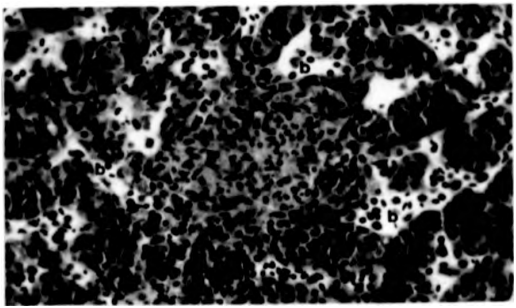


Figure 12. Interstitial granuloma in the mid-kidney of a coho salmon challenged with 10^3 Rs at day 28. The granuloma features a marked lymphohistiocytic infiltrate (a). The interstitium is mildly hypercellular and is composed primarily of large mononuclear-like cells and lymphocytes (b). H & E, 75x.

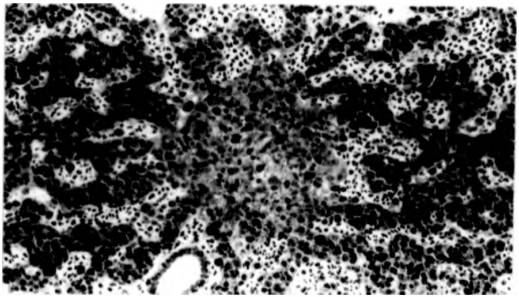
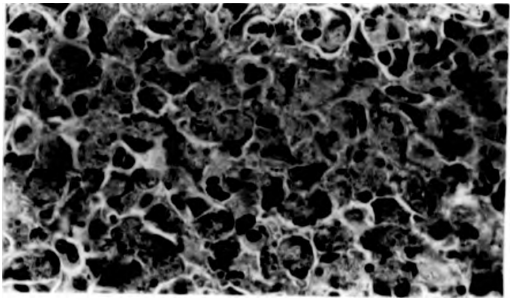
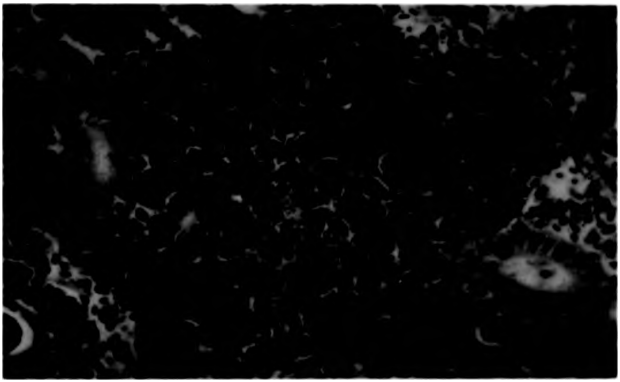


Figure 13. High magnification of the central region of the nidus. Note the marked accumulation of histiocytes ( ). The cytoplasm appears granular due primarily to the phagocytosis and accumulation of Rs cells, as well as cell debris. At this stage of infection (day 28, 10^5 Rs) there is limited necrosis. H & E, 750x.



NUMEROUS ORIGINALS IN COLOUR

Figure 14. Posterior kidney section of coho salmon at 28 days post-challenge with 10^7 Rs. In addition to exuberant extracellular accumulation of Rs (B), there was widespread necrosis of the renal interstitium. Immunoperoxidase, counterstained with Hematoxylin, 75x



Renal Histopathology in Atlantic Salmon

Serial histopathology in Atlantic salmon experimentally infected with Rs revealed disparate perivascular, interstitial, glomerular, and vascular manifestations (Table 4, section 2) of BKD with respect to coho salmon.

Perivascular granulocytosis was infrequent and transient in fish challenged with 10^7 (2 of 6 fish) and 10^6 (2 of 7 fish) Rs at day 7. Moreover, there was a marked absence of granulocytic infiltration throughout the interstitium of Atlantic salmon. Rather, accumulation of large monocyte-like cells and foci of lymphocytes occured within 7 days of challenge.

Circular to ovoid granulomata appear by day 14 within the interstitium of the anterior and posterior kidneys. The granulomata were initially composed of histiocytes, lymphocytes, and transient granulocytes. Macrophages and occasional granulocytes were interspersed within a central area of necrosis, and in approximately 7 days lymphocytes accumulated around the margin of the lesion. Melanomacrophages, initially detected throughout the interstitium, infiltrated and dispersed melanin throughout the matrix of the granuloma by day 14.

By day 28 numerous spindle-shaped fibroblasts were evident along the external margin of the lymphocytes, as well as infiltrating into the centre of the granuloma. A collagenous matrix was deposited around the periphery of the nidus between days 28 and 42 (Figure 15).

By day 28, in fish challenged with 10^7 Rs, the granulomata are multifocal in distribution and markedly enlarged with massive accumulations of extracellular Rs interspersed within a caseonecrotic centre. The cellular component of the granulomata appeared disrupted (thinner), and pyknotic nuclei and karyorrhectic material were dispersed throughout the lesions.

By this stage, fish succumbed within 1 to 2 days.

In fish challenged with 10^4 and 10^3 Rs, there was between days 28 and 42 a marked proliferation of fibroblasts which encircled the lesion and formed a fibrocaseous nodule. Whorls of collagen were deposited within and around the granuloma so that the lesions were subdivided into smaller cellular clusters. Gram and PAS staining revealed a variable number of Rs. In fish with solid granulomata there was usually only scant to no Rs detectable histochemically or immunohistochemically; whereas, in fish with lesions containing a caseonecrotic core, Rs were more numerous.

Direct extension of the renal granuloma ventrally to the swim bladder (Figures 16 and 17) and dorsally to the spinal cord was apparent in many fish. Esophageal involvement was observed, but rarely.

Epithelioid cells appeared between days 28 and 42 and multinucleate (Langhans type) giant cells were evident in 4 of 8 fish within the periphery of the granulomata by day 56 and in increased numbers by day 84 (7 of 9 fish challenged with 10^3 Rs) (Figure 18).

Figure 15. Fibrous granuloma in the posterior kidney of an Atlantic salmon 56 days post challenge with 10^3 Rs. Note the displacement and compression of the excretory components and the relative sparring of the large vascular channels (b). H & E, 75x.

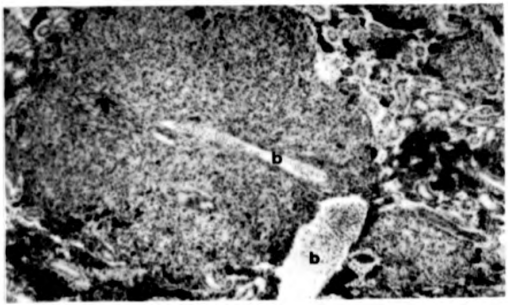


Figure 16. Tuberculoid granuloma in the posterior kidney of an Atlantic salmon challenged with 10^4 Rs. The lesion features a degree of fibrous encapsulation ( ) response with melanomacrophage accumulation. Immunoperoxidase, counterstained with hematoxylin. x75

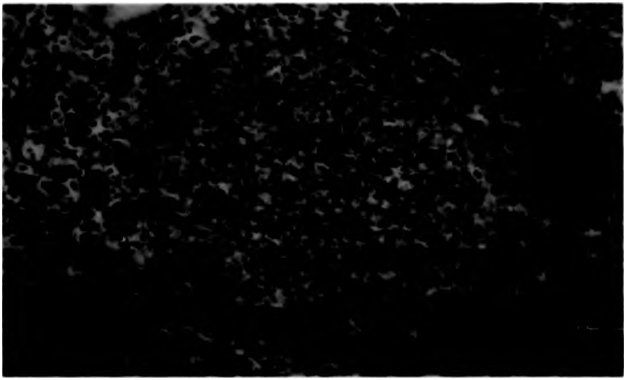


Figure 17. Over the course of infection in Atlantic salmon multifocal to locally extensive areas of inflammation extend from the ventral aspect of the kidney to the swim bladder (E). H & E, 300x

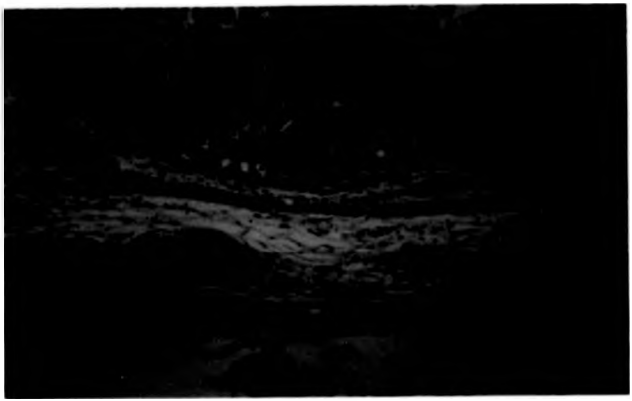
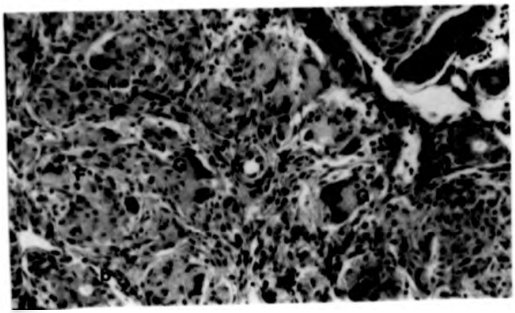


Figure 18. Accumulation of Langerhans type giant cells (G) at the margin of a granuloma in the posterior kidney of an Atlantic salmon 56 days post-challenge with 10^3 Rs. Note the extensive fibrohistiocytic (F) response and neovascularization (b). H & E, 300x



Glomeruli

Histopathology of the excretory component of the posterior kidney revealed pleomorphic glomerular alteration. Lesions varied not only between individuals within a particular challenge cohort, but also within a given histological section. Initial changes to the glomeruli were apparent by day 14 and included prominent nuclei around the margin of the glomerular tuft. Hypercellularity was marked in many glomeruli (Figure 19) and featured cells with segmental nuclei and inapparent nuclear membranes. The basement membrane of the glomeruli occasionally appeared prominent and intensely eosinophilic and occasional, focal, refractile globular deposits occured on the luminal aspect of glomerular capillaries. Frequently, the vasculature was distended and contained a mixed cell population of erythrocytes, granulocytes, and monocytes, and terminally, (day 28 with 10^7 and day 56 with 10^5) it contained septic emboli of Rs (Figure 20) as well as thrombi (Figure 21) in up to 20% of glomeruli in affected kidneys.

Enlargement of Bowman's space occured with hypertrophy of the parietal epithelium. Neither glomerular crescents nor periglomerular fibrosis was detectable in any of the sections. In the absence of ancillary diagnostic tests, such as immunofluorescence (with anti-trout IgM) and electron microscopy, histopathological interpretation of glomerular lesions remains speculative.

Figure 19. Photomicrograph of the posterior kidney of a coho salmon 14 days post-challenge with 10^3 Rs. The glomeruli appear uniformly hypercellular with a mild to moderate expansion of Bowman's space (g). Inflammatory cells are evident in the lumen of the afferent arteriole (i). H & E, 300x

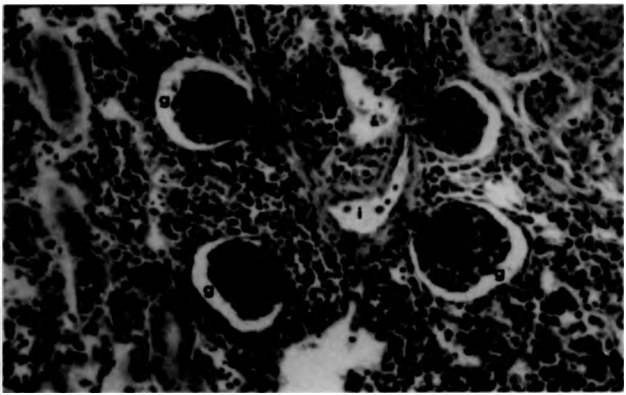


Figure 20. Septic emboli in the microvasculature of the renal glomerulus (s) of a coho salmon challenged with 10^7 Rs, 28 days post-infection. PAS, 750x

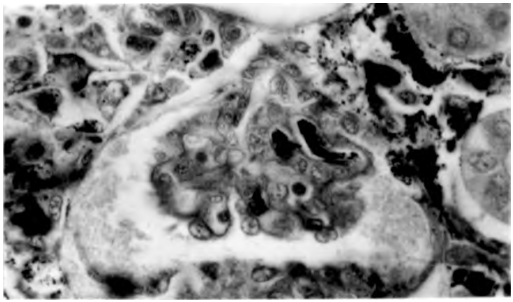
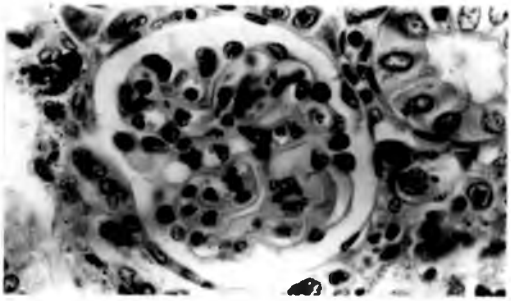
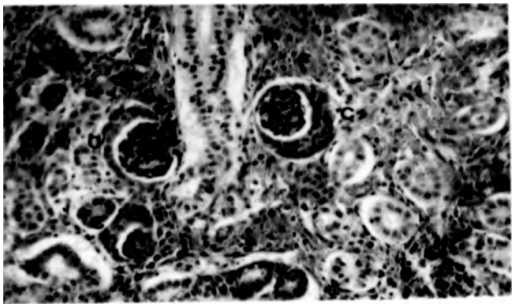


Figure 21. High magnification of a renal glomerulus with intravascular thrombi (t). Coho salmon at day 84 post-infection. H & E, 750x



A profound difference between Atlantic and coho salmon was the frequent appearance (between 7 of 7 and 4-6 of 6 fish) of crescentic glomerulonephritis. Between 60-80% of glomeruli (within a given renal section) featured florid parietal epithelial cell proliferation resulting in the appearance of crescents within Bowman's space (Figure 22). Lymphocytes and histiocytes were occasionally interspersed within the crescent which sometimes attained such large sizes as to compress or obliterate the glomerular tuft. Capillary loops frequently appeared intensely eosinophilic, and hypercellularity of the glomerulus was noted. The periglomerular interstitium sometimes featured a marked histiolymphocytic infiltration, and on other occasions showed marked edema, with scant accumulations of inflammatory cells, or exuberant extracellular Rs. The integrity of the proximal convoluted tubules appeared intact. Histochemical staining for fibrin and collagen revealed only scant staining within glomeruli.

Figure 22. Histological section of an Atlantic salmon challenged with 10^3 Rs at day 28 post-infection. Note the prominent parietal epithelial crescents (c) within Bowman's space. The glomeruli appear hypercellular with mild compression of the tuft. H & E, 300x



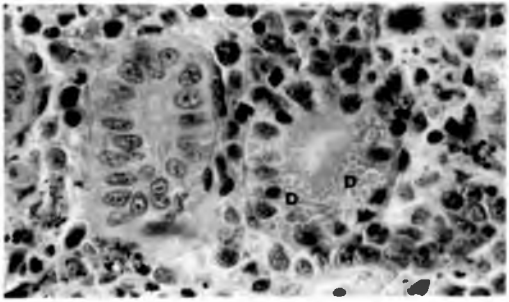
Renal Tubules

By day 14, finely granular eosinophilic material was observed in the lumina of segments of the proximal collecting tubules (PCT) in fish challenged with 10^7 Rs (Table 3, section 1). Epithelial cellular integrity was maintained in the majority of the distal tubules and collecting ducts. By day 28, however, highly refractile , eosinophilic, spherical droplets of variable size appeared in the apical portion of the proximal tubular epithelium; these increased in number and distribution over the course of infection (Figure 23). The droplets stained positive with Martius Scarlet Blue.

Eventually there was segmental disruption of epithelial cells with loss of cellular detail and release of material into the lumen of the tubule. Lymphocyte infiltration within the tubular epithelium was apparent at this stage, but it did not appear to appreciably differ in its intensity relative to that in control fish tubules.

At day 56 in fish challenged with 10^5 Rs, there were focal areas of frank necrosis of segments of the PCT. Cellular cytoplasm appeared refractile, homogeneous, and intensely eosinophilic and nuclei were pyknotic. Denuded segments of tubular epithelium were occasionally found distributed throughout the interstitium.

Figure 23. Histological section of the posterior kidney of an Atlantic salmon demonstrating the marked accumulation of droplets in the apical region of the tubular epithelial cells (D). H & E, 750x



Similar lesions were evident in fish challenged with 10^3 Rs at days 56 and 84. In addition, however, there were intracellular accumulations of irregular spicule-shaped black deposits as well as scant numbers of Rs in segments of the PCT. Basophilic primordia as well as intensely basophilic (regenerative) tubular epithelia were noted in varying numbers in fish at this stage of infection.

Thrombosis

Virtually all coho salmon challenged with Rs developed intravascular thrombosis either as an agonal or terminal phenomenon of BKD (Table 3, section 1). Homogenous, intravascular, refractile masses of varying size were localized primarily in the lumen of vascular sinusoids in the pronephros (Figure 24). In 3 fish systemic thrombosis was apparent.

Histiocytosis appeared to impinge on the vascular adventitia and occasionally extended into the lumina of larger vessels, particularly the caudal vena cava. Exophytic septic thrombi composed of a mixture of fibrin, monocytes, erythrocytes, and intracellular and extracellular Rs were noted. Septic emboli and Rs-laden monocytes, as well as secondary, small mural thrombi were apparent distally in Figure 25.

In Atlantic salmon, the initial hemodynamic and cellular alterations evident in the acute vascular response of coho salmon was not as pronounced. Intravascular thrombosis was rarely apparent in the Atlantic salmon sections examined. Interspecific differences in the histological manifestation of BKD in the kidney of coho and Atlantic salmon are presented in Table 5.

Figure 24. Pronephros of a coho salmon 84 days post-infection with 10^3 Rs. Note the multifocal intravascular thrombi (t), and marked histiocytosis of the interstitium. H & E, 75x

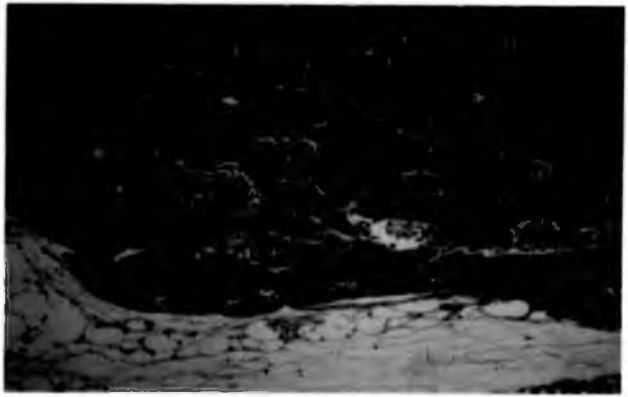


Figure 25. Longitudinal section of the caudal vena cava of a coho salmon. There is a prominent thrombus (T) composed of an admixture of fibrin, intra- and extracellular Rs, and inflammatory cells. Smaller septic emboli (e) are apparent distal to the initial lesion. PAS, 75x

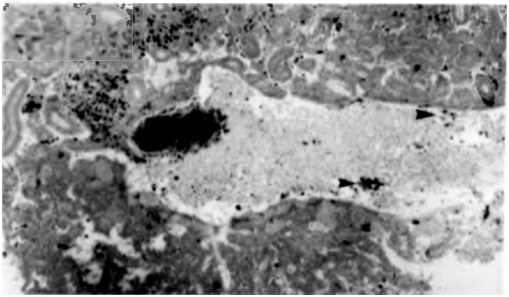


Table 5. Summary of observed renal histopathological differences between coho and Atlantic salmon

Atlantic salmon		Coho Salmon
transient	perivascular neutrophils	prominent
prominent	fibroblast infiltration	rare
rare	histiocytosis	prominent
prominent	tuberculoid formation	rare
frequent	giant cell formation	absent
rare	renal vascular involvement	frequent
rare	thrombosis	frequent
prominent	crescentic glomerulonephritis	rare
rare	renal hyaline droplet formation	frequent
diffuse	extension to swim bladder	focal
prominent	melanomacrophage response	scant

Splenic Histopathology in Coho and Atlantic Salmon

Histopathological changes of the splenic interstitium in coho and Atlantic salmon essentially parallelled those of the kidney (Tables 3 and 4, section 2). Interstitium and ellipsoid changes were minimal until day 14 in fish challenged with 10^7 and day 28 in fish challenged with 10^6 and 10^5 Rs. Initial alterations in all challenge groups included marked accumulations of lymphocytes in the periarteriolar space and in the intravascular compartment which appeared to persist for up to 7 days (Figure 26). The interstitium was moderately cellular and composed primarily of erythrocytes, lymphocytes, mononuclear cells, and stroma. Ellipsoids consisted of 2-3 layers of concentrically arranged macrophages. Pronounced interstitial hypocellularity was apparent in the latter stages of infection and consisted primarily of erythrocytes, prominent stroma, and a marked lack of lymphocytes and monocyte-like cells (Figure 27).

Multifocal, early granulomata with scant accumulations of lymphocytes were present by day 28 in fish challenged with 10^5 Rs. In fish challenged with 10^3 Rs, interstitial splenic hyperplasia, consisting primarily of monocyte-like cells, and focal accumulations of lymphocytes occurred by day 28. Beyond day 56 of infection multifocal granulomata, apparently centred on ellipsoids, were present throughout the interstitium.

Figure 26. Splenic arteriole of a coho salmon 7 days post-injection with 10^7 Rs. There is a marked lymphocytic investment of the arteriole (1). H & E, 300x

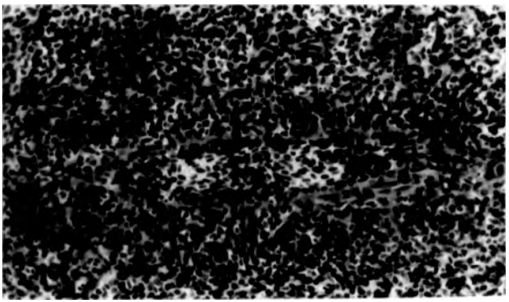


Figure 27. Splenic interstitium of a coho salmon challenged with 10^5 Rs at day 56 of infection. There is a marked hypocellularity with diffuse necrosis and scant accumulation of melanocytes. H & E. 300 x

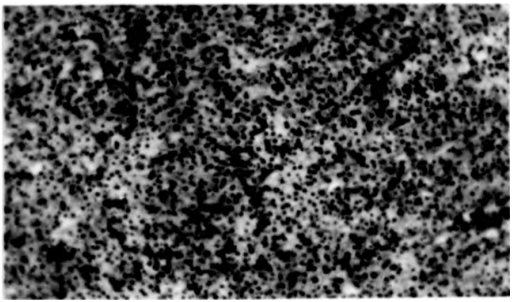
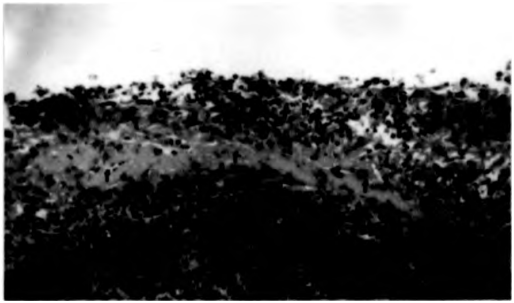


Figure 28. Photomicrograph of fibrinous perisplenitis in coho salmon. The inflammation features a cellular gradient; the basal layer is composed of fibrin (f), overlaid by a lymphohistiocytic infiltrate (l), with a superficial layer of loosely adherent monocytes, neutrophils, fibrin, and bacteria (s). The interstitium features a prominent rim of lymphocytes underlying the affected areas. H & E, 300x



Capsular involvement was initially apparent on day 7 in fish challenged with 10^7 and consisted predominantly of neutrophils admixed with histiocytes and occasional lymphocytes (Figure 28). From Day 28 onwards there was a florid accumulation of extracellular Rs with minimal to no inflammatory response. In fish challenged with 10^5 and 10^3 Rs, there was a profound lymphohistiocytic capsular splenitis.

In fish challenged with 10^5 and 10^3 Rs, the pericapsular inflammation consisted of fairly distinct cellular layers. The basilar layer consisted of fibrin , which was overlaid by a layer of granulation tissue composed of lymphocytes, macrophages, and fibroblasts with pronounced neovascularization. Occasional pyknosis, karyorrhexis, and phagocytes containing nuclear debris were apparent. The superficial layer was initially composed of granulocytes which appeared to slough readily into the abdominal cavity; later, it consisted of either well organised granulation tissue or florid extracellular Rs.

Initially, subscapular congestion was evident; however, after 14-28 days there was a marked accumulation of lymphocytes contiguous to the overlying inflammation. Eventually, mononuclear cells accumulated and there was extension of the overlying granulation response into the outer limits of the splenic interstitium.

In 3 fish, periarteriolar accumulation of the large monocyte-like cells, reminiscent of those seen in the renal interstitium occurred.

Liver Histopathology in Coho and Atlantic Salmon

The onset of hepatic involvement in the pathogenesis of BKD occuured at day 7 for 10^7, day 14 for 10^5, and day 28 for 10^3 Rs challenged fish (Tables 3 and 4, section 3). Capsular

involvement parallelled that previously described for the spleen.

Focal to locally extensive accumulations of fibrin overlying the craniodorsal and cranial aspect of the liver was detected within 7-14 days. Fibrin admixed with a moderate to mild infiltrate of granulocytes (initially), and macrophages (subsequently), occurred in fish challenged with 10^5 Rs by day 14. Fish challenged with 10^3 Rs featured a marked inflammatory infiltrate with scant to limited amounts of fibrin. Over the course of 28 to 56 days post challenge, capsular involvement extended cranio- and caudoventrally. Resolution of fibrin and subsequent capsular fibrosis resulted in adhesions between the liver and pericardium, cranially, and the viscera (pyloric ceacae), caudally.

Parenchymal involvement was minor in all fish. Only 2-4 fish in each challenged group exhibited granulomatous perihepatitis which extended into the superficial layers of the liver, and this occurred only in the terminal stages of infection. In 4 fish, early granulomata were apparent contiguous to central veins. Diffuse lymphocytic infiltration was noted along the hepatic portal triad in 3 fish. In addition, multifocal intravascular thrombosis was discerned in 3 fish (previously mentioned in the renal section).

It is important to note that the initial capsular involvement apposed a similar inflammatory process involving the overlying esophageal serosa.

Abdominal Histopathology in Coho and Atlantic Salmon

Intraperitoneal inoculation of Rs initially yielded focal to multifocal peritonitis, periesophagitis and mural pneumatocystitis (inflammation of the swim bladder)(Tables 3 and 4, section 4).

Within 3 to 7 days of injection, extravascular aggregates of granulocytes appeared either focally (or locally extensively) interspersed between pancreatic acinar, pyloric caecae or adipose tissue. Over the course of the next 14 to 28 days, a marked lymphohistiocytic infiltrate evolved and extended along and around virtually all serosal surfaces. The dorsocranial fossa of the esophagus and ventrolateral margins of the abdominal viscera were 2 sites that consistently demonstrated inflammation.

By day 7 post challenge, focal aggregates of inflammatory histiocytic infiltrate were apparent on the dorsocranial fossa of the abdominal esophagus (Figure 29). The mesovaria, mesotestes, and mesoesophagus radiate from this site, as well as the ultimobracnchial gland and cranial aspect of the swim bladder.

Over the course of infection, the inflammatory response expanded along the dorsolateral aspect of the esophagus, eventually enveloping the esophagus and extending to the dorsocranial aspect of the liver and the caudal surface of the transverse septum (Figures 30 and 31). Progression of the inflammatory response caudally along the esophagus facilitated direct extension or seeding of the ventrolateral (overlying) surface of the swim bladder as well as the cranial aspect of the ovaries and testes.

By day 14, direct extension of the periesophagitis occurred ventrally to the pyloric ceacae, stomach, and anatomically related tissues.

Figure 29. Low power magnification of the pronephros at the level of emergence of the coeliacomesenteric artery (c). Note the relationship of the anterior kidney to the mesoesophagus and mesovaria. Ascites and locally extensive accumulation of inflammatory cells is evident adjacent to the esophagus (a). H & E, 30x

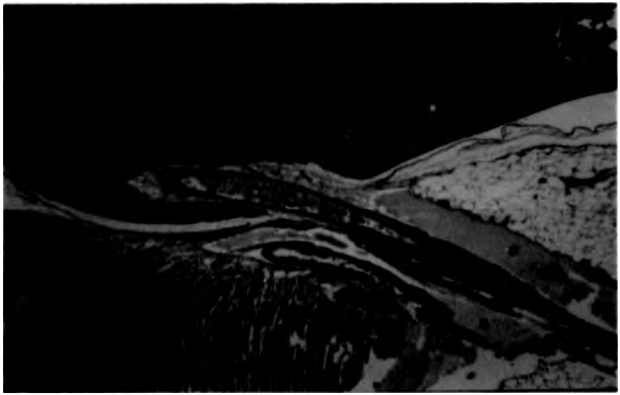


Figure 30. Sagittal section of the pronephros, esophagus, and liver. Note the disseminate granuloma within the renal interstitium and locally extensive involvement of the ventral aspect of the kidney, extending to the esophagus (g). A similar inflammatory response is associated with the liver capsule (l). H & E, 30x

Figure 31. Higher magnification of Figure 30 demonstrating the expansion of the inflammatory response to the ventral aspect of the esophagus, craniodorsal aspect of the liver (c) and dorsal aspect of the transverse septum (d). H & E, 300x

In fish challenged with 10^7 Rs, fulminant extracellular Rs involving virtually the entire abdominal cavity was apparent by day 28. Foci of necrotic pancreatic acinar cells with occasional apoptotic cells was particularly evident in areas with abundant extracellular Rs. In fish challenged with 10^3 Rs, there was by day 56 an organized granulomatous response with minimal to no apparent extracellular Rs (Figure 32). However, in areas with large histiocytic accumulations, central necrosis and exuberant extracellular bacteria were observed.

Granulation tissue eventually destroyed and replaced the exocrine and endocrine pancreatic tissue. Granulomata, however, did not appear to extend into or involve the submucosa of the esophagus, stomach or pyloric ceaca. In 2 fish granulomata within the lamina propria of the colon were noted 56 days post injection and appeared to extend up into the fibrovascular folds of the colonic mucosa. Proliferation of eosinophilic granular cells or accumulation of the monocyte-like cells was not appreciated in any of the sections.

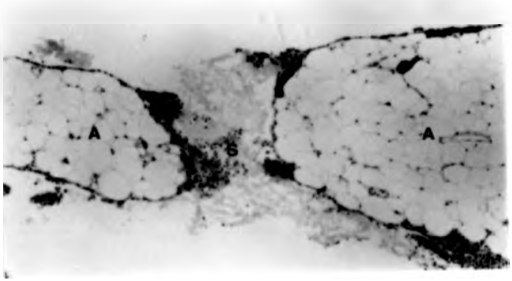
Focal granulomata were apparent at varying levels along the colonic serosal surface.

As the peritonitis developed, concurrent development of a serous, serosanguinous, or suppurative ascites was recognized histologically (and clinically) on day 14 for fish challenged with 10^7, and on day 28 for fish challenged with 10^5 (Figure 33). This reaction was mild with 10^3 Rs . Granulocytes, lymphocytes, and macrophages were interspersed within the proteinaceous fluid.

Eventually the inflammatory response seeded the ventral abdominal peritoneum and extended into the underlying abdominal musculature.

Figure 32. Fibrohistiocytic peritonitis (F) in Atlantic salmon challenged with 10^3 Rs at day 56. Note the absence of fibrin. H & E, 75x

Figure 33. Histological section of the abdominal adipose tissue (A) of a coho salmon 14 days post-injections with 10^7 Rs. Ascites is evident with superficial accumulation of inflammatory cells (S). H & E, 75x



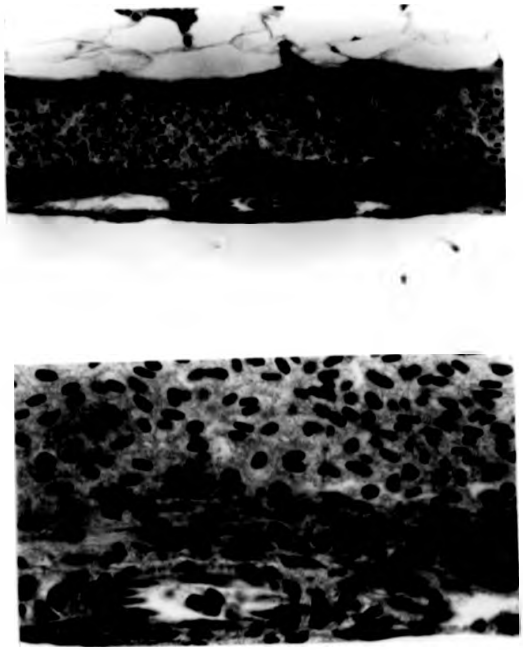
Cardiovascular Histopathology of Coho and Atlantic Salmon

Focal accumulations of macrophages within the papillary layers of the myocardium were present in 1 to 3 of ten fish as early as 3 days post infection (Tables 3 and 4, section 5). These lesions, which were situated at various levels of the trabecular musculature, increased in number and size over the course of infection. Degenerative changes in the ventricular myocardium, including the development of multiple small or solitary large cytoplasmic vacuoles, were apparent terminally in fish challenged with 10^7 and 10^5 Rs.

Pericarditis was minimal and appeared to extend from involved areas of the transverse septum. Initially, macrophage accumulation occurred at the dorsal commissure of the septum, usually adjacent to the site of periesophagitis extension to the liver capsule.

In the acute stage of the infection, profound hemodynamic alterations were apparent. Initially, the abdominal vasculature appeared markedly dilated with margination, pavementing, and extravasation (chemotaxis) of granulocytes (Figure 34). Eventually (by day 7 to 14), this response subsided and lymphocytes, as well as extracellular Rs and macrophages or monocytes replete with Rs were observed within the vasculature lumen.

Figure 34 a and b. Low and high magnification of the splanchnic vasculature of a coho salmon 7 days post-injection with 10^7 Rs. The vessel appears congested with margination, pavementing, and extravasation of neutrophils (N). H & E, 300x and 750x.



Reproductive Histopathology of Coho and Atlantic Salmon

Direct extension of inflammatory infiltrate from the esophageal pedicle to the mesovarium and eventually to ovarian tissues occurred as early as 14 days post-infection and eventually involved 5-9 of the 10 fish depending on the challenge group involved (Tables 3 and 4, section 6). In addition, by day 28 for 10^7, day 56 for 10^5, and day 56 for 10^3 challenged Rs, extracellular accumulations of bacteria extending up from the ventral aspect of the ovaries range from scant to florid amounts; these accumulations were interspersed in the interlamellar (ovigerous) regions. Ovarian follicular cells contiguous to the extracelluar Rs contained a finely granular, intensely basophilic cytoplasm. Special stains demonstrated intracellular Rs (Figure 35).

There was minimal to no vascular or thecal stroma response evident (Figure 36). Ovarian tissue with fulminant extracellular Rs featured some degenerative changes, such as ooplasmic vacuolation and faint staining ooplasm.

Additional histological cross sections at various levels of the abdomen revealed superficial accumulations of extracellular Rs on the ventral and ventrolateral aspects of the gonads (Figure 37). A pronounced histiolymphocytic infiltrate extended along the mesovaria or mesotestes. The eventual outcome was that the ovary or testes adhered to the dorsolateral aspect of the abdominal wall.

Figure 35. Ovarian tissue of coho salmon challenged with 10^3 Rs 56 days post infection. There is marked accumulation of Rs (red stained bacilli) within the cytoplasm of the follicular cells. PAS, 750x

Figure 36. Ovarian tissue with accumulation of macrophages laden with intracellular Rs in the interlamellar spaces. Note the absence of inflammatory response in the thecal vasculature (t). PAS, 750x

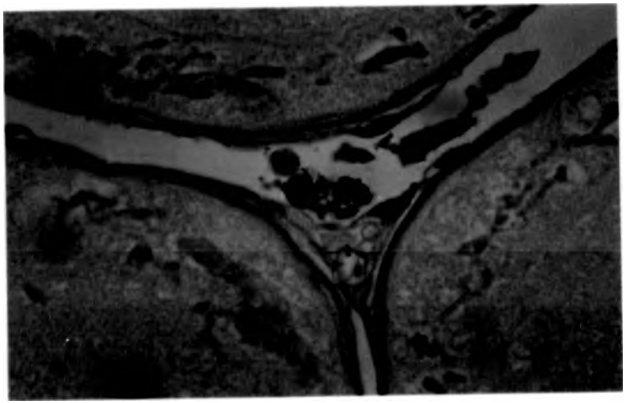
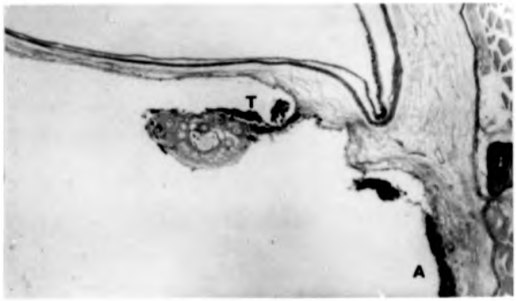


Figure 37. Cross section of a male coho salmon 28 days post-injection with 10^7 Rs. Note the accumulation of Rs along the mesotestes (T) and lateral abdominal wall (A). PAS, 30x



To assess the extent of intra ovum infection additional sections of 32 female fish (with extracellular Rs) were prepared and special stained with PAS and the ABCIP for Rs (polyclonal 1:800). For each fish individual ova were enumerated and intracellular Rs identified; 17 of 847 or 2.0% contain Rs within their ooplasm. It is important to appreciate that this is a relatively insensitive technique and that this prevalence is a gross underestimation.

Neural Pathology in Coho and Atlantic Salmon

Central nervous system involvement appeared to be a terminal phenomenon in the pathogenesis of infection (Table 3 and 4, section 7). Intravascular aggregates of septic embolic, extracellular Rs, and (more frequently) macrophages or monocytes laden with the bacterium were detected within the microvasculature of the tela choroidea of the epithalamus, within the saccus vasculosis (immediately caudal to the pituitary gland)(Figure 38), as well as in the meninx primitiva, optic tectum, hypophysis, and the medial lobe of the hypothalamus (Figure 39). Frequently, overlying ependymal cells acquired a papillary-like appearance. Within the parenchyma of the cerebellum and optic tectum, vascular congestion of the microvasculature was evident.

Granulomatous encephalitis was not apparent in any neural tissue sections examined; however, in 2 fish focal accumulations of extracellular Rs were detected overlying the meninx of the optic tectum with an attendant increase in cerebrospinal fluid and mononuclear cell infiltrate (Figures 40 and 41).

Figure 38. Longitudinal section of the saccus dorsalis of the epithalamus. Septic emboli of intra- and extracellular Rs are evident in the lumen of the vasculature (E). Note that similar emboli are present within the vasculature of the dermal stratum compactum (S). PAS, 30x

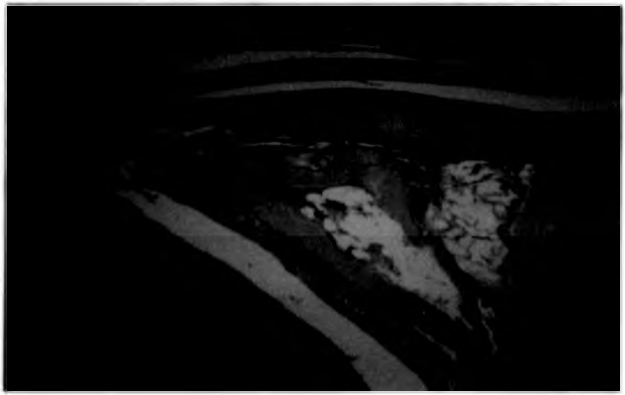


Figure 39. Vascular emboli (e) composed of fibrin, intra-and extracellular Rs, and monocytes, situated below the optic tectum. H & E 75x

Figure 40 a and b. Sagittal section of the otic tectum. There is a marked increase in cerebrospinal fluid overlying the optic tectum (a). Higher magnification demonstrates multifocal extracellular accumulation of Rs with mononuclear infiltrate (b). H& E, 75x and 300x

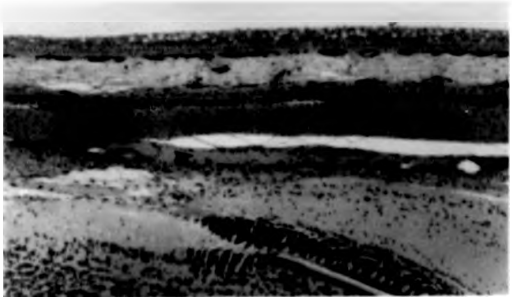
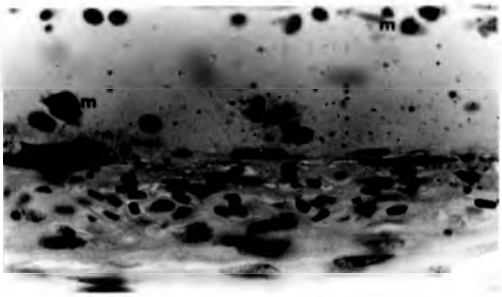


Figure 41. Higher magnification of Figure 40. Note extracellular accumulation of Rs (r), marked mononuclear infiltrate (m) and absence of granulomata formation. H & E, 750x



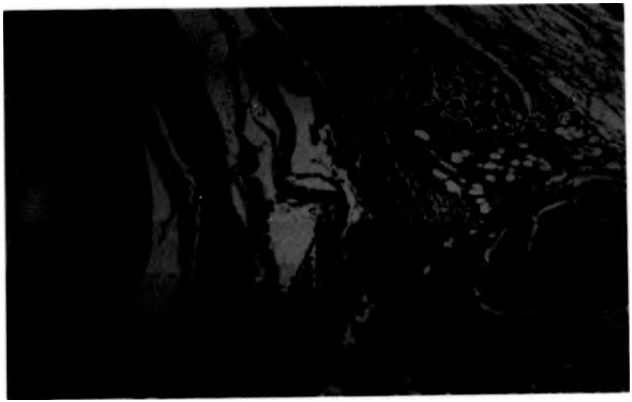
Ocular Histopathology in Coho and Atlantic Salmon

Ocular involvement was apparent by day 28 in coho and Atlantic salmon. Interestingly, 2 of 3 fish challenged with 10^3 Rs featured no apparent systemic involvement (Table 3 and 4, section 8).

Initially, focal to locally extensive accumulations of macrophages laden with Rs were localized in the afferent microvasculature of the choroidea. Subsequent disruption of the choroid vasculature, edema, and focal areas of haemorrhages were evident. In 1 fish, a retrobulbar fibrohistiocytic mass was discerned.

In 2 fish in the terminal stages of infection (day 84, in fish challenged with 10^3 Rs) extensive, unilateral granulation tissue involving the choroid, sclera, extrinsic ocular muscles and optic nerve was evident. Retrograde perineurial expansion of the lesion to the level of the optic chiasma was apparent (Figure 42).

Figure 42. Retrobulbar granulomatous response in coho salmon at day 84 of infection. There is a marked histiocytic accumulation and infiltration around the extrinsic ocular muscles and optic nerve. The lesion extended to the level of the optic chiasma. H & E, 30x



Branchial Histopathology in Coho and Atlantic Salmon

Diffuse haematogenous dissemination of extracellular Rs was noted in the later stages of the infection. In fish challenged with 10^7 and 10^5 Rs, the bacterium was localized in the primary and secondary branchial vasculature in 2-4 of 10 coho salmon (Tables 3 and 4, section 9). Virtually all gill arches were involved. Focal to locally extensive histiocytic accumulations are associated with the secondary lamellae.

Skeletal Musculature

With the exception of direct extension of a histiocytic infiltrate through the ventral aspect of the peritoneum to the underlying skeletal musculature there was a marked absence of muscular involvement in challenged fish (Tables 3 and 4, section 10). Focal accumulation of septic emboli with associated Rs-laden monocytes or macrophages were noted in the vasculature of the stratum compactum on the dorsal aspect of the cranium.

DISCUSSION

Histopathology of Atlantic and coho salmon revealed pleomorphic multisystemic manifestations of BKD which were contingent on inoculum dose, post infection interval, and host species. For example, those fish challenged with 10^7 Rs succumbed within 30 to 42 days with a limited inflammatory response, exuberant extracellular Rs, and severe interstitial necrosis. In contrast, fish challenged with 10^5 and 10^3 Rs died more slowly (100% mortality between day 75 and 93) and exhibited a more elaborate chronic inflammatory process. Therefore, discordant histopathological findings between this investigation and past studies may be attributed, in part, to these variables. Factors shown from past studies to affect pathogenesis of BKD include genetic constitution, nutritional status, season, as well as water temperature and hardness (Smith, 1964; Wedemeyer and Ross, 1973; Withler and Evelyn, 1990; Sakai, Atsuta, and Kobayashi, 1991).

However, notwithstanding the variables in the various studies, histological lesions homologous to those published in case reports and past studies were recognized in the present study and their interspecific variation, prevalence, and attendant sequelae documented.

Comparison of the nephritogenic activity of Rs between coho and Atlantic salmon demonstrated profound differences which may be resolved into the perivascular, interstitial, excretory, and vascular compartments (Table 5).

In Rs-challenged coho salmon, a marked perivascular granulocytosis was apparent by day 3 in up to 6 of 120 fish examined histologically and it persisted for approximately 7 to 10 days. This accumulation of cells was particularly apparent

around the vascular sinusoids and the caudal vena cava, and frequently resulted in the expansion of cords or nests of interstitial lymphomyeloid cells. Evaluation of serial sections suggested that these cells were extravascular (they did not communicate directly with the vascular sinusoids) and that they may migrate through vascular adventitia and endothelia, as in mammalian bone marrow, to enter the cardiovascular system (Jain, 1986). Haematology was not performed in this study; however, a peripheral leucocytosis with neutrophilia has been reported in rainbow trout (Oncorhynchus mykiss) between 2 and 7 days post Rs challenge (Bruno and Munro, 1986b). Expansion of the granulocytopoietic pool in humans with infectious disease has been documented with concomitant reduction and prolongation of the cell cycle of other bone marrow lineages (Dormer, Hultner and Mergenthaler, 1990) This phenomenon may account, in part for the clinical manifestation of anemia with chronic inflammatory processes. In fish, because of the difficulty in resolving progenitor cells, relative cell production rates could not be appreciated.

Studies on the nature of the inflammatory response in fish suggest marked homology in cellular composition with mammalian species. Finn and Nielson (1971) reported a marked initial neutrophilic response with a subsequent recruitment of lymphocytes and macrophages, which is consistent with observations in this investigation.

Functional and anatomical homology of the perivascular interstitium of fish to mammalian bone marrow has been adduced by a number of investigators (Zapata, 1979; Meseguer, Estaban and Angulleiro, 1991). Ultrastructural and histochemical investigations of fish pronephric tissue suggest that in some species white (myeloid) and red (lymphoid) pulp is present

(Sailendri and Muthukkaruppan (1975). Based on the present investigation, this phenomenon may be consistent with coho, and possibly Atlantic salmon.

Extensive investigations have been undertaken with fish to further understand in vitro granulocyte and macrophage function, whereas few studies have focused on the kinetics of granulocytosis (of other cell lineages) in fish affected with BKD or other infectious diseases.

Similar alterations to those of the perivascular compartment are evident within the renal interstitial cell population. Evolution from a heterogeneous cell population to a more homogeneous composition, consisting of small lymphocytes and large mononuclear-like cells, is apparent in Atlantic and coho salmon by day 28. The larger cells have histological features consistent with cells of the myeloid as well as lymphoid lineages and are consistent with those described by Wood and Yasutake (1956). Further ultrastructural and histochemical evaluation is warranted to define this larger cell type due to its prominence in both BKD and marine anaemia (plasmacytoid leukemia)(Kent, Groff, Traxler et al, 1990).

In coho salmon granulomata evolved from an initially suppurative, then pyogranulomatous (predominantly histiocytes with granulocytes interspersed throughout) lesion, and eventually to a histiocytic granuloma. In fish challenged with 10^7 and 10^5 Rs virtually all the macrophages were replete with Rs and the center of the lesion underwent necrosis with liberation and dispersal of extracellular bacteria. A prominant lymphocytic mantle was apparent with granulomata, perhaps reflecting a strong cell mediated immunity. In mammals, immunohistochemistry of granulomata with lymphocyte subset markers indicates that peripheral lymphocytes are thymus derived lymphocytes (L_T)

expressing CD8 leukocyte differentiating antigen; whereas, those lymphocytes interspersed within the centre of the lesion feature CD4 epitopes (Unanue, 1980; Sheffield, 1990). Moreover, profound alterations in the ratio of these cells may account for histiocytic or tuberculoid granulomata in such granulomatous diseases as leprosy (Mycobacterium leprea)(Randhawa, 1990) or sarcoidosis (Mishra, Poulter, Janossy, and James, 1983; Munro, Campbell, Dubois et al, 1988). It is interesting to speculate that such a phenomenon may account for differences between the histiocytic response of the coho salmon and the more tuberculoid response of the Atlantic salmon.

Interestingly, in Atlantic salmon, discrete tuberculoid granulomata develop with epithelioid and giant cell formation. Moreover, recruitment of melanomacrophage centers (the putative antigenic processing centers) contiguous to and around the lesion as well as dispersal of melanin throughout the lesion was apparent. In those fish with an apparently strong cell mediated immune response, fibrous encapsulation ensued and there was limited expansion or extension of the nidus to adjacent tissue. However, in fish with an inadequate response, central caseonecrosis, lack of encapsulation, and dissemination of Rs cells was apparent. A prime example of this extension is the direct extension of Rs from the kidney dorsal to the spinal cord and ventrally to the swim bladder in Atlantic salmon. In coho salmon, a more focal extension was observed and it appeared limited to the dorsal aspect of the esophagus.

As a profound granulomatous response with scant Rs was observed in this study, as well as in past investigations (Bendele and Klontz, 1975), a delayed type hypersensitivity (DTH III) was considered as a possible pathogenic mechanism of BKD.

In Atlantic salmon challenged with 10^1 and to a lesser

extent 10^5 Rs, resolution of the granulomata was evident (as in Bruno, 1986b), whereas in coho salmon a healing response is not apparent in virtually all fish examined. A possible sequelae to the histiocytic versus tuberculoid response in coho salmon was endothelial damage and intravascular coaggulation. Fibrous encapsulation of granulomata in Atlantic salmon appeared to mitigate the extent and involvement of the inflammatory process within the renal vasculature. In coho salmon, marked interstitial hyperplasia due primarily to histiocytes, appeared to impinge on the endothelia, and possibly through secretion of proteolytic enzymes (collagenases and elastases, in particular) may degrade the adventitia, incite endothelial injury, and evoke the coagulation cascade (Ward, 1991).

Coagulopathies have been associated with a number of infectious agents in fish including Vibrio salmonicida (Salte, Nafstad, and Asgard, 1987; Salte and Norberg, 1991), and Aeromonas salmonicida (Salte, Norberg, and Odegaard, 1991), due to endotoxins, as well as to PKX (the etiological agent of proliferative kidney disease) (Clifton-Hadley, Bucke and Richards, 1987) and to Saprolegnia spp (pers. observation) possibly due to mechanical endothelial injury.

Hematocrits performed over the course of the study suggest that in coho salmon with terminal or agonal increases in values from 25-32% to 72-75%, an increase in viscosity may have rendered the blood hypercoagulable and more prone to thrombogenesis. As well, possible loss of antithrombin III through compromised glomerular filtration may also have accounted for the observed thrombosis. In Atlantic salmon anemia was limited to hematocrits of 35-45.

Glomerulonephropathy associated with acute (Young and Chapman, 1978) and chronic (Sami et al, 1992) BKD has been

reported and attributed to immune complex deposition. In this study, without the aid of ancillary tests such as immunofluorescence or electron microscopy, interpretation of glomerular lesions remains speculative. Nevertheless, a previously undescribed crescentic glomerulonephritis, due to proliferation of parietal epithelial cells, was apparent in Atlantic (but not coho) salmon from 28 days onwards.

In man, systemic conditions associated with parietal cell proliferation in the glomerulus include polyarteritis nodosa, systemic lupus erythromatosis, Henoch-Schonlein purpura, Wegener's granulomatosis, hypersensitivity angiitis and Goodpasture's syndrome (Elfenbein, Baluarte, Cubillos-Rojas et al, 1975; Stilmant, Bolto, Sturgill et al, 1979). Primary nephritogenic disease resulting in crescent formation has been described in nonstreptococcal rapidly progressive glomerulonephritis, anti-glomerular basement membrane disease, and membranoproliferative glomerulonephritis (Antonovych and Mostofi, 1980).

Based on recent ultrastructural observations in rainbow trout and brook trout (Young and Chapman, 1978; Sami et al, 1992), and based on comparison with mammalian conditions, the observed crescentic glomerulonephritis may be a sequela to acute membranous glomerulonephritis. In man this condition has been attributed to disruption of the glomerular basement membrane with fibrin excretion and subsequent proliferation of parietal epithelial cells. In this study, appropriate histochemistry revealed that there was scant fibrin deposition in fish glomeruli but that large amounts were present within the apical region of the proximal convoluted tubule epithelium. This suggests some degree of consistency with mammalian conditions. An alternative mechanism may be analogous to the hypocomplementemia in Finnish

Landrace lambs with crescent formation (Maxie, 1985), where an inherited deficiency in serum C3 levels impairs complement-mediated removal of immune complexes within the basement membrane of glomeruli. A membranoproliferative glomerulonephritis ensues.

Atlantic salmon crescents may progress from initially epithelial, then fibroepithelial to eventually fibrous and the sclerotizing glomerulonephritis observed in rainbow trout (Sami et al, 1992) may either be a sequalae to crescentic glomerulonephritis or, alternatively, a separate species-specific entity.

Soluble immune complex formation is recognized in fish infected with BKD and may account for complex deposition in glomeruli (Sami et al 1992). In addition cationic antigens may localize in glomerular basement membranes and in situ complex with antibodies and incite glomerulonephritis (Batsford, 1991).

In either pathogenic mechanism it is important to appreciate that a protein losing nephropathy ensues and a nephrotic-like syndrome may arise. Further investigations into the pathophysiology of BKD are necessary to formulate improved treatment and management strategies.

Clinical chemistry on serial blood samples of fish in this study (unpublished data) suggests that in addition to a marked hypoalbuminemia there is a marked hypogammaglobinemia (approximately 30% normal values), which based on histomorphometry may be attributed to a profound reduction in the lymphoid component of the renal and splenic interstitium. Alternatively, immune comlex formation, inanition, peritonitis, or glomerulonephritis may also account for this phenomenon. A possible virulence factor, protein p57 (Turaga, Wiens and Kaattari, 1987) has been shown to be immunosuppressive in vitro. Alternatively, immune complex formation, inanition, peritonitis,

or a protein lossing glomerulopathy may account for this observation. Interestingly, this reduction in immunoglobulin may account for increased susceptibility of fish to conditions such as vibriosis (Vibrio anguillarum or V ordalii) (Miyake and Evelyn, unpublished data) which elicit more profound humoral protective mechanisms, in vivo (Velji, Albright, and Evelyn, 1990).

Extrarenal involvement in ip injected fish appeared to arise principally in the dorsal aspect of the abdominal cavity. Direct extension of inflammatory infiltrate laden with Rs from the renal interstitium was apparent. In coho salmon this occurred focally, and immediately dorsal to the abdominal esophagus; whereas, in Atlantic salmon locally extensive to diffuse involvement along the length of the swim bladder was apparent.

Tangential sections of the pronephros revealed that the infiltrate extended along the coeliacomesenteric artery (periarteritis). As this is a prime site for localization of inflammatory infiltrate in Rs-infected fish, further (more detailed) studies with serial sections and electron microscopy of this site are warranted to elucidate this mechanism.

The inflammatory infiltrate appeared to expand ventrolaterally to dependent areas of the viscera, possibly due to the effect of gravity and abdominal contractions associated with locomotion (transperitoneal).

Accumulation of neutrophils, monocytes, and possibly reactive mesothelial cells in the ventral quadrant of the abdomen was appreciated and was consistent with previous work on experimental implants in fish (Malty and Summerfelt, 1988).

Direct extension to the liver capsule and transverse septum ensued and eventually enveloped this viscus. By day 14 in 10^7 and day 28 in 10^4 Rs-challenged coho and Atlantic salmon,

a profound fibrinoperitonitis developed. Aspirated fluid grossly appeared cloudy and serous, and cytology confirmed a septic peritonitis with numerous extracellular Rs, monocytes, disintegrated neutrophils, and subnormal protein levels (fibrin <1, normal values 1-4).

A pseudodiphtheritic membrane, as described by Smith (1964), was apparent in fish challenged with 10^7 and 10^5 Rs. In contrast, fish challenged with 10^3 Rs developed a profound fibrohistiocytic response. This observation may discount the role of temperature in the clinical manifestation of BKD (fibrinomembranous or granulomatous)(Smith, 1964) and suggests that bacterial load, degree of vascular injury, and the ability of fish to mount an inflammatory response may be more significant (Finn and Neilson, 1971).

Liver involvement in experimentally challenged fish appeared limited to capsular fibrin- or granulomatous hepatitis. Multifocal to locally extensive areas of inflammatory infiltrate into the superficial regions of the parenchyma were apparent; however, there was a marked absence of vascular or parenchymal involvment.

Similar processes occurred in the splenic capsule. Interstitial alterations, included a marked depletion of lymphoid cells and granulomata centred on ellipsoids, were consistent with those described by Ferguson (1989) for systemic bacteremias.

Extension of the inflammatory infiltrate from the esophagus along the mesovaria to the ovaries in coho, as well as from the swim bladder to ovaries in Atlantic salmon occurred between day 28 and 56. Scant to florid extracellular accumulations of Rs associated with ova were evident from day 28 onwards and appeared to extend up and between the interlamellar trabeculae. These Rs cells appeared to be ascites-borne and they were phagocytized by

follicular cells.

Phagocytosis of Rs by follicular cells is a novel observation and may account for intra ovum recruitment of bacteria via the micropyle or pore canals as proposed by Bruno and Munro (1986a). However, the present study failed to show such recriutment into eggs, possibly due to the insensitivity of the technique, lack of Rs growth in ova, or insufficient sample size.

Interestingly, pathogen penetration into ova is prevented in large part by the zona pellucida in mammals and by the egg shell in birds (Shisong and Wrathall, 1989). Yet with bacteremic states, intra ovum infections may arise from the ovary or oviduct. Mechanisms of penetration involved include either phagocytosis of pathogens by follicular cells or bacterial protease secretion and subsequent penetration by agents (Ng, Edirisinghe, Sathananthan, and Ratnam, 1987; Shimizu, Kondoh, and Tanaka, 1987; Clay and Board, 1991; Koehler, Clark, and Smith, 1991).

In Rs-challenged coho and Atlantic salmon 4-6 gm in size, the prevalence of Rs infected oogonia is approximately 2% in the present study, similar to that of 200 gm rainbow trout (Bruno and Munro, 1986a). Observed differences and reported post-ovulatory infection rates may be due to 1) the use of histology - a relatively insensitive technique for Rs detection, 2) post-ovulatory recruitment of Rs by eggs (Evelyn, Ketcheson and Prosperi-Porta, 1986a) 3) protective mechanisms of the developing egg (a physical membrane barrier, such as the zona radiata or intervening basement membrane, as well as possible antibacterial elements in the ooplasm), or 4) inability of Rs to elaborate proteolytic enzymes (Bandin, Santos, Bruno et al, 1990), or 5) bacterial recruitment before anti-Rs components (if they exist)

may be elaborated. Possible defense mechanism within the ooplasm may include a relatively anaerobic state, ovotransferrin, lectins (Kamiya, Muramoto, Goto et al, 1990), nonspecific or specific antibodies (Antsee, Holt and Pardoe, 1973), as well as possible nutritional deficiencies (due to the fastidious growth requirements of Rs) within the ooplasm. Lysozyme and C reactive protein have been detected in ooplasm of certain fish species; however, Rs appears uniquely resistant to their effect (Fielder and Balco, 1976; Yousif, Albright and Evelyn, 1991).

In addition, vascular involvement in mature fish has been cited in the pathogenesis of intra ovum infection (Armstrong, 1989), whereas, in immature fish there appears to be a marked absence of vascular response to ovarian infection, which may reflect the degree of ovarian perfusion in developing ova, as well as expression of receptors or rate of renewal on the plasma membrane (Tyler, Sumpter, and Handford, 1990).

Another, more recently recognized clinical feature of BKD is pyogranulomatous meningoencephalitis (Speare et al, 1991). Predilections sites include the posterior tela choroidea and the vascularized capsule of the saccus dorsalis of the epithalamus, saccus vasculosus of the hypophysis, and optic chiasma. In this study there was a lack of granulomatous response in the brain or meninges. Intra- and to a lesser extent extracellular Rs and microthrombi were restricted to the vascular lumina of the saccus dorsalis, saccus vasculosis, hypothalamus, and optic tectum and there was no apparent extension to the perivascular central nervous tissue. In 2 fish, extracellular accumulation of Rs, increased amounts of cerebrospinal fluid and a mononuclear cell infiltrate were noted overlying the dorsal aspect of the optic tectum.

Although dissemination of the Rs to the CNS appears to be

primarily haematogenous, in 2 fish a granulomatous response was evident within the mucosa of the nasal papillae and appeared to extend in a retrograde manner along the olfactory nerves to the olfactory bulb and cerebrum. Microabscess formation within the nasal papillae has been previously recognized with Vibrio anguillarum (Traxler and Li, 1972) and this may be a more important portal of infection than previously recognized.

Similar extension (as a sequelae to posterior uveitis) to the optic chiasma was also recognized (Speare et al, 1991) and observed in this study. Retrobulbar granulomatous periophthalmitis was evident in 3 fish in this study between days 56 and 84. Incipient ocular lesions were characterized by focal to locally extensive accumulations of monocytes (macrophages) replete with Rs within the afferent vasculature of the choroidea. Subsequent disruption of the vasculature, edema and hemorrhage occurred and may have facilitated the expansion of the lesion to periocular tissue. No apparent intraocular involvement was noted.

Intravascular localization of extracellular Rs in the branchial vasculature was apparent in 2 to 4 of ten fish on day 56 and, as indicated by Wolke (1975) reflects the extent of metastatic dissemination of bacteria at the nadir of infection.

Skeletal muscle involvement with BKD was not apparent in this study and may reflect a fortuitous site predilection of the bacterium for highly vascularized tissue.

Chapter III

Sequential Histopathology of Bacterial Kidney Disease (*Renibacterium salmoninarum*) in Coho (*Oncorhynchus kisutch*) and Atlantic (*Salmo salar*) Salmon Challenged by Cohabitation

INTRODUCTION

Serial histopathology in coho and Atlantic salmon challenged with 3 levels of Rs (10^7, 10^4, and 10^1) by intraperitoneal injection revealed that the pathogenesis of BKD was dependent, in large part, on the dose of inoculum, species challenged, and sampling interval (Chapter II).

Lesions consistent with past descriptions of natural and experimental Rs infections were observed (chapter II); however, previously undescribed findings, such as renal perivascular granulopoiesis, crescentic glomerulonephritis, intravascular thrombosis, as well as ovarian follicular cell accumulation of Rs, warrant further evaluation as to their occurrence over the course of naturally acquired infections.

Coho and Atlantic salmon challenged with 10^7 Rs incurred 100% mortality between day 32 to 42 post challenge, between day 56 to 64 for 10^4 Rs, and between day 84 to 96 for 10^1 Rs. This relatively rapid mortality may have accounted for the failure to detect some lesions, such as granulomatous or cavitary myositis, dermatitis, eosinophilic granular cell proliferation within the gastrointestinal tract (Wood and Yasutake, 1956) or meningoencephalitis (Speare, Ostland and Ferguson, 1991) in these species, and suggests that experimental (intraperitoneal) challenge may alter the pathogenesis of BKD within the host.

To further resolve the histopathology of BKD in Atlantic and coho salmon, each species was separately challenged, inter alia, by cohabitation with fish infected by Rs-intraperitoneal injection, sampled at monthly intervals to characterize and quantify the histopathological lesions.

Materials and Methods

Coho (Oncorhynchus kisutch) and Atlantic (Salmo salar) salmon between 4-6 gm in size were obtained from Rosewall Creek Hatchery (Big Qualicum stock) and United Hatcheries, respectively. Progeny were derived from Rs-screened brood stock and maintained on well water systems.

Rs challenge inocula were prepared as described by Evelyn, Ketcheson, and Prosperi-Porta (1986a). Rs (isolate 384) was cultured on KDM-2 (Evelyn, 1977), harvested in the log phase of growth, and suspended in sterile peptone-saline. Rs cell concentrations were adjusted to 10^7 and 10^5 Rs/ml of peptone-saline.

Two hundred Atlantic and 200 coho salmon were transferred into separate 250 L flow through, tanks supplied with well water at 8 to 9 C. A sustained cohabitation exposure was established by inoculating, from each cohort, 25 fish with 0.1 ml of 10^7 and 25 fish with 10^5 Rs (Murray, Evelyn, Beacham et al, 1992). The fish challenged by intra-peritoneal injection were fin clipped for later identification and test fish were held in the same tank as the injection-challenged fish.

Fish were fed a maintenance diet (2% of the body weight/daily) and daily mortalities were recorded and frozen for later evaluation.

Each group was sampled every 28 days over a 7 month interval. Ten fish from each tank were killed by immersion in MS 222 and preserved in Davidson's solution for histopathology. Sagittal and parasagittal tissue block of the cranium and abdominal viscera, respectively, were prepared and processed by routine histological techniques (Chapter II). Sections were cut to a thickness of 5 um and stained with hematoxylin and eosin.

Schiff's periodic acid, and Gram's stain, as well as the avidin biotin conjugated immunoperoxidase for Rs (Chapter I)(polyclonal antibody, 1:800) and cytokeratins (AE1 and AE3)(Noga, Dykstra and Wright, 1989). Sections were examined, lesions characterized, and prevalence recorded.

RESULTS

Significant mortalities of coho and Atlantic salmon challenged by cohabitation with Rs injected fish were initially noted on day 96 for the former population and day 112 for the latter. By day 196 post-introduction of infected fish approximately 5% of the coho and 30% of the Atlantic had succumbed to BKD (Figure 43). Histopathology of coho salmon challenged by cohabitation exposure to Rs revealed that the organism occurred initially in the kidney. As in Chapter II, lesions were characterized as afflicting the perivascular, interstitial, excretory and intravascular compartments.

Perivascular accumulation of granulocytes in coho salmon were associated with the caudal vena cava and, to a lesser extent, the pronephric vascular sinusoids (8 of 10 fish on day 28 and 2 of 120 fish on day 56). Multifocal, perivascular accumulations of lymphocytes were evident in all fish on days 28 and 56. Thereafter, large monocyte-like cells appeared, with varying numbers of interspersed lymphocytes; these were detected through to day 196.

In Atlantic salmon, limited perivascular involvement occurred but infiltration of large mononuclear-like cells was apparent by day 56 (Table 6, section 1).

In coho salmon, focal to multifocal granulomata (initially pyogranulomata, then principally histiocytic) were evident in 3

to 5 of 10 fish from day 56 to day 168. An exception was on day 112, when 5 of 10 fish featured focal, 2 of 10 multifocal, and 2 of 10 disseminate granulomata. No extracellular nor intracellular accumulations of Rs were apparent in H and E stained sections.

Solitary to multifocal elliptical cavitations 0.1 to 0.7 cm in size, situated at varying levels of the renal interstitium, appeared in 2 of 10 fish on days 84 and 112, and in 4 of 10 fish on day 140 (Figure 44). The lesion consisted of a central cavity containing an admixture of proteinaceous fluid, degenerate granulocytes and histiocytes (monocytes or macrophages), and a margin of macrophages arranged in concentric lamellae. Histochemistry with Masson's trichrome and immunohistochemistry with cytokeratins failed to
demonstrate fibroplasia or epithelioid cell formation.

In the posterior kidney, due to the severe hypercellularity, the excretory elements appeared to be displaced and compressed (Figure 45). Moreover, hyperplasia of interstitial mononuclear-like cells with no apparent cavitation was noted in 2 to 4 of 10 fish from day 112 to day 168 (Figures 45 and 46).

Resolution of interstitial lesions was apparent in 2 to 3 fish by day 112 and was characterized by a more organized granulomata consisting of peripheral cuboidal-like cells and infiltration of melanomacrophage centers around the lesion (Figure 47). In Atlantic salmon, multifocal tuberculoid granulomata predominated and occurred in 7 to 8 of 10 fish between days 84 and 196 (Table 6, section 1).

In both coho and Atlantic salmon, the renal excretory compartment featured mild to moderate focal glomerular basement membrane thickening in 2 of 10 fish on day 112, 3 of 10 fish on

day 140, and 5 of 10 fish on day 196. Crescentic glomerulonephritis was noted in Atlantic salmon on days 140 and 168.

Degenerative changes in the tubular epithelium, characterized by hyaline droplet formation, apical vacuolation, loss of tubuloepithelial cohesion, and coagulation necrosis, occurred by day 140 in 5 of 10 fish and day 168 in 7 of 10 coho salmon. These lesions are often commensurate with interstitial hyperplasia. In Atlantic salmon, similar tubular epithelial changes appeared on days 140 and 168.

Figure 43. Cumulative mortality of coho and Atlantic salmon challenged by cohabitation with Rs injected fish. Innoculated fish excluded from data.

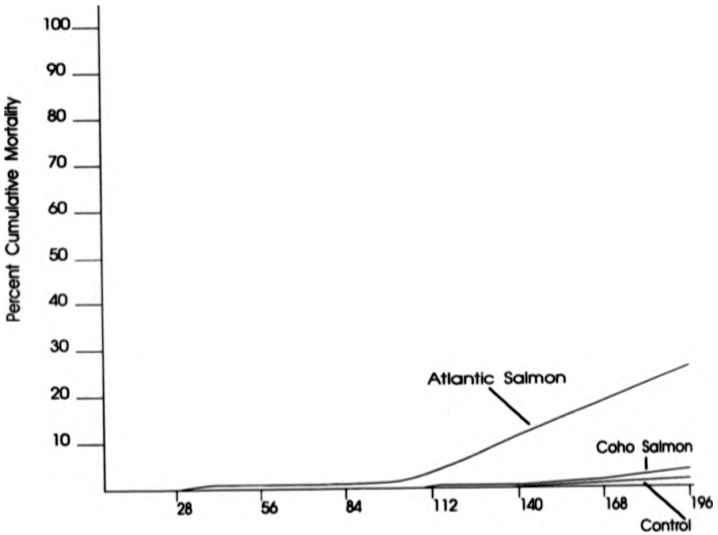


Figure 44. Large pseudocystic lesions in the posterior kidney of coho salmon challenged by cohabitation (P). Note the central area of necrosis with an admixture of fibrin, extracellular Rs, as well as macrophages at varying stages of degeneration. Hematoxylin and Eosin, 30x.

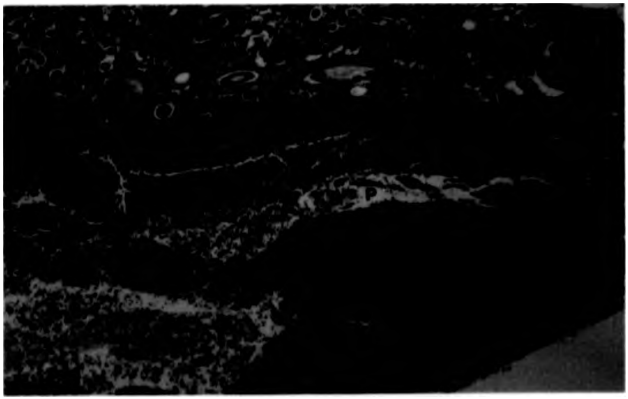


Figure 45. Posterior kidney of coho salmon at day 140. The interstitium is markedly hyperplastic and contains a fairly homogeneous cell population (H). Note the degree of tubular displacement. Hematoxylin and Eosin, 75x.

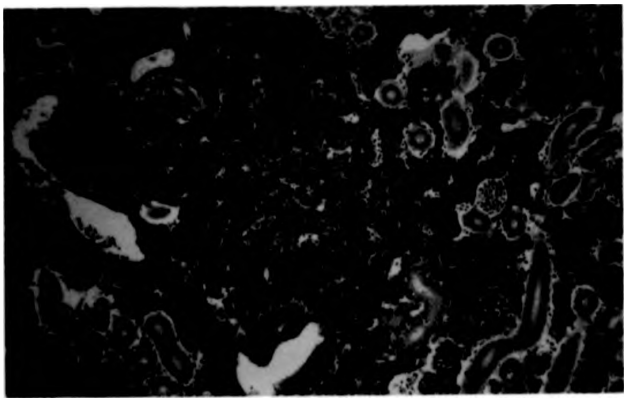


Figure 46. High magnification of Figure 45. The interstitium consists of a fairly uniform population of cells characterized by a large vesicular spherical nucleus and scant amounts of eosinophilic cytoplasm (C). Hematoxlyin and Eosin, 750x.

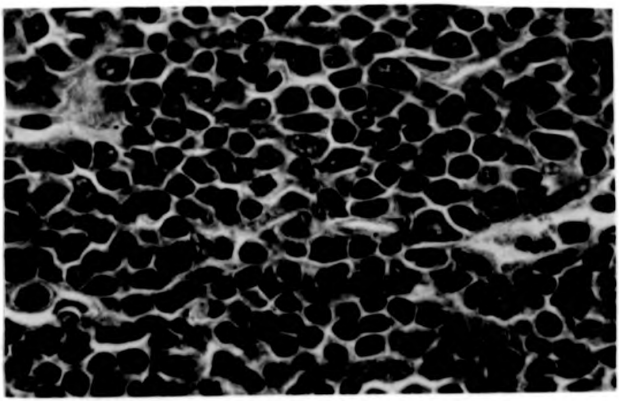
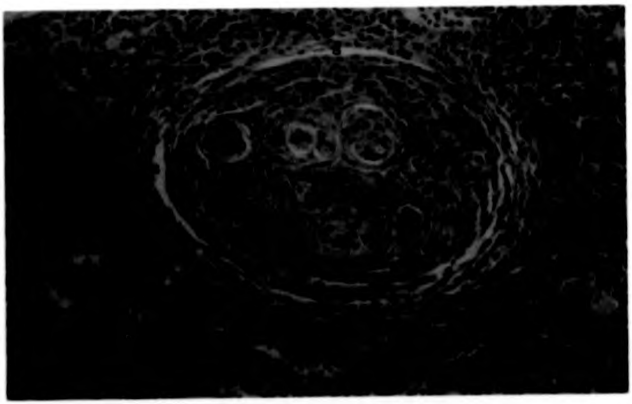


Figure 47. Apparently resolving histiocytic granuloma in the posterior kidney of a coho salmon challenged by cohabitation. Note the concentric arrangement of spindle shaped cells (s), margin of dispersed melanin (m), and infiltration of melanomacrophages (mm) in the perilesional region. Degenerate excretory components are evident in the nidus (e). Hematoxylin and Eosin, 300x.



Between 2 to 3 of 10 coho salmon from day 112 to day 168 featured mild focal to multifocal intravascular thrombosis. Thrombosis in the caudal vena cava or venules was not apparent in any of the Atlantic salmon.

There was a profound lack of extrarenal involvement of BKD over the course of most of this experiment in both coho and Atlantic salmon (Tables 6 and 7, section 4). Notable exceptions to this include the direct extension (rupture) of an inflammatory infiltrate associated with the renal cavitations, ventrally, to the swim bladder in 1 to 2 of 10 coho salmon between days 112 and 140. This direct extension occurred at various levels along the kidney. Periesophagitis with concurrent multifocal peritonitis was noted in 5 of 10 coho on day 196.

In addition, diffuse granulomatous endocarditis was evident in 1 to 2 of 10 coho on days 140 and 168; however, there was no apparent pericarditis and peripheral vascular involvement was limited in both species over the course of exposure.

Granulomatous to pyogranulomatous lesions were also localized to the liver, peritoneum, ovaries, and central nervous system.

In the liver, neither capsular nor subcapsular involvement was apparent in either species until day 196 of the infection (Tables 6 and 7, section 3). Initially, focal to multifocal(locally extensive) predominantly lymphohistiocytic granulomata from 0.5 to 1.2 cm in size were detected within the hepatic parenchyma (Figure 48). Attenuated, spindle shaped fibroblasts were arranged in a fine layer around the margin of the lesion. Hepatocytes, portal triads, and the vasculature adjacent to the nidi frequently appeared compressed.

Mild, focal (Figure 49) to severe diffuse (Figure 50) peritonitis was noted on day 196 in 5 of 10 coho and Atlantic

salmon. The infiltrate extended along the serosal surfaces between the pyloric caecae and associated exocrine acinar tissue and eventually enveloped the viscera.

Ovarian involvement was limited to 3 to 4 of 10 fish on days 140 and 196. Thecal vascular congestion and accumulation of mononuclear cells was evident with diffuse fibrin deposition around the ova (Figure 51).

Central nervous system lesions were apparent in 8 of 10 coho and 2 of 10 Atlantic salmon on day 168 and consisted of focal to multifocal granulomata associated with either the tela choroidea of the saccus dorsalis and epiphysis of the epithalamus, tela choroidea posteria, or within the dorsal aspect of the spinal cord immediately caudal to the vagal lobe (Tables 6 and 7, section 7).

Small cells 7 to 15 um each with an intensely basophilic nucleus and scant cytoplasm radiated in linear arrays from the superficial aspect of the cerebellum (Figure 52) through the molecular to the granular layer and infiltrated along the junction of these 2 cell layers (Figures 53 and 54). Accumulation of eosinophilic granular cells adjacent to the hypophysis was also noted in 4 coho salmon.

In coho salmon, branchial and skeletal muscular involvement was not detected until day 196 and consisted of multifocal granulomatous branchitis and myositis. Retrobulbar granulomatous opthalmitis was detected in only 1 coho salmon on day 168 and 2 coho on day 196.

Comparison of the sequential histopathology in coho and Atlantic salmon resulting from cohabitation with Rs-infected fish is presented in Table 8.

Figure 48. Mild, focal subacute to chronic fibrohistiocytic granuloma in the liver of a coho salmon. Note the massive accumulation of lymphocytes around the margin (l) and their infiltration (i) into the center of the lesion. Hematoxylin and Eosin, 300x.

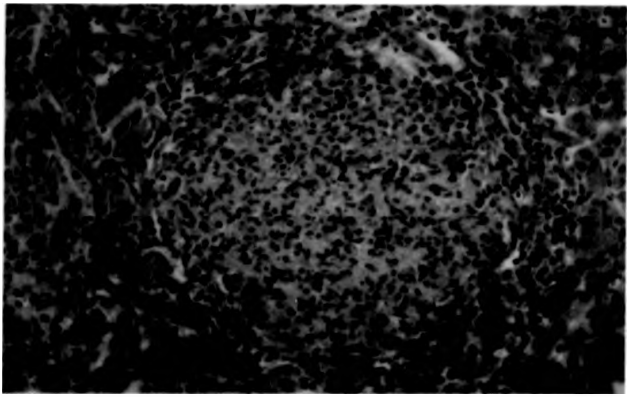


Figure 49. Locally extensive to diffuse, mild lymphohistiocytic infiltration between the pyloric caecae and the pancreatic and associated adipose tissue (I). Note the absence of fibrin within the abdominal cavity and mild hypercellularity (H) of the lamina propria of the pyloric ceacea. Hematoxylin and Eosin 75x.

Figure 50. Severe, diffuse chronic fibrohistiocytic peritonitis (P). Note the massive inflammatory response. The infiltrate has obliterated the adipose tissue and only small islands of exocrine pancreatic tissue persist (a). Hematoxylin and Eosin, 75x.

Figure 51. Early ovarian involvement in coho salmon challenged by cohabitation. An accumulation of erythrocytes and mononuclear cells is evident within the dilated thecal vasculature (t). Fibrin deposits are apparent around the margins of the ova (m). Hematoxylin and Eosin, 750x.

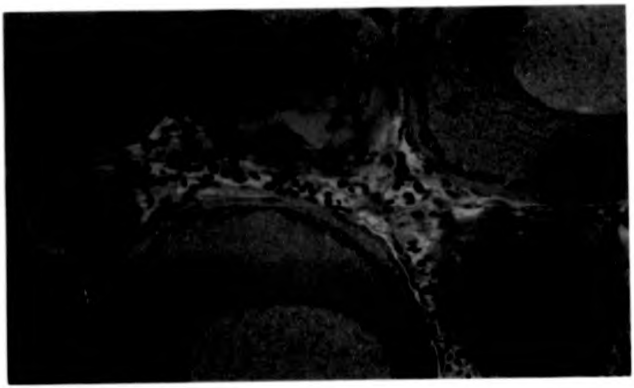


Figure 52. Early involvement of the superficial aspect of the of the cerebellum. Note the intensely basophilic nature of the cellular infiltrate (c). Hematoxylin and Eosin, 300x.

Figure 53. Over time the inflammatory infiltrate extends from the superficial aspect, through the parenchyma to the junction (J) of the molecular (m) and granular (g) layers. Note the degree of vascular involvement (v) in the underlying aspect of the cerebellum. Hematoxylin and Eosin, 190x.

Figure 54. High magnification of the inflammatory infiltrate (I) at the junction of the molecular (M) and granular (G) layers of the cerebellum. Hematoxylin and Eosin, 750x.

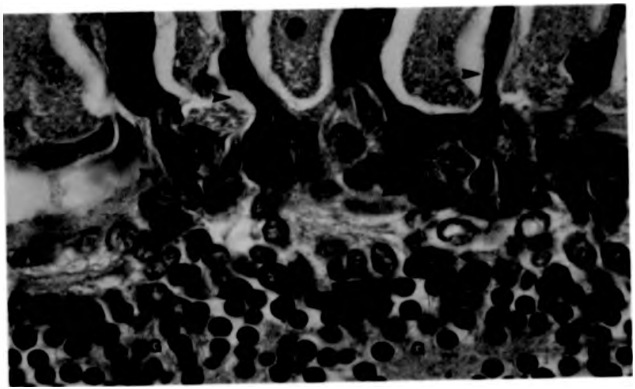


Table 6. Sequential Histopathology of Coho Infected by Cohabitation. Number of lesions/fish examined.

	Day 28	Day 56	Day 84	Day 112
1) Renal				
perivascular				
neutrophils	8/10	2/10	0/10	0/10
lymphocytes	10/10	10/10	0/10	4/9
interstitium				
granuloma				
focal	0/10	2/10	0/10	5/10
multifocal	0/10	2/10	2/10	2/10
diffuse	0/10	0/10	0/10	2/10
free Rs	0/10	0/10	0/10	0/10
blast like				
cells	0/10	10/10	10/10	10/10
pseudocysts	0/10	0/10	2/10	2/10
glomerular	0/10	0/10	0/10	2/10
tubular	0/10	0/10	0/10	0/10
intravascular				
thrombosis	0/10	0/10	0/10	3/10
2) Splenic				
ellipsoids	0/8	0/7	4/6	0/5
capsule	0/8	0/7	0/6	0/5
parenchyma	0/8	0/7	0/6	0/5

Table 6. Sequential Histopathology of Coho Infected by Cohabitation (Cont'd)

	Day 140	Day 168	Day 196
1) Renal			
perivascular			
neutrophils	2/10	1/10	2/10
lymphocytes	1/10	4/10	3/10
interstitium			
granuloma			
focal	3/10	2/10	0/10
multifocal	0/10	2/10	2/10
diffuse	0/10	0/10	4/10
free Rs	0/10	0/10	0/10
blast like cells	10/10	7/10	9/10
pseudocysts	4/10	1/10	0/10
glomerular	3/10	5/10	5/10
tubular	5/10	7/10	2/10
intravascular			
thrombosis	2/10	2/10	3/10
2) Splenic			
ellipsoids	2/8	4/6	1/6
capsule	0/8	0/6	1/6
parenchyma	2/8	1/6	0/6

Table 6. Sequential Histopathology of Coho Infected by Cohabitation (Cont'd)

	Day 28	Day 56	Day 84	Day 112
3) Hepatic				
capsule				
fibrin	0/10	0/10	0/10	0/10
granulomatous	0/10	0/10	0/10	0/10
parenchyma	0/10	0/10	0/10	0/10
4) Gastroenteric				
peritonitis				
focal	0/10	0/10	0/10	2/10
multifocal	0/10	0/10	0/10	0/10
free Rs	0/10	0/10	0/10	0/10
esophagitis	0/10	1/10	0/10	0/10
free Rs	0/10	0/10	0/10	0/10
swim bladder	0/10	0/10	0/10	0/10
5) Cardiovascular				
endocarditis	0/10	0/10	0/10	0/10
pericarditis	0/10	0/10	0/10	0/10
peripheral				
vasculature	0/10	0/10	0/10	0/10
6) Reproductive	0/10	0/10	0/10	0/10
7) Neural	0/10	0/10	0/10	0/10
8) Ocular	0/5	0/4	0/5	0/5
9) Branchial	0/10	0/10	0/10	0/10
10) Skeletal				
Musculature	0/10	0/10	0/10	0/10

Table 6. Sequential Histopathology of Coho Infected by Cohabitation (Cont'd)

	Day 140	Day 168	Day 196
3) Hepatic			
capsule			
fibrin	0/10	0/10	2/10
granulomatous	0/10	0/10	4/10
parenchyma	1/10	3/10	1/10
4) Gastroenteric			
peritonitis			
focal	0/10	0/10	1/10
multifocal	0/10	4/10	5/10
free Rs	0/10	0/10	0/10
esophagitis	2/10	0/10	5/10
free Rs	0/10	0/10	0/10
swim bladder	0/10	0/10	0/10
5) Cardiovascular			
endocarditis	2/10	2/10	2/10
pericarditis	1/10	0/10	1/10
peripheral			
vasculature	0/10	0/10	2/10
6) Reproductive	3/10	0/10	4/10
7) Neural	0/10	8/10	5/10
8) Ocular	0/6	1/6	2/6
9) Branchial	0/10	0/10	0/10
10) Skeletal			
Musculature	0/10	0/10	3/10

Table 7. Sequential Histopathology of Atlantic Salmon Infected by Cohabitation

	Day 28	Day 56	Day 84	Day 112
1) Renal				
perivascular				
neutrophils	1/10	2/10	0/10	2/10
lymphocytes	0/10	9/10	7/10	10/10
interstitium				
granuloma	0/10	0/10	2/10	0/10
focal	0/10	0/10	0/10	0/10
multifocal	0/10	0/10	7/10	8/10
diffuse	0/10	0/10	0/10	0/10
free Rs	0/10	0/10	0/10	0/10
blast like cells	0/10	6/10	9/10	10/10
pseudocysts	0/10	0/10	0/10	0/10
glomerular	0/10	0/10	0/10	2/10
tubular	0/10	0/10	0/10	0/10
intravascular				
thrombosis	0/10	0/10	0/10	0/10
2) Splenic				
ellipsoids	0/7	0/6	0/5	2/3
capsule	0/7	0/6	0/5	0/3
parenchyma	0/7	0/6	0/5	0/3

Table 7. Sequential Histopathology of Atlantic Salmon Infected by Cohabitation (Cont'd)

	Day 140	Day 168	Day 196
1) Renal			
perivascular			
neutrophils	3/10	0/10	1/10
lymphocytes	1/10	0/10	5/10
interstitium			
granuloma			
focal	2/10	2/10	3/10
multifocal	7/10	8/10	3/10
diffuse	0/10	0/10	3/10
free Rs	0/10	0/10	0/10
blast like cells	10/10	10/10	7/10
pseudocysts	0/10	0/10	0/10
glomerular	3/10	6/10	5/10
tubular	1/10	4/10	2/10
intravascular			
thrombosis	0/10	0/10	0/10
2) Splenic			
ellipsoids	0/5	0/8	1/7
capsule	0/5	0/8	0/7
parenchyma	2/5	0/8	1/7

Table 7. Sequential Histopathology of Atlantic Salmon Infected by Cohabitation (Cont'd)

	Day 28	Day 56	Day 84	Day 112
Hepatic				
capsule				
fibrin	0/10	0/10	0/10	0/10
granulomatous	0/10	0/10	0/10	0/10
parenchyma	0/10	0/10	0/10	0/10
4) Gastroenteric				
peritonitis				
focal	0/10	1/10	0/10	0/10
multifocal	0/10	0/10	0/10	0/10
free Rs	0/10	0/10	0/10	0/10
esophagitis	0/10	1/10	0/10	0/10
free Rs	0/10	0/10	0/10	0/10
swim bladder	0/10	0/10	0/10	0/10
5) Cardiovascular				
endocarditis	0/10	0/10	0/10	0/10
pericarditis	0/10	0/10	0/10	0/10
peripheral				
vasculature	0/10	0/10	0/10	0/10
6) Reproductive	0/10	0/10	0/10	0/10
7) Neural	0/10	0/10	0/10	0/10
8) Ocular	0/4	0/6	0/6	0/5
9) Branchial	0/10	0/10	0/10	0/10
10) Skeletal				
Musculature	0/10	0/10	0/10	0/10

3)

Table 7. Sequential Histopathology of Atlantic Salmon Infected by Cohabitation (Cont'd)

	Day 140	Day 168	Day 196
3) Hepatic			
capsule			
fibrin	0/10	0/10	0/10
granulomatous	0/10	0/10	0/10
parenchyma	0/10	1/10	0/10
4) Gastroenteric			
peritonitis			
focal	0/10	0/10	0/10
multifocal	0/10	0/10	1/10
free Rs	0/10	0/10	0/10
esophagitis	0/10	0/10	0/10
free Rs	0/10	0/10	0/10
swim bladder	3/10	3/10	0/10
5) Cardiovascular			
endocarditis	0/10	2/10	2/10
pericarditis	0/10	1/10	0/10
peripheral			
vasculature	0/10	0/10	2/10
6) Reproductive	0/10	1/10	1/10
7) Neural	0/10	0/10	3/10
8) Ocular	0/6	0/6	0/8
9) Branchial	0/10	0/10	0/10
10) Skeletal			
Musculature	0/10	0/10	0/10

Table 8 Comparison of the Sequential Histopathology of Atlantic and Coho Salmon Challenged with Rs by Cohabitation

	Coho	Atlantic
1) Renal		
perivascular		
neutrophils	present	rare
interstitium		
granuloma	histiocytic	tuberculoid
free Rs	absent	absent
mononuclear cell	present	present
pseudocyst	present	absent
tubular involvement	limited	limited
intravascular thrombosis	limited	absent
2) Splenic		
ellipsoids	rare	rare
capsule	absent	absent
parenchyma	absent	present
3) Hepatic		
capsule		
fibrin	limited	absent
granulomatous	limited	absent
parenchyma	limited	limited
4) Gastroenteric		
peritonitis		
multifocal	rare	absent
free Rs	absent	absent
esophagitis	limited	absent
free Rs	absent	absent
swim bladder	absent	rare
5) Cardiovascular		
endocarditis	diffuse	diffuse
pericarditis	absent	absent
peripheral vasculature	limited	absent
6) Reproductive	rare	absent
7) Neural	present	rare
8) Ocular	present	rare
9) Branchial	absent	absent
10) Skeletal Musculature	limited	absent

DISCUSSION

Past studies on the histopathology of BKD have focused on either case reports of (natural) epizootics (Wood and Yasutake, 1956; Smith, 1964; Hendricks and Leek, 1977; Ellis, Novtony, and Harrell, 1978; Hoffman, Popp, and van de Graaff, 1984) or on serial samples of experimentally challenged fish (Young and Chapman, 1978; Bruno, 1986b). Interpretation of these results, however, is difficult due to a number of intrinsic (age, species, strain, genetic composition, intercurrent infection, or nutritional status) and extrinsic (water quality, challenge dose, or stocking density) variables involved in the studies. To circumvent these difficulties, coho and Atlantic salmon with a known disease history and stock origin were challenged, inter-alia, by cohabitation with Rs-inoculated fish to mimic the natural recruitment and pathogenesis of infection. The fish were then serially sampled for histopathological evaluation.

In Chapter II, fish succumbed to BKD between 32 and 96 days post-Rs injection. In this investigation, histopathological expression of BKD and significant mortalities attributed to Rs were not incurred until 164 to 196 days, a prepatent interval more consistent with natural infections (Murray et al, 1992). Interestingly, in contrast to what has been experienced in the salt water (ongrowing) phase of production (Brackett, Newbound, Coombs et al, 1990), Atlantic salmon (4-6 gm in size) in the freshwater stage appeared more susceptible to Rs infection than coho (Figure 43). This observation may be attributed to the observed cresentic glomerulonephritis or some other, yet undefined pathogenic mechanism in Atlantic salmon.

Challenge by cohabitation yielded a marked delay in the onset of histopathological expression of BKD, as well as a

distinct renal site predilection in comparison to inoculated fish (Tables 9 and 10).

Incipient lesions were uniform and similar to those described in Chapter II (Table 5); however, the progression from an acute or subacute to a chronic stage resulted in a number of novel sequelae.

Preliminary histological features of BKD detected in coho and Atlantic salmon included the sequential perivascular accumulation of granulocytes, lymphocytes, and mononuclear-like cells around the caudal vena cava and pronephric sinusoids. The transition between successive cell lineages extended over a longer time interval than in injected fish and featured a more segmental distribution (Chapter II).

Lymphocytes were more prominent and persisted for longer periods in coho and Atlantic salmon challenged by cohabitation than by ip injection. Klontz (1972) reported that fish inoculated with bacterin demonstrated numerous lymphocytes within the pronephric interstitium, apparently in response to antigenic stimulation. Moreover, improved cell-mediated immunity (CMI) (Nikl, Albright, and Evelyn, 1991) and a peripheral lymphocytosis (Bruno and Munro, 1986b) have been reported in fish challenged with formalinized and viable Rs, respectively.

Despite the foregoing, in vitro experiments have suggested that an immunosuppressive factor (p57) is produced by Rs (Turaga, Wiens, and Kattaari, 1987) and in vivo studies with fish challenged by ip inoculation of Rs resulted in a profound depletion of lymphocytes (within lymphoid elements), hypogammaglobinemia, and exuberant extracellular Rs (Chapter II). These results suggest autochthonous or allochthonous factors may abrogate the host response to infection and exacerbate its pathogenesis in ip challenged fish. A further appreciation of

lymphocyte heterogeneity and cytokine interaction with immune and inflammatory cells, particularly macrophages or monocytes, may resolve this phenomenon.

In rainbow trout (Oncorhynchus mykiss), nonspecific cell mediated immunity has a profound effect against the pleurocercoids of Diplostomum spathaceum (the etiological agent of diplostomosis)(Hoglund and Thuvander, 1990). In vitro this effect has been demonstrated to be dependent on the ratio of activated macrophages to parasitic larvae (Whyte, Chappell, and Secombes, 1989). In fish challenged by ip injection, the ratio of Rs cells to macrophages may be such that nonspecific defense mechanisms of the host are overwhelmed. In contrast, fish challenged by cohabitation may benefit from a more favorable Rs/macrophage ratio and thus have a greater opportunity for antigen processing and for elaborating an inflammatory response to ward off infection.

In coho salmon challenged by cohabitation, histiocytic granulomata were detected in 2 to 4 of 10 fish over the course of infection; the granulocytes appeared to be more well differentiated than those observed in fish challenged by ip injection (Chapter II). Melanin granules were more frequently interspersed around and within nidi, macrophages acquired a more cuboidal appearance, and intra- and extracellular Rs occurred in lower numbers.

A markedly hyperplastic interstitium, composed predominantly of mononuclear-like cells (Chapter II) was noted in 2 to 3 of 10 fish on day 144 and 168. Displacement and compression of the excretory elements of the posterior kidney and degenerative changes in the renal tubular epithelium were also apparent. Periarteriolar investment of these hyperplastic cells also occurred in the spleen of 3 coho salmon. These changes are

similar to incipient stages of marine anemia (MA) (plasmacytoid leukemia)(Kent, Groff, Traxler et al, 1990) and further evaluation of the histogenesis of both BKD and MA is warranted.

A novel sequela, limited to coho salmon challenged by cohabitation, was the development of focal to multifocal, unilocular pseudocysts within the renal interstitium. Balouet and Baudin Laurencin (1986) noted cystic nodules in rainbow trout (Oncorhynchus mykiss) and turbot (Scophthalmus maximus) 30 to 90 days post-injection with inert (nonantigenic) particulate matter (talc). A pathogenic mechanism was not adduced. By analogy with mammalian pseudocysts, it seems likely that hypersecretion of proteolytic enzymes, or dys-inhibition of regulatory factors (alfa-1-anti trypsin) accounts for these lesions (Cotran, Kumar and Robbins, 1989), or alternatively, expansion of lesions may excede their vascular supply and infarction may ensue.

Although the onset of histological expression and mortalities was protracted in fish challenged by cohabitation, the histogenesis and prevalence of renal interstitial granulomata in Atlantic salmon was similar to that observed in fish challenged by ip injection of 10^3 Rs (Chapter II). Histopathology of the abdominal cavity indicated a profound lack of peritoneal involvement until day 196 in fish challenged to Rs by cohabitation (involvement was noted on day 20 by ip injection). Lesions were infrequent and limited primarily to the parenchyma of the viscera.

In the spleen, with the exception of immature granulomata in 2 on day 140, few pathological changes were noted. Periarteriolar accumulation of lymphocytes, interstitial depletion of lymphocytes, and ellipsoid hyperplasia were not detected. These lesions may have occurred later, or alternatively, may have occurred in a transient manner and thus

escaped detection due to the sampling intervals.

Similarly, focal to multifocal granulomata in the parenchyma of the liver were detected in 1 to 3 of 10 fish on days 140, 168 and 196. This localization was consistent with an hematogenous dissemination of Rs (either via the hepatic artery or the hepatic portal system). In ip challenged fish it was the result of direct extension or seeding from adjacent tissue (Chapter II). A renal, rather than hepatic, site predilection is attributed to lack of Kupfer's cells in the liver and localization of the mononuclear phagocytic system in the vascular sinusoids of the kidney (Ferguson, 1989).

Periesophagitis was limited to 2 of 10 fish at day 140 and 5 of 10 fish at day 196. Nevertheless, the focal to multifocal distribution of inflammatory infiltrate suggests an hematogenous dissemination. Expansion of the infiltrate along serosal surfaces was consistent with the observations of Bruno (1986b).

Cardiovascular involvement was limited to 1 to 2 of 10 fish in each of the species. In comparison to rainbow trout (O mykiss) and plaice (Pleuronectes platessa) (Ferguson, 1975; Roberts, 1989), the endocardium of these salmonid species did not appear to be as actively phagocytic. A sparse mononuclear phagocytic population was evident and frequently these cells were replete with Rs. After 28 to 56 days, diffuse accumulations of macrophages or monocytes occurred on the myocardial trabeculae, suggesting a post-challenge recruitment of phagocytes.

Vascular seeding, rather than direct extension or seeding of Rs from adjacent tissue, also appeared to occur in the ovaries and testes of coho and Atlantic salmon challenged by cohabitation. Septic emboli, microthrombi, and inflammatory infiltrates were evident within the thecal microvasculature and consistent with observations of Armstrong (1989) and Bruno and

Munro (1986a). It seems likely that in the acute infections, follicular cells phagocytize Rs from contaminated ascites (Chapter II); whereas, in chronic infections, Rs may localize to the gonads via the circulatory system. Special stains failed to demonstrate Rs within oogonia of fish challenged by cohabitation.

As with ovarian tissue, profound differences between ip injected and cohabitation challenged fish were observed in the central nervous system (CNS). In addition to the intravascular localization of Rs reported by Speare et al (1991) and in Chapter II, 3 lesions were consistently observed in coho salmon. The first was a granulomatous meningitis overlying the optic tectum, the second was a profound (possibly granulomatous) encephalitis of the molecular and granular layers of the cerebellum; and the third was a marked accumulation of eosinophilic granular cells associated with the hypophysis.

CNS lesions appear to arise from thrombo-emboli (often with multisystemic involvement) in acute septicemia, as with infectious thrombo-embolic meningoencephalitis (ITEME)(Haemophilus somnus) in cattle (Harris and Janzen, 1989). The recent recognition of Rs-induced meningoencephalitis in Pacific and Atlantic salmon production stocks has been attributed to a post-treatment effect, development of a new strain or tropism of Rs, as well as intercurrent infection with some other pathogen. In this study, severe encephalitis was observed in nontreated fish challenged with an Rs isolated in 1985, which suggests that the CNS form of BKD may have been present in past cases and previously unrecognized or, alternatively, some yet defined environmental or husbandry factor may have contributed to the emergence of this tissue tropism. It is important to note that in mammals renal metabolic dysfunctions have been noted to induce encephalopathies (Cotran et al, 1989).

Dissemination of Rs to skeletal muscle, observed in this experiment, was consistent with that of natural infections (Klontz, 1983) and appeared to occur along fascial planes or via the microvasculature.

Table 9 Comparison of the Sequential Histopathology in Coho Salmon Challenged with Rs by Intraperitoneal Injection and Cohabitation

	Injection	Cohabitation
1) Renal		
perivascular		
neutrophils	prominent	occasional
interstitium		
granulomata	histiocytic	histiocytic
free Rs	present	absent
blast like cell	present	present
pseudocyst	absent	present
tubular involvement	hyaline	limited
intravascular thrombosis	present	limited
2) Splenic		
ellipsoids	prominent	rare
capsule	present	absent
parenchyma	absent	present
3) Hepatic		
capsule		
fibrin	present	limited
granulomata	present	limited
parenchyma	limited	present
4) Gastroenteric		
peritonitis		
multifocal	present	limited
free Rs	present	absent
esophagitis	present	limited
free Rs	present	absent
swim bladder	present	rare
5) Cardiovascular		
endocarditis	focal	diffuse
pericarditis	diffuse	rare
peripheral vasculature	present	rare
6) Reproductive	extensive	rare
7) Neural	present	present
8) Ocular	present	rare
9) Branchial	present	absent
10) Skeletal Musculature	absent	limited

Table 10 Comparison of the Sequential Histopathology of Atlantic Salmon Challenged with Rs by Intraperitoneal Injection and Cohabitation

	Injection	Cohabitation
1) Renal		
perivascular		
neutrophils	prominent	occasional
interstitium		
granuloma	tuberculoid	tuberculoid
free Rs	present	absent
blast like cell	present	present
pseudocyst	absent	absent
tubular involvement	limited	limited
intravascular thrombosis	absent	absent
2) Splenic		
ellipsoids	prominent	rare
capsule	present	absent
parenchyma	absent	present
3) Hepatic		
capsule	present	absent
fibrin	present	absent
granulomatous	present	absent
parenchyma	rare	present
4) Gastroenteric		
peritonitis		
multifocal	present	rare
free Rs	present	absent
esophagitis	limited	absent
free Rs	limited	absent
swim bladder	present	rare
5) Cardiovascular		
endocarditis	focal	diffuse
pericarditis	focal	absent
peripheral vasculature	present	absent
6) Reproductive	extensive	limited
7) Neural	present	limited
8) Ocular	present	rare
9) Branchial	present	absent
10) Skeletal Musculature	absent	absent

Chapter IV

Corticosteroid Modulation of the Inflammatory Response to Bacterial Kidney Disease (*Renibacterium salmoninarum*) in Coho and Atlantic Salmon

Introduction

Serial histopathology of coho and Atlantic salmon challenged with Renibacterium salmoninarum by intraperitoneal injection revealed a profound cell mediated immune response (Wood and Yasutake, 1956; Bruno, 1986; Chapter II; Chapter III). Histopathological expression of BKD was characterized as principally histiocytic in coho and tuberculoid in Atlantic salmon, both forms contingent on the interaction of 2 cell lineages, the lymphocytic and myeloid (monocytes and macrophages).

In humans and experimental animals glucocorticosteroids have been employed to further refine the understanding of cellular interactions and protective mechanisms to a number of different pathogens. In vitro and in vivo studies have demonstrated that antimicrobial activity of corticosteroids treated macrophages could be restored with administration of gamma interferon against Listeria and Salmonella, but not Nocardia, or Aspergillus (North, 1971; Rinehart, Sagone, Balcerzak et al, 1975; Rook, Steele, Ainsworth and Leveton, 1987). These observations were attributed to complex cellular interactions and subcellular metabolism with corticosteroids.

In fish, past studies on immunosuppression and disease susceptibility have focused principally on the humeral response to a number of environmental or physical stressors or to the administration of corticosteroids (Anderson, Robertson, and Dixon, 1982; Ruglys, 1985; Anderson, 1990, Rainger, Rowley and Pettitt, 1992). In more recent work by MacArthur, Fletcher, Pirie et al (1983), Kent and Hedrick (1987), and Johnson and Albright (unpublished data) with cortisol implanted fish (for a sustained release of corticosteroid), featured a profound

inhibition of macrophage recruitment, granuloma formation and resolution of lesions.

To further appreciate the inflammatory response of coho and Atlantic salmon to Rs, this study was undertaken to ascertain the effect of chronic glucocorticosteroid administration on the histogenesis of BKD.

Materials and Methods

Coho and Atlantic salmon, 4 to 6 gm in size were obtained from Rosewall Creek Hatchery and United Hatchery, respectively and transported to the Pacific Biological station (Nanaimo, British Columbia). On arrival, individuals from each species were randomly divided into 4 groups of 50 fish and placed in 50 l flow through pot tanks supplied with dechlorinated municipal water.

After 1 week acclimation, individual groups were anaesthetized, 2 control groups from each species were ip injected with sterile peptone-saline and 2 challenge groups were implanted with a synthetic glucocorticosteroid. The anti-inflammatory was prepared as an emulsion of liquefied coconut butter (melted at 60 C) and hydrocortisol (Sigma) at a concentration of 25 mg/ml. Individual fish were administered 0.1 ml ip, for an immunosuppressive dose of 0.50 mg/g (Kent and Hedrick, 1987).

After a 1 week recovery period, fish were again anesthetized with MS222 and from each species, 1 corticosteroid and 1 peptone-saline treated group were challenged ip with 0.1 ml of 10^4 Rs, the 2 remaining cohorts were inoculated with 0.1 ml sterile peptone saline.

Fish were retained in individual pot tanks, fed a daily

ration of 2% body weight, and mortalities were recorded and removed for later microbiological evaluation.

On days 7, 28, 42, and 56 between 3 to 5 fish were sampled for histopathology. Fish were killed in a solution of MS222, the ventral abdominal musculature incised, then preserved in Davidson's solution. Parasaggital blocks of the abdomen and saggital blocks of the cranium were prepared and processed by routine histological techniques. Sections were cut to a thickness of 5 um and stained with Hematoxylin and Eosin, Schiff's Periodic Acid, Martius Scarlet Blue, and Masson's Trichrome. Slides were evaluated histologically, lesions characterized and quantified as in chapter 1.

To assay cortisol titers, on day 28, 5 fish from each cohort were euthanized in MS222 and 0.5 ml blood obtained in heparinized microhematocrit tubes (Sigma) by caudal venipuncture. Individual samples were centrifuged at 6500 rpm for 5 minutes, the plasma decanted and stored at -70 C. Prepared samples were forwarded to the Western College of Veterinary Medicine and an enzyme immunoassay technique was employed to determine plasma cortisol titers (Munro and Stabenfeldt, 1985) .

Results

Coho and Atlantic salmon immunosuppressed by exogenous corticosteroids incured an earlier onset and greater rate of mortality when challenged with 10^5 Rs (Figure 55), than immunocompetent fish challenged with the same level of bacteria (Figure 56)(Chapter II).

Figure 55. Mortality curve of immunosuppressed coho and Atlantic salmon challenged with 10^5 Rs

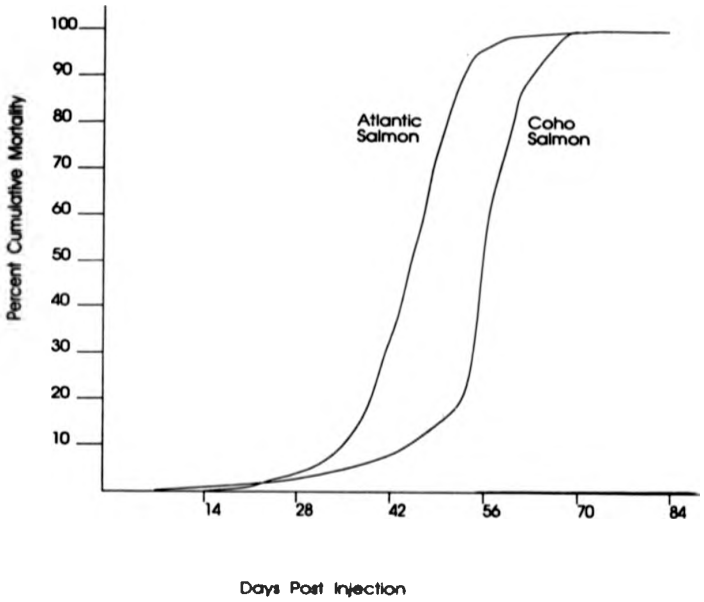
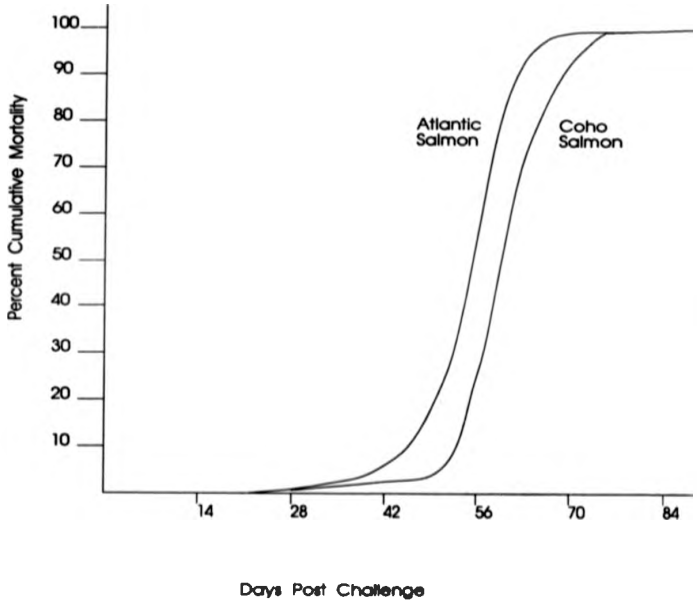


Figure 56 Cumulative mortalities of coho and Atlantic salmon challenged ip with 10^5 Rs (Chapter II)



Histopathology of coho and Atlantic salmon 7 days post-Rs infection revealed multifocal to disseminated granulomata composed predominantly of neutrophils, interspersed lymphocytes and melanin granules (Tables 11 and 12, number 1)(Figure 57). There was a variable number of lymphocytes in the perilesional tissue. Scant to minimal infiltration was evident around the nidus with minimal accumulation of lymphocytes in the lumen of the pronephric sinusoids adjacent to the granuloma as in chapter II. The interstitium is mildly hyperplastic and composed predominantly of the large mononuclear-like cells (Chapter II), lymphomyeloid progenitor cells and a variable number of lymphocytes. Melanomacrophage recruitment contiguous to granulomata was occasionally noted in both coho and Atlantic salmon. Extrarenal involvement was minimal (Tables 11 and 12, numbers 2-10).

By day 28, there was a profound histiocytic response in the renal interstitium of the coho salmon. Granulomata were irregular and expansive. Macrophages infiltration varied from locally extensive to diffuse involvement with replacement of virtually all the hematopoietic elements. In 6 of 9 fish, on day 28 and 5 of 7 fish on day 42, exuberant extracellular and intracellular Rs growth was apparent (Figue 58).

On days 28 and 42, in 4 to 5 fish necrotic cells 7 to 10 um in size with pyknotic nuclei and refractile cytoplasm are evident in nidi and throughout the interstitium (Figure 59). These cell frequently were the only lineage affected and morphologically were consistent with lymphocytes.

In contrast to the histiocytic inflammation of coho salmon, Atlantic salmon featured a more tuberculoid granulomatous response (Chapter II).

Figure 57 Histopathological section of the posterior kidney of an immunosuppressed coho salmon 7 days post-infection with 10^4 Rs. Note the intracellular accumulation of Rs within phagocytes ( ) and the proteinaceous material within Bowman's space (P). H and E. 300x

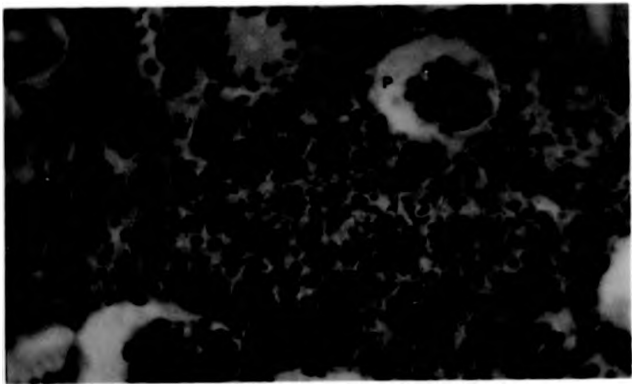


Figure 58. Posterior kidney of an immunosuppressed coho salmon on day 28. Note the exuberant intra- and extracellular growth of Rs (R). H and E. 300x

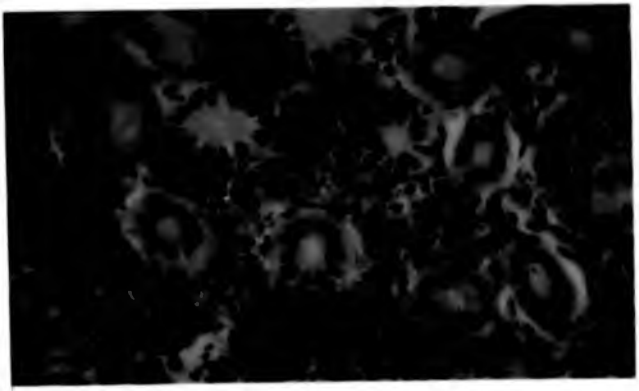
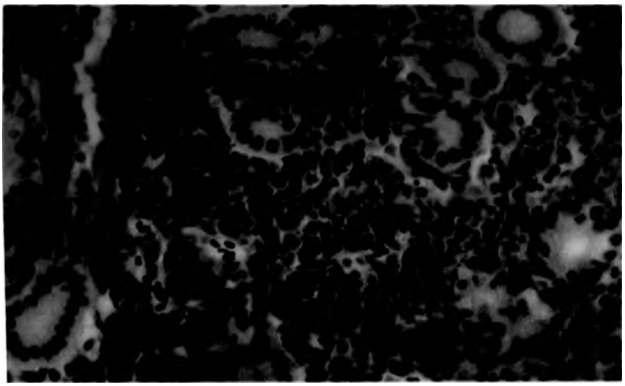


Figure 59. Photomicrograph of the posterior kidney of an immunosuppressed coho salmon 28 days post-infection. Note intravascular macrophages with phagocytized nuclear debris (a) and interstitial karyorrhexis and pyknosis ( ). H and E. 300x



The incipient stages of Rs infections in Atlantic salmon were consistent with those described for the coho. The majority of fish (6 of 7 fish on day 28 and 5 of 7 fish on day 42) featured multifocal granulomata (Table 12, number 1). On comparison to the inflammatory response noted in chapter II, these lesions were composed primarily of fibrin, extracellular ground substance, neutrophils, macrophages and lymphocytes. Fibroplasia was infrequently observed. The lesions progressed and consisted predominantly of histiocytes and eventually florid Rs growth was noted within phagocytes; necrosis ensued and bacteria were liberated in the interstitium. Neutrophils, histiocytes and occasional lymphocytes were scattered throughout the Rs. The perilesional area featured abundant karyorrhectic debris.

Administration of corticosteroids also results in a marked reduction of fibrosis and Langerhans giant cell formation in Atlantic salmon (Table 12, section 1).

In immunosuppressed fish challenged with 10^4 Rs involvement of the excretory component was histologically consistent lesions described in Chapter II. However, in the acute stage of infection accumulation of finely granular eosinophilic material was evident in Bowman's space, suggestive of some form of glomerular injury and excretion of proteinaceous filtrate. This glomerulopathy may be a corticosteroid induced injury or a direct effect of the Rs pathogen.

Extrarenal involvement in immunosuppressed coho and Atlantic salmon occurred earlier, more frequently and more diffusely than in immunocompetent fish. Florid extracellular accumulations of Rs (occasionally admixed with fibrin) were apparent on the serosal surface of the viscera with a paucity of inflammatory response (Figures 60 and 61).

Plasma cortisol levels of fish sampled on day 28 were 18 nmol/ml for control fish, 123 nmol/ml for implanted coho, and 185 nmol/ml for Atlantic salmon.

Figure 60. Florid extracellular accumulation of bacteria admixed with fibrin on the serosal surface of the viscera in an immunosuppressed coho salmon 28 days post-Rs infection (R). Note the paucity of inflammatory infiltrate within the lumen of the microvasculature ( ). H and E. 300x

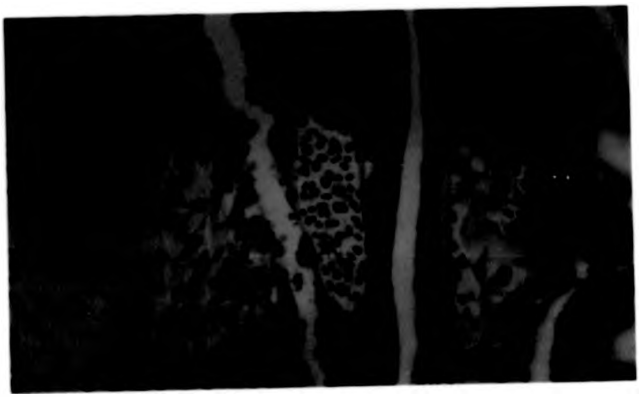


Figure 61. Massive accumulation of Rs and fibrin associated with the microvasculature of the abdominal adipose tissue ( ). Note the scant inflammatory infiltrate composed predominantly of mononuclear cells and lymphocytes (S). H and E. 300x

Table 11 Immunosuppressed Coho salmon challenged with 10^4 Rs (Fractional nos= no. fish with indicated feature/no. of fish examined)

	Day 7	Day 28	Day 42
1) Renal			
perivascular			
neutrophils	0/5	0/8	0/7
interstitium			
hypocellular	3/5	7/16	1/6
blast-like			
cells	5/5	8/9	4/6
granuloma			
focal	6/7	4/17	1/7
multifocal	0/7	15/17	6/7
glomerular	1/5	2/8	2/5
tubular	0/5	4/14	2/4
intra-vascular			
thrombosis	0/10	0/8	0/7
2) Splenic			
sinusoids	1/3	1/4	2/5
capsule	0/3	1/4	2/5
parenchyma	1/3	2/4	2/5
3) Hepatic			
capsule			
fibrin	0/10	8/12	3/8
granulomatous	0/10	2/12	2/8
free Rs	0/10	5/12	4/8
parenchyma	0/10	3/12	1/8

Table 11 Immunosuppressed Coho salmon challenged with 10^5 Rs

	Day 7	Day 28	Day 42
4) Gastrointestinal			
peritonitis			
focal	0/5	2/8	0/7
multifocal	0/5	3/8	0/7
diffuse	0/5	5/8	3/6
free Rs	0/5	7/8	6/7
esophagitis	1/4	1/8	1/4
free Rs	0/4	5/8	3/4
swim bladder	0/4	3/6	3/7
5) Cardiovascular			
endocarditis	0/5	2/8	0/6
pericarditis	0/5	0/8	2/6
peripheral vasculature	0/5	0/7	0/6
6) Reproductive	0/5	0/6	0/7
7) Neural	0/5	2/6	2/7
8) Ocular	0/2	0/3	0/3
9) Branchial	0/5	0/8	0/7
10) Skeletal			
Musculature	0/7	0/16	0/6

Table 12 Immunosuppressed Atlantic salmon challenged with 10^5 Rs (Fractional nos= no. fish with indicated feature/no. of fish examined)

	Day 7	Day 28	Day 42	1)
Renal				
perivascular				
neutrophils	0/5	0/7	0/7	
interstitium				
granuloma				
focal	4/8	0/7	0/7	
multifocal	0/8	6/7	5/7	
fibrosis	0/8	0/7	2/7	
glomerular	0/8	2/7	3/7	
tubular	0/8	2/6	4/6	
intra-vascular				
thrombosis	0/8	0/6	1/7	
2) Splenic				
ellipsoids	0/4	2/4	2/5	
capsule	0/4	3/4	5/5	
parenchyma	1/4	3/4	5/5	
3) Hepatic				
capsule				
fibrin	0/5	3/6	0/7	
granulomatous	0/5	2/6	3/7	
free Rs	0/5	3/6	5/7	
parenchyma	0/5	0/6	0/7	
perivascular cuffing	0/5	0/6	1/7	

Table 12 Immunosuppressed Atlantic salmon challenged with 10^1 Rs

	Day 7	Day 28	Day 42
4) Gastrointestinal			
peritonitis			
focal	2/7	0/7	0/7
multifocal	0/7	5/7	3/7
free Rs	0/7	5/7	3/6
esophagitis	3/4	4/5	1/5
free Rs	0/4	3/5	3/5
swim bladder	3/3	3/5	3/7
5) Cardiovascular			
endocarditis	2/4	0/7	0/7
pericarditis	0/4	0/7	0/7
peripheral			
vasculature	0/7	0/8	0/7
6) Reproductive	1/4	2/7	1/7
7) Neural	0/5	2/7	2/7
8) Ocular	0/2	0/3	1/5
9) Branchial	0/5	0/7	0/7
10) Skeletal			
Musculature	0/5	0/7	0/7

DISCUSSION

Administration of suprapharmacologic doses of corticosteroid in coho and Atlantic salmon yielded an earlier onset and increased mortality rate when challenged with Rs than immunocompetent fish with a comparable level (Chapter II). These data were consistent with studies employing acute stressors, such as handling or transportation (Strange, Schreck and Ewing, 1978; Ellsaesser and Clem, 1986; Robertson, Thomas, Arnold and Trant, 1987), which resulted in a marked increase in mortality rates, as well as reduced mean time to death (Maule and Schreck, 1990). The prime factor in the pathogenesis of these infectious disease is immunosuppression (Barton and Iwama, 1991).

In a similar manner, serial histopathology of cortisol implanted coho and Atlantic salmon challenged with 10^3 Rs revealed a profound modulation of the inflammatory response which may be attributed to the immunosuppressive effects (cell mediated) of glucocorticosteroids (Table 13).

In the early stages of infection the two major differences between immunosuppressed and immunocompetent coho and Atlantic salmon were the multifocal distribution of granulomata and lack of lymphocytic response in the perilesional tissue.

Impaired macrophage bactericidal activity may facilitate seeding of blood-borne Rs within the pronephric interstitium. Angilidis, Baudin-Laurincin and Youinou (1987) have demonstrated a profound reduction in chemiluminescence activity of pronephric phagocytes isolated from stressed rainbow trout.

In man and animals suprapharmacologic doses of corticosteroids result in a profound reduction <u>in vivo</u> in monocyte bactericidal activity (Rook et al, 1987), random migration, chemotaxis (Rinehart et al, 1974), antigen processing

and cell mediated hypersensitivity response (Salmon and Higgs, 1987).

Histiocytosis with exuberant intra- and extracellular proliferation of Rs was observed by day 28 in immunosuppressed coho and Atlantic salmon and may be attributed to monocyte dysfunction as well as impaired cell mediated immunity (MacArthur et al, 1983; Angilidis et al, 1987).

On days 28 and 42, necrosis of cells within the interstitium of immunosuppressed fish was noted and consisted of predominantly cells of the lymphoctic series. Lymphocytolytic activity has been noted with glucocorticoids administered to mice and rabbits (whereas man, guinea pigs and rhesus monkeys appeared resistant)(Frenkel and Havenhill, 1963; Claman, Moorhead and Benner, 1971) and was considered a contributing factor in the observed peripheral lymphopenia in immunosuppressed fish (McLeay, 1973).

Reduced levels of circulative lymphocytes have also been attributed to redistribution of cells from the vasculature to lymphoreticular compartments. In 3 of 5 fish on day 7 and 4 of 9 fish on day 28, however, the renal interstitium appeared markedly hypocellular and the calibre of the sinusoids enlarged. Peters, Nubgen, Raabe and Mock (1991) noted that in addition to lymphocytolysis and redistribution lymphopenia, atypical maturation may also have accounted for this observation. Interestingly, glucocorticosteroid modulate growth factors in mammals (GM-CS, IL-3)(Bowen and Fauci, 1988) and may, by analogy, contribute to this phenomenon in fish.

In immunosuppressed Atlantic salmon incipient stages of BKD were consistent with those described in Chapter II. However, by day 28 granulomata appeared to be irregular and progressive with no evidence of resolution. As with coho salmon it is interesting

to speculate that Rs growth in Atlantic salmon was not limited in immunosuppressed fish macrophages (or histiocytes) because these phagocytes were not immunologically primed.

In higher vertebrates tuberculoid granulomata are due to a number of etiopathogeneses which require macrophages, lymphocytes and secreted immunomodulatory factors (cytokines). Lymphocytes are characteristically scattered throughout the nidus or encircle the lesion in the form of a lymphocytic mantle. In mammals, the lymphocytes in the centre of the granulomata are CD4 helper/inducer cells; whereas, those which encircle the lesion are CD8 suppressor/cytotoxic cells (Sheffield, 1990; Chapter II).

The CD4 cells recognize novel antigens via class II major histocompatibility complex (MHC), and secrete lymphokines, such as gamma-interferon (IF) and interleukin-2 (IL-2), which activate macrophages and restrict replication of intracellular pathogens. In contrast, the CD8 suppressor/cytotoxic cells recognize antigens associated with class I MHC and initiate lysis of target cells expressing bacterial antigens.

In mammals and lower vertebrates, glucocorticosteroids preferentially inhibit CD4 helper/inducer lymphocyte activity (Bowen and Fauci, 1988; Hakim, 1988). As a result, on antigenic stimulation cytokine secretion may be impaired and macrophage activation inhibited. This mechanism may account for the rapid accumulation of intra- and extracellular Rs in corticosteroid treated fish versus immunocompetent fish (Table 13). Moreover, with elevated cortisol levels CD8 cell function may be unaffected or even enhanced, resulting in excessive lysis of viable uninfected host tissue. Further evaluation of this phenomenon is warranted to appreciate the possible contribution of these cells and cytokines in the pathogenesis of granulomatous processes in fish.

Immunosuppressed Atlantic salmon featured a marked reduction of fibrosis and Langhans giant cell formation. With the former, administration of anti-inflammatory agents has resulted in a profound inhibition of collagen synthesis in humans (in both normal and inflamed tissue) at the level of protocollagen gene transcription (Cutroneo, DiPetrillo and Cutroneo, 1990); whereas, in the latter, glucocorticosteroids abrogate giant cell formation in vitro through inhibition of lymphokine mediated fusion (macrophage fusion factor)(Galino, 1984).

Extrarenal involvement in immunosuppressed coho and Atlantic salmon featured a profound lack of inflammatory response and occurred earlier, more frequently and more diffusely than in immunocompetent fish. This observation is consistent with those of MacArthur et al (1983) and may be attributed to glucocorticosteroid-induced inhibition of eicosanoic acid synthesis (Rainger et al, 1992) and lack of synthesis of chemotactic factors, such as IL-B4.

Plasma cortisol levels of fish sampled on day 28 were consistent with a state of immunosuppression (Kent and Hedrick, 1987). Control fish had a titer of 18 mg/ml, implanted coho 123 mg/ml, and Atlantic salmon 185 mg/ml.

As serum electrophoresis of Rs-infected coho and Atlantic salmon revealed a profound hypogammaglobinemia (Chapter II), additional plasma samples were obtained from 10^5 Rs infected (not cortisol implanted) coho and Atlantic salmon on day 28 for cortisol titer determination. Plasma cortisol levels were between 49 and 70 mg/ml, considered above the immunosuppressive threshold (Kent and Hedrick, 1987). Immunosuppressive states associated with infectious disease processes in fish have been described (Barton and Iwama, 1991), so BKD related immune

dysfunctions may be attributed not only to the bacterial p57 antigen, but also to endogenous host factors.

Past studies on the modulation of the humoral or cell mediated response in fish have relied almost exclusively on plasma cortisol titers to assess the extent of immunosuppression. Recent work by Pickering and Pottinger, (1989), however, suggests that there are considerable interspecific differences in lymphocyte sensitivity to plasma cortisol titers. Future studies may therefore require ancillary tests such as chemiluminescence or mitogen response to ensure a state of immunosuppression is present in test fish.

Table 13 Comparison Histological Features of Naive and Immunosuppressed Coho and Atlantic Salmon

	Rs+Cortisol	Rs-Cortisol
Renal		
perivascular neutrophils		
coho	-	++
Atlantic salmon	-	-
interstitium		
hypocellular		
coho	++	-
Atlantic salmon	+	-
mononuclear-like cells		
coho	++	++
Atlantic salmon	++	++
early granuloma		
multifocal distribution		
coho	++	+-
Atlantic salmon	++	+
interstitial fibrosis		
coho	-	-
Atlantic salmon	-	++
multinucleated giant cells		
coho	-	-
Atlantic salmon	-	++
excretory		
glomerulonephritis		
coho	++	+
Atlantic salmon	++	+
crescentic glomerulonephritis		
coho	+-	-
Atlantic salmon	++	++
intra-vascular		
thrombosis		
coho	+	++
Atlantic salmon	+	-
lymphocytes		
coho	-	+
Atlantic salmon	-	+
activated macrophages		
coho	++	+
Atlantic salmon	++	+
coho	+-	++
Atlantic salmon	+-	++

Chapter V

Evaluation of Soluble, Cell Wall-Associated, and Somatic Constituents of *Renibacterium salmoninarum*, as Potential Inflammogens in the Pathogenesis of Bacterial Kidney Disease

Introduction

Bacterial kidney disease (BKD) is a chronic insidious infectious condition of salmonids caused by a Gram-positive, facultative intracellular pathogen, Renibacterium salmoninarum (Rs). Histopathological expression of BKD is multisystemic, and ranges from a predominantly histiocytic response in coho salmon, to a tuberculoid granulomatous response in Atlantic salmon (Chapter II and III; Wood and Yasutake, 1956; Bruno, 1986b).

Past studies on Rs virulence determinants have focused primarily on an extracellular or cell wall associated protein, p57, which is readily detected in infected fish tissues (particularly the kidney and spleen) and, in vitro, in the supernatant of broth cultures. The p57 protein is the principal component of the extracelluar proteins produced by Rs and, biochemically, it is characterized as a hydrophobic, anionic protein with an isoelectric point of 5 (Turuga, Wiens, and Kaattari, 1987; Bruno, 1988; Rockey, Gilkey, Wiens and Kaattari, 1991a).

Pathogenic mechanisms attributed to p57 include in vitro immunosuppression (Turuga et al, 1987), hemagglutination (Bruno and Munro, 1986b), and leukagglutination (Weins and Kaattari, 1991). A putative hemolytic factor contributing to clinical manifestation of anemia has been adduced (Bruno and Munro, 1986b) and subsequently discounted (Bandin, Santos, Bruno, et al, 1990). Reduced hematocrits (PCV) or hemoglobin levels in vivo may be attributed to the pathophysiologic mechanisms associated with anemia of chronic disease. Agglutination of spermatozoa, as well as an ability to bind fibronectin are also reported. The role of p57 in the pathogenesis of BKD (in vivo), however, remains speculative.

Somatic constituents of Rs have also been studied, but they have been employed primarily for taxonomic purposes (Stackebrandt, Wehmeyer, Nader and Fieldler, 1988). Studies on their pathogenic or antigen properties are lacking.
Trypsonized bacterial cell walls consist of polysaccharides (up to 60% dry weight) which are covalently bound to peptidoglycans (Fiedler and Draxl, 1986). The principle carbohydrate is galactose, in a furanoid configuration, with smaller amounts of rhamnose, N-acetylglucosamine, and N-acetylfucosamine (Kusser and Fiedler, 1983). The presence of N-acetylfucosamine, coupled with a unique polypeptide bridge, composed of glutamic acid, lysine, alanine, and glycine at a molar ratio of 1:1:4:1 supports the unique taxonomic status of Rs.

The inflammogenic or immunogenic potential of cell wall components of a number of pathogenic bacteria is recognized and has significantly contributed to the understanding of the role of these components in the pathogenesis of infection. Indeed, such substances have been used in novel disease treatments and in vaccine development.

To investigate the contribution of somatic and soluble fractions of Rs in the histogenesis of BKD, 4 Rs fractions were obtained and inoculated into coho and Atlantic salmon.

Materials and Methods

To evaluate the inflammogenic (pathogenic) potential of soluble and cell wall associated, as well as somatic constituents of Rs, 4 bacterial fractions were kindly provided by Microtek R&D (Sydney, British Columbia) and injected ip into coho and Atlantic salmon. Fractions were prepared by initially culturing *Renibacterium salmoninarum* (strain MT312) in serum free

broth at 14 to 15 C and, harvesting the Rs cells in the log phase of growth (after approximately 140 hours; OD of 3). Due to commercial interests, detailed methodology for fraction preparation are not available.

Fraction 1 (lot 0720C) consisted of purified extracellular proteins, including p57 and its derivatives, and was prepared by ammonium sulfate precipitation of the proteins from the broth (Kusser, unpublished data). The proteins were characterized by SDS-PAGE, lyophilized, and stored frozen at -70 C. A challenge solution was prepared by dissolving 6 mg of these proteins into 10 ml of physiological saline (0.9% NaCl).

Fraction 2 (lot 0727 C), the p57 antigen, was extracted from Rs cells with 6 M urea (Kusser et al, unpublished data) and defined by SDS-PAGE, hemagglutinin titer, and protein concentration. A 2 mg aliquot was dissolved in 10 ml physiologic saline.

Fraction 3 (lot 0810C), the lipooligosaccharides, was prepared by a modification of the technique of Galanos, Luberitz, and Westphal (1979) and was characterized by SDS-PAGE, total sugar content, and Ouchterlony double diffusion with Concanavalin A (Kusser, unpublished data). The 2 ml phenol extract was dissolved into 10 ml physiologic saline.

Fraction 4 (lot 0511C), polysaccharide-free peptidoglycan was prepared by first solubilizing the cell walls with sodium dodecyl sulphate (SDS), and then by removing covalently bound polysaccharides by formamide extraction (Kusser, unpublished data). The peptidoglycan was defined by SDS-PAGE and assayed for polysaccharides by a phenol-sulfuric acid technique. A challenge peptidoglycan solution was prepared by combining 2 ml of extract with 8 ml of physiological saline.

Two hundred and fifty coho salmon, 4-6 gm is size, from

Rosewall Creek Hatchery and 250 Atlantic salmon, 4-6 gm in size, from United Hatcheries were transported to the Pacific Biological Station (PBS). On arrival each species was randomly divided into 5 groups of 50 individuals and maintained in 50 l pot tanks supplied with dechlorinated municipal water between 5-7 C. After a 1 week acclimation period individual groups of fish were anesthetized and injected ip with 0.1 ml of 1 of the 4 fractions. Control fish received 0.1 ml ip of physiological saline. All fish were similarly rechallenged with the respective fraction 28 days later.

Five fish from each group were euthanized and evaluated histologically every 28 days after the first injection. Fish were preserved in Davidson's solution, and parasaggital blocks of the viscera and saggital blocks of the cranium prepared. Tissues were processed in an automated tissue processor, embedded in paraffin, sectioned to a thickness of 5 um, stained with Hematoxylin and Eosin, and evaluated histopathologically.

Table 14. Purified fractions of Renibacterium salmoninarum injected into coho and Atlantic salmon.

Fraction	Lot Number	Amount	Dose
1. p57	0727C	2 mg	0.003 mg/gm fish
2. Extracellular proteins	0727C	6 mg	0.10 mg/g fish
3. lipooligosaccharide	0810C	2 ml	0.003 mg/gm fish
4. Peptidoglycan	0511C	2 ml	0.003 mg/gm fish

RESULTS

The histopathologic profile of Rs soluble, cell wall associated and somatic constituents is presented in Table 14. Fish challenged with the 6 molar urea extract, p57, extracellular proteins of Rs (p57 and its degradative products), and the lipooligosaccharide extract failed to elicit an inflammatory response over the course of the experiment.

Histopathology of the formamide extract, fraction 4, however, revealed focal to multifocal renal interstitial pyogranuloma in 2 to 3 of 5 coho and 1 to 2 Atlantic salmon on days 56 and 84 (Table 15). The lesions were ovoid to circular eosinophilic areas composed initially of neutrophils and eventually lymphohistiocytes (Figure 62). The interstitium was mildly hyperplastic and composed of predominantly mononuclear-like cells and lymphocytes. No extrarenal involvement is apparent.

It is important to note that these lesions are consistent with the incipient stages of BKD (Wood and Yasutake, 1956; Chapter II and III) and, for this experiment, fish were derived from Rs screened stocks, and subsampled for Rs prior to challenge. In addition, special staining with Gram's, PAS, and ABCIP (Chapter I) failed to demonstrate Rs in histological sections of affected tissues.

Table 15. Prevalence of Histopathological Lesions in Coho and Atlantic Salmon Challenged with various Renibacterium salmoninarum fractions.

Fraction	Days Post-Injection				
	0	28	56	84	112
p57					
Coho	0/5	0/5	0/5	0/5	0/5
Atlantic	0/5	0/5	0/5	0/5	0/5
Extracellular Proteins					
Coho	0/5	0/5	0/5	0/5	0/5
Atlantic	0/5	0/5	0/5	0/5	0/5
Lipooligosaccharide					
Coho	0/5	0/5	0/5	0/5	0/5
Atlantic	0/5	0/5	0/5	0/5	0/5
Peptidoglycan					
Coho	0/5	0/5	3/5	2/5	0/5
Atlantic	0/5	0/5	1/5	2/5	0/5

Figure 62. Histological section of the pronephros of a coho salmon injected with the peptidoglycan fraction. Note the nidus within the renal interstitium. Hematoxylin and Eosin, 300x.

Discussion

The principle *in vitro* virulence factor of Rs, the 6 molar urea extract, p57 (F antigen) did not elicit any appreciable histological alterations in ip challenged fish. This phenomenon may be attributable to a number of Rs intrinsic and extrinsic factors. For example, autologous (Rockey, Turaga, Wiens, et al, 1991b; Griffiths and Lynch, 1991), as well as heterologous (fish serum or tissue derived) serine proteases may have inactivated the p57 antigen before its virulence may have been exerted, *in vivo*. Moreover, after injection, the antigen may have been absorbed systemically, either protein bound or biotransformed (into an innocuous metabolite), then excreted without pathogenic consequence. In addition, the p57 protein may not be a virulence factor *in vivo*, or if p57 does have pathogenic properties, its effect in tissue may be evanescent.

Observations that monoclonal antibodies developed against p57 inhibit activity, *in vitro*, suggest that an immune response by challenged fish may abrogate the *in vivo* effects of this protein (Wiens and Kaattari, 1991). Serum antibody levels to p57 were not determined in this investigation, but these fish were young, derived from Rs-free parents and raised in well water, and thus there was no reason to expect p57 neutralizing antibodies in the fish at the start of the experiment. To further evaluate the pathogenesis of the antigen, future investigations should evaluate host antibody response. Autoagglutination or nonspecific cell absorption of p57 may also interfere with its pathogenic effects.

Fish challenged with extracellular proteins of Rs (p57 and its degradative products) also failed to elicit an inflammatory response over the course of the experiment (Table 15). This

observation is in direct contrast to an *in vivo* experiment with 9 to 12 gm Atlantic salmon, where a purified extracellular toxin from the culture supernatant of Rs (type AC 33209) proved to be highly lethal (Shieh, 1988). It is highly likely, however, that Shieh's results are based on a fast-growing contaminant because he used a 24 hour broth culture as the source of his toxin. In addition, most pathogenic features attributed to Rs (immunosuppression, hemolysis, DNAase, proteolysis) should yield subacute to chronic rather than acute manifestations. Further investigation into the pathogenesis of this putative toxin is warranted.

It is important to note that while histopathological lesions were not apparent in the lipooligosaccharide extract in challenged coho and Atlantic salmon, this is the only fraction to react with concanavalin A in Ouchterlony double diffusion (Kusser, unpublished data), which suggests a possible immunogenic potential with this fraction. Chemical analysis suggests that this fraction is a lipooligomannan, homologous to lipoarabanomannans, potent B cell immunogens of most *Mycobacterium* species (Gaylord, Brennan, Young, and Buchanan, 1987; Puzo, 1990; Harboe, Wiker, and Nagai, 1992). It may therefore stimulate antibody production.

Conversely, and by analogy with other lipoteichoic acids, this fraction may function as a cell wall autolysin, an ion exchange factor, as a mediator of cell to cell or cell to substrate interaction, or as a binder of fibronectin (Suttcliffe and Shaw, 1991). Moreover, *in vitro* activity similar to O antigens of Gram-negative bacterial lipopolysaccharides, such as nonspecific suppression of thymus derived lymphocyte (L_T) activity, inhibition of antigen stimulation or processing by peripheral cells may also occur (Brennan, 1989) and should by

evaluated in fish as possible virulence mechanisms.

It is interesting to speculate that because the lipooligomannan of Rs may extend from the cell membrane, across the cell wall to an exposed site on the cell surface, this orientation may facilitate lectin mediated phagocytosis by mannose or N-acetylglucosamine receptors recently recognized on the plasma membrane of nonspecific cells within the pronephros (Dannevig, Struksnaes, Skogh, et al, 1990). Further evaluation of the immunogenic potential of this fraction for subunit vaccine development, as well as a potential virulence or pathogenic factors in vivo are necessary.

A granulomatous inflammatory process was noted in 2 to 3 of 5 coho and 1 to 2 of 5 Atlantic salmon challenged with the formamide extract, peptidoglycan. Numerous nonspecific systemic, as well as specific focal inflammatory processes are attributed to the cell wall peptidoglycan (PG) of bacteria. Pathogens, such as Mycobacterium tuberculosis, M lepriae, Borrelia burgdorferi, Eubacterium aerofaciens, and Bifidobacteria adolescentis, have PG's which are strongly inflammogenic, poorly biodegradable, and persist in host tissue for protracted periods (Rastogi and David, 1988; Fox, 1990; Harzenberg, Klasen, Kool, et al, 1992). Enzymes catabolizing PG in mammals include lysozyme, N-acetyl muramyl-2-aniline amidase, peptidase, and glucosaminase (Fox, 1990). Resistance to lysozyme, for example by O-acetylation of the PG in Streptococcus gonococcus (Fleming, Wallsmith and Rosenthal, 1986) or substitution of N-acetyl muramic acid by other N-acetyl derivatives (Brennan, 1989) in M tuberculosis occurs. It is possible that some such a mechanism explains Rs resistance to lysozyme (Fryer and Sanders, 1981), and facilitates PG persistence within the renal interstitium where it can incite granuloma formation.

In tuberculosis (M tuberculosis), granulomatous responses are induced by a variety of cell wall compounds, including mycolic acid containing glycolipids (which are not present in Rs), glycopeptidolipid mycosides, Wax-D, cord factor (trehalose dimycolate) and the cell wall skeleton, composed primarily of arabinogalactan and PG (Puzo, cited by Rastogi and David, 1988). A similar inflammatory phenomenon is observed with Streptococcal cell wall PG, which elicits a marked leukocytosis, characterized by a neutrophilia and monocytosis (Wells, Hightower, and Parks et al, 1989). A strong cell mediated immunity is imperative for development of these lesions (Unanue, 1980).

By day 112, none of the PG-injected coho and Atlantic salmon exhibited granulomatous lesions indicating that catabolism or redistribution and elimination of the PG had occurred by this stage.

It is interesting to note that past studies on virulence determinants and pathogenic mechanisms of another major fish pathogen, Aeromonas salmonicida (As), the etiological agent of furunculosis, have similarly resolved cell wall associated and extracellular factors (Munro, 1984). The former have been characterized as 1) a 57 kD, water insoluble, adhesin molecule analogous to the fimbriae of K88 Echerichia coli, which may also confer protection against complement and 2) a 49 kD hydrophobic, nonenzymatic surface protein. Whereas, the latter is composed of heterogeneous extracellular proteases (ECP), including hemolysins and a leukocidin, as well as lipopolysaccharide (the Gram-negative equivalent to peptidoglycans of Gram-positive bacteria)(Austin and Austin, 1987).

Preliminary investigations by Ellis, Hastings and Munro (1981) revealed that fish challenged with the ECP developed lesions consistent with those of furunculosis and spurred a

considerable amount of *in vitro* and to a lesser extent *in vivo* research to further characterize these factors. However, subsequent studies with attenuated strains of As (with loss of the A layer, plasmids or ECP and retained virulence)(Titball and Munn, 1985) have since discounted the sole role of ECPs in the pathogenesis of furunculosis and suggested a synergy of multiple factors (Munn and Trust, 1984).

It is important to appreciate from work conducted on As that there may be a multiplicity of bacterial virulence factors involved in the pathogenesis of BKD. Past studies on Rs have focused almost exclusively on the p57. Further resolution and sequencing of cell wall components and evaluation of inherent immunogenic and inflammagenic activity (in specific pathogen free stock) may enhance our understanding of the pathogenesis of this disease.

GENERAL CONCLUSION

Bacterial kidney disease (BKD) is a multisystemic pyogranulomatous condition afflicting salmonid species. The etiological agent of BKD, Renibacterium salmoninarum (Rs) is a Gram-positive coccobacillus with varying degrees of endemnicty in production facilities (Fryer and Saunders, 1981).

Diagnosis of BKD is often contingent on a combination of techniques, including Gram's stain, direct or indirect immunofluorescence, as well as serology or histopathology.

To circumvent inherent limitations in conventional histochemical and immunochemical techniques, an avidin biotin conjugated immunoperoxidase (ABCIP) was developed for localization of Rs in histological sections. One rabbit polyclonal and four commercially available mouse monoclonal antibodies were evaluated. Comparison of this technique with Gram's stain, PAS, hematoxylin and eosin, and DFAT (with rabbit polyclonal anti-Rs), revealed improved Rs detection with the ABCIP. Rs antigens proved stable in tissues prepared in formalin, Bouin's, and Davidson's fixatives, and relatively labile in tissues fixed in solutions of potassium dichromate (Chapter I).

Due to the permanent nature of stained preparation, high resolution (even at the subcellular levels) and ability to appreciate tissue architecture, the ABCIP was employed as a adjunct to histochemical techniques to determine the histogenesis of BKD in coho and Atlantic salmon.

To ascertain the spatial and temporal distribution of lesions associated with BKD, coho and Atlantic salmon were challenged with 3 levels of Rs (10^3, 10^5, and 10^7) and serially sampled until all individuals had succumbed to the infection.

Histopathology revealed a renal site predilection with lesions resolved into perivascular, interstitial, excretory and intravascular compartments (Chapter II).

Alterations in the cellular composition of the perivascular region of coho salmon were consistent with human bone marrow kinetics associated with inflammatory processes. Cells evolved from initially granulocytes, to a mixed neutrophil, lymphocyte composite, and eventually to a homogeneous population of large mononuclear-like cells.

Cells were observed extending from the perivascular compartment across the adventitia to the lumen of the caudal vena cava. This mechanism may account for the previously described peripheral leukocytosis associated with BKD, characterized initially by a neutrophilia and subsequently by monocytosis and lymphocytosis (Bruno and Munro, 1986b).

The nature of the granulomatous response of coho and Atlantic salmon was also characterized. The coho salmon featured a histiocytic granulomata, whereas Atlantic salmon exhibited a more tuberculoid response. These observations were attributed to interactions and relative proportions of macrophages and putative T lymphocytes (L_T). Langerhans giant cell were noted contiguous to renal interstitial granulomata in Atlantic salmon, but were not present in coho salmon.

Pleomorphic alterations were observed in the excretory compartment of coho and Atlantic salmon. Glomerular hypercellularity (associated with inflammatory infiltrate), focal to segmental thickening of the endothelial wall, as well as distension and accumulation of proteinaceous material within Bowman's space were detected. In Atlantic salmon a crescentic glomerulonephritis (due to parietal cell hyperplasia) was consistently detected in the acute and subacute stages of

infection and with special stains focal to diffuse linear fibrin deposits within the endothelia (as well as in the apical region of cells in the proximal convoluted tubules) were delineated.

Vascular involvement was apparent in coho salmon and infrequently detected in Atlantic salmon. Microthrombi were localized in the lumen of the pronephric vasculature and septic thrombi and emboli were noted in the caudal vena cava. These lesions were attributed to possible endothelial injury associated with infection and inflammation, hyperviscosity of the blood, cardiovascular or septic shock, as well as excretion of anticoagulant factors through compromised renal glomeruli.

Extrarenal dissemination of the inflammatory response in coho salmon appeared to arise from ventral aspect of the pronephros along the coeliacomesenteric artery (associated with the mesoesophagus). It extended ventrolaterally to involve the transverse septum, pericardium, liver capsule and associated viscera.

Fibrinous and fibrohistiocytic peritonitis were noted in fish. These lesions, however, appeared more dependent on the level of Rs challenge (and vascular injury) than on water temperature, as previously advocated (Smith, 1964).

Ovarian involvement arose through either direct extension along the mesovaria or seeding by septic ascites. Rs was detected in follicular cells of primordial oogonia and may provide a portal to establish intra ovum infections (Bruno and Munro, 1986a).

Septic thrombi and phagocytes frequently with intra- and extracellular Rs, were noted in the vasculature of the brain. There was scant respiratory and skeletal muscle involvement in experimentally challenged fish.

Because intraperitoneal injection may circumvent normal

defense barriers and antigen processing mechanisms in challenged fish, an experiment was undertaken to expose fish to infection by cohabitation with Rs injected fish (to naturally acquire infection) and serially sampled for histopathology.

Histological lesions of the incipient stages of BKD in coho and Atlantic salmon challenged by cohabitation were consistent with those fish challenged by intraperitoneal injection. Prime differences in the manifestation of BKD include, renal and hepatic pseudocyst formation, severe renal interstitial hyperplasia (similar to a plasmacytoid leukemia in chinook and Atlantic salmon, Marine Anemia), granulomatous encephalitis, lack of peritoneal involvement, parenchymal rather than serosal involvement of the viscera, as well as granulomatous branchitis and myositis. Frequently, lesions within a given sample interval were heterogeneous and reflected multiple pathogenic processes.

To further resolve the nature of the inflammatory response of fish to BKD, immunosuppressed coho and Atlantic salmon were challenged with 10^3 Rs. Expansive, poorly organized granulomata, with exuberant intra- and extracellular Rs growth were noted in both coho and Atlantic salmon. In man, impaired bactericidal activity, reduced random migration and antigen processing have been reported with macrophages administration of supratherapeutic doses of glucocorticosteroids which may account for the observed lesions in coho salmon. Whereas, in Atlantic salmon, it is interesting to speculate that preferential inhibition of CD4 cells (a subset of L_y's expressing CD4 epitopes) may impede immunological priming of macrophages and abrogate the tubercle formation. Florid extrarenal proliferation of Rs with a paucity of inflammatory infiltrate may be attributed to impaired prostaglandin and leukotriene (particularly LTB4) synthesis.

To evaluate the inflammogenic potential of somatic, cell-wall associated and soluble factors of Rs, 4 purified fractions, including the extracellular proteins, the p57, the lipooligomannan, and the peptidoglycan (PG) were obtained and challenged by intraperitoneal injection of coho and Atlantic salmon.

Past studies on virulence factors and pathogenic mechanisms of Rs have focused almost exclusively on the protein p57, a hydrophobic, anionic protein with an isoelectric point of 5. This protein has been shown in vitro to be immunosuppressive, hemagglutonating, and leukagglutonating. In this investigation no lesions were apparent in fish challenged with the p57, extracellular proteins, or lipooligomannan. However, mild, multifocal pyogranulomata were noted in the renal interstitium of coho and Atlantic salmon challenged with the PG.

In a number of mammalian pathogens, including Mycobacterium tuberculosis, M leprae, Borrelia burgdorfori, Eubacterium aerofaciens, and Bificobacteria adolescentis, purified PG fractions were strongly inflammogenic, poorly biodegradable and persisted for prolong periods. Mammalian enzymes which catabolize PGs include lysozyme, N-acetyl muramyl-2-aniline amidase, peptidase, and glucosaminase (Fox, 1990). Resistance to lysozyme, for example by N-acetyl substitution or O-acetylation of PG occurs. It is possible that some such mechanism may account for Rs resistance to lysozyme (Fryer and Saunders, 1981) and facilitates PG persistence within the renal interstitium where it can incite granulomata formation.

Past investigations of another major fish pathogen, Aeromonas salmonicida(As), the etiological agent of furunculosis, have similarly resolved cell wall associated and extracellular virulence factors. On demonstration that lesions histologically

consistent with furunculosis may be elicited with administration of extracellular proteases (ECP)(Ellis, Hasting, and Munro, 1984), considerable *in vitro* work was spurred to further characterize these factors. Subsequent research, however, discounted the sole role of ECPs in the pathogenesis of furunculosis and a multiplicity of factors were adduced (Munn, 1984).

It is important to appreciate from this work that for Rs multiple bacterial virulence factors may contribute to the pathogenesis of BKD. Further fractionation and sequencing of Rs of cell wall components of Rs and evaluation of their inherent immunogenic and inflammogenic activity may enhance our understanding of the pathogenesis of this disease.

FUTURE CONSIDERATIONS

Sequential histopathology of Rs infected coho and Atlantic salmon revealed that histologic expression of BKD was dependent on inoculum dose, method of infection, (ip versus cohabitation), sampling interval, as well as species challenged. Additional factors which warrant evaluation include the effect of water temperature, challenge by gavage, and challenge age (comparison of the nature of the inflammatory response in fry, fingerlings, and adults, which is known to be profoundly different in mammals). Preliminary observations (Raverty, unpublished data) suggested a reduced rate of inflammatory infiltration, mobilization of intracellular Rs to primordial lymphoreticular cells and high mortalities in experimentally or naturally infected coho and Atlantic salmon.

In addition, ancillary tests, such as haematology (complete blood count, prothrombin time, partial thromboplastin time), clinical chemistry, serum electrophoresis (to quantify serum proteins and immunoglobulin titers), immunofluorescence (IgM and C3 to determine onset and progression of glomerulonephritis), as well as determination of the humoral response to Rs and tissue p57 levels in fish experimentally infected with varying levels of Rs and naturally by cohabitation may contribute further to the understanding of the immunopathology and pathophysiology of BKD.

Histopathology of BKD in this study suggested that contrary to past observations, Rs is not relatively nonpathogenic/avirulent (Armstrong, 1989), but rather that the bacterium is highly pathogenic/virulent and may elaborate toxins, similar to M tuberculosis (Cotran, Kumar and Robbins, 1989). Progression from subacute to chronic stages of infection without resolution of BKD may result in (or from) depletion of tissue Rs growth inhibitors (Daly and Stevenson, 1988), potentiated

bacterial proliferation, and exo- or endotoxin secretion. Investigations to identify, further characterize, and quantify these substances in fish tissues are warranted.

Granulomatous lesions within the renal interstitium varied from primarily histiocytic in coho to more tuberculoid in Atlantic salmon. In humans afflicted with leprosy (M leprea) and to a lesser extent, sarcoidosis, histiocytic versus tuberculoid manifestations are contingent on the ratio of associated CD4:CD8 L_x's, which express MHC-I and MHC-II receptors, respectively (Sheffield, 1990).

Recent demonstration of homologous gene sequences for MHC in carp and mice by the PCR technique may facilitate identification of these two T cell subpopulations (if they occur in fish) either by in situ hybridization with the carp gene sequences for MHC-I and MHC-II, or alternatively, these sequences may be cloned into E coli, their proteins expressed (MHC-I and MHC-II), (evaluated for functional homology to mammalian MHC), and monoclonal antibodies developed either for immunohistochemistry or cell flow cytometry. Efforts are presently underway to develop monoclonal antibodies against L_x epitopes, and if successful these monoclonal antibodies may be used to further characterize the composition and kinetics of the inflammatory infiltrate associated with BKD (already 1 clone specific to CD 8 cells for rabbits has been shown by immunofluoresence to cross react with a specific subpopulation of L_x's in fish)(P Levine, unpublished data). Mammalian leukocyte associated antigens (CD) should be assessed for potential cross reactivity and specificity to piscine cell lineages. Lectin histochemistry with PNA may also prove a valuable adjunct for macrophage detection.

The yet undefined, monocyte-like cells within the renal

interstitium in coho and Atlantic salmon in subacute and chronic stages of BKD should be further characterized ultrastructurally, as well as histochemically (including stains for beta-galactosidase, acid phosphatase, alpha-1-antitrypsin, chymotrypsin, and methyl green pyronine).

Ultrastructural investigations of coho and Atlantic salmon macrophages infected either in vitro (with varying levels of Rs) and in vivo, should be performed for subcellular localization of Rs. Preliminary studies suggest that Rs is distributed throughout the cytoplasm of macrophages from coho salmon and retained within phagolysosomes in brook trout and Atlantic salmon (Young and Chapman, 1978). Insight into possible mechanisms for intracellular persistence may be adduced by comparison to known intracellular pathogens of mammals.

In addition, in vitro experiments may be undertaken to assess potential modulation of macrophage (and to a lesser extent granulocytes) chemotaxis, phagocytosis, and intracellular killing from Rs infected and control fish to ascertain the effect of intracellular parasitism on phagocytic function.

Comparison of the histological observations in this study to inflammatory reactions (processes) in mammals, (such as perivascular granulopoiesis and the composition of the inflammatory infiltrate) suggests that cytokines, analogous to GM-CSF, IL1, IL3, and TNF may modulate inflammation to a greater extent than previously appreciated in fish. Further research into the interaction of these cells and associated soluble factors may provide insight into the cell mediated immune response in fish and how this relates to BKD.

For example, complement mediated opsonization and phagocytosis of Rs by macrophages has been recently demonstrated (Rose and Levine, 1992). However, the effect of complement

factor C5a on chemotaxis, C5a and C3a on vascular permeability, or C5b-9 on cell lysis of Rs (although unlikely) remain obscure. Moreover, recent demonstration of a 30-60 nm capsule associated with the cell wall of Rs (Dubreuil, Lallier and Jacques, 1990), suggests that anticapsular antibodies may also be elaborated prior to opsonization. The contribution of the acute phase reactent, C reactive protein, should also be investigated, particularly as it may initiate the complement cascade and modulate the peracute and acute stages of infection.

Interestingly, genetic resistance to M tuberculosis and other intracellular pathogens in mice has been attributed to the non-H-2 gene and Bcg gene (Orrell, Brett, Ivanyi, Coghill, et al, 1992). Past genetic studies in fish susceptibility to BKD have focused principally on the plasma protein, transferrin (Suzumoto, Schreck, McIntyre, 1977; Winter, Schreck, and McIntyre, 1980), and in the future should also address intrinsic variation in macrophage activation and function.

Observations by Ketcheson and Evelyn (unpubl data) suggest that corticosteroid administration post-Rs challenge yields no difference in mortality rate on comparison to control, nonimmunosuppressed, Rs challenged fish. In chapter IV, however, coho and Atlantic salmon inoculated with Rs 1 week after corticosteroid administration, featured an earlier onset and greater rate of mortality than immunocompetent Rs challenged fish. This observation may be attributed to the pharmacokinetics of corticosteroids. Pretreatment of fish with cortisone inhibits phospholipase A$_2$ activity and prevents elaboration of leukotriennes, prostaglandins, and a number of immune and inflammatory mediators associated with the peracute and acute stages of infection, prior to Rs challenge; whereas, Rs challenged fish, subsequently inoculated with corticosteroids,

have already elaborated eicosanoic acid derivatives and exerted their immunophysiologic effect. It would be interesting to recover macrophages from the first group (immunosuppressed, then Rs challenged) and ascertain if macrophage function is restored with the addition of gamma-IFN.

Nevertheless, the present results suggest that vaccination, perhaps either by modified live vaccines or liposomal delivery, may be efficacious in conferring protective immunity. Development of anti-idiotypic antibody vaccines, however, should be discouraged as protracted, elevated levels of circulating primary antibodies with intercurrent BKD may exacerbate immune complex formation and glomerulonephritis.

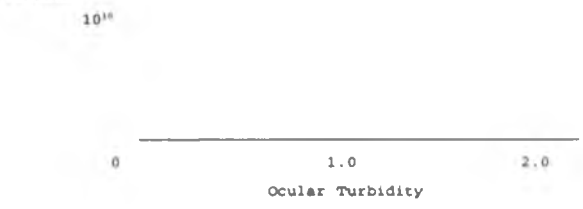
Preliminary investigation of extracellular, cell wall associated, and somatic constituents of Rs suggested that only the peptidoglycan (PG) fraction is inflammogenic.

Failure to detect morphological changes in p57 challenged fish may be a dose related phenomenon, autocatalytic activity, some other yet define mechanism, or the p57 may not be inflammogenic <u>in vivo</u> (this should be distinguished from immunogenic activity). Further investigations should challenge fish with p57 either within liposomes or, alternatively (as the p57 is a potent agglutinin) agglutinated to latex beads to ensure the molecule is presented to antigen processing cells. Moreover, as significantly higher antibody titers have been elicited by particle bound (HGG and latex beads), rather than solubilized antigen (Bridges and Manning, 1991), future investigations on the immunogenic and immunomodulatory activity of p57 should evaluate both forms of Ag presentation. The ELIZA (Turaga, Wiens, and Kaattari, 1987) or ABCIP (Chapter I) with monoclonals specific for p57 may be employed for titration and visualization, respectively, of the antigen in tissue.

Discrete granulomata typical of BKD were not detected in the PG challenged fish; however, multifocal, mild granulomata were apparent. This observation may be related to the amount of antigen administered and further evaluation with higher doses of Rs PG are necessary. Enzyme digestion of the PG, and subsequent sequencing may provide valuable insight into potential pathogenic mechanisms, as well as possible avenues for chemotherapeutic and vaccine development.

In addition to its inherent microbiological idiosyncrasies, Rs may be an invaluable model to elucidate the immune and inflammatory processes in fish.

Appendix I. Growth Curve of Renibacterium salmoninarum. A value of 10^{10} Rs was determined to have an ocular density of 1.0 at a wavelength of 540 nm.



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DEDICATION

This work is dedicated to Dr A C MacNeill, aquatic veterinary specialist, and Dr G R Bell, aquatic microbiologist, for their contributions to the biomedical understanding of aquatic species and inspiration for the pursuit of excellence.

NUMEROUS ORIGINALS IN COLOUR