Bacterial Kidney Disease in salmonid fish

Development of methods to assess immune functions in salmonid fish during infection by Renibacterium salmoninarum

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**To the memory
of my grandmother
Hildur.**

Abstract

Bacterial kidney disease (BKD) is a chronic systemic infection of salmonid fish. BKD has been a main threat to salmonid health, in aquaculture as well as in feral fish populations, all over the world during recent decades. Various disease manifestations have been reported, from the characteristic swollen kidney containing white nodules, to more diffuse signs and even of asymptomatic carriers. Regular screening programmes have been the most important possibility to prevent transmission, since no effective antibiotics or prophylactic therapy are available. BKD is caused by the Gram-positive bacterium *Renibacterium salmoninarum,* a slow growing and fastidious diplobacilli. Diagnosis has been complicated due to the difficulties in bacterial cultivation. *R. salmoninarum* can survive intracellularly in the phagocytic cells of the fish, resulting in a granulomatous reaction, similar to that in tuberculosis of mammals. Progress in the development of prophylactic methods to control BKD requires knowledge of basic immune functions in salmonid fish. In this thesis, techniques have been developed to study the immune response and immune functions in salmonid fish during the progression of BKD.

An enzyme-linked immunosorbent assay (ELISA) was developed to detect *R. salmoninarum* antigens in fish kidney. The negative-positive threshold value in the ELISA was determined based on the results of more than 400 kidney samples negative for BKD. The sensitivity of the ELISA method was tested on samples from populations with clinical or enzootic BKD. An improvement in the possibility to detect BKD in fish populations by the ELISA was demonstrated when compared with bacterial cultivation.

Monoclonal antibodies to salmonid Ig were used in an ELISA for determination of the humoral response to *R. salmoninarum* by a non-lethal sampling. Elevated antibody levels were measured in populations of rainbow trout and salmon with clinical BKD. No correlation, of the antibody level and the occurrence of *R. salmoninarum* antigens in the kidney of individual fish, was found. A cell-mediated immune response was measured in rainbow trout with experimentally induced BKD by an *in vitro* stimulation of the lymphocyte population by *R. salmoninarum* antigens. Three monoclonal antibodies (Mabs) 1C2, 7A5/20 and 8H4/23 to lymphocytes of rainbow trout were developed. The Mab 1C2-positive lymphocyte population of spleen was indicated to have an influence on the immune response to BKD.

Key words: Bacterial kidney disease; *Renibacterium salmoninarum*; enzyme-linked immunosobent assay (ELISA); iscome; lymphocytes; monoclonal antibodies; cell-mediated immunity

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Bakteriell njurinflammation hos laxfisk

Utveckling av metoder för att studera immun funktioner hos laxfisk vid infektion med Renibacterium salmoninarum

Sammanfattning

Bakteriell njurinflammation (BKD) är en kronisk, systemisk infektion hos laxfisk. BKD förekommer över hela världen och drabbar vild så väl som odlad fisk. Sjukdoms symptomen kan vara varierande, vanligt är att njuren är svälld med grå-vita fläckar av varierande storlek. Vita fläckar kan också förekomma på mjälten, hjärtat och på levern. Fiskar kan också vara infekterade utan att visa tecken på sjukdom. Regelbundna hälsokontroll-undersökningar är den viktigaste möjligheten att begränsa BKD, eftersom varken antibiotika eller något funktionellt vaccin finns tillgängligt. BKD orsakas av *Renibacterium salmoninarum,* en Gram-positiv stavbakterie. Diagnostik av sjukdomen har försvårats då *R. salmoninarum* kräver komplexa odlingsmedier och växer mycket långsamt. *R. salmoninarum* kan överleva och till och med föröka sig i fiskens fagocyterande celler. Desssa celler, som ingår i det immunologiska försvaret, har annars till uppgift att förgöra inkräktande agens. Granulom bildas, genom en immunologisk reaktion hos däggdjur, vid sjukdomar som *tex.* tuberkulos orsakad av intracellulärt förekommande bakterier. Likartad granulombildning utvecklas vid BKD. För att utveckla förebyggande metoder för att kontrollera BKD, krävs ökade kunskaper om hur fiskars immunsystem fungerar. I denna avhandling har tekniker utvecklats, för att studera immunsvaret och immunfunktioner hos laxfisk vid infektion med *R. salmoninarum*.

En immunologisk teknik (enzyme-linked immunosorbent assay, ELISA) har utvecklats för att spåra bakterien *R. salmoninarum* direkt i fiskens njure. Mer än 400 njurprover ifrån fiskar som inte är infekterade med BKD har testats, för att bestämma variationen i de negativa proverna, och därmed gränsvärdet för ett negativt/positivt prov. Känsligheten i metoden har utvärderats i prover ifrån fiskpopulationer där BKD tidigare konstaterats. Den utvecklade ELISA metoden har förbättrat möjligheten att spåra BKD i fiskpopulationer vid jämförelse med odling av bakterien.

Monoklonala markörer användes för att spåra antikroppar riktade mot *R. salmoninarum* i laxfiskars blod. Med denna metod studerades fiskens humorala antikroppssvar vid BKD. Hos däggdjur används liknande teknik i hälsokontrollprogram som ett mått på smittan i en population. Förhöjda antikroppsnivåer uppmättes också i fiskpopulationer med både regnbågslax (*Oncorhynchus mykiss*) och lax (*Salmo salar*) infekterade med *R. salmoninarum*. Någon korrelation mellan individuella fiskars antikroppssvar och halten av *R. salmoninarum* i njuren kunde dock ej fastställas, i enlighet med andra undersökningar. Detta indikerar att det cell-medierade immun (CMI) svaret är betydelsefullt för skydd mot BKD. Det specifika immunförsvaret hos fisk, liksom hos däggdjur, regleras av lymfocyter. Tre monoklonala antikroppar (MAs), 1C2, 7A5/20 och 8H4/23 som identifierar lymfocyter hos regnbågslax har framställts i denna avhandling. Den lymfocyt-population som identifierades av MA 1C2 indikerade att delta i immunsvaret vid BKD. En *in vitro* metod användes för att studera CMI svaret vid BKD. Lymfocyter ifrån fiskar som smittats med BKD stimulerades till proliferation utav värme-avdödade eller fixerade *R. salmoninarum*. Denna metod kan användas för att studera påverkan på CMI svaret i samband med utvecklandet av vacciner.

Contents

Appendix

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I.   Jansson, E., Hongslo, T., Höglund, J. and Ljungberg, O. 1996. Comparative   evaluation of bacterial culture and two ELISA techniques for the detection of *Renibacterium salmoninarum* antigens in salmonid kidney tissues. Disease of Aquatic Organisms 27: 197-206

II.   Jansson, E. and Ljungberg, O. 1998. Detection of humoral antibodies to *Renibacterium salmoninarum* in rainbow trout *Oncorhynchus mykiss* and Atlantic salmon *Salmo salar* challenged by immersion and in naturally infected populations. Disease of Aquatic Organisms 33: 93-99

III.   Jansson, E., Grönvik, K-O., Johanisson, A., Näslund, K., Westergren, E., Pilström, L. Monoclonal antibodies to lymphocytes of rainbow trout (*Oncorhynchus mykiss*). Submitted.

IV.   Jansson, E., Hongslo, T., Johanisson, A., Pilström, L., Timmusk, S. and Norrgren, L. Bacterial kidney disease as a model for studies of cell-mediated immunity in rainbow trout, (*Oncorhynchus mykiss)*. Submitted.

Abbreviations

BKD	bacterial kidney disease
C3	component of the complement system
CD	cluster of differentiation
CHSE	chinook salmon embryo
CMI	cell-mediated immunity
Con A	concanavalin A
DNA	deoxyribonucleic acid
DTH	delayed-type hypersensitivity
EGC	eosinophilic granular cell
ELISA	enzyme-linked immunosorbent assay
EPC	epithelioma papillosum cyprini
FCM	flow cytometry
G/C	guanine/cytosine
IFAT	indirect fluorescent antibody test
Ig	immunoglobulin
IL	interleukin
kDa	kilo Dalton
KDM	kidney disease medium
KDM-C	kidney disease medium charcoal
LAK	lymphokine-activated killer cell
LPS	lipopolysaccharide
Mab	monoclonal antibody
MAF	macrophage activating factor
MGC	multinucleated giant cell
MHC	major histocompatibility complex
MLR	mixed leukocyte reaction
NCC	non-specific cytotoxic cell
NK	natural killer cell
OD	optical density
PFA	paraformaldehyde
p57	major cell surface protein of *R. salmoninarum*
RNA	ribonucleic acid
RTG-2	rainbow trout gonad
SKDM	selective kidney disease medium
SI	stimulation indices
SV	sequevars
TCR	T-lymphocyte receptor

Introduction

Bacterial Kidney Disease

The first reports of a new disease in salmon (*Salmo salar*) from the rivers Aberdeenshire Dee and Spey in Scotland (Furunculosis Committee, 1933; Smith, 1964) and from hatchery reared brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*) in the Western United States (Belding & Merrill, 1935) in the early 1930s describe a complex disease mainly affecting the kidneys. Lesions indicative of metabolic disturbances and of reactions against an infectious agent were described. The occurrence of small Gram-positive rods was early demonstrated in smears from the kidney, however the bacteria could not be propagated with conventional techniques for fish pathogenic bacteria. Koch´s postulate could be fulfilled, initially by injection of emulsion from internal organs containing the Gram-positive bacteria but later also with cultivated bacteria, and the disease was referred to as Dee disease, "white boil disease" or as Kidney disease. Comparison of the clinical picture, the pathologic lesions and characterization of the infective bacteria finally confirmed the identical aetiology of the diseases (Earp *et al*., 1953; Smith, 1964). The disease is today known as Bacterial Kidney Disease (BKD) and the aetiological agent is *Renibacterium salmoninarum* (Sanders & Fryer, 1980).

   Limited survival of *R. salmoninarum* in water, up to a few weeks, indicates that the bacterium is an obligate pathogen of fish, either in clinically diseased fish or in latent carrier fish (Evelyn, 1993). BKD has only been described in salmonid fish although the propagation of *R. salmoninarum* has been demonstrated in other fish species, *e.g*., cyprinids (Sakai *et al*., 1989) and in sablefish *Anoplopoma fimbria* (Bell *et al*., 1990).

   BKD is a serious threat to wild as well as cultivated salmonid fish. The disease is reported from North and South America, in several countries in Europe (Denmark, England, Finland, France, Germany, Iceland, Norway, Scotland, Sweden, *etc*.) and in Japan (Kinkelin, 1974; Fryer & Sanders, 1981; Hoffmann *et al*., 1984; Kimura & Awakura, 1977; Fryer & Lannan, 1993). Disease problems are reported to be especially serious in areas where Pacific salmon occur, *i.e*., the Pacific Northwest and the Great Lakes region of the United States.

Pathology

External lesions
Loss of balance is seen in *R. salmoninarum* infected fish. Exophthalmia, superficial blebs or blisters of the skin and even the formation of cavitations in the

musculature are reported from infected salmonids. The blebs and cavitations are described to contain a white, yellowish or haemorrhagic fluid. Pale gills, distended abdomen, petechiae and haemorrhagic areas around the fins and the lateral line are also common external indications of BKD (Belding & Merrill, 1935; Earp *et al.*, 1953; Bruno, 1986; Evelyn, 1993).

Internal lesions

The fish kidney is an organ with several functions: endocrinal, excretorial, haematopoietical and phagocytic, and is the organ in which macroscopic lesions most often are observed in connection with *R. salmoninarum* infection. The kidney is generally swollen and greyish-white nodules of various sizes are seen on the surface of the organ. Similar nodules can be observed in spleen, heart and in the liver. The spleen might be increased in size and the liver bright in colour. Petechial haemorrhages of the muscle lining the peritoneum are described and also an accumulation of ascitic fluid in the abdominal cavity. In advanced cases, extensive kidney damage is obvious since the whole organ is enlarged, greyish in colour and necrotic, Fig.1, (Belding & Merrill, 1935; Wood & Yasutake, 1956; Bell, 1961; Fryer & Sanders, 1981; Bruno, 1986). An opaque membrane, covering internal organs is described, especially in fish held at low temperatures (below 9° C). The membrane consists of fibrin and leukocytes (Smith, 1964).
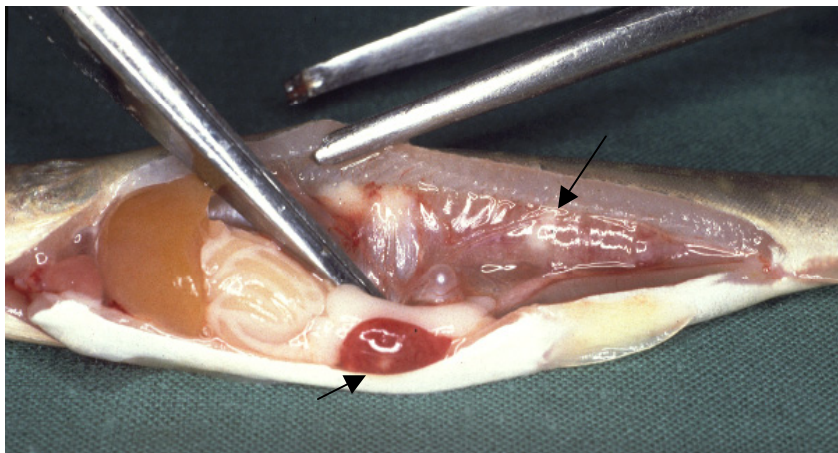

Figure 1. Arctic char with Bacterial Kidney Disease. The kidney is enlarged and greyish in colour. White nodular lesions are observed in kidney and spleen (arrows).
Photo: National Veterinary Institute.

Histopathology

BKD is histologically described as a chronic, granulomatous, systemic inflammation, similar to that in tuberculosis of mammals (Snieszko & Griffin, 1955; Wood & Yasutake, 1956). The chronic inflammations in fish are characterized by an increased amount of melanomacrophages and dispersed melanin (Bruno, 1986). Focal necroses, surrounded by granulomatous tissue of epithelioid and lymphoid cells, with different degrees of encapsulation, are typical observations, Fig 2.
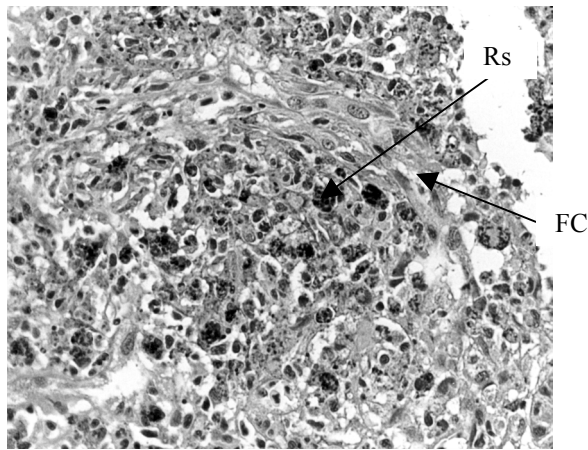

Figure 2. Kidney of rainbow trout infected with *R. salmoninarum* (Paper I). Granulomatous lesion with macrophages containing *R. salmoninarum* (Rs), surrounded by fibrous capsule (FC), immunohistochemical staining, X 250. Photo: Paul Mbuthia

Granulomas develop as a result of activated macrophages that adhere closely to each other and form the epithelioid appearance. Several activated macrophages can fuse to a multinucleated giant cell (MGC; Secombes, 1985). MGC is described in chronic inflammations in fish of fungal or parasitic origin (Ferguson, 1989) and also in BKD infected fish (Snieszko & Griffin, 1955; Wood & Yasutake, 1956). MGC were also observed in the experimental challenges in the present study, Fig 3. Activated macrophages and MGCs release high amounts of lytic enzymes into the tissue that cause tissue necrosis. The granulomatous lesions appear to arise in the connective tissue between the parenchymal cells. The haematopoietic tissue of the anterior kidney was indicated to be affected initially, followed by extensive tissue destruction in the posterior, mainly excretory, part. The glomeruli and tubules of the nephron are affected. Hypercellular glomeruli and occlusion of Bowman`s space containing filamentous or granular material are reported (Sami *et al*., 1992). Eosinophilic granules in proximal tubules are described after several bacterial infections in fish and also in connection with BKD (Young & Chapman, 1978). It is possible that excess proteineous content is absorbed into the luminal fluid of the tubules. Extensive myocarditis (Wood & Yasutake, 1956; Bruno, 1986) and brain lesions in the form of meningitis or

encephalitis may develop in connection with BKD, also in the apparent absence of systemic lesions (Speare, 1997). The Gram-positive diplobacilli occurs extracellularly as well as intracellularly in association with the granulomas.
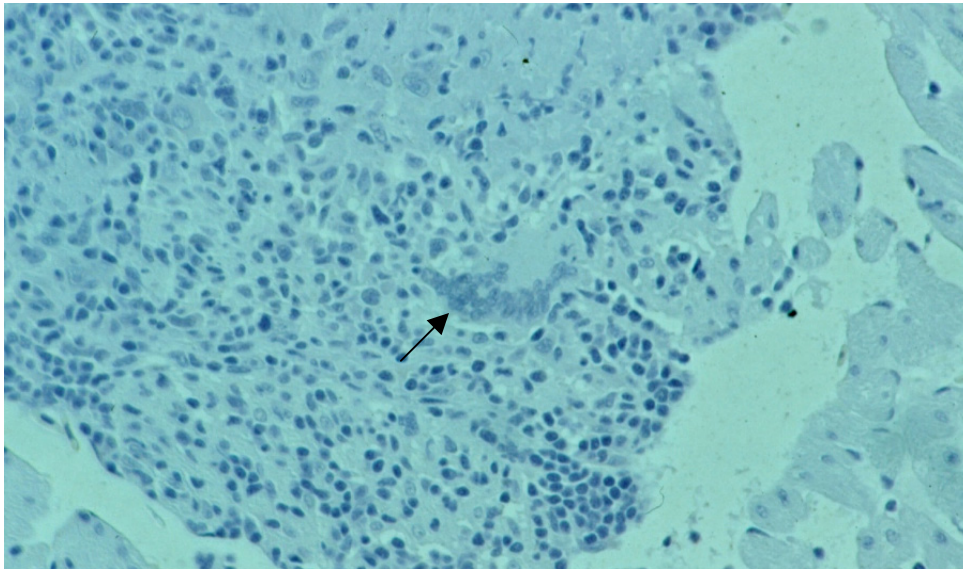

Figure 3. Heart of rainbow trout infected with *R. salmoninarum* (Paper I), multinucleated giant cell (arrow), HE X 400.
Photo: Paul Mbuthia.

Immune response

Humoral immune response
Extracellular bacteria are usually effectively destructed due to the induction of specific antibodies that bind to surface antigens of the bacterium, a process that increases phagocytotic activity. Antibodies may also induce the complement system to further trigger the inflammatory response by the production of effector molecules such as chemoattractants for phagocytes. The antibody response to *R. salmoninarum* has been shown to be directed predominantely to the major cell surface, p57 protein (Getchell *et al*., 1985) by (Bartholomew *et al*., 1991). This protein is secreted in large quantities into fish blood during infection (Turaga *et al*., 1987b).

Cell-mediated immune response
The intracellular survival of *R. salmoninarum* and the morphological lesions characterized by an accumulation of macrophages in an epitheloid structure to a granuloma indicate that an induction of the cell-mediated immune (CMI) response is necessary for recovery, as in mammals. The infiltration of lymphocytes in the

granulomatous tissue and observations of recession of injury further support a CMI activation (Munro & Bruno, 1988). Studies of CMI functions in fish are, however, limited due to scarcity of markers available for the participating cell populations.

Pathogenesis

Transmission

Feeding of *R. salmoninarum* infected flesh and viscera to juvenile chinook salmon resulted in complete transmission of BKD (Fryer & Sanders, 1981), indicating uptake through the intestinal wall. Subsequently, the pasteurisation of fish feed prevents this route of transmission. An increased vulnerability to predation has however, been demonstrated in fish with elevated *R. salmoninarum* levels (Mesa *et al*., 1998).

  Disease transmission is also possible by contact fish to fish (horizontally, (Bell *et al*., 1984; Murray *et al*., 1992)). Penetration through intact skin has been demonstrated for *Vibrio anguillarum* and the epithelial cells of the gills are the primary sites of entry for several fish pathogens (Evelyn, 1996). Since there are indications of intracellular survival of *R. salmoninarum* also in non-phagocytic cells (Bruno, 1986), these appear to be possible routes of entrance.

  Transmission of BKD from brood stock to egg (vertically) is a further effective route of transfer (Bullock *et al*., 1978; Evelyn *et al*., 1984). The bacteria are located inside the egg, in the yolk, protected from any egg surface disinfecting agents. High amounts of *R. salmoninarum* in the coelomic fluid are the main source of infection of the eggs, as the bacterium is small enough to be transmitted by the micropyle into the egg. There are also indications of infection before ovulation.

Possible virulence factors

The chronic nature of BKD does not indicate the contribution of potent toxins to the pathogenesis. DNA degradation, haemolytic and proteolytic activity was demonstrated *in vitro* (Austin & Rodgers, 1980; Daly & Stevenson, 1985; Bruno & Munro, 1986b) and injection of high concentrations of acetone precipitates of supernatants after culture was found lethal to fingerling Atlantic salmon (Shieh, 1988). The haemolytic activity was attributed to a 65 kDa polypeptide and the gene was designated hly (Grayson *et al*., 1995). Evaluation of the extracellular products of 10 strains from different geographic origins, however, did not suggest any haemolytic or cytotoxic activities *in vivo* (Bandin *et al*., 1991). A potential virulence factor is the 57 kDa hydrophobic cell-surface protein (p57, F antigen (Getchell *et al*., 1985) with haemagglutinating (Daly & Stevenson, 1990) and leucoagglutinating (Wiens & Kaattari, 1991) properties. Low-virulent strains of *R. salmoninarum* demonstrate a considerably reduced expression of p57 on the

bacterial cell surface (Bruno, 1990; O´Farrell *et al*., 2000). The gene encoding p57, msa (Chien *et al*., 1992), is however found in both virulent and attenuated strains and both types release p57 into culture supernatants at a similar level (O´Farrell & Strom, 1999). The attenuated strain was not able to propagate in the salmonid kidney (O´Farrell *et al*., 2000). The soluble *R. salmoninarum* surface proteins suppress the salmonid specific antibody response (Turaga *et al*., 1987a). The suppression was attributed to the p57 protein, but also a surface protein of 22 kDa (Fredriksen *et al*., 1997).

Immune complex formation

The abundance of the p57 antigen in the bloodstream and the subsequently production of antibodies to this protein might result in the formation of immune complexes of antigens and antibodies. In mammals, the immune complexes can be distributed to different tissues and initiate the activation of complement, inducing the Type III hypersensitivity reaction. Phagocytic activity in mammals is stimulated by opsonins, such as the C3 derivative C3b, of the complement system. The deposition of C3b on the cell surface of bacteria or on immune complexes facilitates their binding to complement receptors on phagocytic cells and erythrocytes (Payne *et al*., 1987; Payne & Horwitz, 1987; Joiner, 1988). Erythrocytes are important in the destruction of immune complexes, by carrying the C3b coated complexes to the spleen or liver for destruction. Haematological studies of Atlantic salmon and rainbow trout demonstrated a reduction in the red cell count. The reduction in cell numbers was suggested to depend on an accumulation of the erythrocytes in the spleen, supported by an observed splenomegaly (Bruno & Munro, 1986a). An increased frequency of splenomegaly was recorded in BKD infected rainbow trout compared with that in salmon (Bruno, 1986), and was also found in the experimental challenges in the present study (data not shown).

The pathogenesis of the poststreptococcal glomerulonephritis in mammals develops due to the deposition of immune complexes in the kidney. The immune complexes cannot be phagocytosed, as they are attached to the basement membrane of the glomeruli, resulting in tissue damage due to the release of lytic enzymes deliberated by attracted neutrophilic granulocytes and further to several types of damage to renal function. The epithelial surface of the glomerual basement membrane of BKD infected rainbow trout contained immunoglobulin-positive deposits, suggested to be similar kinds of immune complexes (Sami *et al*., 1992). Complement-mediated opsonisation and phagocytosis of *R. salmoninarum* have been demonstrated. Incubation of *R. salmoninarum* in rainbow trout serum was required for adherence. The accessibility of C3b on the surface of *R. salmoninarum* to complement receptors on phagocytes was demonstrated (Rose & Levine, 1992).

Environmental factors and susceptibility

Pathogenesis and mortalities in connection with BKD are affected by environmental conditions such as temperature (Sanders *et al*., 1978), salinity (Fryer & Sanders, 1981) and stress (Mesa *et al*., 1998). The macroscopical and morphological lesions described are suggested to be a combination of the effect of the bacterium itself and the result of the immunological response of the fish to the infection (Young & Chapman, 1978; Fryer & Lannan, 1993). Studies of the pathogenesis of BKD have been complicated due to differences in susceptibility between salmonid species. Pink salmon (*Oncorhynchus gorbushca),* sockeye salmon *(O. nerka*) and chinook salmon *(O. tshawytscha)* are regarded as the most sensitive species demonstrated by more aggressive infections connected with higher mortalities. Atlantic salmon (*Salmo salar*) and brown trout are fairly resistant and rainbow trout (*O. mykiss)* is considered to be relatively resistant *(*Sanders *et al., 1978;* Sakai *et al*., 1991; Dale *et al., 1997;* Starliper *et al*., 1997). The resistant species, however, usually exhibit a more pronounced encapsulation of the lesions (Belding & Merrill, 1935; Evelyn, 1993).

Renibacterium salmoninarum

Morphology

R. salmoninarum is a non-motile rod, 0.3-1.0 by 1.0 to1.5 μm, often occurring in pairs. The bacterium is Gram-positive and non-alcohol fast and does not produce endospores (Goodfellow *et al*., 1985).

Cultivation

R. salmoninarum is aerobic and fastidious in its growth requirements.
The first successful propagation of the bacteria was achieved on chicken embryo tissue. Several different agar-media were tested for sub-cultivation and growth was possible on Dorset's medium used for cultivation of *Mycobacterium tuberculosis.* Modifications of Dorset's medium, mainly by the addition of L-cysteine, greatly increased the possibility to isolate the kidney disease bacterium. Although several modifications of different agar media for *R. salmoninarum* such as L-cysteine supplemented Mueller Hinton medium (Wolf & Dunbar, 1959), Kidney Disease Medium (KDM; Evelyn, 1977) and Charcoal agar (KDM-C; Daly & Stevenson, 1985), the bacteria grows slowly and secondary bacterial infections are a serious problem in diagnostics. The selective kidney disease medium (SKDM) containing cycloheximide, cycloserine, polymyxin B sulphate and oxolinic acid has been found useful to reduce contaminations by other bacteria and fungi (Austin *et al*., 1983). The optimal temperature for cultivation is 15-18° C, no growth is possible at 30° C. Incubation times for the primary isolation of *R. salmoninarum* on SKDM was studied and initial growth of the organism could be detected as long as 19 weeks post-inoculation of the kidney sample (Benediktsdóttir *et al*., 1991). Fish tissues have been shown to have an inhibitory

effect on the growth of *R. salmoninarum* (Evelyn *et al*., 1981; Daly & Stevenson, 1988). The toxicity was associated with the soluble fraction of the kidney homogenate and could subsequently be removed by washing. Growth of *R. salmoninarum* is possible in serum-free broth, consisting of 1% peptone, 1% yeast extract and 0.1% L-cysteine, suitable for growing large batches of bacteria (Daly & Stevenson, 1993).

Recent studies have demonstrated the possibility to cultivate *R. salmoninarum* also in cell cultures. The bacteria were propagated intracellularly in the fish cell lines (EPC, CHSE and RTG-2; McIntosh *et al*., 1997; González *et al*., 1999). Cultivation in cell culture was found to be a useful technique for production of the p57 antigen, and EPC cultivated *R. salmoninarum* was more efficiently adapted to rainbow trout after intraperitoneal injection than *R. salmoninarum* cultivated on agar media.

Biochemical reactions
R. salmoninarum is inactive in conventional biochemical tests based on the utilization of different carbohydrates for growth. The API-ZYM test has been suitable for identification, since this is an enzymatic test for which propagation of the bacterium is not necessary (Goodfellow *et al*., 1985; Austin & Austin, 1987). A strong catalase positive reaction, a negative oxidase test and proteolytic activity are described (Ordal & Earp, 1956; Goodfellow *et al*., 1985). The bacterium is β-haemolytic but the activity declines after prolonged cultivation (Bruno & Munro, 1986b).

Cell wall
In the Gram-negative bacterium the cell wall is multilayered, in the Gram-positive bacterium, in contrast, the cell wall consists of a single, thick, continuous layer. The mechanical strength of the cell wall is achieved by peptidoglycan, which, in most Gram-positive bacteria, is the major polymer of the cell wall, associated with polysaccharides and the teichoic acids. The amount of peptidoglycans in the cell wall of *R. salmoninarum* is, however, low. The peptidoglycan is of the A3α type, containing lysine with N-acetylmuramyl residues linked by phosphodiester bridges to the polysaccharide. The polysaccharide is the major component of the cell wall (60-70% of the dry weight) and contains galactose as the major sugar component and the unique amino sugar N-acetyl-fucosamine (Kusser & Fiedler, 1983; Fiedler & Draxl, 1986). The covalently linked peptidoglycan to the polysaccharide was suggested to account for the rigid strength of the cell wall of *R. salmoninarum* that not is possible to lyse by use of lysozyme. Electron microscopic studies revealed the presence of a capsule surrounding *R. salmoninarum* (Dubreuil *et al*., 1990). The surface has been demonstrated to be hydrophobic and to have haemagglutinating properties (Daly & Stevenson, 1987). The hemagglutinin was demonstrated to be identical with the soluble heat stable

p57 antigen (Daly & Stevenson, 1990) that is the target antigen in several immunological diagnostic assays (Daly, 1989).

Taxonomy

Based on the morphological appearance of pleomorphic short rods, which frequently occur in pairs, the kidney disease bacterium was initially considered to be a species of the genus *Corynebacterium* (Ordal & Earp, 1956). A new genus was proposed after studies of the cell wall composition and the guanine/cytosine (G/C) content (53 %) of DNA (Sanders & Fryer, 1980). *Renibacterium salmoninarum* sp. nov. was suggested for the new species (type strain Lea-1-74; ATCC 33209). Chemical and microbiological studies demonstrated a distinct and homogeneous species (Goodfellow *et al*., 1985; Bruno & Munro, 1986b) and serological analysis indicated serologic homology (Bullock *et al*., 1974; Austin & Rodgers, 1980; Getchell *et al*., 1985).

Comparison of the 16S ribosomal RNA with different species of Gram-positive bacteria of several genus classified *R. salmoninarum* into a member of the *Arthrobacter-Micrococcus* subline of actinomycetes, releated to *Arthrobacter*, *Micrococcus, Jonesia, Brevibacterium etc*. (Stackebrandt *et al*., 1988). Phylogenetic investigations supported the classification to the high G/C eubacterial subdivision (Gutenberger *et al*., 1991) consistent with the reevaluation of the mol percent G/C that was determined to be 55.5 (Banner *et al*., 1991).

The uniformity of the species has rendered epizootiologic studies difficult to accomplish. The 16S-23S ribosomal DNA region was found highly conserved. Complementary analysis by using randomly amplified polymorphic DNA (RAPD) allowed, however, differentiation between isolates of diverse host and geographic origin (Grayson *et al*., 1999; Grayson *et al*., 2000). Four different intergenic spacer sequences between 16S and 23S rRNA genes (sequevars, SV) were identified. The dominating, highly conserved SV 1 was present in strains from Canada, Norway, Sweden, the United Kingdom and the United States. SV 2 was detected in isolates from Iceland and Japan, SV 3 from Canada and SV 4 from Norway and Scotland. Polymerase Chain Reaction (PCR) amplification of the spacer regions separating tRNA genes provides a further technique for differentiation of isolates in epizootiological studies (Alexander *et al*., 2001).

Diagnosis

BKD in clinical disease

Characteristic indications of advanced cases of BKD are the enlarged greyish kidney and the occurrence of white nodules in internal organs such as kidney, heart, liver and spleen. The characteristic lesions are verified by Gram-staining of smears and by immunological techniques, since cultivation of the bacteria is very time-consuming due to the slow growth. Histologically, BKD is characterized as a

systemic, chronic, granulomatous inflammation. The Gram-positive bacteria are difficult to distinguish in fixed organs by standard Gram staining and the modified Ollet´s Gram stain, Lillie´s allocrome stain or PAS are recommended to demonstrate the intracellular occurrence (Midtlyng *et al.*, 2000). Immunohistochemical techniques have been developed and a specific identification of the bacterium in combination with observations of the morphological lesions is possible (Jansson *et al.*, 1991; Evensen *et al.*, 1994). Chronic inflammations in fish are characterized by an increased amount of melanomacrophages and dispersed melanin (Bruno, 1986). The melanin pigments are difficult to distinguish from intact bacteria in immunohistochemistry due to a similarity in size and in colour of the oxidation product of commonly used chromogens. Intense bleaching by a strong hydrogen peroxide solution was efficiently applied to avoid this problem without any effect in immunoreactivity of the identified epitopes (Jansson *et al.*, 1991).

BKD in covert infection

Various manifestation of BKD is reported form the obvious, clinical disease described above to more diffuse lesions and even of asymptomatic carriers (latent carriers) without any signs of disease. The identification of *R. salmoninarum* in latent carriers fish has been an important task since the main defence against the disease is avoidance. The slow growth of the organism increases the risk for overgrowth by contaminating microbes, resulting in a false negative interpretation. Cultivation of *R. salmoninarum* on selective agar has been used in fish health screening programmes, but the prolonged incubation time of at least 12 weeks that is necessary to detect covert infections (Benediktsdóttir *et al.*, 1991), is epizootiologically unsatisfactory.

Immunological techniques have been developed; such as enzyme-linked immunosorbent assay (ELISA), dot blot and Western blot for detection of *R. salmoninarum* antigens in internal organs (Sakai *et al.*, 1987; Pascho & Mulcahy, 1987; Griffiths *et al.*, 1991; Hsu & Bowser, 1991; Rockey *et al.*, 1991; Gudmundsdottir *et al.*, 1993; Olea *et al.*, 1993; Jansson *et al.*, 1996, Paper I). These techniques offer a higher sensitivity than cultivation and especially the ELISA technique is suitable for analyses of the numerous samples necessary in monitoring during health control programmes. Common among immunological techniques applied is the utilisation of antigen-antibody affinity to detect and visualise the presence of the antigens. The strength of the antibody-epitope interaction (affinity) and the stability of the antibody-antigen complex (avidity) are crucial for the efficiency of the immunological techniques. The sensitivity and specificity depend on the quality of the antibodies employed. Monoclonal antibodies, produced by a single B-cell line, used to be superior in specificity since only a single epitope is identified and all antibodies are identical. The polyclonal antibodies, obtained from several B-cell lines, react with various epitopes on the antigen and consequently have several targets for binding, resulting in a higher potential for recognition.

Molecular techniques, such as the dot-blot assay for detection of ribosomal RNA from *R. salmoninarum* (Mattsson *et al*., 1993) and PCR-assays for amplification and identification of unique sequences of the bacterial nucleic acids have been developed (Brown *et al*., 1994; León *et al*., 1994; Magnússon *et al*., 1994; Miriam *et al*., 1997; Chase & Pascho, 1998; Pascho *et al*., 1998; Rhodes *et al*., 1998; Cook & Lynch, 1999; Heldtander *et al*. manuscript in preparation). These techniques are valuable as complementary techniques for conformation of BKD, especially since only small amounts of samples are necessary for analysis and good sensitivity is demonstrated.

Disease control

Antibiotic treatment has only temporary effect, possibly due to the intracellular survival of the bacteria, and is not recommended as therapy. Regularly screening programmes to prevent horizontal, as well as vertical transmission of BKD have been the most important possibility of control (Elliott *et al*., 1989; Fryer & Lannan, 1993). In areas with high prevalence of BKD, horizontal transmission has been reduced by rearing eggs and juvenile fish in separate groups according to the infection level of the female parent (Elliott *et al*., 1995). Erythromycin injection of brood stock, before spawning, has reduced the transmission of *R. salmoninarum* to the eggs. This might, however, increase the risk for selection of erythromycin-resistant bacteria (Evelyn *et al*., 1986). Brood stock culling, the destruction of gametes from BKD classified parents, has been demonstrated to reduce the prevalence of the disease (Gudmundsdottir *et al*., 2000). This requires a sensitive technique for identification of carriers, and a careful separation of the eggs until the diagnosis is established.

Vaccination experiments have so far had limited progress. Recent investigations, however, have demonstrated that oral administration of *R. salmoninarum* expressing low levels of cell associated p57, resulted in an extension of the mean time to death after challenge (Piganelli *et al*., 1999). The protection was not related to the humoral antibody level. This supports earlier histopathological indications of an involvement of the cell-mediated immune response in recovery, due to the intracellular survival and the composition of inflammatory cells in connection with signs of regression (Munro & Bruno, 1988). Advances in the control of BKD depend on an understanding of the pathogenesis of BKD and knowledge of basic immune functions in fish (OIE, 2000).

The immune system of fish

Knowledge of immune functions in fish is limited although advancements have been made during recent decades, as a consequence of the increase of the aquaculture industry. As in the immune system of mammals, both innate and adaptive immune functions exist, which efficiently defend the host against different kinds of pathogens such as viruses, bacteria and parasites. Fish and mammals diverged from each other millions of years ago and have quite different manners of living. Undoubtedly, there will be differences in the organization of the immune system.

Innate immunity
Physiochemical barriers such as scales and epithelial surfaces (on gills, skin and gut) with secreted mucus are the primary defence line against the various infective agents the fish constantly have to encounter. Non-specific cytotoxic cells (NCC), monocytes/ macrophages, granulocytes, and eosinophilic granular cells (EGC) are involved in the non-specific cellular defence (Secombes, 1996). Humoral substances and cell secretions of complement, C-reactive protein, interferon, lysozyme, toxins, transferrin, lectins, *etc.*, contribute to natural resistance against infectious agents (Yano, 1996; Ingram, 1998). The NCCs, reactive to xenobiotic grafts (tissue from a different species than the recipient) and also to infective parasites, are suggested to be especially important at low temperatures when the specific immune response activity is reduced. No cytoplasmic granules are observed inside these lymphocyte-like cells and direct cell contact is necessary for killing (Secombes, 1996).

An acute inflammatory response in fish, as in mammals, is characterized by accumulation of phagocytic cells such as neutrophilic granulocytes (neutrophils) and monocytes in the blood, and an increase of fluid, phagocytes and lymphocytes at the site of infection. The migration of the phagocytes to the area is stimulated by chemoattractants such as chemokines and chemotactic agents. The expression of adhesion molecules on cells in the affected area is important for the migration in mammals, yet to be studied in fish. The EGCs, that are located in the gills, gut, meninges, skin, that surround major blood vessels are considered to have functions similar to the mammalian mast cells and contribute to the influx of serum components to the affected area. Such a component is C-reactive protein that binds to the polysaccharides of the bacterial cell wall, thereby activating the complement system to lysis or phagocytosis of the pathogen. The inflammatory cells appear during the first 3-4 days after initiation of infection and immediately start to internalise, kill and digest the infective agents. The adherence of an organism to selective receptors on the membrane of a phagocyte influenced by complement factors, initiates the internalisation process. Adherence induces the formation of pseudopodia that enclose the agent in a phagosome for fusion with a

lysosome to form a phagolysosome. Hydrogen peroxide, hydrolytic enzymes, lysozyme, oxygen-free radicals, peroxidase, *etc.* from the lysosome digest the internalised material. Intracellular enzymes are also released into the tissue during the phagocytic lysis inducing hemorrhagic liquefaction.

Encapsulated bacteria adhere poorly and are thereby less readily internalised. Hydrophobic bacteria, however, are more likely to be taken up by the macrophages (van Oss, 1978). The hydrophobicity and the leukocyte agglutinating activity of *R. salmoninarum*, attributed to the p57 protein, indicates similarities of p57 with proteinaceous adhesins with affinity to cellular receptors that might contribute to the attachment (Daly & Stevenson, 1987; Bruno, 1988; Wiens & Kaattari, 1991).

Characteristics of the non-specific defence are the fast response, without a specificity or memory part, with activity also at low temperatures. Cells and mediators of the specific response may, however, stimulate the participants of the non-specific defence to a more potent activity, especially during prolonged infections as during persistent infections. The respiratory burst of activated macrophages generates reactive oxygen and nitrogen intermediates with increased antimicrobial and cytotoxic activities.

Adaptive immunity
Lymphoid cells of the adaptive immune system of mammals can be distinguished as B- and T-lymphocytes (B- and T-cells, respectively) according to their function, maturation and phenotype. Antibodies are produced by activated B-cells and T-cells express T-cell receptors (TCR). T-helper cells (generally $CD4^+$ T-cells) are the main regulators of the immune response and cytotoxic T-cells (mainly $CD8^+$ T-cells) can be activated to eliminate altered self-cells. The first reaction to a foreign molecule is the initiation of a clonal expansion of the reactive lymphoid cell population. This specific proliferate response, as well as, variable genes for specific receptors were evolutionarily, initially described in the vertebrates. The adaptive immune functions appear to have arisen during the stage of fishes in the evolution (Pasquier & Litman, 2000).

Markers to salmonid immunoglobulin have been developed (DeLuca *et al.*, 1983; Thuvander *et al.*, 1990). Lymphocytes expressing immunoglobulin on the surface (Ig^+ lymphocytes) have been regarded as a homologue population to the B-cell population since this population responded to a mammalian B-cell mitogen. The Ig^- lymphoid population was stimulated by T-cell mitogens. However, no markers to the TCR have been developed although the α- and β gene sequences of the TCR have been found conserved (Partula *et al.*, 1995; Partula *et al.*, 1996).

Cell mediated immunity (CMI), the ability to transfer a specific response by living cells, is mediated by the T-cell. Transplantation studies of fish have

demonstrated that the initial reaction to allogenic graft (tissue from an individual with a different genetic constitution but of the same species) results in an infiltration of lymphocytes and macrophages to the transplantate. The infiltration results in breakdown of the melanophores in the epidermis. Similar reactions are induced after a secondary transplantation from the donor, however the destruction of the graft is now more efficient. In mammals, the allograft destruction is mediated by cytotoxic CD8$^+$ T-cells. These cells recognize foreign antigens in association with altered major histocompatibility complex (MHC) antigens of class I. MHC class I is expressed on all nucleus-containing cells of an individual. The occurrence of CD8$^+$ T-cells in fish has been indicated since CD8 transcription has been demonstrated in rainbow trout (Hansen & Strassburger, 2000). Recently, two kinds of cells with specific cytotoxic activity to alloantigenic cells have been described in fish. Two different kinds of response with respect to antigen recognition and cytotoxic mechanisms were demonstrated, although both populations expressed TCR Vα and Vβ genes and held lytic granules (Zhou *et al.*, 2001).

The mixed leukocyte reaction (MLR) is a response of CD4$^+$ T-cell to a non-compatible MHC class II molecule on an antigen-presenting cell. The activated CD4$^+$ T cell produces interleukin 2 (IL-2) that stimulates the proliferation of all T-cells expressing the appropriate receptor for IL-2. Irradiation of a T-cell population makes the cells unable to proliferate after stimulation, but have still the ability to produce cytokines for stimulation of another untreated population. Mammalian T-cells are the responders in the MLR but cannot act as stimulators. Separation of the Ig$^+$ and the Ig$^-$ lymphocyte population of channel catfish (*Ictalurus punctatus*) demonstrated that the Ig$^-$ lymphocyte population was the responding population, however the Ig$^-$ population could also act as stimulators. This indicates the occurrence of two functionally different subpopulations included in the Ig$^-$ population (Miller *et al.*, 1986).

Temperature and immune functions
Immune reactions in fish depend, not unexpectedly, on the ambient water temperature. Low temperatures of acclimation of the fish, as well as the *in vitro* temperature of the assay, suppressed the proliferate response to a T-cell mitogen. The permissive temperature interval designed to obtain an optimal immune response to a B-cell mitogen remained unaffected by both *in vivo* and *in vitro* temperature (Clem *et al.*, 1984). Impairment of T-cell functions at low temperatures was suggested to depend on changes in the T-cell plasma membrane (Bly & Clem, 1992).

Immunity to persistent infections

Phagocytosis of R. salmoninarum

Ultrastructural studies demonstrated that *R. salmoninarum* could survive and multiply intracellularly in the fish macrophages (Young & Chapman, 1978; (Gutenberger *et al.*, 1997), Fig.4. The bacteria survived inside the macrophages for up to 4 days *in vitro* and were thereafter killed. Survival in rainbow trout macrophages was suggested to depend on the ability to resist killing and not by inhibition of the macrophages capacity to kill (Bandin *et al.*, 1993). Opsonization with immune serum increased the intracellular survival, indicating that both complement and antibodies have an effect on the resistance to bactericidal activity (Bandin *et al.*, 1995).
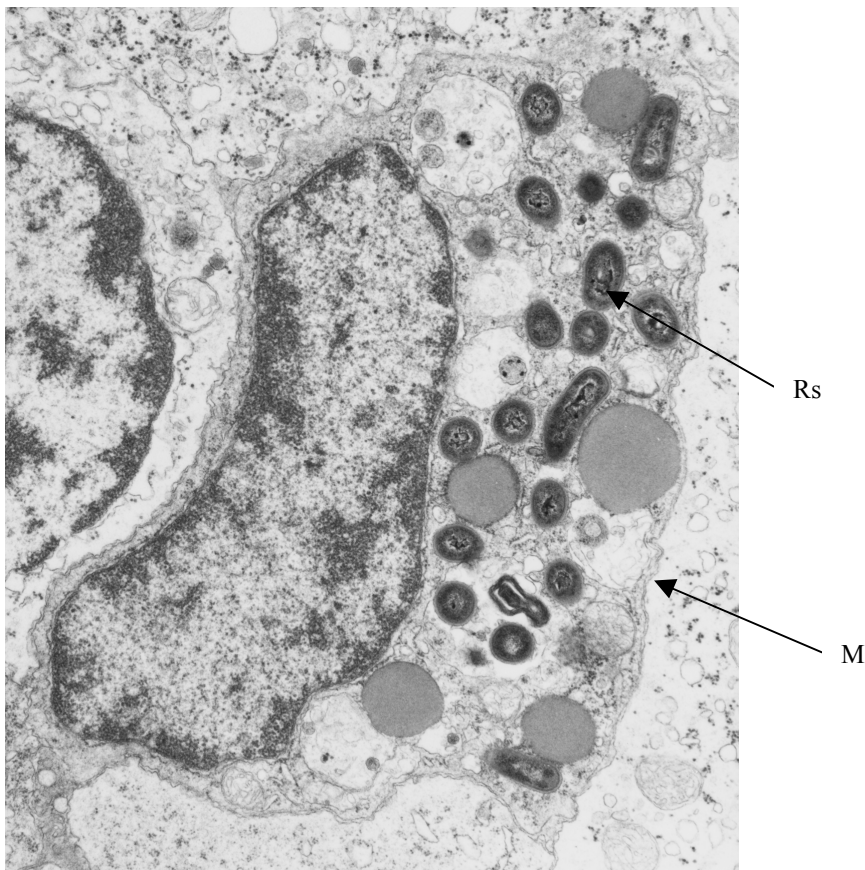

Figure 4. Macrophage (M) of rainbow trout kidney containing *R. salmoninarum* (Rs), EM. *R. salmoninarum* bacteria occur inside phagosomes but also free in the cytoplasm. Photo: Leif Norrgren.

Macrophage activating factor (MAF), containing cytokines such as interferon-γ, with an ability to initiate the CMI response was demonstrated to increase the phagocytic and the respiratory burst activity to *R. salmoninarum*. The increased

killing capacity was an indication that a CMI response will have the potential to eliminate the infection (Hardie *et al*., 1996).

Defence against intracellular bacteria in mammals

CMI response, in the form of the delayed-type hypersensitivity (DTH) reaction, is important in mammals in the defence against intracellular pathogens such as *Mycobacterium tuberculosis, Mycobacterium leprae* and *Listeria monocytogenes*. Antigen-specific T-cells initiate a cascade of cell interactions, resulting in an incredible amplification and activation of macrophages, the main effector cells of the DTH response, as a result of the persistent infection. The accumulated macrophages adhere closely to each other and form an epitheloid cell structure of a granuloma, with ability to demarcate the infection. Although this kind of response effectively kills the infective bacteria, the immunological reaction itself may result in detrimental, extensive tissue damage, due to the release of high amounts of lytic enzymes that promote tissue necrosis.

Delayed-type hypersensitivity reactions in fish

Protection against the facultative intracellular haemoflagellate *Cryptobia salmositica* in rainbow trout requires an activation of the humoral as well as the CMI response. The DTH reaction was indicated *in vivo*, by a local infiltration of mononuclear cells and *in vitro* by the inhibition of macrophage migration by antigens from the parasite (Thomas & Woo, 1990). The DTH reaction has also been induced in rainbow trout after injection of killed *M. tuberculosis* or of the fish pathogen *M. salmoniphilum (M. fortuitum)*. These studies indicated differences in the binding and processing of bacterial antigens, since the typical DTH response was only observed after injection of whole bacterial cells and not by injection of the soluble proteins as is possible in the mammalian DTH reaction (Bartos & Sommer, 1981). The indications of cross-reactivity to bacterial antigens and the influence of the temperature on the response demonstrate divergences to the mechanisms known from endothermic vertebrates.

Aims

The aim of the present thesis was to develop methods to study immune response and immune functions in salmonid fish during the progression of Bacterial Kidney Disease (BKD). Immunological techniques to follow the infection level were evaluated because these may offer high sensitivity and also a rational design for experimental and epizootiological investigations.

Materials and methods

The materials and methods used during the course of the present studies are summarized below. For a more comprehensive description, see Papers I-IV.

Fish (I-IV)

Rainbow trout (*Oncorhynchus mykiss;* Papers I-IV) and Atlantic salmon (*Salmo salar;* Papers I and II) were kept in tanks (250 or 1200 l) supplied with continuously flowing well water at a temperature of 10± 2 ºC. A photoperiod of 12 h light and 12 h dark was used. The fish originated from a local fish farm that was declared free from bacterial or viral fish diseases by The Swedish Fish Health Control Programme. Fish designed to be included in the experimental infections (Papers I-II and IV) were tested for the occurrence of soluble *Renibacterium salmoninarum* antigens by ELISA immediately upon arrival (as described in Paper I). The fish were acclimatized to laboratory conditions for at least 3 weeks before use in any experiment.

Experimental challenge (I, II and IV)

Randomly selected subgroups of fish were divided into 250 l tanks for immersion for 30 min. in oxygenated water containing 10^8 *R.salmoninarum*/ml, determined by viable count. Fish in the control groups were treated in a similar way, except for the addition of bacteria. The temperature was elevated for all groups in connection with the immersions and held at 14±3 ºC throughout the experimental period. The occurrence of *R .salmoninarum* antigens in kidney and the humoral antibody response to *R. salmoninarum* antigens was followed for up to 8 weeks in Paper I, 12 weeks in Paper II and up to 31 weeks in Paper IV. In Paper IV the lymphocyte population of spleen was also regularly characterized by flow

cytometry and the *in vitro* lymphocyte stimulation test applied to measure response after stimulation by mitogens and *R. salmoninarum* antigen preparations.

Sampling in fish farms (I and II)

Blood and/or kidney samples were collected from rainbow trout, Atlantic salmon, Arctic char (*Salvelinus alpinus*) and from brown trout (*Salmo trutta)* from fish farms declared free from BKD or with enzootic or clinical BKD. Kidney samples from all collected fish were tested for bacterial culture (Papers I and II).

Bacterial culture (I, II and IV)

Kidney samples were homogenized in peptone-saline.
The pellet after centrifugation was spread on SKDM or KDM-C-agar, in order to detect *R. salmoninarum* and also on Blood agar (5% horse-blood) to identify infection by common fish pathogenic bacteria. Occurrences of *Flavobacterium psycrophilia or F. columnaris* were tested on KDM-C agar since this medium has been found convenient for isolation of these species (experiences at Department of Fish, National Veterinary Institute). Strains of *R. salmoninarum* were verified by Gram- and immunofluorescence staining of smears and further by catalase, oxidase and the API-zym test. Further bacterial fish pathogens were identified by conventional biochemical tests.

Preparation of polyclonal R. salmoninarum antibodies (I)

R. salmoninarum antigens for immunization of rabbits were prepared from strain 4/86 from the culture collection of the National Veterinary Institute, isolated from a rainbow trout with clinical BKD. Iscoms were prepared from detergent-extracted and sonicated antigens that were mixed with cholesterol and Quil A and dialysed against PBS. Hemagglutinin was extracted by washing in distilled water at a pH of 5.5 according to (Daly & Stevenson, 1990). Rabbits were immunized by intramuscular injection of the iscom preparation or with extracted hemagglutinin in Freund´s adjuvant. Rabbit IgG was purified from serum on a Protein G column (Pierce, Rockford, IL, USA) dialysed against PBS and freeze-dried. Specificity of the IgG were tested by Western-immunoblotting and both preparations resulted in antibodies with the main reactivity to the 57 kDa but also reactive with further 2 molecules of lower size.

Preparation of monoclonal antibodies to rainbow trout lymphocytes (III)

Mice were immunized with synthetic peptides, prepared from selected parts of the α- and β- gene sequences of the T-cell receptor of rainbow trout, coupled to ovalbumin and mixed with Freund´s adjuvant by intraperitoneal injections. Mice were also immunized with immune complexes of lymphocytes and the monoclonal antibody 1C2, obtained after immunization with the synthetic peptides. Hybridoma cultures were produced following standard procedures (Köhler & Milstein, 1975; Köhler & Milstein, 1976): Splenocytes from immunized mice were fused with the myeloma cell line Sp2/0 in polyethylene glycol (Sigma, St. Louis, USA) and seeded in microtitre plates with thymocytes as feeder cells. The hybridoma supernatants were tested for the presence of antibodies to the lymphocytes by ELISA and/or by flow cytometry.

Enzyme-Linked Immunosorbent Assay (ELISA)

Detection of R. salmoninarum antigens (I, II and IV)

Kidney samples for detection of *R. salmoninarum* antigen were homogenized in PBS and the solvent HemoDe (Fisher Scientific, Pittsburg, USA). The samples were heated to 98±2 °C and the aqueous phase after centrifugation was tested in the ELISA. Microtiter plates (Nunc, Roskilde, Denmark) coated with rabbit IgG to *R. salmoninarum* antigens were blocked with 1% bovine serum albumin (Sigma, St. Louis, USA). Samples and controls were added in duplicate and affinity-purified, peroxidase-labelled goat anti-*R. salmoninarum* (Kirkegaard & Perry laboratories Inc., Gaithersburg United States) used as conjugate. The enzymatic reaction was visualized by tetramethybenzidine (Kebo, Stockholm, Sweden, TMB) as substrate and optical density values (OD) were measured at 450 nm. Saline, containing 0.5 % Tween 20, were used for washing between each step in the ELISA and incubations were performed at 24 ºC. Optimal dilutions for the assay (antibodies for coating, samples and conjugate) were initially determined.

   The negative-positive threshold value of the ELISA was defined as the mean OD value plus 3 standard deviations of samples collected from populations classified as free of BKD, verified by bacterial culture. All samples that exceeded the threshold value were retested as above, but also on wells coated with normal rabbit IgG, as a control of false positive reactions.

Detection of humoral antibodies (II and IV)

Microtitre plates were coated with heat-treated *R. salmoninarum* (100 ºC, 15 min.). Fish serum diluted 1:100 were added in duplicate and the plates were incubated at 15 ºC overnight. Bound antibodies were detected by monoclonal

antibodies (Mabs) to salmonid Ig (Clones 1.14 or 4C10; DeLuca *et al*., 1983; Thuvander *et al*., 1990) followed by a rabbit anti-mouse Ig-HRP conjugate and the substrate TMB. Incubations with Mabs and conjugate were performed at 37 ºC, with washing and OD measurements as above. Total Ig levels were determined on microtitre plates coated with rabbit antibodies to salmonid Ig (Thuvander *et al*., 1987) and subsequently performed in accordance with the ELISA for detection of antibodies to *R. salmoninarum,* except that serum samples were tested at dilutions of 1:32 000. For determination of the amount of bound Ig (in both assays), serum samples were compared with a standard of serial dilutions of affinity-purified rainbow trout Ig.

Preparation of leucocytes (III and IV)

Blood was collected from the caudal vessel into heparinized vacutainer tubes and was diluted in Leibowitz cell culture medium (L15) supplemented with gentamicin and glutamine. Dilute blood was layered on 54% Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) and centrifuged at 400g for 10 minutes. The leucocytes were obtained at the interface between the Percoll and the plasma. The leucocytes were washed and the procedure was repeated. Spleen and thymus tissue in L15 were pressed through metal nets, further dispersed through a syringe and subsequently separated on Percoll as above.

Flow cytometry (III and IV)

Leucocytes were incubated at 4 ºC with hybridoma supernatant in microtitre plates. The cells were washed twice in PBS and incubated with fluorescein isothiocyanate (FITC) conjugated rabbit anti-mouse Ig antibodies (DAKO A/S, Copenhagen, Denmark). Purified Mabs, directly coupled to 7-amino-4-methylcoumarin-3-acetic acid (AMCA), FITC or biotin (revealed by streptavidin-phycoerythrin PE, Pharmingen, San Diego, United States) were used in the double-staining experiments. After washing, the samples were analysed in a flow cytometer (FACStar^{PLUS}, Becton Dickinson Immunocytometry Systems, San José, United States). Size, granularity and fluorescence were collected for each cell. For each individual sample 20 000 events were acquired and the proportion of labelled cells was determined after gating for cells that corresponded in size and granulation to the lymphocyte population.

Lymphocyte stimulation test (III and IV)

Leucocytes were added to sterile microtitre plates (1x10^6 /well). The mitogens Lipopolysaccharide (LPS, Sigma, St.Louis, USA) or Concanavalin A (Con A, Pharmacia Biotech Uppsala, Sweden) were added to final concentrations of 50 and 5 µg/ml L15. *R. salmoninarum* antigens were prepared from strain 4/86,

cultivated in broth (Daly & Stevenson, 1993). The bacteria were washed and resuspended in PBS for heat treatment in an autoclave (98±2 °C for 20 min) or in freshly prepared paraformaldehyde (PFA) for fixation. The supernatant, after the first centrifugation, was heat-treated as above in order to obtain the *R. salmoninarum* growth initiating factor(s) (Evelyn *et al.*, 1990). Mitogens and antigens were added in triplicates for each fish, incubated at 20 °C, pulsed with 3 H-thymidine after four days and harvested after further 24 h onto glass-fibre filters in a Betaplate harvester (LKB Wallace, Mountain View, United States). Radioactivity was measured in a beta counter (LKB Wallace). Results are expressed as stimulation indices (SI): mean counts per minute (cpm) of stimulated cultures/mean cpm of non-stimulated cultures.

Statistical analysis

Differences in the efficiency as capture antibodies in the ELISA between the iscom and hemagglutinin induced antibodies, respectively, were compared by the Mann-Whitney Test. The Mann-Whitney test was also used for calculation of differences in *R. salmoninarum* antigen levels and antibody response between challenged and control groups. The Spearman R correlation test was used for comparison between the antibody response and the occurrence of *R. salmoninarum* antigens in the kidney of individual fish. Percentages of fluorescent cells in flow cytometry are expressed as the mean value±SE of data from separate experiments with cells either from a single fish or from pools of 2-5 fish. A p-value <0.05 was required for determination of significant differences between groups.

Results and discussion

ELISA for detection of R. salmoninarum antigens (I)

A polyclonal ELISA for detection of the heat-stable 57 kDa antigen (antigen F) in salmonid kidney samples was developed. The capture antibodies used in the ELISA was obtained after immunization of rabbits with *R. salmoninarum* antigens incorporated into immunostimulating complexes (iscoms). Iscoms have been demonstrated to be effective as immunogens due to a favourable presentation of the antigens but also by an adjuvant function (Dalsgaard, 1978). Iscoms, complexes build of cholesterol and Quil A (the extract of the bark from the tree *Quillaja saponaria* Molina), interact with hydrophobic substances, such as amphipatic proteins (Morein *et al.*, 1984). The hydrophobic properties of the *R.salmoninarum* hemagglutinin (Daly & Stevenson, 1987; Bruno, 1988) seem to have contributed to the efficient immunogen preparation for immunization. The

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iscom-induced antibodies were superior as capture antibodies in the ELISA when compared with *R.salmoninarum* hemagglutinin induced antibodies. The negative-positive threshold value in the ELISA was determined to 0.1 by analysis of samples obtained from regularly monitored fish farms considered to be free from BKD. The specificity of the ELISA was determined to 99.7% when compared with bacterial culture. The sensitivity of the assay was comparable and even indicated to be better than cultivation under optimal conditions, in samples collected from populations with clinical or enzootic BKD. However, 20% of the samples that were intended for bacterial culture have to be excluded from the comparison due to overgrowth by contaminating microbes. There was no indication of any disturbances in the analysis of the contaminated samples in the ELISA analysis. The identification of the heat-stable p57 *R. salmoninarum* soluble antigen is a suitable target for recognition, since the release of the antigen into tissues during infection increases the accessibility of antigens for the capture antibodies. This might explain the higher sensitivity in ELISA techniques compared with cultivation. Naturally infected fish kidneys estimated to contain 10^3 *R. salmoninarum*/gram kidney were classified positive by the ELISA. The level of the p57 protein could be followed in both rainbow trout and salmon with experimentally induced BKD.
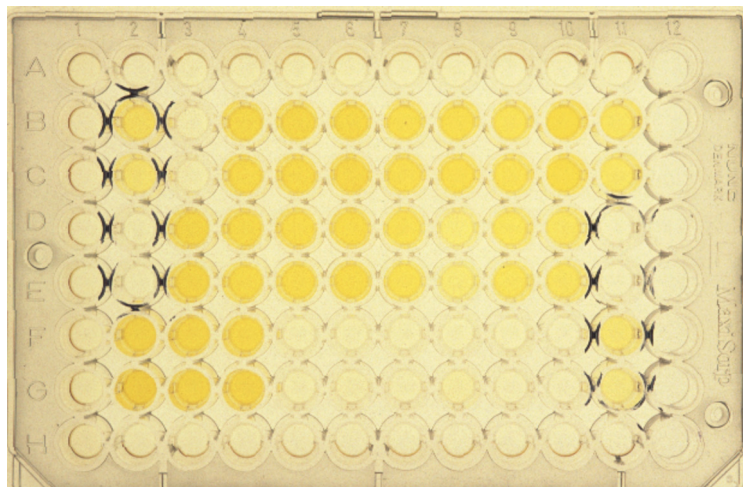

Figure 5. ELISA for detection of the p57 protein of *R. salmoninarum.*
Kidney samples are incubated in wells of microtitre plates, and
p57 are captured and identified by use of specific antibodies. Wells containing
samples with *R. salmoninarum* are stained yellow, wells without *R.salmoninarum*
are unstained. Photo: National Veterinary Institute.

ELISA for detection of antibodies to R. salmoninarum antigens in salmonid sera (II)

Monoclonal antibodies to salmonid Ig were used in an ELISA for determination of the humoral response to heat-stable *R. salmoninarum* antigens. Elevated antibody levels to *R. salmoninarum* were measured in experimentally challenged rainbow trout (*Oncorhynchus mykiss*) and salmon (*Salmo salar*). A slow antibody response was found as only 3% were classified as positive 4 weeks after challenge, although the experimental temperature was considered to be sufficient for antibody development. The proportion of seropositive fish was, however, increased and 72% were classified positive at the 8-week sampling. Elevated antibody levels were also measured in naturally exposed populations with clinical BKD. The antibody ELISA method was found useful to follow the progression of BKD at the population level in controlled experimental situations, as blood samples can be analysed without first killing the fish. The long induction time required for the antibody response and elevated OD values also in individual non-exposed fish show that the technique not could be used for general fish health monitoring.

Monoclonal antibodies to lymphocytes of rainbow trout (III)

Three monoclonal antibodies (Mabs) to lymphocytes of rainbow trout were developed. Mab 1C2, obtained after immunization by a synthetic peptide homologue to a part of the β-chain of the T cell receptor of rainbow trout, identified a protein of about 40 and also of 55-60 kDa. Immunizations by immune complexes of lymphocytes coupled to Mab 1C2 resulted in Mabs 7A5/20 and Mab 8H4/23, respectively. Mab 7A5/20 identified an antigen of about 75-80 kDa and Mab 8H4/23 proteins of 70, 100 and 150 kDa. Flow cytometry analysis of the lymphocyte population of blood and spleen, after double staining with the developed Mabs and the Ig$^+$ marker 1.14 (DeLuca *et al*., 1983), demonstrated identification of subpopulations of both Ig$^+$ and Ig$^-$ lymphocytes. Mab 1C2 labelled 18%, Mab 7A5/20 identified 9% and Mab 8H4/23 was reactive with 85% of the lymphoid population of spleen in flow cytometry (FCM) analysis. The proportion of Ig$^-$ lymphocytes was increased when Ig$^+$ lymphocytes were eliminated by immunomagnetic separation. Mabs 1C2 and 8H4/23 labelled 9 and 98%, respectively, of the lymphocytes in thymus. These results show that the developed Mabs identify different lymphocyte subpopulations than the Ig$^+$ marker 1.14. Thrombocytes are difficult to distinguish from lymphocytes with FCM in fish due to similarities in size and granulation. No cross-reactivity was found in double-staining experiments with Mabs 1C2, 7A5/20, 8H4/23 and thrombocyte markers of rainbow trout. The T-cell population is the dominating lymphocyte population in mammals. The proportion of Ig$^+$ lymphocytes in blood of rainbow trout was determined to 52 % in the present investigation. Fc receptors binding the Fc domain of the antibody are important in the regulation of the immune

response in mammals. Indications of similar receptors on leucocytes of salmonids are described (O´Dowd *et al.*, 1998). Molecular characterization of lymphoid cells in channel catfish indicates that several subpopulations of cytotoxic effector cells exist, both with the expression of T-cell receptor genes or without, but also with Fc bound Ig on the surface (Stuge *et al.*, 2001). These findings indicate that the Ig^+ lymphocyte population not is identical with the B-cell population.

Mab 8H4/23, suggested to be a universal lymphocyte marker, labelled lymphoid cells in the peripheral parts of the thymus. Distinctly appearing structures become visible after the staining, structures with a shape similar to that in nurse cells described in mammals. These thymocyte-epithelial-cell complexes have been described *in vitro* and ultrastructurally *in situ* also in rainbow trout (Flano *et al.*, 1996). The demonstrated applicability of Mab 8H4/23 in immunohistochemical investigations Fig.6, suggests that it could be useful in studies of the ontogeny of rainbow trout lymphoid cells.
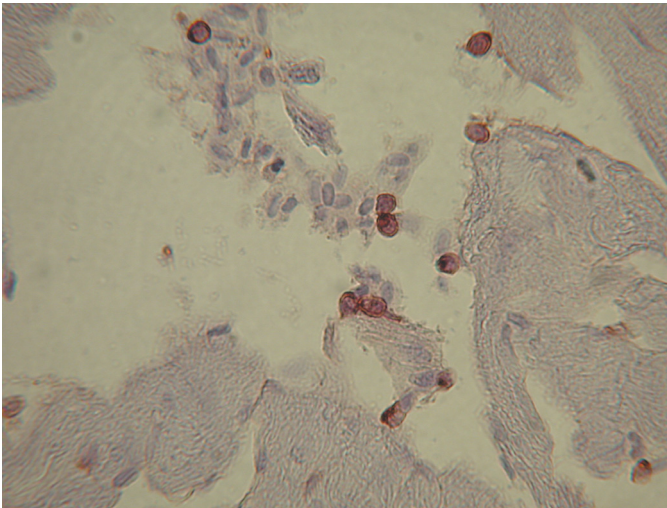
Figure 6. Lymphocytes (red) immunohistochemically stained by use of Mab 8H4/23. Heart, X400. Photo: National Veterinary Institute.

Immune response and phenotypic characterization of the lymphocyte population in BKD challenged rainbow trout (IV)

An activation of the immune response to BKD was demonstrated since the incidence as well as the occurrence of soluble *R. salmoninarum* antigens in kidney was reduced from the initial sampling 4 weeks after immersion to the sampling at 10 weeks after challenge. The increase of the total- and the *R. salmoninarum* specific humoral response from the 4th to the 10th week was not the

main reason for the reduction in the infection level, since there was no correlation on the individual level. The splenocytic response to the T-cell mitogen Concanavalin A was reduced during the same period but no effect on the B mitogen response was found. The proportion of Ig^+ lymphocytes was not affected throughout the study. This suggests an effect on the Ig^- lymphocyte population during the course of BKD. *R. salmoninarum* antigen preparations induced an increased *in vitro* stimulation of the lymphocyte population at the initial sampling 4 weeks after challenge. No stimulation was found in non-challenged fish. Stimulation indices (SI) were determined to 6 and 3 respectively for heat-stable and paraformaldehyde (PFA) fixed *R. salmoninarum.* No stimulation was found in the samplings 5, 6 and 10 weeks after challenge. The lymphocyte marker Mab 1C2 (Paper III) identified 29% of the lymphocytes in spleen at the initial sampling, followed by a reduction to 7% in subsequent samplings. The following increase of the 1C2 population again after injection of *R. salmoninarum* antigen supports the proposal that the 1C2 positive lymphocytes are involved in the response to BKD. In mammals, the initial response to a systemic infection is located to the spleen by an activation of B- and T-cells to effector cells. It is possible that the initially elevated level of the 1C2 population and the subsequent reduction reflect a similar induction of effector cells for migration to affected organs. The kidney is an important organ for immune functions in fish. Elevated *R. salmoninarum* antigen levels in the kidney after injection of the antigens indicate that the antigens were processed there. The injection of *R. salmoninarum* antigens into challenged fish induced a stronger antibody response compared with the injection in unchallenged fish, demonstrating an anamnestic response.

General discussion

For several decades BKD has been a main threat to salmonid health in aquaculture as well as in feral fish populations. The slow growth and the fastidious requirements have made studies of the organism difficult to accomplish, but this has also long been an obstacle in the diagnostics of BKD, especially to identify latent carriers. Aggravating circumstances contribute to the limited understanding of immune functions in salmonid fish. Knowledge of immune functions in vertebrates is based on studies of mammalian immune systems.

 In the present thesis, a polyclonal enzyme-linked immunosorbent assay (ELISA) has been developed to detect soluble *R. salmoninarum* antigens in the salmonid kidney. Since a high sensitivity is required in the identification of carrier fish, a polyclonal based assay has the advantage of several epitopes possible for identification and thereby a higher potential for detection. The ELISA method was found more convenient than the traditional cultivation technique for screening of wild brood fish due to a faster diagnosis. The high sensitivity and

specificity of the technique make the assay important in survey control programmes.

Rainbow trout was chosen in the present studies of the immune response and immune functions during the progression of BKD. This species belongs to the more resistant salmonid species and obviously has evolved mechanisms to control the infection. Establishment of BKD by immersion was used, since this technique represents a more natural route of disease transmission, than the commonly used injection. The immersion technique allows stimulation of skin and mucous layers of the fish, barriers that might be necessary to stimulate in order to activate immune defence mechanisms. An activation of the immune response was also demonstrated, since the incidence as well as the occurrence of soluble *R. salmoninarum* antigens in kidney, was reduced from the 4th to the 10th week sampling. Simultaneously the humoral response to *R. salmoninarum* was increased, indicating an effect of the humoral response on recovery, possibly against extracellular bacteria. No correlation of the *R. salmoninarum* antigen and the amounts of specific humoral antibody at an individual level was, however, found. This study, as in previous investigations (Paterson *et al.*, 1981), demonstrates that other immune functions also have to be activated. The T-cell mitogen response in spleen was reduced during the recovery period. No effect on the B-cell mitogen response was found. This indicates involvement of the T-mitogen responding population in the defence. The CMI activation could be demonstrated since *R. salmoninarum* antigens induced an *in vitro* proliferation of lymphocytes in the spleen at the initial sampling. Simultaneously, the Mab 1C2 positive lymphocyte population was elevated in the spleen. This was followed by a reduction in subsequent samplings and a further increase after an intraperitoneal *R. salmoninarum* injection. This indicates that the Mab 1C2-population are involved in the defence against *R. salmoninarum.*

The Ig^+ population of blood and spleen was the major lymphoid population in the present studies, as in previous investigations (Thuvander, 1990). The Ig^+ population is not necessarily identical with the B-cell population. Recent molecular investigations of natural killer (NK)-like cells of channel catfish indicate the occurrence of cytotoxic effector cells that express Fc receptors for Ig (Shen *et al.*, 2002). NK cells of mammals are known to be effector cells with cytotoxic activity and with the capability of lymphokine production. Distinct from the cytolytic T-cells they do not rearrange their TCR genes or express the TCR-CD3 on the surface and therefore do not react with processed antigens on an antigen presenting cell. The NK cells that express Fc receptors have, however, the capacity to act in a specific manner. The Fc part of an antibody, already bound to a target cell by its Fab region, might bind to the Fc receptor, thereby accomplishing antibody-dependent cytotoxicity. The cytotoxic activity of the NK cell can be increased during influence of lymphokines, and thereby develops to a lymphokine-activated killer (LAK) cell, a cell type so far not demonstrated in fish. Mammalian NK cells demonstrate allogeneic activity since they react with

foreign MHC. NK cells and T cells have been suggested to originate from a common precursor as a murine NK cell population has been described to have the potential to become NK cells or T cells depending on the *in vivo* microenvironment (Davison, 1996). Mab 1C2, obtained after immunization with a homologue peptide to the β-chain of the TCR, resulting in identification of a molecule at a similar size as the β-chain, indicates recognition of a β-chain expressing lymphoid cell. However, the double staining of a minor proportion of the 1C2 population with an Ig+ marker shows that the identified population could not be classified as a true T-lymphocyte population. A key issue for investigation is if the detected Ig on the cell surface is Fc receptor bound Ig or if the lymphoid cell itself produces the Ig. Molecular characterization of the 1C2 population should reveal whether these cells transcribe Ig at a RNA level. Functional assays to evaluate any cytotoxic capacity of the 1C2 population should consequently also be performed.

R. salmoninarum is an exceptional example of an organism that has evolved mechanisms to evade the defence mechanisms of the host. There appear to be no powerful toxins, ensuring the possibility of continued existence in carrier fish. The vertical transmission secures transmission of the bacterium further to the next generation. The intracellular survival in phagocytes contributes to an effective transport through the body. The kidney, an important organ for phagocytic activity, is the target organ for the disease. The macrophages are suitable for survival of intracellular bacteria such as *R. salmoninarum* since they are effective in internalising the bacteria, have limited antibacterial activity if not stimulated and have a lifetime of up to several months. The bacteria are inside the macrophage, protected from humoral defence mechanisms of the host. An activation of the macrophages induced by the CMI response requires stimulation of both B- and T-cells in mammals. Vaccination by use of attenuated bacterial strains has had progress against persistent infections in mammals. The avirulent *R. salmoninarum* strain MT 239 (Bruno, 1990) and two nutritionally mutant strains were used as live vaccines in Atlantic salmon and protection was demonstrated by a reduced mortality in the vaccinated groups after intraperitoneal challenge (Daly *et al*., 2001). MT 239 does not express the cell surface associated p57 protein at a similar level as virulent strains. There is, however, no difference in the ability to produce p57 into cell culture medium. Two copies of the gene for p57, msa, are sequenced from both avirulent and virulent strains. Similar levels of msa RNA are also transcribed (O´Farrell & Strom, 1999). The two nutritionally mutant strains express cell surface p57, but are both avirulent, indicating a further factor in addition to p57 involved in virulence. Further studies of these attenuated strains are necessary to avoid risks of reversion to a virulent state.

Stimulation of a cytotoxic cell response requires that the antigens are administrated intracellularly for processing and expression on the cell surface, in connection with MHC-molecules. Iscoms appear to fuse with the plasma membrane of the antigen-presenting cells and thereby deliver the antigens

intracellularly. Since the soluble antigens from *R. salmoninarum* incorporated into iscoms were efficient immunogens to induce a response to the p57 protein complex in rabbits, it is possible that this immunogen might be effective for immunization of fish against BKD.

It is obvious that a protective immune response to BKD is possible. The influence of stress on the immune response is well documented. Adequate husbandry including regulatory health controls, well-balanced fodders and water quality are factors that must be considered in order to reduce the effects of the disease. The lymphocyte stimulation test used in the present thesis was able to detect CMI activities in *R. salmoninarum* exposed rainbow trout, and has the potential to be an available *in vitro* test to assess immune response in connection with BKD. The test has to be established also in other salmonid species and also in connection with protective immunity. An *in vitro* test would be a convenient method to assess immune response to variable immunization regimes that could reduce the use of experimental infections.

Conclusions

The following methods were developed and used in studies of the immune response and of immune functions in salmonid fish during the progression of BKD:

A polyclonal enzyme-linked immunosorbent assay (ELISA) has been developed for detection of heat-stable soluble antigens of *R. salmoninarum* in salmonid kidney. The developed ELISA has improved the possibility to detect BKD in experimental studies as well as in epizootiological investigations.

A monoclonal-based ELISA for detection of humoral antibodies to heat-stable *R. salmoninarum* antigen was used. Elevated antibody levels were demonstrated in experimentally challenged rainbow trout and Atlantic salmon. There was no correlation of the antibody level and the occurrence of *R. salmoninarum* antigens in the kidney of individual fish. It was found that serological assays have a limited use in fish epizootiological investigations, due to influences in the antibody developments by environmental factors.

The lymphoid population of rainbow trout consist of Ig^+ and Ig^- lymphocytes. By means of the three monoclonal antibodies (Mabs) 1C2, 7A5/20 and 8H4/23, developed in the present study, new subpopulations of the lymphoid population of blood and spleen could be identified. The new subpopulations consist of both Ig^+ and Ig^- lymphocytes. By means of Mab 1C2 and 8H4/23, two subpopulations of the lymphoid population in the thymus could also be recognized. Phenotypic characterization of the lymphoid population of spleen indicated that the 1C2 population was involved in the immune response to BKD.

A protective immune response to *R. salmoninarum* was demonstrated in BKD challenged rainbow trout. Both a humoral and a CMI response were demonstrated. Similarity with the mammalian immune system was demonstrated, since a secondary immunization resulted in an anamnestic response. The possibility to measure a CMI response to *R. salmoninarum* demonstrate that this kind of *in vitro* test can be used to assess the immune response to several kinds of immunogens and immunization regimes for BKD.

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