Carp Erythrodermatitis:
host defense-pathogen interaction

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Carp Erythrodermatitis: *host defense-pathogen interaction*

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Proefschrift

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STELLINGEN

1- Breeding programs to enhance fish disease resistance should focus on improving 1st or 2nd lines of defense.

This thesis

2- Bacterial LPS is erroneously considered non-toxic to fish.

This thesis

3- Modulation of Aeromonas salmonicida ECP production by carp serum factors at an early stage in the disease process contributes to carp susceptibility differences.

This thesis

4- The metaphor, which describes metastatic cells as decathlon winners, seems highly unlikely, since cancer cells must in fact pass many hurdles but are not always successful once they reach metastatic sites.

Garth Nicolson. 1982. Cancer metastasis, organ colonization and the cell surface properties of malignant cells. Biochem. Biophys. Acta, 695: 113-176

5- To understand how cells function, one must look at them as a highly social multicellular community, rather than considering them in isolation as the key to solving biological problems.

E.B. Wilson. 1925. The Cell in Development and Heredity, 3rd Ed., Macmillan, Inc.

6- Adoptive specific cancer immunotherapy with tumour infiltrating lymphocytes has little chance to lead to true remissions, since tumour is often a heterogeneous population of cells.

S.A. Rosenberg. 1988. Immunotherapy of patients with advanced cancer using interleukin-2 alone or in combination with lymphokine activated killer cells. In: Important Advances in Oncology. V.T. DeVita et al. (eds). pp. 217-257.

7- Since therapy often fails to kill the last tumour cells, the focus of therapy should not be on eradication but rather on the reestablishment of normal regulatory mechanisms.

8 - Our education system leaves little place for human sensitivity and thereby stunts individual creativity.

9- Every intention that has not yet produced its effect is an event that has not yet come to completion.

G. Zukav. 1989. The Seat of the Soul. Simon and Schuster Inc.

10- The mass media jeopardize the success of new medical therapies.

11- Cure for cancer is not possible unless patients recognize their active role in the healing process.

12- The withdrawal of military forces in the west by Russia was compensated by the opening of a MacDonald in Moscow.

13- Intuition is at the basis of any creative act.

Stellingen behorend bij het proefschrift " Carp erythrodermatitis: host defense-pathogen interaction" van C.N. Pourreau
Wageningen, 1 juni 1990

PREFACE

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CONTENTS

Abbreviations

A.	Aeromonas
ACE	additional cell envelope
A-layer	additional (cell surface) layer
α_1-AT	alpha-1-antitrypsin
α_2-M	alpha-2-macroglobulin
B-cell/T-cell	bone-marrow or thymus derived lymphocyte
BKD	bacterial kidney disease
C'	complement
CE	carp erythrodermatitis
CFU	colony forming units
ECP	extracellular products
GCAT	glycerophospholipid:cholesterol acyltransferase
Ig-G	immunoglobulin-G
IL-1	interleukin-1
IL-2	interleukin-2
i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous
kD	kilo dalton
LD_{50}	lethal dose for 50% of the tested organisms
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MW	molecular weight
(SDS)-PAGE	(sodium dodecyl sulphate) polyacrylamide gel electrophoresis
PHA	phytohaemagglutinin
PBS	phosphate buffered saline
PMN	polymorphonuclear granulocyte
PS	polysaccharide
(S)RBC	(sheep) red blood cells
Tf	transferrin
TPA	tissue plasminogen activator
TSA/TSB	tryptic soy agar/ tryptic soy broth
UPA	urokinase type plasminogen activator
WBC	white blood cells

Chapter 1

INTRODUCTION

A. HISTORICAL CONSIDERATION

Aeromonas salmonicida is one of the oldest described fish pathogens. Emmerich and Weibel (1894) isolated the bacterium from diseased brown trout from a hatchery in Germany, and named it "Bacillus der Forellenseuche".

The origin of the pathogen in Europe or in North America remains uncertain, but importation of cultured trout contributed to its rapid spread throughout Europe and the United States. The first organized attempts to deal with the problem date from 1929, as the seriousness of the situation became increasingly evident (Austin and Austin, 1987). Unfortunately, this has not prevented the spread of A. salmonicida, which presently has a worldwide distribution.

Traditionally, A. salmonicida was thought to be exclusively a salmonid pathogen. However, the host range of the pathogen has greatly expanded to include many non-salmonids species as well (Table 1).

B. CHARACTERIZATION OF THE PATHOGEN

Aeromonas salmonicida is currently placed in the bacterial family Vibrionaceae (Bauman and Schubert, 1984). However, recent molecular genetic analysis suggests that Aeromonas ssp. is sufficiently distinct from that of Vibrionaceae to represent a distinct family of eurobacteria. Aeromonadacae fam. nova has been proposed (Colwell et al., 1986). A. salmonicida is further characterized as a psychrophilic, non-motile, non-sporulating, gram-negative, rod-shaped bacterium (0.5-1 by 0.7-2 μm). It is an oxidase positive, facultative anaerobe, which generally produces a brown water soluble pigment especially in the presence of tyrosine (Griffin et al., 1953 b; O'Leary et al., 1956; Donlon et al., 1983; Popoff, 1984).

B.1 Typical and atypical strains:

Physiological in vitro characteristics allowed a further subdivision of the species into 3 subgroups; ssp. salmonicida for the typical strains, producers of brown pigment and generally found in - and pathogenic to - salmonids, and two subspecies achromogenes (Smith, 1963) and masoucida (Kimura, 1969 a,b) for atypical, non-pigment producers (Schubert, 1974).

Table 1. Non-salmonid fish species reported to be infected by A. salmonicida.

Species	Authors
Minnow	Williamson, 1929
Goldfish	Mawdeskey-Thomas, 1969
Lamprey	Hall, 1963
Sablefish	Evelyn, 1971
Carp	Bootsma et al., 1977
Perch	Bucke, 1979
Catfish	McCarthy, 1980
Bream	
Roach	
Dace	
Chub	
Tench	
Pike	
Bullheads	
Sculpin	
Whitefish	
American eel	Hayasaka and Sullivan, 1981
Atlantic cod	Morrison et al., 1984
Sand-eels	Dalsgaard and Paulen, 1986

McCarthy and Roberts (1980) argued that the subspecies achromogenes should also include ssp. masoucida, and proposed that a new spp. nova be considered, based on phenotypic and genotypic analysis of over 150 isolates. This new classification however, was not adopted by the "Approved lists of bacterial names" (Skerman et al., 1980). Haemophilus piscium, implicated in ulcerative furunculosis, was later included in the spp. achromogenes (Paterson et al., 1980; Trust et al., 1980).

Atypical subspecies, particularly spp. masoucida are closely related to A. hydrophila, insofar as possessing similar physiological and growth characteristics. However, A. salmonicida spp. masoucida is non-motile, sensitive to A. salmonicida bacteriophages, possesses an antigenic

component specific for A. salmonicida and has a DNA homology of 103 % with A. salmonicida (MacInnes et al., 1979).

A. salmonicida strains were shown to be serologically homogenous (Karlsson 1964, Hahnel et al., 1983; Popoff, 1984), but shared extracellular antigens common to A. hydrophila (Liu, 1961; Bullock, 1966). Karlsson (1964) indicated that extracellular cross-reacting antigens were thermolabile and heat-stabile whole cell components. O-antigen side chains of A. salmonicida were found to be specific and uniform by electrophoretic analysis (Aoki and Trust, 1984; Chart et al., 1984; Evenberg et al., 1985), and by electroblotting and immunodetection techniques (Pyle and Cipriano, 1986; Evenberg et al., 1985). Also, agglutination assays showed rabbit antiserum to A. salmonicida lipopolysaccharides (LPS) to be highly specific (Pyle and Cipriano, 1986).

Criteria used to separate typical from atypical strains of A. salmonicida have been: failure to reduce nitrate, failure to produce indole and lysine decarboxylase, absence of positive Voges Proskauer reaction, and ability to ferment sugars (for review see Austin and Austin, 1987). McCarthy (1980) and McCarthy and Roberts (1980) argued that the original description of atypical strains, as reported by Schubert (1974) was based upon data from only a few isolates. The existence of many aberrant strains, some of which do not fit in any of the 3 subgroups, is now well established from a wide range of fish hosts and geographic locations (McCarthy, 1980).

B.2 Smooth, rough and intermediate colony types:

A characteristic trait of A. salmonicida is its ability to dissociate into different colony types (i.e."rough", "smooth" and intermediate) upon long term storage or suboptimal culture conditions (Duff, 1939). Electron microscopy studies later showed that "rough" and "smooth" forms could be distinguished by the presence or absence of an additional external layer (Fig. 1) (Udey, 1977; Cipriano, 1983b; Evenberg, 1988).

This feature has been extensively discussed in connection with pathogenicity (McCraw, 1952; Udey and Fryer, 1978; Ishiguro et al., 1981; Rogers et al., 1981; Evenberg et al., 1982; Phipps et al., 1983; Trust et al., 1983; Kay et al., 1984; Stewart et al., 1986). Duff had already in 1937 reported a loss in pathogenicity after several months of maintenance in artificial culture medium. The loss was accompanied by a change in the

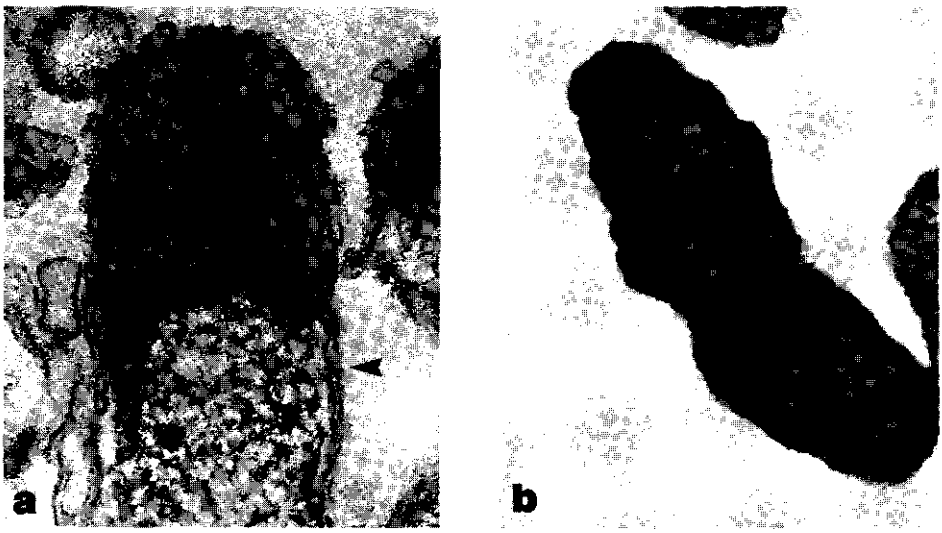

Fig.1. Electron micrographs showing the difference between the virulent V234/81 (a) and the avirulent V75/93 (b) strains of atypical A. salmonicida. The arrow shows the additional cell surface layer (A-layer).

morphological appearance of colonies on nutrient agar from glistening, convex and translucent resembling the original stock culture, to strongly convex, distinctly opaque and cream-colored forms. An intermediate type was also described. The "smooth" form was reported to be less stabile in prolonged storage and pathogenic, while the "rough" form was more stabile, but non-pathogenic. Surprisingly, the current accepted view is that the "rough" colony type is in fact virulent, whereas the "smooth" form is not.

Later studies also showed morphological differences upon growth in broth medium (Evenberg, 1988). "Rough" cultures tend to autoagglutinate at the bottom of the culture vial, leaving a clear culture broth, whereas "smooth" cultures remain turbid from non-agglutinating cells. These properties again were found to be associated with virulence and the presence of the additional paracrystalline layer for the "rough" form and avirulence and absence of the layer for the "smooth" variant.

Similarly, a loss of the external layer either spontaneously or after suboptimal in vitro culturing, caused a concomitant loss of agglutination and of virulence (Kay et al., 1981, 1984). Atypical strains of A. salmonicida also appeared to be more stabile than the typical strains with

respect to the preservation of the outer protein, upon suboptimal culture conditions (Evenberg, 1982).

More recently, a virulent and autoagglutinating strain was found which lacked the A-layer (Smith et al., 1982; Ward et al., 1985). No suggestions were made by the authors concerning the surface membrane properties and the autoagglutination and virulence of this strain. Furthermore, the reverse notion that auto-agglutination of A-layer positive cells are necessarily virulent is now known not necessarily to be true, since several A-layer positive strains (mutants) of A. salmonicida have been found to be avirulent (Hackett et al., 1984; Evenberg, 1988).

B.3 Virulence factors:

Initial histopathological studies carried out in the 1930's by the Furunculosis Committee led to two key observations that have since directed the research on the pathogenicity of A. salmonicida, namely that infected fish showed proteolysis of muscle tissues and were leukopenic (Mackie et al., 1935). They hypothesized, as a result of detailed clinical observations, that the pathogenic process caused by the bacterium could be explained by a prolific growth of the microorganism in the blood and tissues of the fish. This, in turn, interfered with blood supply, resulting in anoxic cell necrosis and ultimately death.

The virulence factors of A. salmonicida have been the subject of much research and controversy. However, most workers now agree that several factors contribute to the pathogenicity of A. salmonicida and that a combination of cell-associated and extracellular products is responsible for the difference in virulence among the various strains. Additionally, the intricate interaction between the bacterium and its specific host plays a major role in the outcome of an infection.

B.3.1 The A-layer

A paracrystalline hydrophobic protein covers the cell surface of most virulent A. salmonicida strains (Evenberg et al., 1982 a,b; Stewart et al., 1986). The presence of this protein, described as A-protein, ACE protein or A-layer by various authors, certainly contributes to the virulence of A. salmononicida. By increasing the cell surface hydrophobicity, the A-layer is suggested to facilitate the attachment to fish epithelial and

phagocytic cells (Udey and Fryer, 1978; Trust et al., 1983; Parker and Munn, 1985; Sakai, 1986).

Evenberg (1988) speculated that hydrophobicity might allow the invading pathogen to cover itself with certain host proteins in a similar way to Staphylococcus aureus. Indeed, A-layer positive strains of A. salmonicida significantly absorb mammalian immunoglobulin (i.e. IgG) (Phipps and Kay, 1988). Serum immunoglobulin depletion seen in A. salmonicida infected fish, was suggested to result from immunoglobulin adherence, in addition to proteolytic attack by bacterial and inflammatory products (Evenberg, 1988).

Another property ascribed to the A-layer involves iron sequestration. Typical A. salmonicida strains appear to posses two iron uptake mechanisms, one involving a direct transferrin iron interaction, and another involving a siderophore (Chart and Trust, 1983). Atypical strains require heme for growth in culture, and appear to be less efficient than virulent strains at sequestering iron under the iron-limiting conditions of the host (Ishiguro et al., 1986).

Finally, the A-layer was suggested to serve to as a physical barrier against bactericidal activity of fish serum complement (Munn et al., 1982; Sakai, 1985) and lysozymal attack within host phagocytic cells (Munn and Trust, 1984; Olivier et al., 1986).

B.3.2 Extracellular products (ECP)

Observed pathological changes during A. salmonicida infections suggested to early investigators that cytolytic and proteolytic enzymes where involved in the pathogenicity of the bacterium. Injection into fish of crude virulent A. salmonicida supernatants and later, purified material from culture supernatant induced the characteristic skin lesions and showed a similar pathology and mortality as live bacteria (Munro et al., 1980; Ellis et al., 1981; Cipriano, 1983b; Shieh, 1982; Sakai, 1985). ECP from atypical A. salmonicida cultures also were found to induce the characteristic symptoms of the disease in carp (Pol et al., 1980).

Studies have demonstrated that A. salmonicida releases several ECP into culture supernatant. The amount of products released was shown to vary greatly depending on the bacterial strain, and culture conditions such as medium composition, pH (Cipriano et al., 1981; Nomura and Saito, 1982) and oxygen supply (Fyfe and Munro, 1986; Fyfe et al., 1988).

Proteases

Proteases have aroused substantial research interest as virulence factors and several investigators performing detailed biochemical analysis have described several proteases varying in molecular weights, optimum pH-ranges, and substrate and inhibitor specificities (Shieh and MacLean, 1975; Mellergaard, 1983; Sheeran et al., 1984; Tajima et al., 1984; Hastings and Ellis, 1985).

The release of serine proteases with caseinase, gelatinase, elastase, and collagenase activities have been reported for typical and atypical strains of A. salmonicida (Shieh and MacLean, 1975; Sakai, 1977; 1985, Smith et al., 1982; Mellergaard, 1983; Hastings and Ellis, 1985). Various isolates differ in the production and quantity of extracellular proteases as revealed by isoelectric focussing (Hastings and Ellis, 1985) and SDS-PAGE.

Leucocytolysin

The absence of a leucocyte response and of phagocytosis at the site of infection and a marked leucopenia after experimental bacterial challenge, suggested to Griffin (1953) and later, to Klontz et al. (1966) the involvement of a leucocytolysin. Fuller et al. (1977) isolated a glycoprotein from bacterial culture supernatant, which was leucocytolytic in vitro, and induced leucopenia when injected into trout. The cytolysin was not further characterized and there is now some doubt as to whether the reported cytolytic activity is the result of a single substance, since no further biochemical analysis was performed (Evenberg, 1988). In fact, Cipriano et al. (1981) resolved the extracellular bacterial products into four fractions by ion exchange chromatography. One fraction showed proteolytic and cytolytic activities in vitro and induced lesions and mortality when injected in rainbow trout. SDS-PAGE analysis showed that this fraction consisted of at least six different proteins (Cipriano, 1983 b).

Hemolysins

Titball and Munn (1981, 1985 a,b) have shown that certain typical A. salmonicida strains are able to produce two different hemolysins; a broad-spectrum hemolysin with maximum activity against horse erythrocytes (H-lysin) and another which is more specifically active against trout

erythrocytes (T-lysin). An extracellular proteolytic cleavage of these hemolysins by a caseinase seems to be required for the complete activation of these proteins (Titball and Munn, 1985 a,b).

Evenberg (1988) suggested that hemolysins and proteases, produced as extracellular products may act together with the specific porphyrin-binding properties of the A-layer, to supply the invading pathogen with an essential iron supply in an iron-limiting environment.

An extracellular lethal toxin produced by Aeromonas salmonicida has recently been purified by ion exchange chromatography (Lee and Ellis, 1989 a,b). The toxin was described as a glycerophospholipid: cholesterol acyltransferase (GCAT) of molecular weight 25 kD, which in its active state is aggregated with LPS (MW 2000 kD). The toxin was further described as a hemolysin (T-lysin), a leucocytolysin, and a cytolysin. Lipopolysaccharide was shown not to influence the GCAT activity of the enzymatic complex, but rather increased its hemolytic and toxic properties. Lipopolysaccharide was said to protect GCAT against proteolytic inactivation. This toxin was probably similar to the T-lysin purified by Titball and Munn (1985), which also was found to contain GCAT and had a molecular weight of 23-25 kD (Buckley et al., 1982).

Outer membrane fragments: "Blebs"

The outer membrane of A. salmonicida was described by Evenberg et al. (1982, 1985) as consisting of membrane proteins, lipoprotein, lipopolysaccharides and phospholipids. The A-layer when present covers most of the bacterial cell surface. It comprises regularly arranged subunits of additional cell envelope protein (ACE), complexed with the lipopolysaccharides. Acidic polysaccharide antigen (PS-antigen) is inserted in the outer membrane and is also exposed onto the cell surface.

Differences among A. salmonicida strains have been attributed to the absence of the oligosaccharide chain (O-chain), the A-layer and/or the PS-antigen in some mutants. These changes were shown to correlate with the different morphological properties in culture, described earlier (Evenberg, 1988).

The A-layer and the LPS of A. salmonicida are structurally and antigenically highly conserved in the various isolates, and easily accessible to antibodies (Evenberg et al., 1982, 1985; Chart et al., 1984;

Pyle and Cipriano, 1986).

Lipopolysaccharide of avirulent and particularly virulent A. salmonicida is released in significant quantities in culture medium (Anderson, 1973; McIntyre et al., 1980). The release of LPS "blebs" is thought to result from an overproduction of outer membrane components. "Blebbing" was said to be related either to packaging of enzymes or their introduction into the membrane of host cells. Munro et al. (1980) also proposed that the release of large amounts of LPS as decoys, may serve as a bacterial defense mechanism against host phagocyte, antibody and complement attack.

C. DESCRIPTION OF THE DISEASE

Fijan (1972) is credited with the name carp erythrodermatitis (CE). He demonstrated that the disease was caused by a transmissible, antibiotic-sensitive, organism which manifests itself as a skin infection. Bootsma et al. (1977) isolated the organism from skin lesions in mirror carp in Yugoslavia, and subsequently identified it as an "atypical" strain of A. salmonicida by comparison with the typical strains found in salmonids. CE was considered to be a chronic to acute disease, which varied in morbidity and mortality depending on host resistance (age, species), environmental

Fig. 2. Characteristic hemorrhagic surface lesion in carp lethally infected with atypical V234/81 A. salmonicida.

factors (season, water quality and temperature), and bacterial challenge (virulence, and level of infection) (Evenberg, 1988).

Typical A. salmonicida infections in salmonids are known to occur when water temperature exceeds 16°C, and cause focal necrosis, skin abscesses, intestinal inflammation, and hemorrhages of fins, gills, and internal organs (McCraw, 1952; McCarthy and Roberts, 1980). Atypical strains of the bacterium are found at all water temperatures and cause a somewhat different pathology in carp (Fig. 2). The first clinical signs of CE are white "epithelial tufts", or fine shreds of superficial skin layers appearing at the site of injury on the skin surface. A hemorrhagic zone then develops between the dermis and the epidermis accompanied by dermal necrosis and scale loss (Bootsma et al., 1977). The breakdown of superficial tissue leads to a characteristic circular lesion with a hemorrhagic zone, surrounding a central necrotic area, which is ultimately invaded by opportunistic pathogens (Bootsma et al., 1977).

After lethal experimental challenge, a terminal stage is reached by 7-10 days at a water temperature of 20°C. At that stage, a generalized edema (i.e. scale protrusion, popped-eyes, abdominal swelling) is accompanied by darkening in color, loss of appetite and of weight, and ultimately secondary fungal or bacterial infections.

Gross clinical changes in infected carp are accompanied by a reduction in total serum proteins, with a decrease in osmotic value of the vascular fluid, and a reduction of the interstitial fluid resorption (Pol et al., 1980; Evenberg, 1988). Loss of proteins through vascular leaking may be due to histamine release (Ellis, 1981), impaired synthesis of serum proteins (liver damage), anorexia (Evenberg, 1988), and/or non-specific proteolysis (Ellis, 1981). In addition, carp become anemic, with subsequent decoloration and shrinking of the spleen and kidney and experience an increased respiration rate.

In the most chronic form of CE, contraction of collagen tissue forming the scar, results in serious deformity, which considerably reduces the commercial value of the fish (Fijan, 1972).

D. HOST DEFENSE MECHANISMS

Teleost fish possess a complex defense system, generally comparable to the avian and mammalian systems, which enables the animal to protect itself

against foreign substances and pathogens in a hostile environment. These protective mechanisms are either non-specific or specific (Rijkers, 1980; Lamers, 1985).

A first line of defense in fish includes physical and chemical barriers. While the skin prevents penetration, serum factors such as transferrin induces nutritional deprivation, and protease inhibitors, lectins and complement immediately neutralize foreign substances (Fänge et al., 1976; Suzumoto et al., 1977; Ellis et al., 1981; Ingram, 1980; Munro et al., 1980; Fletcher, 1982).

A second line of defense can be readily induced upon infection. Protective substances produced (i.e. C-reactive proteins) are not specific and show no memory formation. Also involved in second line defense reactions, are phagocytosis, natural non-specific cytotoxicity, and inflammation (Ramos and Smith, 1978, Ingram, 1980).

A third line of defense involves responses of the immune system. Cellular and humoral components are usually specific and their response leads to memory formation. Teleost leucocytes (i.e. lymphocytes, plasma cells, mononuclear phagocytes and granulocytes) and their respective functions have been described by various authors (Lehmann and Stürenberg, 1975, 1981; Fergusson, 1976; Ellis, 1977; Weinberg, 1978; Davina et al., 1980; Boomker, 1981, Smith and Braun-Nesje, 1982; Warr and Simon, 1983; Clem et al., 1984; Grondel and Harmsen, 1984; Bayne, 1986; Chung and Secombes, 1987, 1988; Rombout et al., 1989). The regulation of these cells is under the control of cytokines, such as IL-1 and IL-2 (Grondel and Harmsen, 1984). Studies on functional heterogeneity and determination of surface markers have shown lymphocyte subpopulations in fish, as is described in mammals (Secombes et al., 1983; Miller et al., 1987)

Teleosts are capable of producing antibodies (Corbel, 1975) and to mount primary and secondary immune responses to a variety of antigens (Rijkers, 1980, Lamers, 1985). In most fish, plasma cells secrete serum Ig in a tetrameric form (Shelton and Smith, 1970; Acton et al., 1971). However, there are also indications for the existance of low molecular weight immunoglobulins in serum and secretions (Lobb and Clem, 1981; Rombout, personal communication).

Cell-mediated immunity in fish has not been completed elucidated, but there is an increasing amount of data available on specific cellular immune

responses in fish. Transplantation immunity (graft rejection) has been shown by several authors (Hildemann, 1957, 1970; Botham et al., 1980; Botham and Manning, 1986; Rijkers, 1982; Avtalion et al., 1988). The mixed leucocyte response (Etlinger et al., 1977; Caspi and Avtalion, 1984; Kaastrup et al., 1988), MHC-restricted cytotoxicity (Verlhac and Deschaux, pers. comm.), and T-B cell cooperation (Avtalion et al., 1975; Cuchen and Clem, 1977) have all been described in fish.

Finally, it is important to realize that the kinetics of the immune response in fish is greatly influenced by environmental factors such as water temperature, oxygen supply, and pH, as well as toxicants (Avtalion, 1981; Anderson et al., 1984). There are now indications for the existence of a major histocompatibility locus in carp (Kaastrup et al., 1989).

D.1 Absence of leucocyte infiltration and leucopenia

The absence of leucocyte infiltration at the foci of infection and marked leucopenia in fish infected with A. salmonicida suggested to several investigators that the bacterium produced a leucocytolysin in vivo (Mackie and Menzies, 1938; Field et al., 1944; Griffin, 1953b; Klontz et al., 1966). Additionally, Blake (1935) showed the presence of "free bacteria" and little phagocytosis in the blood of infected fish. Fuller et al. (1977) then reported the isolation of a leucocytolytic glycoprotein from bacterial culture supernatant. Later, Cipriano et al. (1981) extracted 4 fractions from bacterial culture supernatants by ion exchange chromatography. Fraction 2, equivalent to the virulence factor isolated by Fuller et al. (1977), possessed not only leucocytolytic, but also proteolytic activity.

According to Cipriano et al. (1981), the leucocytic factor, contained within fraction 2 of the bacterial supernatant, contributed to virulence not only by its leucocytic properties, but also as a non-specific cytotoxin capable of generating endogenous pathological changes.

The currently accepted view is that the leucocytic and proteolytic activities of A. salmonicida are dual expressions of the same factor (Cipriano et al., 1981; Lee and Ellis, 1989).

D.2 Phagocytosis and the A-layer

The A-layer was found to correspond with strong agglutinating properties of the organism and to the adhesion to fish tissue culture cells

(Udey and Fryer, 1978; Sakai, 1986). Several possible functions for the A-layer have been put forward: 1) the hydrophobicity of the A-layer would provide bacterial cells with an enhanced ability to associate with phagocytic monocytes in absence of opsonization (Trust et al., 1983), 2) A-layer positive bacteria would be insensitive to killing by lysozomal enzymes and would be able to multiply within fish phagocytes (Munn and Trust, 1984, Olivier et al. 1986) and 3) these bacterial cells would survive in phagocytic organs such as the spleen, following an experimental challenge (Munn and Trust, 1984).

Work on the structure and possible role of the A-layer led several authors to conclude that the presence of this external membrane component was necessary for bacterial virulence (Udey and Fryer, 1978, Ishiguro et al., 1981). However, bacterial strains (mutants) which possess the A-layer have been identified, but are not virulent (Hackett et al., 1984; Evenberg, 1988).

Olivier et al. (1985) succeeded in conferring protection of coho salmon against an A. salmonicida challenge by treatment with Freund's complete adjuvant alone. They demonstrated that the adjuvant potentiated the defense mechanism non-specifically, by increasing the number of activated macrophages which showed phagocytic and bactericidal activity against virulent bacterial cells (Olivier et al., 1985). Their findings were in accordance with Munn and Trust (1984) who postulated that virulent A-layer positive cells must be able to survive and proliferate within non-activated phagocytes.

On the other hand, several authors showed that trout macrophages were able to ingest and to kill A. salmonicida cells in vitro (Olivier et al., 1986; Graham et al., 1988; Secombes, 1989), suggesting bacterial strain differences or genetic variation in fish. Unfortunately, these authors did not specify if the bacterial strains used were A-layer positive or not.

D.3 Degranulation of granulocytes

Degranulation of eosinophilic granulocytes of the lower intestin and the rectum of trout was shown to be induced by intraperitoneal injection of A. salmonicida extracellular products (Ellis, 1985; Vallejo and Ellis, 1989). The authors described the eosinophilic response as being similar to the anaphylactic granule extrusion of mammalian mast cells, in that varying

degrees of granule vacuolation and loss of electron density were seen. However, the response differed from that of mammalian cells, in that degranulation involved the release of intact electron lucent granules and subsequent extracellular granule desintegration. Direct cytotoxicity was not involved in the response and the cells recovered shortly after degranulation. Vallejo and Ellis (1989) postulated that bacterial and/or complement proteases were responsible for the eosinophilic granule release.

Pronephros cells collected from carp injected intraperitoneally with A. salmonicida ECP show a population of mostly very young granulocytes (i.e. few cytoplasmic granules and an extensive endoplasmic reticulum), which suggest an increased emigration of mature cells out of the lymphoid organ into the periphery, but a degranulation of mature granulocytes can not be excluded (Figures 3 and 4; Pourreau and Rombout, unpublished). A rapid turnover of granulocytes is typical of an acute inflammatory state.

D.4 Neutralizing activity of serum

Munro et al. (1980) and Ellis et al. (1981) reported on the neutralizing activity of normal trout serum against toxic A. salmonicida ECP. The serum components involved and the mechanism of that neutralization were not described in these studies.

D.4.1 Role of antibodies

Duff (1942) injected trout with heat- or chloroform inactivated whole bacterial cells and succeeded in raising serum antibodies and protection against a subsequent lethal bacterial challenge. This study stimulated many investigators to pursue similar lines of research.

Passive immunization was achieved using trout serum prepared against either killed whole bacteria (Spence et al., 1965), ECP (Cipriano, 1982a) or a combination of the two (Cipriano, 1983a). Antisera against virulent or avirulent bacterial cells and more recently, against purified protease were also raised in rabbits and subsequently protected salmon (McCarthy et al., 1983, Olivier et al., 1985) and trout (Ellis et al., 1987 a,b).

Active immunization on the other hand, gave inconsistent results. Injection of whole cells elicited high but unprotective antibodies raised mostly against the bacterial membrane LPS (Cipriano, 1982 b; Evenberg, 1988). Cipriano (1983b) successfully protected brook trout by injecting

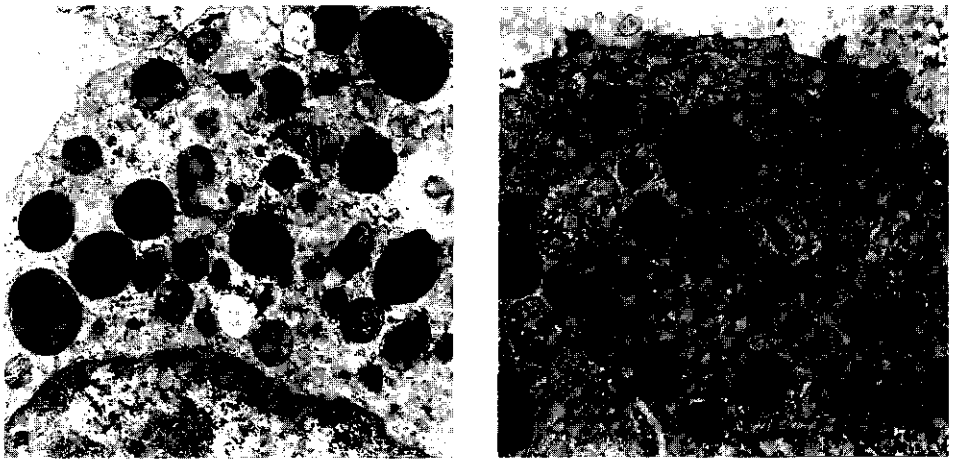

Fig. 3. Scanning electron microscopic detail of a basophil in pronephros of carp 6 days after injection of a) bacterial culture medium without ECP (Magnification 28500x) and b) A. salmonicida culture supernatant (Magnification 28500x).

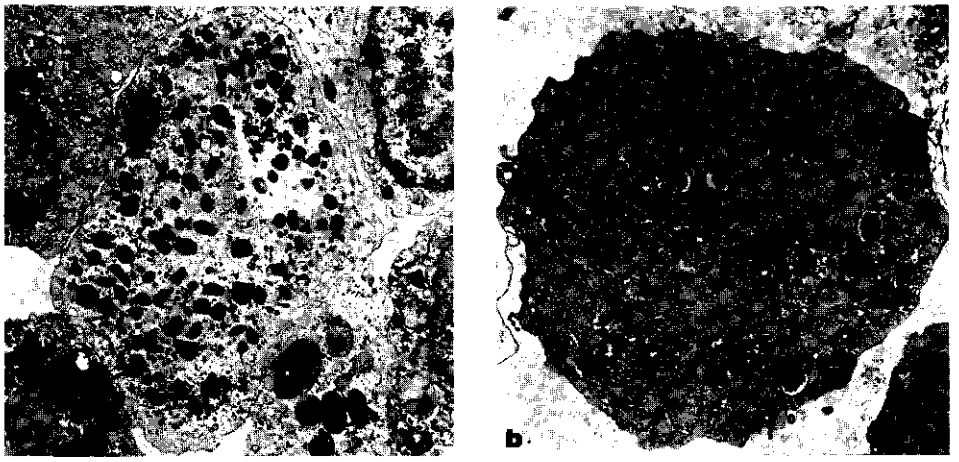

Fig. 4. Scanning electron microscopic detail of a neutrophil in pronephros of carp 6 days after injection of a) bacterial culture medium without ECP (Magnification 13700x) and b) A. salmonicida culture supernatant. Note the relative low number of granules and the well developed endoplasmic reticulum in the cytoplasm (Magnification 13700x).

the fish with immune rainbow trout serum, but serum from Atlantic salmon was unprotective, despite its high agglutinating antibody titers.

Ellis et al. (1987 a,b) could not elicit the production of specific antibodies against purified bacterial protease. Other investigators did however succeed, but found these antibodies not to be protective (Austin and Austin, 1987).

Cipriano (1982a) found that virulent and avirulent bacterial cultures gave similar agglutinin titers and protective ability. In contrast, McCarthy et al. (1983) found that only virulent variants to be protective and ascribed this protection to the presence of the A-layer.

Most studies have reported immunization efficacy in terms of the humoral antibody response. However, the presence of humoral antibodies has now repeatedly been shown not to correlate necessarily with protection. Therefore, results of many early attempts at protecting fish against the bacterium are retrospectively difficult to interpret.

D.4.2 Role of complement

Serum complement was proposed to serve as a major bacterial cell opsonin and to be involved in ECP neutralization (Sakai, 1984 a,b). Indeed, Sakai (1984b) showed in vitro that complement enhanced phagocytosis and bactericidal activity of trout leucocytes against virulent A. salmonicida cells. Activation of the "classical" complement pathway was said to be a result of this opsonization. On the contrary, Olivier et al. (1986) reported later, that addition of specific antibody to complement did not enhance the in vitro phagocytic and bactericidal activity of coho salmon macrophages. Contradictions between these two authors could involve bacterial strain differences.

In fact, the extracellular A-layer of A. salmonicida seems to protect bacterial cells from the bactericidal action of complement. Munn and Trust (1984) showed that A-layer positive cells were resistant to complement attack in the presence of specific antibody in trout serum.

In contrast to bacterial cells, neutralization of A. salmonicida ECP was said to occur via the "alternative" complement pathway (Sakai, 1984 a). Sakai showed, that trout serum complement activity decreased as ECP was neutralized. He also showed that ECP, preincubated with trout serum was not lethal to the fish as opposed to untreated ECP. This preincubated serum

showed a low proteolytic activity and complement was depleted.

D.4.3 Role of transferrin

Bacteria have an absolute requirement for iron. As an iron-binding and transport protein, transferrin limits the amount of iron in the blood-stream, thus making it unavailable for the bacteria during an infection. Iron has been shown to play an important role in fish pathology, by enhancing the growth of invading micro-organisms (Weinberg, 1974). On the other hand, when iron was deleted, the host defense was found to be favored.

Suzumoto et al. (1977) reported that salmon exhibiting different serum transferrin genotypes, differed in their resistance to an infection with Bacterial Kidney Disease (BKD). This difference in resistance, was suggested to lie in the avidity of the transferrin genotypes for iron. Transferrin polymorphism has been reported in serum of several marine and fresh water fish species (for review, see Kirpchnikov, 1973), including carp (Creyssel and Richard, 1966; Valenta et al., 1968, 1976; Balakhnin et al., 1972), but the significance of the transferrin genotypes in disease resistance was not reported by these authors.

D.4.4. Role of anti-protease

While some investigators argue that complement is responsible for the neutralization of toxic ECP, Munro et al. (1980) and later also Grisley et al. (1984) and Ellis and Grisley (1985) suggested that an α-globulin in trout serum may play that role. Ellis (1987) showed that A. salmonicida protease was inhibited by α_2-macroglobulin of rainbow trout serum, and that this inhibitor accounted for about 9% of the total trypsin inhibiting capacity of the serum. Alpha$_2$-macroglobulin was said to have a defensive function against A. salmonicida ECP. On the other hand, the resistance of bacterial protease to neutralization by fish serum inhibitors was thought by Ellis (1987) to represent a bacterial survival strategy.

E. CONTROL MEASURES

E.1 Husbandry and control of livestock transport

During the first half of the 20th century, the Kennett Valley Fisheries Association in the United States and the Furunculosis Committee in England were founded and funds were raised to tackle the problem of A. salmonicida

outbreaks in salmonid hatcheries. These organizations evaluated the extent of the disease and implemented the first control attempts by restricting transport and systematically controlling fish and eggs upon arrival at receiving farms. However, it later became clear that the disease was not restricted to salmonids (see Table 1), nor limited to hatcheries. Strict husbandry practices, such as maintenance of good water quality, and routine desinfection policies, implemented at that time remain to this day the best preventive measures against A. salmonicida outbreaks in fish farms (Herman, 1972, McCarthy and Roberts, 1980).

E.2 Antimicrobials

With the development of antimicrobial compounds for human and veterinary medicine, many of these products were tested for the treatment of infected salmonids. Sulphonamides, oxilinic acid, oxytetracyclines, chloramphenicol, and flumequine proved to be efficacious against A. salmonicida (Gutsell, 1946; Snieszko, 1958; Endo et al., 1973; McCarthy et al., 1974; Gayer et al., 1980; Michel et al., 1980; Austin et al., 1983). Chemotherapeutic agents continue to be necessary for preventing heavy mortalities during disease outbreaks, but systematic reliance on these compounds is now avoided because of bacterial resistance (Snieszko and Bullock, 1957; Aoki et al., 1983; Toranzo et al., 1983; Mitoma et al., 1984; Hedges et al., 1985), or suppression of the immune system (Rijkers et al., 1980; Grondel, 1986). Another serious limitation in the use of these compounds is their long retention times in fish tissues (Snieszko and Friddle, 1951; McCarthy and Roberts, 1980; Grondel et al., 1989), which could make these fish unsuitable for human consumption.

E.3 Vaccination

Attempts to develop an efficacious vaccine against A. salmonicida have been extensive and regained interest after the awareness of bacterial chemoresistance. The early success of Duff (1942) in eliciting protection of trout could unfortunately not be reproduced by subsequent investigators. Numerous inconsistent results obtained by various workers pointed out that the precise composition of the vaccine, the mode of administration, the age and the physiological state of the fish are of critical importance (Evenberg, 1988). To date, inactivated whole cells, soluble extracts, and

attenuated live vaccines have been tested as vaccine candidates and have shown a reasonable protection, when injected in salmon (Cipriano and Starliper, 1982). Injection of a commercial bacterin ("Furogen", Aqua Health, Charlottetown, Canada) reduced the mortality from 13-36% in controls to 3-10% in vaccinated trout during large scale field trials in different areas of the world (W.D. Patterson, pers. communication). Evenberg et al. (1988) also succeeded in immunizing carp with a vaccine preparation consisting of concentrated, detoxified culture supernatant. Vaccination by injection is only practical and economical in fish farms, when relatively large fish are treated. The problems encountered so far in the development of an efficacious vaccine against A. salmonicida were multifold. As mentioned earlier, most investigators have measured efficacy in terms of levels of circulating antibody levels, which is now known not necessarily to correlate with protection. Cipriano (1983) showed indeed, that the serum toxin-neutralization titer correlated with protection of trout against the bacterium, whereas the serum agglutinin titer did not. Other difficulties encountered in vaccine development against A. salmonicida have been: a lack of knowledge of protective antigens, inconsistent experimental challenge methods to ascertain protective efficacy in the field, difficulty in maintaining virulent bacterial strains in culture, and low immunogenicity of prepared purified vaccines.

E.4 Genetic differences in resistance

Breeding programs to improve salmonid resistance to A. salmonicida have been established since the early 1900's. As fish farming becomes more intensified and prophylactic measures take on a greater importance for disease control, selection methods play an increasing role.

Intrastrain variability in resistance to A. salmonicida has been demonstrated mostly for trout and salmon. In particular, it was found that different strains of common brook trout and brown trout differ from each other in their resistance to furunculosis (Embody and Hayford, 1925; Hayford and Embody, 1930; Snieszko, 1958; Ehlinger, 1964, 1977). After three generations of selection of common brook trout, based on resistance to the bacterium, a three-fold reduction in mortality from furunculosis, together with a doubling of the body size was achieved (Embody and Hayford, 1925).

In the United States, a planned selection program for producing genetically resistant strains of salmon and trout to furunculosis began in 1952 (Austin and Austin, 1987). The work on brook trout was stopped because of an octomytosis outbreak, but was resumed shortly after by Ehlinger (1964). Different U.S. and Canadian artificial fish breeding facilities were exposed to furunculosis. Less resistant fish were eliminated. This procedure was repeated over several generations for 16 years. A comparison between selected and control fish revealed 80% improved resistance to the disease after experimental challenge and 16% improvement during a natural disease outbreak. Selection did not further increase resistance and fish ultimately became susceptible to Gill Disease (Ehlinger, 1964).

Cipriano (1983b) demonstrated that serum from a resistant rainbow trout strain could passively protect brook trout from an experimental challenge with virulent bacteria. In contrast, the passive immunization of brook trout with serum from susceptible atlantic salmon was not protective. In another study, Cipriano (1983a) showed that it was the toxin-neutralization activity of the trout serum, rather than the agglutinin titer which conferred protection to the fish.

Cipriano and Heartwell (1986) showed that mucus precipitin activity correlated with the resistance of 3 strains of brown trout to A. salmonicida. Fish selected for a high level of mucus precipitin activity, produced progeny that were more resistant to furunculosis than those produced by random mating.

More recently, Sövényi et al. (1988) compared the susceptibility to infection with A. salmonicida of a cross between a Hungarian and a Japanese carp breed with that of the parental lines. Growth and condition factor of the hybrid carp were significantly higher than the parents. Following artificial challenge with the bacterium, the hybrid morbidity was half that of the original stock. Even though selective breeding to improve disease resistance of cultured fish has been practiced for a long time, insufficient knowledge of parameters involved in disease resistance may have limited the success of these programs.

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Chapter 2

RATIONALE AND SCOPE OF THIS THESIS

Most of the studies on A. salmonicida since Duff's early work on trout vaccination have been initiated to try to elicit protection of salmonids against the bacterium either experimentally or in the field, rather than aimed at understanding the mechanisms involved in the pathogenicity. Faced with disappointing results, a demand for a more systematic approach to the control of A. salmonicida has become necessary.

The objectives of this thesis were therefore to study specifically the host defense/pathogen interaction, in an attempt to understand the disease process in carp and to propose some avenues for future research.

Carp was chosen as our host fish species because of its high susceptibility to A. salmonicida. Also, despite the fact that carp erythrodermatitis is of major concern in many hatcheries worldwide, where carp are raised for sport fisheries, for human consumption and as ornamental fish, there has been very little published information concerning the disease.

In the work presented here, the disease process was studied from two sides, i.e. the pathogen and the host. In particular, the ability of the bacterium and/ or its ECP to interfere with the specific and non-specific defense systems of carp was investigated, as well as the defense reaction of carp to the bacterial aggression. This interaction between pathogen and host usually leads to the death of the fish. The mechanisms involved in the disease process will be clarified.

In the third chapter of this thesis, two classic in vivo models were used to assess the impact of the bacterium on the humoral and the cellular immune system of carp, i.e. antibody production against a foreign antigen and skin allograft rejection, respectively.

In the fourth chapter, the effects of cell-associated and of extracellular products (ECP) of A. salmonicida on the proliferative response of carp leucocytes were studied in vitro.

In the fifth chapter, serum complement activation by bacterial cells

and ECP was investigated *in vitro*.

In the sixth chapter, we report on preliminary results concerning the role of carp serum transferrin in the disease process and suggest some avenues for future research.

Results in the previous studies lead us in chapter seven to focus on the role of serum antiprotease in the resistance of carp to *A. salmonicida*. Several genetic strains of carp were selected and monitored for their serum antiprotease levels during an experimental infection.

Finally, a summary of the results and a comprehensive description of the disease process is given in chapter eight.

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Chapter 3

DOES AEROMONAS SALMONICIDA AFFECT THE IMMUNE SYSTEM OF CARP, CYPRINUS CARPIO L.?

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ABSTRACT

Pourreau, C.N., Evenberg, D., De Raadt, W.M., Van Mechelen, J.A.N. and Van Muiswinkel W.B., 1986. Does Aeromonas salmonicida affect the immune system of carp, Cyprinus carpio L.? Vet. Immunol. Immunopathol., 12: 331-338.

Aeromonas salmonicida is a significant bacterial pathogen of cyprinid and salmonid fishes causing the systemic disease furunculosis. Several observations led us to believe that A. salmonicida was able to evade or suppress the immune system of the fish: 1) injection of whole bacteria or surface antigens was unsuccessful at protecting fish against lethal challenges; 2) memory did not develop in survivors of sublethal infections; 3) diseased fish often carried other opportunistic bacterial pathogens in addition to A. salmonicida, and 4) serum protein and particularly immunoglobulin significantly decreased during A. salmonicida infections.
We tested the ability of fish sublethally infected with virulent and avirulent A. salmonicida to mount a humoral immune response to sheep erythrocytes and found fewer plaque forming cells in the pronephros and lower serum anti-SRBC antibodies in infected fish as compared to controls.
We also monitored the cellular immune response of diseased fish by skin allograft rejection and found an enhancement of the response that increased as the disease progressed. However, the extend of inflammation was reduced in infected fish as compared to non-infected animals. At this moment these preliminary observations are difficult to explain. Our future research will focuss more specifically on cell populations that may be affected by A. salmonicida.

INTRODUCTION

Aeromonas salmonicida, the etiologic agent of furunculosis in trout and salmon, carp erythrodermatitis, and ulcerative disease of goldfish, has been a world wide economic threat to intensive culture of these fishes for over fifty years (Herman, 1968, McCarthy and Roberts, 1980, McCraw, 1952). Attempts to protect fish

against A. salmonicida using oral or immersion vaccines (Paterson and Fryer, 1974, Smith et al., 1980, Udey and Fryer, 1978), injection of surface antigens (Evenberg et al., 1984) or toxoids (Austin and Rogers, 1981, Klontz et al., 1966, Spence et al., 1965) has often been unsuccessful (Paterson, 1981). More specific studies on the interaction between the bacterium and its host have therefore been initiated (i.e. mechanism of bacterial virulence, and defense response of the fish)

We now know that certain strains of A. salmonicida possess an additional cell envelope protein (A-layer), first described by Udey and Fryer (1978), which is important for virulence (Munn et al., 1982, Trust et al., 1980, Udey and Fryer, 1978), autoagglutination (Evenberg et al., 1982), and protection of underlying cell wall components from bactericidal activity of fish serum (Munn et al., 1982, Paterson, 1981). The bacteria produce toxins in vivo which have proteolytic (Mellegaard, 1983, Munro et al., 1980, Shieh and McLean, 1975, Sheeran and Smith, 1981, Tajima et al., 1984), haemolytic (Karlsson, 1962, Munro et al., 1980, Nomura and Saito, 1982, Titball and Munn, 1981), and leucocytolytic activities (Fuller et al., 1977, Munro et al., 1980).

Fish respond immunologically to the bacterium and its extracellular products (ECP) by producing specific antibodies directed against cell surface antigens and extracellular products (Anderson, 1974, Anderson et al., 1979, Cipriano, 1982, Krantz et al., 1963, Michel, 1979, Udey and Fryer, 1978, Weber and Zwicker, 1979). However the presence of agglutinins is not necessarily indicative of protection (Cipriano, 1982, 1983, Michel, 1979). Normal fish serum is also capable of neutralizing the extracellular products (ECP) via activation of complement by extracellular proteases (Sakai, 1984).

Recent observations by Evenberg et al. (in this issue) led us to believe that the bacterium was able to suppress the immune response of the fish: 1) injection of surface antigens was unsuccessful at protecting fish against subsequent lethal challenges (Evenberg et al., 1984), 2) memory did not develop in survivors of sublethal infections, and 3) diseased fish carried not only A. salmonicida but also other opportunistic pathogens such as A. hydrophila and Pseudomonas ssp. Evenberg also showed that serum proteins and particularly immunoglobulins significantly decreased during A. salmonicida infections. We have started to investigate how A. salmonicida evades or alters the defense system of carp, Cyprinus carpio. These are the first results of the study.

MATERIALS AND METHODS

Fish

Mirror carp, Cyprinus carpio, six months of age and 50 to 60 g in weight, were acclimated for two weeks in laboratory aquaria, supplied with fresh water at 16-18 °C and fed commercial dry pellet (K30, Trouw) daily.

Bacteria

The virulent autoagglutinating strain (V234/81) causative agent of carp erythrodermatitis, the avirulent auto-agglutinating strain (V75/93) and avirulent non-autoagglutinating strain (126-68) were provided by the Department of Molecular Cell Biology, University of Utrecht, and were cultured as described by Evenberg et al. (1981).

Humoral and cellular immune response

Fish were injected intramuscularly with a suspension of 10^7, and a month later 10^9, sheep red blood cells (SRBC) in PBS. The fish were challenged by an injection under a dorsal scale of 10 ul of A. salmonicida (2×10^4 cells/fish) in glycine diluent, 10 days prior to the booster SRBC injection. The diluent consisted of 240 mM glyxine, 30 mM NaCl, 3 mM Na_2HPO_4, 0.004 % sodium oleate and 0.04 % BRIJ 58 (Serva), pH 6.8. Control fish received diluent alone. The number of direct plaque forming cells (PFC) in the pronephros and the serum haemagglutination titer against SRBC were determined using standard techniques.

Groups of 10 fish were injected with a sublethal dose of virulent A. salmonicida (2×10^4 cells/fish) in diluent and received first set skin allografts at 5, 8, 11, 14, 17, and 20 days post Aeromonas injection. Controls were injected with diluent and grafted after 11 days. The appearance of the grafts was examined every two days for vascularization, haemorrhage and melanophore destruction. The day at which the grafts showed 100% pigment cell destruction (Median survival time, MST) was recorded for each fish.

RESULTS AND DISCUSSION

Aeromonas salmonicida suppressed the humoral immune response of carp to sheep erythrocytes. Fish sublethally infected with virulent and avirulent strains both showed lower numbers of plaque forming cells in the pronephros (13% and 22% less) than non infected controls (Fig. 1). Infected fish also produced less serum antibodies to sheep cells (38% reduction with the virulent strain and 60% with the avirulent

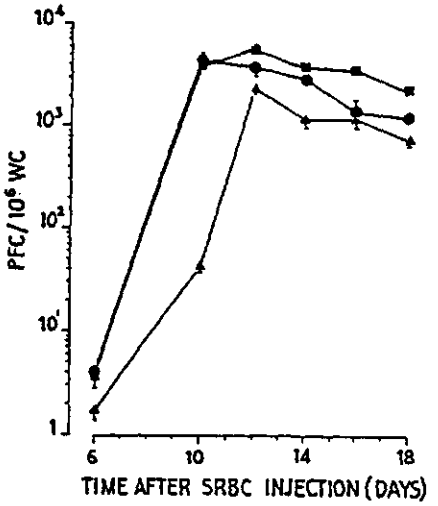

Fig. 1.

Kinetics of the secondary PFC response against 10^9 SRBC in the pronephros of carp at 16-18 °C. Fish were injected under a dorsal scale with PBS (■); $2x10^4$ avirulent (▲); or $2x10^4$ virulent (●). A. salmonicida ten days prior to SRBC. Points represent arithmetic means ± 1 SE (n=6).

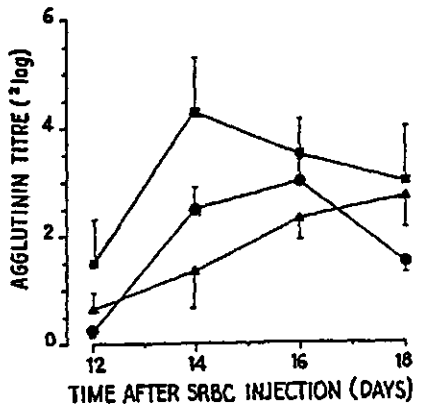

Fig. 2.

Kinetics of the secondary agglutinating antibody response against 10^9 SRBC at 16-18 °C in carp injected under a dorsal scale with PBS (■); $2x10^4$ avirulent (▲); or $2x10^4$ virulent (●). A. salmonicida ten days prior to SRBC. Points represent arithmetic means ± 1 SE (n=6).

strain 14 days after the booster SRBC injection (Fig. 2). In the case of fish infected with virulent A. salmonicida, this was not a delayed response to the antigen since antibody titers tapered off by 18 days. The same was not seen with fish infected with avirulent A. salmonicida. Antibody titers against formalin killed A. salmonicida cells were not significantly higher than natural antibody levels in sera of non-infected fish after 18 days (natural antibody titers:16-32 and anti-A. salmonicida titers in infected fish: 32-64).

Leucopenia and an increase in large lymphocytes was observed in infected fish. This result agrees with observations made by Klontz et al. (1966). The leucocytolytic factor released by the bacteria in vivo might destroy the mature white blood cells in circulation and lymphoid organs to such an extend as to suppress antibody production. The blood clotting time was also reduced 20-25 days post-infection with both virulent and avirulent A. salmonicida.

Results obtained with skin allografts showed that the rejection was accelerated in infected fish as compared to control. This phenomenon was more pronounced as the disease progressed up to 20 days (Fig. 3). By 14 days after injection of virulent A. salmonicida the MST of grafts was 59% shorter than normal. Since total pigment destruction was used as the criteria for graft rejection, we need to determine if the accelerated reaction was due to an enhanced cellular immune response or a non-specific toxic action of aggressins released by the bacteria on cells of the skin.

Aeromonas-infected fish showed a marked suppression of the inflammatory response to the skin allografts. This result is consistent with observations made by Munro et al. (1980), and Klontz et al. (1966). Klontz suggested that the lack of leucocytic response was due to the destruction of white blood cells by the leucocytolytic factor secreted by A. salmonicida and a depression of hematopoiesis. Above observations suggest that the avirulent strain V75/93 possessed other potential virulence factor(s) besides the A-layer, although it did not induce mortality at a dose of 10^6 CFU/fish.

We are now focusing our attention on the effect of A. salmonicida on complement activity, phagocytosis in vivo and the effect of ECP from cell culture supernatants on immune cells in vitro.

ACKNOWLEDGEMENTS

I thank P. de Graaf (Dept. of Molecular Cell Biology, University of Utrecht) for providing the bacterial strains and technical assistance. We are also grateful to Mr W.J.A. Valen for drawing the figures and to Mrs H. Vertregt for typing the manuscript.

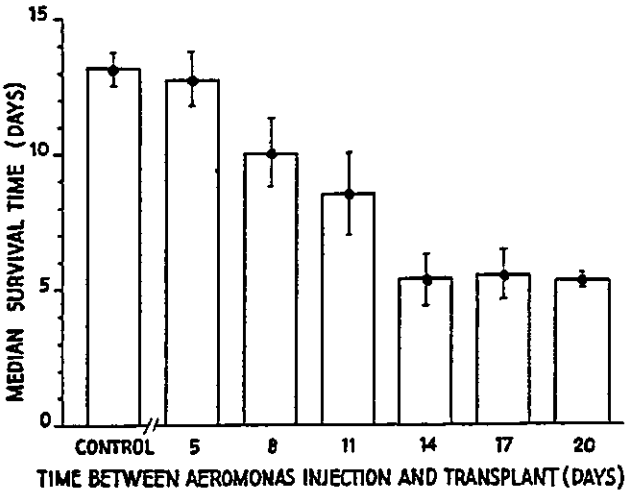

Fig. 3. Median survival time of first set skin allografts in fish grafted 5, 8, 11, 14, 17, and 20 days after sublethal injection of $2x10^4$ virulent (●). A salmonicida per fish. Points represent arithmetic means ± 1 SE (n=10).

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Chapter 4

MODULATION OF THE MITOGENIC PHA RESPONSE OF CARP, *CYPRINUS CARPIO* L., BY EXTRACELLULAR PRODUCTS OF *AEROMONAS SALMONICIDA*

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ABSTRACT

The influence of bacteria-free supernatants from cultures of atypical virulent (V234/81, autoagglutinating, A-layer positive) and avirulent (126/68, non-agglutinating, A-layer negative) strains of A. salmonicida, obtained after different culture times in yeast-tryptone broth at 20°C, was tested on the PHA response of carp pronephric leucocytes in vitro. Supernatants from virulent cultures modulated the response, whereas avirulent supernatants had no effect. The response was enhanced (400%) by supernatant from early virulent cultures (20 h), but severely depressed (<3%) by later ones (96 h). The effects were dose-dependent. Inhibitory activity of 96-h supernatant was lost by heating (70°C, 30 min.) suggesting that the inhibiting factors are all proteinaceous.

Heated 96-h supernatant was as stimulatory as early supernatant. Stimulation of leucocytes also occurred in the absence of PHA with early and heat-treated 96-h supernatants, but at a tenth of the level, suggesting that only stimulated cells (blasts) might respond to the substance(s) present in supernatants. Membrane fragments from virulent and avirulent bacteria, and purified LPS from virulent bacteria were stimulatory with or without PHA. Endotoxin-free, heat-treated, 96-h culture supernatants were also stimulatory, suggesting that an additional mitogenic factor(s), other than LPS, is present in the supernatants. The modulating in vitro effects of extracellular products from A. salmonicida might explain the immunosuppression seen during later stages of erythrodermatitis in vivo.

INTRODUCTION

The virulence factors of Aeromonas salmonicida, the aetiologic agent of furunculosis in salmonids, or erythrodermatitis and ulcerative disease in cyprinids, have been the subject of considerable interest. Udey and Fryer (1978) first demonstrated that virulent, typical, chromogenes strains were autoagglutinating, and possessed an additional layer (A-layer) surrounding the outer membrane, whereas avirulent strains were non-agglutinating and lacked the A-layer. The A-layer of typical and atypical strains was then characterized and associated with resistance to serum,

killing, adhesion to fish tissues, and haemagglutination (Trust et al., 1980, 1982; Hamilton et al., 1981; Ishiguro et al., 1981; Kay et al., 1981; Evenberg et al., 1982; Munn et al., 1982; Cipriano, 1983; Phipps et al., 1983; Evenberg et al., 1985; Van Alstine et al., 1986).

Typical A. salmonicida strains were found to release several extracellular toxins in culture supernatants during growth. These include: two proteases [a serine protease characterized by Shieh and McLean (1975) and Mellergaard (1983) and a metallo-enzyme (Sheeran and Smith, 1981; Sheeran et al., 1983)], two haemolysins [H-lysin and T-lysin, described by Titball and Munn (1981, 1983, 1985), and Nomura and Saito (1982)], a leucocytic factor identified as a glycoprotein by Fuller et al. (1977), and a glycerophospholipid: cholesterol acyltransferase (GCAT) (MacIntyre et al., 1979; Buckley et al., 1982). The extracellular products (ECP) of atypical A. salmonicida have been little studied. Hastings and Ellis (1984) reported that their strains produced only one of the two proteases (the metallo enzyme) in culture. We found that a subcutaneous or intramuscular injection of our virulent A. salmonicida achromogenes strain, as well as its ECP, is acutely lethal to carp (unpubl. results).

The production and activity of proteolytic and haemolytic toxins by typical A. salmonicida isolates, as well as our atypical strains, were found to be influenced by nutrient sources, time of culture, temperature of incubation (Shieh and MacLean, 1975; Nomura and Saito, 1982; Sakai, 1985), fish serum (Shieh, 1984; Sakai, 1985; Titball and Munn, 1985), and protease inhibitors (Shieh and MacLean, 1975; Sheeran and Smith, 1981; Mellergaard, 1983; Hasting and Ellis, 1984). Also, recent work (unpubl. results) has shown variability in the production of extracellular products by our atypical virulent and avirulent A. salmonicida, as was reported for typical strains (Titball and Munn, 1985). In addition to toxins, virulent and avirulent A. salmonicida achromogenes strains were also shown to release high and low molecular weight LPS into culture supernatants (Evenberg et al., 1985).

Our study was initiated to investigate the in vitro effects of supernatants of virulent and avirulent A. salmonicida achromogenes cultures on the mitogenic response of carp leucocytes. We report both stimulatory and inhibitory activities in supernatants from different culture times, and discuss possible factors responsible for these effects.

MATERIALS AND METHODS

Animals. Carp (Cyprinus carpio L.) from the A4 x W1 line, 8-12 months of age, and weighing about 200g, were bred and kept in laboratory aquaria supplied with aerated, running fresh water at 20°C. They were fed with pelleted dry food (K30, Trouw and Co., Putten, The Netherlands) at a rate of 1% of their body weight per day by means of a "Starflinger" automatic feeder.

Bacteria. The atypical virulent (LD50: 10^4 bacteria ml^{-1}) autoagglutinating, A-layer positive strain (V234/81), causative agent of carp erythrodermatitis, and the avirulent (non-lethal) non-agglutinating, A-layer negative strain (126/68), were provided on blood agar plates by Dr. D. Evenberg, Dept. of Molecular Cell Biology, University of Utrecht, The Netherlands. The origin and isolation of these strains was described by Evenberg et al. (1985).

Growth of the bacteria and preparation of culture supernatants. Three colonies of virulent or avirulent A.salmonicida were isolated from the agar plates, inoculated in 20 ml of Luthian broth (yeast-tryptone broth), and grown for 24 h at 20°C on a rotating shaker (Gyrotory shaker, model G2, New Brunswick Sci. Co., Edison, New Jersey, USA) at 75 r/min. Fresh broth was inoculated with 2% of this preculture and grown for the appropriate culture time as above. Optical density readings at 660 nm indicated that the virulent cultures reached a plateau by 72 h, and the avirulent strain by 48 h. The cultures were then centrifuged at 10 000 x g for 20 min, for separation of bacteria from crude culture supernatants.

Crude supernatants were collected immediately after bacterial growth, filter-sterilized using 0.22 μm filters (Schleicher and Schüll, Dassel, FRG), pH adjusted to 7.4, and stored at -20°C until used. Heated supernatants consisted of crude supernatants heated in a water bath at 70°C for 30 min. Endotoxin-free supernatants were obtained by passing crude supernatants through "Detoxy-gel" columns (Pierce Chemical Co., Rockford, Illinois), and subsequently filter-sterilized.

Preparation of bacterial membrane fragments and lipopolysaccharides. Virulent or avirulent bacteria from several cultures were centrifuged, resuspended in sterile phosphate buffered saline (PBS), freeze-thawed several times to disrupt bacterial membranes, washed three times and resuspended to a 1% solution using sterile medium (RPMI-1640, Flow, Irvine, Scotland), and stored at -20°C until used as bacterial membrane fragments. Isolation of LPS from virulent A. salmonicida cultures was done by the hot phenol extraction method of Wesphal et al. (1952) using washed bacterial cells. The LPS was freeze-dried and resuspended in PBS to the appropriate concentrations before use.

Cell Cultures. Culture medium (RPMI-1640) was buffered with 2.1 $\mu g\ ml^{-1}$ NaHCO3 (pH 7.4), diluted to 270 mosmol with distilled water, and supplemented with 2 mM L-glutamine (Merck, Darmstadt, FRG). Pronephric leucocytes were isolated as described by Grondel and Boesten (1982). Briefly, single cell suspensions, obtained by teasing the tissue through a nylon mesh, were washed twice and resuspended to 5×10^6 cells ml^{-1} with ice-cold, supplemented, culture medium. Cell viability always exceeded 90% and erythrocyte contamination was lower than 10%.

Cells were cultured in round-bottom microtitre plates (M24, Greiner, Nurtinger, FRG) in the presence of 0.5% heat-inactivated pooled carp serum (PCS) and 6.5 $\mu g\ ml^{-1}$ phytohaemagglutinin (PHA-P, Difco, Detroit, USA), bacterial membrane fragments, LPS, or culture supernatants at the desired concentrations, or RPMI medium as control, up to final volume of 0.2 ml per well. Microtitre plates were incubated for 4 days at 28°C in a humidified atmosphere containing 5% CO_2.

Measurement of the DNA synthesis. Cell growth was evaluated as the amount of ^{3}H-thymidine (^{3}H-TdR) incorporated into DNA after a 16-h pulse with 0.4 μCi ^{3}H-TdR (specific activity 5 Ci $mmol^{-1}$, Radio chemical Centre, Amersham, U.K.). Results were expressed as the arithmetic mean cpm ±1 S.D. of triplicate cultures. The Student's t-test was used for statistical evaluations.

Measurement of lipopolysaccharides. The presence of lipopolysaccharide levels was measured using the Limulus amoebocyte lysate test (E-Toxate, Sigma Chem. Co, St. Louis, Missouri, USA).

RESULTS

Effect of supernatants from virulent A. salmonicida cultures on the PHA-response of carp leucocytes.

Results (Fig 1) showed that early culture supernatants (12 h) stimulated the mitogen response at low concentrations (2.5 and 5%) but were inhibitory to the cells at higher doses (25 and 50%). The inhibition progressively increased with supernatants from later cultures up to 96 h. High concentrations of culture medium alone were not inhibitory to the cells. The pH of the supernatants was adjusted to 7.4 before adding to cell cultures. This suggests that the inhibitory factor(s), released by the bacteria during growth accumulated in culture supernatants. Supernatants from avirulent cultures showed no significant stimulation. Inhibition was seen only at the highest concentration (50%) for earlier supernatants, and 25 and 50% for

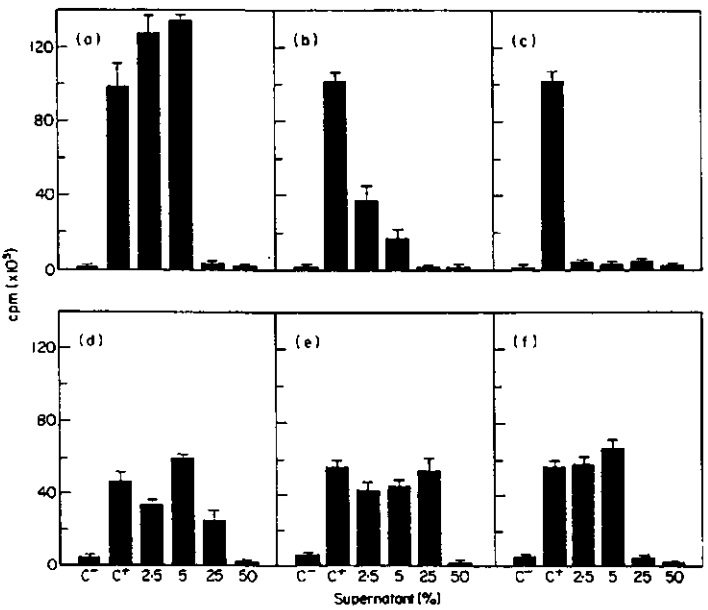

Fig. 1. Effects of supernatant from virulent A. salmonicida cultures of (a) 12 h, (b) 48 h and (c) 96 h and avirulent cultures of (d) 12 h, (e) 48 h and (f) 96 h, on the PHA response of carp pronephric leucocytes. C^-, non-stimulated controls; C^+, PHA stimulated controls. Results are expressed as arithmetic mean cpm ± 1 S.D. of triplicate cultures.

96-h supernatants. Avirulent bacteria seemed to produce about one-tenth of the amount of inhibitory factor(s) produced by the virulent one, even though they grew much more rapidly, as found by optical density readings (results not shown).

Effect of heated supernatants.

When leucocytes were exposed to heated (70°C, 30 min), or non-heated supernatants (Fig. 2), results with early culture supernatants [Fig. 2(a)] showed no significant difference between heated and non-heated material, indicating that the stimulatory factor(s) was heat-resistent. A stimulation of about four times control values (without supernatant) was seen in both groups at supernatant concentrations of 10% and it dropped thereafter. In contrast, marked differences were seen when heated 96-h supernatants were used [Fig. 2(b)]. These supernatants were as stimulatory as 20-h supernatants (55 x 10^3 cpm. for heated 96-h supernatant, v. 60 x 10^3 cpm for crude 20-h supernatant), whereas their non-heated counterparts were strongly inhibitory, except for the lowest concentration (1%) of crude 96 hr supernatant which was stimulatory. The stimulation of heat-treated supernatants was obtained at lower concentrations (2.5%) than with 20-h supernatant (10%).

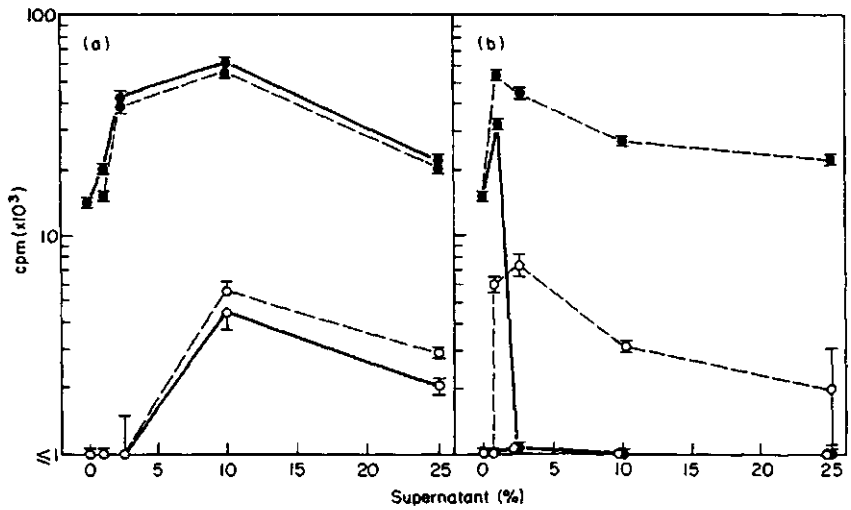

Fig. 2. Effects of crude (——) and heat-treated (---) supernatants from (a) 20-h and (b) 96-h A. salmonicida cultures on PHA-stimulated (●) and non-stimulated (○) pronephric leucocytes of carp. Results are expressed as arithmetic mean cpm ± S.D. of triplicate cultures.

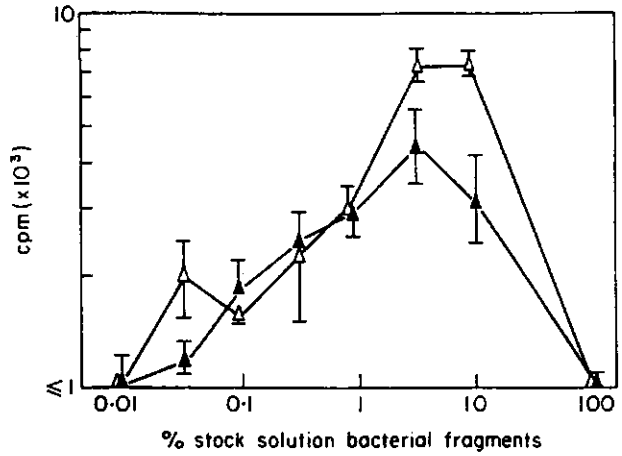

Fig. 3. Effects of membrane fragments from virulent (▲) and avirulent (△) A. salmonicida on the pronephric leucocytes of carp. Control cultures gave stimulations of <200 cpm. Results are expressed as arithmetic mean cpm ± 1 S.D. of triplicate cultures.

Tests using early (20-h) and late (96-h) supernatants without PHA (Figs 2,3) showed that early and heat-treated culture supernatants were mitogenic without PHA, indicating that the factor(s), in itself was stimulatory to carp leucocytes, but the stimulation seen was always about one-tenth of that obtained in the presence of PHA. Inhibition by 96-h crude supernatant was equal, with or without PHA, except for the lowest supernatant concentration.

Effect of free membrane fragments from virulent and avirulent bacteria in absence of PHA.

A stimulation similar to or slightly lower than that seen with early and late heat-treated supernatants, was obtained at endotoxin concentrations of about 1% w/v (Fig 3). Avirulent bacterial fragments were slightly more stimulatory than virulent ones (7×10^3 cpm for avirulent fragments, v. 4.5×10^3 cpm for virulent ones).

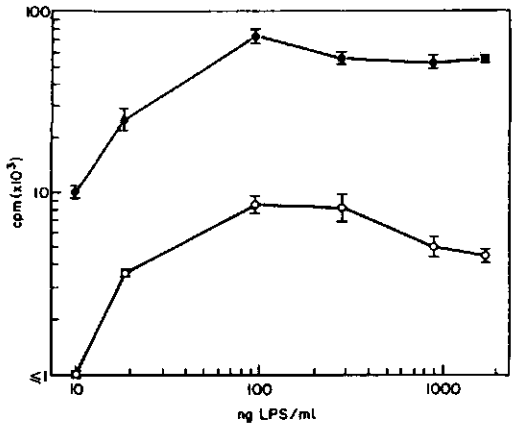

Fig. 4. Effects of purified LPS from virulent A. salmonicida on the PHA-treated (•) and non-treated (○) pronephric leucocytes of carp. Control PHA-treated cultures and non-PHA-treated cultures gave stimulations of 10^4 and <200 cpm, respectively. Results are expressed as arithmetic mean cpm ± 1 S.D. of triplicate cultures.

Exposure of PHA- and non-PHA-treated cells to purified LPS from the virulent A. salmonicida strain.

All LPS concentrations were stimulatory with maximum values, seven times higher than controls (LPS-free), of 8.5 x 10^3 cpm without PHA, and 70 x 10^3 cpm with PHA (Fig 4). Again, the stimulation with PHA was much higher than that of non-PHA-treated cultures, suggesting that A. salmonicida LPS may stimulate different cell populations or different membrane receptors on the same cells. Maxima obtained with purified LPS were slightly higher than those obtained with supernatants or bacterial fragments.

Effect of LPS-free supernatants of virulent strains.

Leucocytes were exposed to heated or non-heated, endotoxin or endotoxin-free, 96-h culture supernatants (Fig. 5). Crude (non-heated, with endotoxin) supernatant was stimulatory only at the lowest concentration (1%) and inhibitory thereafter, as seen previously. Endotoxin-free crude supernatant (non-heated) was also stimulatory at low concentrations. The stimulation was slightly lower than that observed with endotoxin. Heated supernatants, with or without endotoxin, were about four times more stimulatory than their non-heated counterparts. No significant difference was seen using endotoxin or endotoxin-free supernatants.

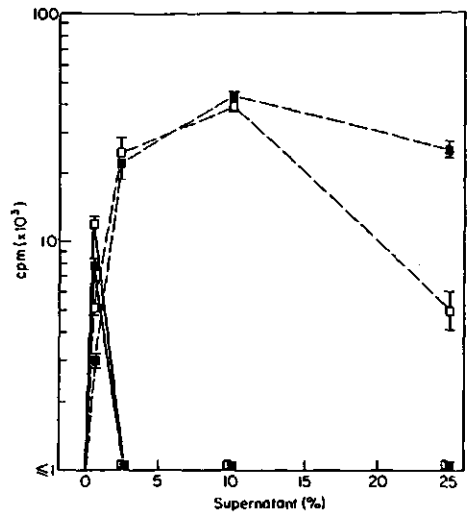

Fig. 5. Effects of crude (——) and heat-treated (---) supernatant with (■) or without (□) endotoxin on PHA-stimulated pronephric leucocytes of carp. Results are expressed as arithmetic mean cpm ± 1 S.D. of triplicate cultures.

DISCUSSION

This in vitro study clearly showed the existence of both stimulatory and inhibitory activities for carp leucocytes in virulent culture supernatants. The stimulation was not seen, however, with supernatants from avirulent cultures, and inhibition was about one-tenth of that observed with the virulent bacteria. Heating virulent 96-h supernatants showed that the opposite effects were caused by different factors. The stimulatory factor(s) was already present in early virulent supernatants (12 and 20 h), and seemed to accumulate in the media during the growth of the bacteria up to 96 h. It was resistant to heating at 70°C for 30 min and was stimulatory over a large range of supernatant concentrations.

The stimulation observed in the presence of PHA was about ten times higher than that seen without PHA in all of our assays. An additive effect of PHA and culture supernatant could not account for such a large stimulation difference, suggesting that an interleukin-like effect may be involved, where only stimulated cells (blasts) may respond to the substances present in the supernatants. Interleukin activity has been

described in carp by Caspi and Avtalion (1984) and Grondel and Harmsen (1984). A similar effect might be involved in the in vivo antibody response of salmonids to killed A. salmonicida cells emulsified in Freund's complete adjuvant. In mammals, a number of agents have been shown to stimulate interleukin (IL1) production by mononuclear cells. Among the most potent IL1 inducers are endotoxins (lipopolysaccharides and lipid A) of Gram-negative bacteria, which stimulate IL1 production with picogram amounts per millilitre (Loppnow et al., 1986). LPS from E. coli was known to stimulate proliferation of salmonid and cyprinid leucocytes in vitro (Grondel and Boesten, 1982; Kaattari and Yui, 1987). Kaattari and Yui (1987) suggested that polyclonal activation was involved, with release of interleukins in culture media. The destruction of mononuclear cells by extracellular toxins of A. salmonicida in vivo (Fuller et al., 1977) could seriously compromise the ability of the fish to build a protective immune response to the pathogen in a natural challenge with live bacteria.

Paterson and Fryer (1974) and Olivier et al. (1985) showed that endotoxin of A. salmonicida chromogenes was antigenic, with or without adjuvant, to salmonids even at low concentrations. We also found that bacterial membrane fragments of virulent and avirulent bacteria, as well as LPS from virulent bacteria, were stimulatory to carp leucocytes in vitro. Cipriano and Pyle (1985) reported that agglutinins raised in trout against culture supernatants were probably directed against endotoxin (LPS) of his ECF1 fraction. McIntyre et al. (1980) and Paterson (1981) also showed that membrane fragments (blebs) were found in supernatants of A. salmonicida cultures. These fragments, shown to contain phospholipids, lipopolysaccharides, and traces amounts of GCAT enzyme (McIntyre et al., 1980), were sloughed into the culture media as a result of cell autolysis (Paterson and Fryer, 1974). The presence of LPS in both virulent and avirulent culture supernatants was confirmed in our study using the Limulus amoebocyte lysate assay. We found LPS in early and late virulent and avirulent supernatants. Endotoxin was stimulatory at very low concentrations and was never toxic to the leucocytes at the concentrations used in our assay, as was reported in trout by Wedemeyer and Ross (1968). LPS concentrations above 1 $\mu g\ ml^{-1}$ would have been sufficient to elicit a stimulation of carp leucocytes, as was reported by Grondel and Boesten (1982) with E. coli LPS.

Our *in vitro* results indicated that only virulent culture supernatants were stimulatory to carp leucocytes. Avirulent bacteria, which also release LPS into the culture supernatants (as seen using the *Limulus* amoebocyte lysate assay), never showed leucocyte stimulation in the mitogen assay. This suggests that virulent bacteria might release different, more mitogenic LPS than avirulent bacteria, that the release of LPS by virulent bacteria may be enhanced because of autolysing bacteria during autoagglutination, or that virulent bacteria may produce another mitogenic material in addition to LPS, which is either not released by avirulent bacteria or released in lower quantities. Parmely et al. (1984) showed that proteolytic enzymes of *P. aeruginosa* were mitogenic to human T cells *in vitro*. It is possible that a similar activation of carp leucocytes by proteases of *A. salmonicida* may occur.

In order to remove LPS from our 96-h culture supernatants, these were passed through a 'Detoxy-gel' column which specifically removes LPS (but not proteins) from bacterial culture supernatants. The LPS content of the 'detoxified' supernatants (tested using the *Limulus* amoebocyte lysate assay) was not higher than background values found in bacteria-free culture media (<9 ng ml^{-1}). These supernatants were still stimulatory to carp leucocytes, indicating that mitogenic material other than LPS was present in the culture supernatants. The nature of this material is still to be determined.

The inhibitory activity of virulent and avirulent 96-h culture supernatants to carp leucocytes was probably due to extracellular toxins released by the bacteria during growth, as known for typical virulent and avirulent *A. salmonicida* (Sakai, 1977, 1985; Titball and Munn, 1985). Our virulent supernatants were, however, found to be ten times more inhibitory than the avirulent ones. ECP of virulent strains are known to be released after 24 h of growth in culture (Shieh and MacLean, 1975; Sheeran and Smith, 1981; Hasting and Ellis, 1984; Titball and Munn, 1981, 1985; Nomura and Saito, 1982; Fuller et al., 1977). The same seems to be true for the atypical strains used, since early culture supernatants were not inhibitory to the cells. That the inhibitory factor(s) in our virulent culture supernatant was heat-labile suggests again that a proteinaceous toxin(s) was responsible for the suppressive effect. Previous work had shown that crude supernatant of our virulent atypical strain (but not the avirulent

one) was lethal to carp, and that sublethal doses reproduced the pathological signs of the disease, as reported for typical strains, (Munro et al., 1980; Ellis et al., 1981; Shieh, 1982; Fyfe et al., 1986). Inhibition of 96-h crude supernatant was the same with or without PHA, except for 1% supernatant concentration which was stimulatory in the presence of PHA. This concentration might have been a threshold level for inhibitory activity, beyond which direct toxicity to the leucocytes might have occurred. We had previous evidence, using the trypan blue exclusion method, that supernatant from our virulent strain killed leucocytes in vitro.

Further studies, possibly using purified toxins, or specific inhibitors, should allow determination of what extracellular products stimulate or inhibit the leucocytes in vitro. This could provide us with a better insight into the pathogenicity caused by A. salmonicida.

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Chapter 5

GENETIC VARIATION IN CARP (*CYPRINUS CARPIO*, L.) COMPLEMENT ACTIVATION BY *AEROMONAS SALMONICIDA* AND ITS EXTRACELLULAR PRODUCTS

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ABSTRACT

Complement activation and depletion in sera from 5 strains of carp (1 Polish line, 1 Hungarian line, 1 Israeli line and 2 Dutch lines) was obtained *in vitro* using *A. salmonicida* and its extracellular products (ECP). Washed cells, crude or LPS-free culture supernatant and purified *A. salmonicida* LPS could be used as complement activators. Depletion of complement factors by bacterial cells was more pronounced with immune, than with normal serum. A significant difference in complement activation was seen among the carp strains using UV - treated bacterial cells. This could be an explanation for the genetic differences in disease susceptibility observed in these carp lines.

INTRODUCTION

Aeromonas salmonicida, the aetiologic agent of furunculosis in salmonids (for reviews see 1-3) and carp erythrodermatitis and ulcer disease in cyprinids and other fish is one of the most devastating bacterial pathogens of fish (3-5). Methods available for the control of these diseases in fish culture systems have traditionally been prophylactic desinfection practices and the use of antimicrobials for therapy. Recently, the appearance of resistant bacterial strains to widely used drugs such as tetracyclines, oxilinic acid and sulphonamides has led to great concern.

Efforts to develop an effective vaccine against typical or atypical strains of *A. salmonicida* have been extensive since the work of Duff in 1942 (6). However, successes have been hampered by difficulties in maintaining stable strain characteristics in culture, uncertainty concerning antigenic determinants, and lack of reliable challenge methods. Surviving the first infection with *A. salmonicida* still remains crucial to the survival from the disease. Certain fish species have been known for many years to be naturally resistant to *A. salmonicida*, while others, on the other hand, are very susceptible to the bacterium (7-14).

Breeding disease-resistant fish was initiated by Embody and Hayford (11) who successfully decreased the susceptibility of brook trout to furunculosis. More recently, Cipriano (15) showed that serum from rainbow trout,

which are naturally resistant to furunculosis, could protect passively immunized brook trout from challenge with a virulent culture. The neutralization of toxic extracellular products produced by A. salmonicida was believed to contribute to the protective effect of rainbow trout serum. Sakai (16) also investigated the inactivation of these products by rainbow trout serum and suggested that complement (C') was implicated in the neutralization. Studies on the activation of C' in several fish species have shown many similarities with the mammalian C' system (16-28). Using mostly non-pathogenic antigens to activate C', indications for the classical and alternative pathways have been demonstrated in fish (29).

In this study, we showed that carp C' could be activated by A. salmonicida cells and by extracellular products released by the bacteria in culture. We also showed that an innate difference in C' activation existed among 5 genetic strains of carp, differing in their susceptibility to carp erythrodermatitis.

MATERIALS AND METHODS

Fish. Five carp strains were tested for serum C' activation by A. salmonicida, its extracellular products (ECP) or purified lipopolysaccharide (LPS). These strains were: 1. A Polish line R3, fifth generation of conventional inbreeding (full-sib mating) from the Fish Culture Exp. Station-Golysz, selected for growth rate; 2. A Hungarian line R8, fourth generation of conventional inbreeding from the Fisheries Institute-Szarvas, selected for production results and high body shape; 3. An Israeli line A4, first generation of gynogenetic offspring from the DOR-70 line, Fish and Aquaculture Research Station-Dor, selected for growth rate and absence of inbreeding depression (30); 4. A Dutch line W49, second generation of gynogenetic offspring from carp obtained from the Organization for Improvement of Inland Fisheries (OVB), Nieuwegein; 5. Another Dutch line WW, first generation gynogenetic offspring also from a female carp from the OVB, Nieuwegein. Gynogenetic fish were produced by using either τ-irradiated sperm (A4 + W49) or UV-irradiated sperm (WW). Other details of the gynogenesis technique were described before (31, 32). In our experi-

ments fish, 1-2 years old and weighing 300-700 g, were kept from hatching onwards in standard aquaria supplied with aerated, filtered and UV-sterilized water at 18-19 °C. Fish were fed (K30, Trouw and Co., Putten, The Netherlands) at a rate of 1.5% of their body weight/day and adapted to their experimental aquaria at least a month before the experiments started. Immune fish, which had survived 5 sublethal challenges with virulent A. salmonicida V234/81 were kept under strict isolation conditions.

Serum. Blood was obtained by caudal puncture from MS-222 anaesthetized fish, and allowed to clot at room temperature for 1 h. Sera were carefully collected to avoid RBC contamination, rapidly frozen, and stored separately at - 80°C. Sera of 4-5 fish from each carp line were pooled before use. If needed, heat-inactivation of C' activity was done by incubation of the sera in a waterbath at 50 °C for 20 min.

Bacteria. The atypical virulent auto-agglutinating, A-layer positive A. salmonicida strain (V234/81), causative agent of carp erythrodermatitis was provided on blood agar plates by D. Evenberg (Dept. of Molecular Cell Biology, University of Utrecht, The Netherlands). The origin and the isolation of this strain was described previously (33).

UV-treated bacteria. Three colonies of V234/81 from a blood plate were inoculated in 10 ml of Luthian tryptone-yeast broth (LB) and grown at 20 °C on a rotating shaker for 24 h (Gyrotory shaker, model G2, New Brunswick Sci. Co., Edison, New Jersey, USA). Fifty ml of fresh LB was inoculated with 2 ml of the bacterial preculture and grown for 72 h at 20 °C on the shaker at 75 rpm. After 72 h, the culture ($\pm 10^9$ cells ml^{-1}) was transferred under sterile conditions to a petridish on a rotator (Framo Gerätetechnik M21/1, Eisenbach, FRG). The culture, stirred at 50 rpm using a small magnet was exposed to a U.V. source (Philips, TUV 15W, G15T8) placed 5 cm above the culture for 20 min. A drop of this UV-irradiated culture was inoculated onto a Coomassi Brilliant Blue (CBB) - LB agar plate to test the efficacy of the UV- source in preventing further bacterial growth. UV-treated V234-/81 were then washed 3 times in VBS++ and kept in 10 ml quantities at 4 °C until use.

Freeze-thawed bacteria. After culturing (as described above), the bacteria suspension was divided into 1 ml aliquots and rapidly freeze-thawed 3 times using liquid nitrogen, to disrupt the cells. The disrupted cell suspensions were kept at 4 °C until use.

Preparation of lipopolysaccharide (LPS). A. salmonicida from a 96 h LB culture was obtained according to a modified version of the method of Westphal and Jann (34, 35).

Crude extracellular products (ECP). Crude ECP of a 96 h culture was obtained by centrifugation of the bacterial suspension (10^5 g, 20 min) and filter-sterilization of the supernatant (0.2 µm membrane filters, Schleicher and Schull, Dassex, FDR), to remove all remaining bacteria. Small aliquots of ECP were then rapidly frozen using liquid nitrogen and stored at - 80°C until use.

Heat-inactivated ECP. Crude ECP was heated at 70°C for 40 min in a waterbath. Heat inactivated ECP was then rapidly frozen and kept at -80 °C until use.

LPS-free ECP. Endotoxin-free ECP was obtained by passing crude supernatant through 'Detoxy-gel' columns (Pierce Chemicals Co., Rockfort, Illinois, USA). ECP was subsequently filter-sterilized, rapidly frozen, and kept at -80°C until use. The LPS content of the ECP was determined by Limulus Amaebocyte Lysate assay to be $\leq$ 9 ng ml^{-1} (E-Toxate, Sigma, St. Louis, Missouri, USA).

Chicken or carp RBC suspensions. Citrate chicken or carp blood was collected on the day of the haemolytic assay and centrifuged at 1800 rpm, for 10 min at 4 °C. Plasma was collected and the cells were carefully washed 3 times in Veronal buffered saline with additional Ca^{++} and Mg^{++} (VBS^{++}, 25). A 2 % cell suspension was kept on ice until use.

Activation of carp C'. The haemolytic assay used in this study was originally developed by Kaastrup and Koch (23) and further modified to measure C' activation by A. salmonicida cells or ECP. The following

bacterial products were tested as activators of carp serum C':

- washed UV-treated or freeze-thawed A. salmonicida cells ($7,5 \times 10^6$ washed cells ml^{-1} VBS++)
- A. salmonicida LPS diluted with VBS++ to concentrations of $9,5 \times 10^{-2}$ mg ml^{-1}, 2.38×10^{-2} mg ml^{-1}, 1.18×10^{-2} mg ml^{-1}, $5,9 \times 100^{-3}$ mg ml^{-1} or 0 mg ml^{-1} (control).

Each of these products (75 μl) were incubated separately with pooled carp serum (75 μl) from one of the five lines of carp, for 2 h at 28°C in a waterbath. One hundred μl of the bacterial product/serum mixtures were then serial diluted (1:1 to 1:256) with VBS++ in round bottom microtiter wells. To each well, 50 μl of a 2 % washed chicken RBC suspension was added. The plate was shaken (Dynatech - microtiter, Varishaker, U.K.) for 10 sec, incubated at 37°C for 20 min (Inventum BK 41, The Netherlands), shaken again (10 sec) and incubated for another 20 min at 37°C . The plate was then shaken for the third time (10 sec), and centrifuged using a microtiter plate centrifuge (Heraeus Christ, Labofuge II, Osterode am Harz, FRG) for 5 min at 800-1000 rpm to spin down the remaining blood cells. The supernatant in each well was carefully pipeted into clean flat bottom microtiter wells. The haemolysis (percent lysis of chicken red blood cells) was read using a microtiter plate photospectrometer (Easy reader EAR-400, SLT-Lab instruments, Groding, Austria) at the absorbance of 492 nm. Blanks were run without bacterial cells and without LPS. All experiments were done in duplicate.

Modified haemolytic assay for testing C' activation by crude, heat-inactivated and LPS-free ECP. A. salmonicida ECP is haemolytic against a broad spectrum of RBC. To avoid this problem, two modified assays were used in evaluating carp C' activation by ECP:

1. The ECP/serum ratio was lowered to 1:4. This ratio which gave no haemolysis of RBC, was subsequently used for testing serum C'activation.
2. The haemolytic activity of ECP was neutralized by a 2 h pre-incubation with normal carp serum at 28°C. Neutralization of the haemolytic activity of ECP was checked by incubating carp RBC (from the same carp from which the serum was collected) in the ECP/serum solution. In this autologous system, haemolysis of the RBC did not occur, confirming ECP inactivation by carp serum. Fifty μl of serum-neutralized ECP was then incubated with

50 µl of fresh serum from the same carp. The haemolytic test was then run as described above using chicken RBC as target cells. The same procedure was followed to test C' depletion by crude, heat-inactivated, and LPS-free ECP.

RESULTS

Activation of C' by UV-treated bacteria. Sera from the 5 strains of carp (R3, R8, W49, WW and A4) were equally able to lyse chicken RBC in vitro. However, when these sera were pre-incubated with UV-treated A. salmonicida, significant differences in their lytic potential were noted (Fig. 1). Complement activation and thus, depletion of C' factors during pre-incubation with bacterial cells, was highest using serum from the Polish strain R3 and the Dutch strain WW, and lowest for the Israeli strain A4 and the Dutch strain W49. The Hungarian line R8 was intermediate. VBS++ alone was neither haemolytic, before nor after pre-incubation with bacterial cells.

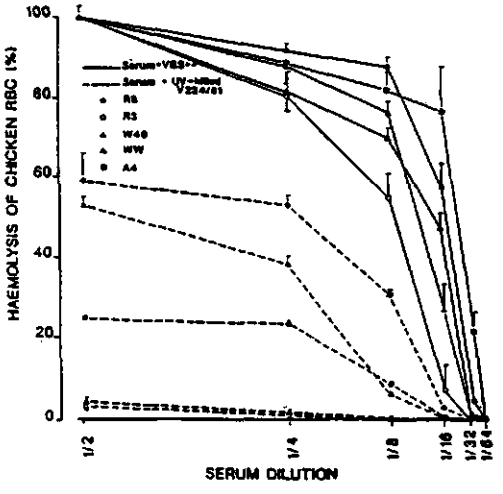

Fig.1. Depletion of complement (C') factors in sera from 5 carp strains R8, R3, W49, WW and A4 by A. salmonicida (V234/81). VBS^{++} = Veronal buffered saline with additional Mg^{++} and Ca^{++}.

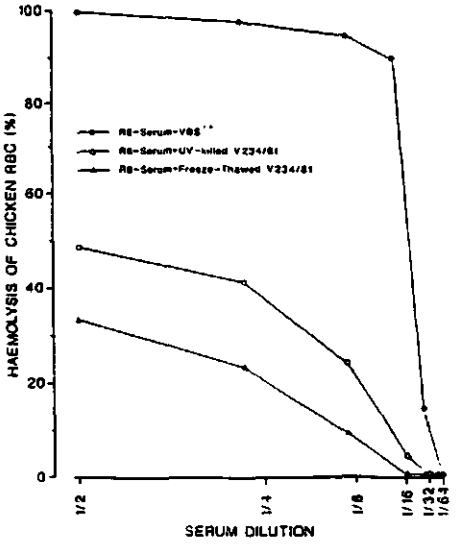

Fig.2. Depletion of serum C' factors by UV-treated or freeze-thawed A. salmonicida cells (V234/ 81).

UV-treated versus freeze-thawed bacteria. Freeze-thawed bacteria elicited a higher activation of carp C' than did UV-treated cells (Fig. 2). In this study, bacteria were not inactivated using formalin, to avoid aggressive treatment of the cells, which might have altered their ability to serve as C' activators.

Complement activation of normal or immune serum by bacterial cells. In order to determine whether the presence of anti-A. salmonicida antibodies in sera, might have an influence on C' activation by bacterial cells, sera from normal or immune fishes from all lines were pooled and tested for C' activation by bacterial cells (Fig. 3). No significant difference in haemolytic activity against chicken RBC was seen between normal and immune serum (Fig. 3). After pre-incubation of the sera with bacterial cells, immune serum showed a clear reduction in haemolytic potential as compared with normal serum. However, under the conditions of the assay the difference was not significant. As expected, heat-inactivated serum (normal or immune) was not influenced by bacterial cells. Heated sera alone did not show any lytic activity.

Activation of carp C' by LPS. LPS from A. salmonicida was purified and tested at different concentrations in the C' haemolytic assay. Results showed that carp C' was activated by LPS in a dose-dependent way (Fig. 4). Maximum C'activation was obtained at an LPS concentration of 95 ng ml-1.

Activation of C' by ECP. Crude ECP (at a serum/ECP ratio of $< 4:1$) was able to activate carp C' dose-dependently (Fig. 5). Removal of LPS ($\triangleq$9 ng ml^{-1}) from the ECP lead to the same maximum C' activation as crude ECP. Crude ECP, neutralized by a pre-incubation at room temperature for 2 h and checked no longer to be haemolytic to chicken RBC, was shown also to activate carp complement, but to a lesser extend than did crude or LPS-free ECP (Fig. 6).

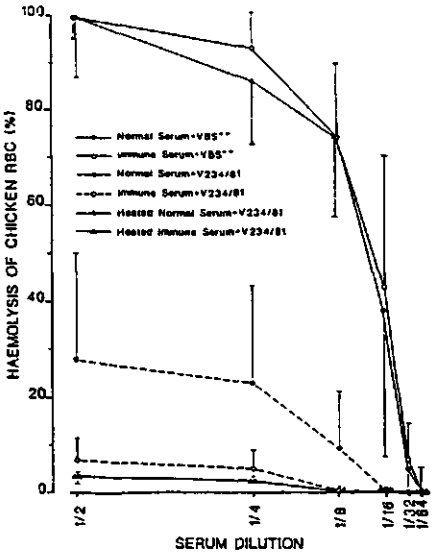

Fig.3. Depletion of C' factors in normal and immune sera pre-incubated with VBS $^{++}$ or bacterial cells (V234/81).

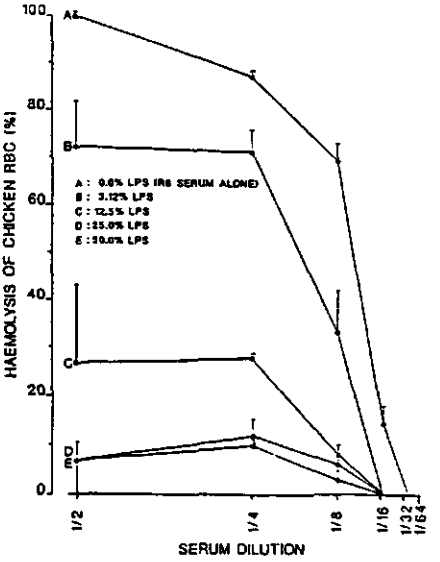

Fig.4. Depletion of carp C' factors by purified lipopolysaccharide (LPS) from atypical A. salmonicida.

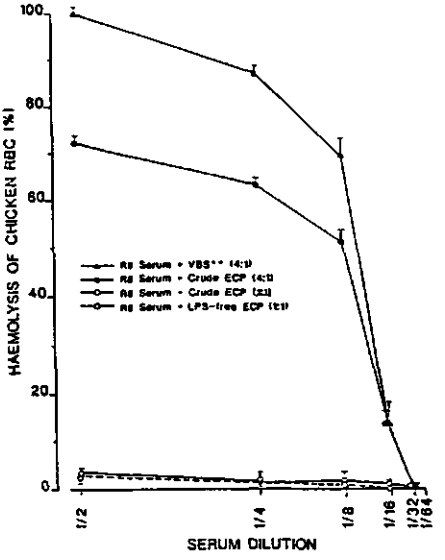

Fig.5. Depletion of carp C' factors by VBS $^{++}$, crude extracellular products (ECP), or LPS-free ECP.

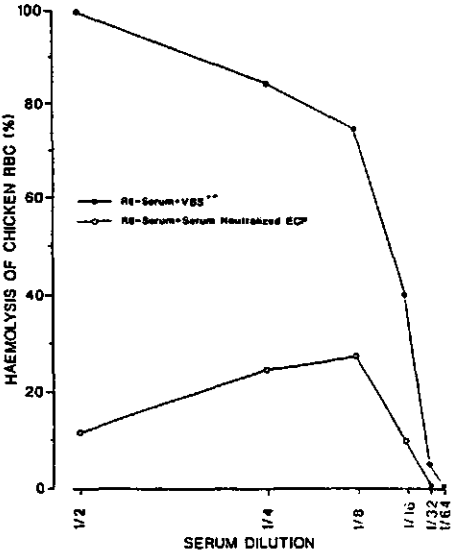

Fig.6. Depletion of carp C' factors by serum neutralized ECP.

DISCUSSION

Carp serum C' activation was obtained in vitro using a virulent strain of atypical A. salmonicida causing carp erythrodermatitis. Both washed bacteria and cell-free culture supernatant could deplete carp serum of active complement factors. UV-treated whole cells were a more potent C' activator than cell fragments obtained by freeze-thawing bacterial cells. Most virulent A. salmonicida, including this strain, possess an additional outer layer (A-layer) which has been shown to mask surface antigens partially (33). This layer, however, does not seem to hinder carp C' activation.

It seems reasonable to suggest that the C' activation was independent of specific anti-A. salmonicida antibodies since levels of serum anti-A. salmonicida antibodies in healthy fish have been previously checked not to be above background levels. It remains possible, that background cross-reactive serum antibodies, present in healthy carp continously exposed to aquatic bacteria may play a role in C' activation. Different sources of LPS have been used by several authors to activate an alternative-like C' pathway in fish (22, 23, 33). We showed in figure 4, that purified LPS from our atypical strain of A. salmonicida activated carp C' in a dose-dependent way. Almost complete C' depletion was obtained at an LPS concentration of 95 μg ml-1. Lipopolysaccharides from the bacterial cell wall is known to be abundantly released into the culture medium (33).

In addition to LPS, the supernatant of a 72-96 h culture of atypical A. salmonicida also has haemolytic and proteolytic activities (unpublished data), which in typical A. salmonicida strains have been attributed to several extracellular enzymes (36, 37). Proteases have been shown in mammals and in fish to activate the alternative C' pathway (16, 38, 39). Since LPS-free supernatant was also able to activate C' in our study, it seems reasonable to conclude that in addition to LPS, proteases of atypical A. salmonicida are activators of carp C'. Release of large quantities of these products by the bacteria in vivo could rapidly lead to C' depletion at a distance from the micro-organisms and thus avoidance of C'-mediated bacterial attack. This escape mechanism in favor of the bacteria could seriously compromise host defenses against this, as well as other

pathogens. To aggravate the situation, an open lesion is created by proteolysis of skin tissues by bacterial toxins, facilitating access for opportunistic micro-organisms. If, as suggested by several investigators (16, 40, 41), serum is implicated in the inactivation of A. salmonicida proteases, C'-depleted fish may then be left to rely on alternative protease neutralization mechanisms, such as serum alpha-globulins (36, 41), which have been shown to be present in carp serum (41).

Complement activation by atypical A. salmonicida cells was found to vary significantly between the 5 strains of carp tested. Whereas sera of the Polish strain R3 could be totally depleted of active C' components by bacterial cells, sera of gynogenetic lines A4 (Israeli) and W49 (Dutch) were only partially depleted. The molecular basis of the variation in C' activation as described in this study is not yet known. Several of the C' factors belonging to both alternative and classical route (factor B, C2, C3 and C4) are known to show genetic polymorphism and linkage to the major histocompatibility complex (MHC) in mammals (42, 43). Some evidence exists for the presence of factor B and C2 in carp (23, 44, 45). It is too early to say whether these proteins also show polymorphism in fish.

Previous experiments (data to be published) have shown that the Polish R3 and Hungarian R8 lines were relatively resistant to a lethal challenge with virulent atypical A. salmonicida. The Dutch line WW showed intermediate survival time after challenge. The Israeli line A4 and the Dutch line W49 were more susceptible to the bacteria. A correlation was found between C' activation by A. salmonicida cells and the resistance of these fish lines to a lethal challenge by the bacteria in vivo. The role of C' as a bactericidal and/or neutralizing agent as proposed by several authors (16, 40, 41) seems in agreement with our findings. Fish exhibiting low initial C' activation potential, could from the start be less able to counteract the initial bacterial attack. Further work to define alternative mechanisms of neutralization of A. salmonicida toxins in vivo is in progress and may provide additional insight as to innate susceptibility differences among carp lines.

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Chapter 6

PRELIMINARY OBSERVATIONS ON THE ROLE OF TRANSFERRIN IN THE PATHOGENICITY OF CARP (CYPRINUS CARPIO L.) ERYTHRODERMATITIS

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ABSTRACT

The electrophoretic analysis of sera from 4 selected carp lines resulted in 4 distinctive transferrin (Tf) variants; GG was found in the R3 and A4 carp, DD was carried by the Polish carp line R3 and some of the Hungarian R8 and Israeli A4 carp, GD was found both in the Polish R8 and Dutch A4 lines, and F'C was seen only in the Dutch W49 line. The relative frequency of each Tf genotype showed an unexpected low occurrence of the GG genotype in the offspring of R8 carp (GD x GD).

A possible correlation between the Tf genotypes of the carp and their susceptibility to A. salmonicida was investigated. The DD and GD genotypes were found frequently in the relatively more resistant carp lines R3 and R8. These genotypes may correspond with a lower susceptibity to the bacterium, while the F'C genotype, found in more susceptible W49 carp, may correspond with higher disease susceptibility.

Neither iron-binding capacity, nor relative serum Tf concentration seemed to correlate with susceptibility to the bacteria. Further work, involving crosses between carp carrying various Tf genotypes is in progress.

INTRODUCTION

Successful pathogens must obtain essential growth factors, in addition to withstanding host defense mechanisms. In particular, most bacteria have an absolute requirement for iron. It has been shown to play a role in pathology, by enhancing growth and virulence of the invading micro-organism (Suzumoto et al., 1977). Transferrin (Tf), as an iron-binding and transport protein, limits the amount of free iron in the bloodstream, thus making it unavailable for bacterial use in the case of infection (Suzumoto et al., 1977). Weinberg (1974) showed that if supplemental iron is given to fish, microbial growth is enhanced, whereas host defense is favored, if it is deleted.

In mammals, Tf generally exibits polymorphism (Manwell and Baker, 1970). This polymorphism has also been described in several fresh water and marine fish species (for review, see Kirpichnikov, 1973), including carp (Creyssel et al., 1964, 1966; Balakhnin et al., 1972; Valenta et al., 1976). Creyssel et al. (1964, 1966) found a three-allelic Tf system in the serum of carp.

Valenta and Kalal (1968) and Balakhnin and Galagen (1972) demonstrated the existence of 4 Tf variants. Reichenbach-Klinke (1973) described up to 4 electrophoretically different Tf zones in carp sera and suggested them to be under genetic control. Finally, Valenta et al. (1976) showed the existence of seven Tf variants in carp, which were designated with the letters A through G, according to their electrophoretic mobility. Genetic analysis revealed a simple Tf codominant autosomal inheritance and a single Tf locus.

Suzumoto et al. (1977) reported that Coho salmon carrying different Tf genotypes differed in resistance to an infection with bacterial kidney disease (BKD). The variable resistance of fish to BKD was suggested to correlate with the avidity of the different Tf genotypes for iron.

Variation in the susceptibility of different genetic lines of carp to Aeromonas salmonicida, a major bacterial fish pathogen, has been reported (Pourreau et al., 1990). Several studies have been initiated, in order to determine the basis for these differences.

An additional cell envelope (ACE) protein in virulent typical A. salmonicida has been shown to be involved in iron sequestration. It appears to possess a binding site for porphyrins, in particular protoporphyrin and hemin (Kay et al., 1985). Typical strains possess two uptake mechanisms, a direct uptake mechanism from Tf-iron complexes and an indirect mechanism involving a siderophore (Chart and Trust, 1983). Atypical A. salmonicida strains, responsible for causing the disease Carp Erythrodermatitis, are less effective in competing for iron, as compared with the typical variants causing disease in salmonids (Evenberg, 1988). Because of the bacteriostatic nature of Tf, it is plausible that certain Tf genotypes in carp may confer greater ability to bind iron and result in increased disease resistance. Transferrin patterns, iron-binding capacity, and serum iron concentration were therefore monitored in 4 genetic carp lines, selected for known susceptibility (high or low) to A. salmonicida, in order to find a possible correlation between Tf genotypes and susceptibility to the bacterium.

MATERIALS AND METHODS

Fish. Four different genetic carp lines were used in the experiments. These were: 1. A Polish line R3, fifth generation of conventional inbreeding (full-sib mating) from the Fish Culture Exp. Station-Golysz, selected for growth rate; 2. A Hungarian line R8, fourth generation of conventional inbreeding from the Fisheries Institute-Szarvas, selected for production and high body shape; 3. An Israeli line A4, first generation of gynogenetic offspring from the DOR-70 line, Fish and Aquaculture Research Station-Dor, selected for growth rate and absence of inbreeding depression (Wohlfarth et al., 1980); and 4. A Dutch line W49, second generation of gynogenetic offspring from carp obtained from the Organisation for Improvement of Inland Fisheries (OVB), Nieuwegein. Gynogenetic fish were produced by using γ-irratiated sperm (A4 + W49). Other details of the gynogenesis technique were described before (Van Muiswinkel et al., 1986, Komen et al., 1988).

In these experiments, fish weighing 300-700 g (1-2 years old), were kept from hatching in standard aquaria supplied with aerated, filtered and UV sterilized water at 18°C. Fish were fed carp pellets (K30, Trouw and Co., Putten, The Netherlands) at a rate of 1.5% of their body weight/day and adapted to their experimental aquaria at least 3 weeks before the experiments started.

Bacteria. The atypical virulent auto-agglutinating, A-layer positive A. salmonicida strain (V234/81), causative agent of Carp Erythrodermatitis (CE) was provided on blood agar plates by Evenberg (Dept. of Molecular Cell Biology, University of Utrecht, The Netherlands). The origin and isolation of this strain has been described by Evenberg et al. (1985). Three colonies of virulent A. salmonicida were isolated from the agar plates, inoculated in 20 ml of Luthian broth (yeast-tryptone broth) and grown for 24 h at 20°C on a rotating shaker (Gyrotory shaker, model G2, New Brunswick Sci. Co., Edison, New Jersey, U.S.A.) at 75 r/min. Fresh broth was inoculated with 2% of this preculture and grown for 30 h corresponding to the bacterial exponential growth phase.

Analysis of Tf genotypes. Blood was drawn from the caudal vein of each experimental carp, two weeks before a lethal bacterial challenge (i.e.

$2x10^6$ CFU ml^{-1}) and from it's parents. Serum was obtained and stored frozen at -80°C until use. Serum proteins were separated by means of vertical polyacrylamide gel electrophoresis (PAGE) at room temperature, with a 0.066 M tris-borate solution (pH 9.0) as electrode buffer. PAGE was done in the absence of SDS. A 4% polyacrylamide gel, with a 10% separation gel were used. Transferrin bands were visualized after specific staining with Nitroso-R-salt (Mueller et al., 1962). Routine gel staining for protein was carried out with Coomassie Blue.

Serum transferrin concentration. The relative amount of serum Tf was measured by gelscan computer analysis (LKB 2400 Gelscan XL), as the surface area under the curve of the Tf bands in the polyacrylamide gels.

Serum iron concentration and total iron-binding capacity (TIBC). Serum iron concentration and TIBC were determined, using the spectrophotometric procedures described by Persijn et al. (1971). The reagents used for these assays were provided by Sigma Diagnostics Co. (Dorset, England).

Statistics. Differences between carp lines and Tf genotypes were tested using a Bonferroni-test (SAS, 1985), allowing for comparison of lines and Tf genotypes with unequal sample sizes.

RESULTS AND DISCUSSION

The electrophoretic analysis of the sera from the 4 carp lines resulted in 4 distinctive Tf variants (Fig. 1), which were designated according to the nomenclature of Valenta et. al. (1976) (Table 1). Molecular weights of Tf types were around 70 kD (Boon, 1987).

The GG genotype was found in the R8 and A4 carp. The DD genotype was carried by the Polish carp line R3 and the Hungarian line R8, the GD genotype was found both in the Polish R3 and the Israeli A4 lines, and the F'C genotype was seen only in the Dutch W49 line. The 100% homozygosity in the gynogenetic offspring of the W49 mother was surprising. It suggests an very high crossing-over frequency during meiosis.

The relative frequency of each Tf genotype showed an unexpected low

occurrence of the GG genotype in the offspring of the R8 carp (GD x GD). A similar situation was seen in the mating DE x EG of Valenta et al. (1976), where a very low frequency of progeny with the DG phenotype was seen, suggesting that certain Tf genotypes might be selected against.

In a previous study, differences in susceptibility to A. salmonicida were found among genetically different carp lines (Pourreau et al., 1990). In particular, the Polish R3 and Hungarian R8 carp lines were found to be relatively resistant to the bacterium, whereas the Israeli A4 and the Dutch W49 lines were more susceptible to the bacterium. These data were therefore used to investigate a possible correlation between the Tf genotypes of the carp and their disease susceptibility. The DD genotype, which was found in the relatively resistant carp lines R3 and R8, may correspond with a lower

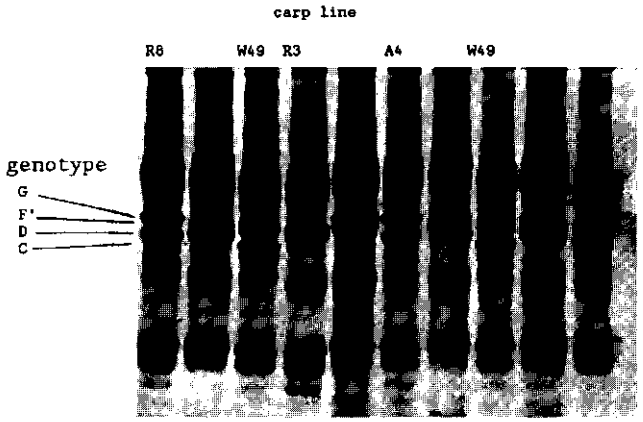

Fig.1 Immunoelectrophoretic analysis of Transferrin (Tf) genotypes of 4 different genetic carp lines. Nomenclature of Tf genotypes according to Valenta et al. (1976).

Table 1. Transferrin (Tf) genotypes[a] in the serum of 4 selected carp lines.

carp line	Tf genotype parents	Tf genotype in offspring				
		% observed (expected)				
		GG	GD	F'C	DD	n[b]
R3	DD x DD	0	0	0	100(100)	69
R8	GD x GD	11(25)	60(50)	0	29(25)	48
W49	F'C	0	0	100	0	10
A4	GD	54	3	0	43	91

[a] Nomenclature of Tf genotypes according to Valenta et al. (1976).
[b] Number of animals tested

susceptibity to the bacterium, while the F'C genotype, found in more susceptible W49 carp, could correspond with higher disease susceptibility. Results obtained with the GD genotype were difficult to interpret. Polish carp (i.e. R8) with this genotype had a significantly lower susceptibility to A. salmonicida, than carp from the Israeli A4 line with the same genotype.

Results on the iron-binding capacity of sera from fish with the various genotypes, showed a higher binding potential for the DD gynotype, which was found to be carried by the more resistant Polish line R3 and Hungarian line R8, but also by 43% of the A4 animals (Table 2). The lowest iron-binding

Table 2.Serum iron concentration and TIBC of 4 transferrin (Tf) genotypes.

Tf genotype[a]	no. of fish	mean serum iron concentration (mol l^{-1})	mean TIBC (mol l^{-1})
DD	6	115.8 ± 33.4	169.2 ± 39.4
GD	6	73.7 ± 54.2	113.5 ± 72.0
F'C	6	37.5 ± 20.5	66.0 ± 19.2
GG	4	24.8 ± 12.2	48.5 ± 10.5

[a] Nomenclature of Tf genotypes according to Valenta et al. (1976).

capacity was seen for the GG genotype, which was found to be present in the Hungarian (R8) and Israeli (A4) carp. These results are difficult to interpret at this point.

Table 3. Relative transferrin (Tf) concentration in the serum of carp carrying the different Tf genotypes.

Tf-genotype	mean area under Tf-curve ± SE	n[a]
DD	1620 ± 324	15
GD	1259 ± 668	6
F'C	1552 ± 307	10
GG	1620 ± 327	6

[a] number of carp sera tested.

The relative concentration of Tf in the serum of the different carp did not seem to correlate with susceptibility to the bacterium (Table 3). In particular, the DD and F'C genotypes, carried by the more resistant R3 and the susceptible W49 carp lines respectively, did not significantly differ in their serum Tf concentrations.

In conclusion, these preliminary results have identified several Tf genotypes in 4 carp lines selected for their low or high susceptibility to A. salmonicida, some of which may correlate with disease susceptibility. Further work, involving crosses between carp carrying the Tf genotypes DD and GG is in progress. One must keep in mind however, that resistance to A. salmonicida is probably multifactorial and involves more than one genetic trait.

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Chapter 7

GENETIC VARIATION IN CARP (CYPRINUS CARPIO, L.) SUSCEPTIBILITY TO AEROMONAS SALMONICIDA: ROLE OF SERUM ANTIPROTEASES

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ABSTRACT

Pourreau, C.N., Tuin, F.C.M., Verweij, M.A.P., Pilarczyk, A., George, F., and Van Muiswinkel, W.B. 1990. Genetic variation in carp (Cyprinus carpio, L.) susceptibility to Aeromonas salmonicida: Role of serum antiproteases, submitted.

Five genetic lines of carp were tested for their susceptibility to virulent, atypical Aeromonas salmonicida: A Polish, 5th generation line R3, a Hungarian 4th generation line R8, an Israeli 1st generation gynogenetic line A4 , and 2 Dutch gynogenetic, 1st and 2nd generation lines WW and W49, respectively. A challenge experiment showed differences in susceptibility among the 5 carp lines: 2 relatively susceptible lines (A4 and W49), an intermediate line (WW) and 2 more resistant lines (R3 and R8) were found.

Carp from 4 of the lines were given another challenge with A. salmonicida and monitored at regular time intervals for their serum antiprotease levels. Serum α_1-antitrypsin (α_1-AT) levels remained at baseline levels in all carp throughout the infection, suggesting that this antiprotease is not involved in the neutralization of A. salmonicida extracellular products (ECP). In contrast, serum α_2-macroglobulin (α_2-M) levels dropped drastically in all of the carp, shortly after bacterial challenge. The recovery of antiprotease was generally very slow. Serum α_2-M levels never returned to baseline levels, i.e. maximum levels reached by the surviving fish were 21% of control level for W49, 55% for A4, 66% for WW and 75% for R3. Results presented in this study suggested that carp serum α_2-M was involved in the bacterial exotoxin neutralization, since the serum antiprotease levels correlated with the disease kinetics in the various carp lines, but was not sufficient to prevent death in all animals. The presence of a 150 kD lytic band on zymograms of serum samples from only the more resistant R3 carp suggested that a specific antiprotease, through complex formation with the 90 kD bacterial exotoxin, may be involved in ECP neutralization.

INTRODUCTION

Studies on the pathogenicity of A. salmonicida have identified several extracellular products (ECP) implicated directly or indirectly in the virulence of the bacterium in fish. These include: cytolysins (Fuller et

al., 1977; Cipriano, 1983), hemolysins (Titball and Munn, 1981, 1985), proteases (Shieh and McLean, 1975; Sakai, 1977, 1985; Sheeran and Smith, 1981; Mellegaard, 1983) and membrane components (Evenberg, 1988). Different strains of A. salmonicida are known to vary in the production and release of these ECP, leading to differences in virulence (Hasting and Ellis, 1985).

Prior studies showed that injection of bacteria-free culture supernatant in fish reproduces the pathology caused by live bacteria (Munro et al., 1980; Ellis, 1981; Sakai, 1985). We further showed that injection of crude or lipopolysaccharide-free culture supernatant from our virulent atypical strain also leads to the carp erythrodermatitis disease (i.e. development of the characteristic hemorrhagic and necrotic surface lesions, lethargy, anemia, internal bleeding and edema) and a rapid death of the fish. Bleeding of carp at various stages of disease progression showed that the coagulation system becomes perturbed. In vitro work showed also that ECP activates carp serum complement and depletes serum of active bactericidal factors (Pourreau et al., 1990). Finally, carp serum proteins, including serum immunoglobulins were shown to decrease drastically during an infection (Evenberg et al., 1986). These observations strongly suggest that the severe disturbance of homeostasis may be due to uncontrolled proteolytic enzymes in the serum of diseased carp. Several investigators working with ECP from typical A. salmonicida strains in salmonids reached similar conclusions (Pol et al., 1980; Ellis and Grisley, 1985; Shieh, 1982; Sakai, 1985).

In mammals, it is well documentated that serum protease levels can rise at sites of inflammation, due not only to the continuous production of bacterial enzymes (Barett and Starkey, 1973; Duswald et al., 1985) but also from the release of lysosomal proteinases by activated polymorphonuclear (PMN) cells (Duswald et al., 1985; Frits et al., 1986; Barbey-Morel et al., 1987) and macrophages (Wahl et al., 1974; Ossana et al., 1986). Plasma proteinase inhibitors produced by hepatocytes and macrophages (Guillin and Bezeaud, 1985; Barbey-Morel, 1987; Perlmutter and Punsel, 1988) serve to control the activity of these enzymes and thus regulate the coagulation, fibrinolytic, complement, and kinin systems (Carrell, 1986). In certain inflammatory disease states, however, rapid inactivation or consumption of many of the plasma proteinase inhibitors is known to occur (Ossana et al.,

1986). Irreversible damage can then occur by either degradation of connective tissue and muscular components, attack of serum proteins or alteration of cellular functions (Travis et al., 1980; Baggiolini et al., 1980; Havemann and Gramse, 1984).

Several investigators have shown that trout serum inactivates the proteolytic activity of A. salmonicida (Ellis et al., 1981; Sakai, 1984). Sakai (1984) attributed this neutralizing activity to serum complement. Grisley et al. (1984) and Ellis (1987) demonstrated the presence of an α_2-macroglobulin (α_2-M) with antiprotease activity in rainbow trout serum. Ellis (1984) further reported the presence of at least three antitrypsin factors in trout serum, one of which was identified as α_2-M. In inhibition assays using human α_1-antitrypsin (α_1-AT) and bovine α_2-macroglobulin (α_2-M), he showed that α_2-M was able to neutralize A. salmonicida protease, whereas the bacterial protease was resistant to the action of α_1-AT. Inhibition of trout serum antitrypsin activity was obtained by preincubation of the serum with A. salmonicida protease. The author concluded that only serum α_2-M neutralized the bacterial protease. The specific role of carp serum antiprotease in the neutralization of A. salmonicida ECP was therefore investigated in carp experimentally infected with the bacterium.

Our objectives in this study were threefold:

1. To evaluate genetic differences in susceptibility to A. salmonicida among selected carp lines.
2. To monitor the levels of α_1-AT and α_2-M in sera of experimentally infected carp and to compare the outcome of these serum inhibitor studies with the extend of disease susceptibility of the different lines.
3. To investigate the presence of any other protease inhibitor(s) in the serum of carp.

MATERIALS AND METHODS

Fish. Five different carp lines were tested for their susceptibility to a lethal A. salmonicida challenge. These were: 1. A Polish line R3, fifth generation of conventional inbreeding (full-sib mating) from the Fish Culture Exp. Station-Golysz, selected for growth rate; 2. A Hungarian line

R8, fourth generation of conventional inbreeding from the Fisheries Institute-Szarvas, selected for production and high body shape; 3. An Israeli line A4, first generation of gynogenetic offspring from the DOR-70 line, Fish and Aquaculture Research Station-Dor, selected for growth rate and absence of inbreeding depression (Wohlfarth et al., 1980); 4. A Dutch line W49, second generation of gynogenetic offspring from carp obtained from the Organisation for Improvement of Inland Fisheries (OVB), Nieuwegein; 5. Another Dutch line WW, first generation gynogenetic offspring also from a female carp from the OVB, Nieuwegein. Gynogenetic fish were produced by using either γ-irratiated sperm (A4 + W49) or UV-irradiated sperm (WW). Other details of the gynogenesis technique were described before (Van Muiswinkel et al., 1986, Komen et al., 1988).

In our experiments, fish weighing 300-700 g (1-2 years old), were kept from hatching in standard aquaria supplied with aerated, filtered and UV sterilized water at 18°C. Fish were fed carp pellets (K30, Trouw and Co., Putten, The Netherlands) at a rate of 1.5% of their body weight/day and adapted to their experimental aquaria at least 1 month before the experiments started.

Bacteria. The atypical virulent auto-agglutinating, A-layer positive A. salmonicida strain (V234/81), causative agent of carp erythrodermatitis (CE), was provided on blood agar plates by Evenberg (Dept. of Molecular Cell Biology, University of Utrecht, The Netherlands). The origin and isolation of this strain has been described by Evenberg et al. (1985). Three colonies of virulent A. salmonicida were isolated from the agar plates, inoculated in 20 ml of Luthian broth (yeast-tryptone broth) and grown for 24 h at 20°C on a rotating shaker (Gyrotory shaker, model G2, New Brunswick Sci. Co., Edison, New Jersey, U.S.A.) at 75 r min^{-1}. Fresh broth was inoculated with 2% of this preculture and grown for 30 h corresponding to the bacterial exponential growth phase.

Experimental infection of carp. Controlled, experimental infection was achieved by carefully inoculating 5-10 μl of an appropriately diluted bacterial culture (i.e. $2x10^4$ and $2x10^6$ CFU ml^{-1} for "low" lethal and lethal challenges, respectively) using a gas-chromatographic Hamilton microliter syringe for accurate dose delivery. To avoid dermal damage, the needle was

carefully inserted between two dorsal scales. This technique gives reliable challenges in 1-2 year old carps (Evenberg et al., 1988). For the disease susceptibility experiment, 10 carp per group were given a lethal challenge and monitored regularly for mortality. The challenge was repeated 3 times.

Serum. Blood was obtained by caudal puncture from MS-222 (Sandoz) anaesthetized fish, and allowed to clot at room temperature for 1 h. Sera were carefully collected to avoid RBC contamination, rapidly frozen using liquid nitrogen, and stored in small aliquots at - 80°C until use.

Immuno-electrophoresis. Carp and human sera were run in an immuno-electrophoresis assay (Scheidegger, 1955) against monoclonal anti-human α_1-AT and α_2-M, using Nordic equipment (Tilburg, The Netherlands). After standard staining procedures with coomassie blue, precipitation lines of identity between human and carp antiproteases were read.

Determination of serum antiprotease. Blood from the A4, W49, WW and R3 carp lines was collected immediately after bacterial challenge and 1, 5, 7, 10, and 13 days post-sublethal challenge with A. salmonicida. Blood from PBS-injected controls was collected immediately after challenge. Sera from 10 fish from the 4 selected genetic lines were obtained at each time period, frozen separately, and pooled before running the antiprotease assays.

Serum samples were then analyzed for α_1-AT and α_2-M by preincubation with a LAS-R reference α_1-AT and α_2-M monoclonal antisera and read using a Hyland PDQ™ laser Nephelometer (both from Hyland Diagnostics, Cooper Biomedical Inc., Malvern, USA) adapted to a Hewlett Packard HP 9815 computer (Cooper biomedical, Malvern, USA).

Zymography analysis. Serum from 5 carp from each of the 4 genetic lines, was obtained immediately (controls) and 6 days after bacterial challenge, and run in a zymography assay previously described by Granelli-Piperno and Reich (1978). Serum samples were submitted to SDS polyacrylamide gels (9%, 20 mA, 90 min). The stacking and separating gels contained 4 and 9% acrylamide, respectively. SDS was then eliminated from the gels, using a 2.5% triton solution. The gel was then applied onto another agarose gel, containing 2 mg ml^{-1} Grade L human fibrinogen (KabiVitröm, Stockholm,

Sweden), 5 $\mu g\ ml^{-1}$ human plasminogen (KabiVitröm, Stockholm, Sweden), 0.06 IU ml^{-1} bovine thrombin (Diagnostica, Stago, Asnières, France), 1.25% agarose Type VII (Sigma Chemicals, St. Louis, MO, USA) in PBS-NaN3, all in final concentrations. In this assay, the plasminogen activators present in the samples activated the plasminogen into plasmin, which in turn degraded fibrin. Clear lysis zones appeared on the opaque fibrin background, corresponding to the molecular weights of the activators, free or complexed to anti-activators.

RESULTS

Susceptibility of the carp lines to A. salmonicida. Carp from the 5 genetic lines were shown to have different susceptibilities to A. salmonicida (Fig. 1). The maximum survival time of the carp varied from 8 days for the W49 line to over 20 days for the R8 line. Fifty percent survival time was a more accurate measure of disease susceptibility, because of the increasing

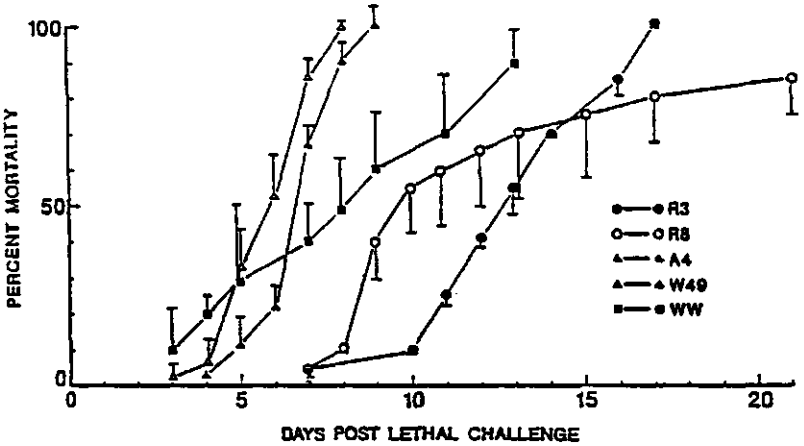

Fig.1. Differences in susceptibility of 5 genetic carp lines to a lethal challenge with virulent atypical A. salmonicida: A Polish 5th generation line R3; A Hungarian, 4th generation line R8; an Israeli, 1st generation, gynogenetic, line A4; a Dutch, 2nd generation, gynogenetic line W49; and a Dutch, 1st generation, gynogenetic line WW.

mortality among the infected fish. It varied from 6 days for the line W49 to 12.5 days for R3. The most susceptible carp (i.e. W49 and A4) all died within a short time from each other. There was much more variability in the survival time of the more resistant carp (i.e. R8). At this challenge intensity, all the fish ultimately died from the infection. A repeat of the experiment gave essentially the same results.

Carp serum α_1-AT and α_2-M. Using immuno-electrophoresis, precipitin lines of identity were seen between carp and human α_1-AT and α_2-M, using anti-human-α_1-AT and -α_2-M monoclonal antibodies. Human anti-inhibitors were therefore used to monitor carp serum antiproteases in the subsequent assays.

Carp serum α_1-AT levels after bacterial challenge. The baseline (PBS injected) levels of serum α_1-AT for the 4 carp lines varied between 37,9 mg dl^{-1} for A4 and 35,6 mg dl^{-1} for WW. Serum antiprotease levels of infected carp remained at baseline levels throughout the experimental period of 8 days in all carp (Table 1).

Table 1. Serum levels of α_1-antitrypsin (mg dl^{-1}) in carp experimentally infected with A. salmonicida.

carp lines	baseline (PBS controls)	day 2 after challenge	day 4 after challenge	day 6 after challenge	day 8 after challenge
W49	37.5	37.5	37.9	37.5	37.5
WW	35.6	37.9	38.0	37.9	38.0
A4	37.9	38.7	35.5	38.7	35.6
R3	36.8	39.0	38.7	39.0	39.1

Carp serum α_2-M levels after bacterial challenge. The baseline levels of α_2-M were not significantly different among the 4 carp lines, i.e. 13.1 mg dl^{-1} for WW, 16.05 mg dl^{-1} for W49, 15.7 mg dl^{-1} for A4, and 14.0 mg dl^{-1} for R3. A rapid and important drop in α_2-M level was seen in all infected fish, 24 hrs post-challenge (Fig. 2). Serum antiprotease levels rose again, but very slowly. The kinetics of recovery of the serum inhibitors varied between the 4 carp lines. Alpha$_2$-M levels of the carp line A4 remained at a critically low level for at least 5 days post-challenge, time at which the mortality rate increased dramatically (Table 2). After a rapid initial

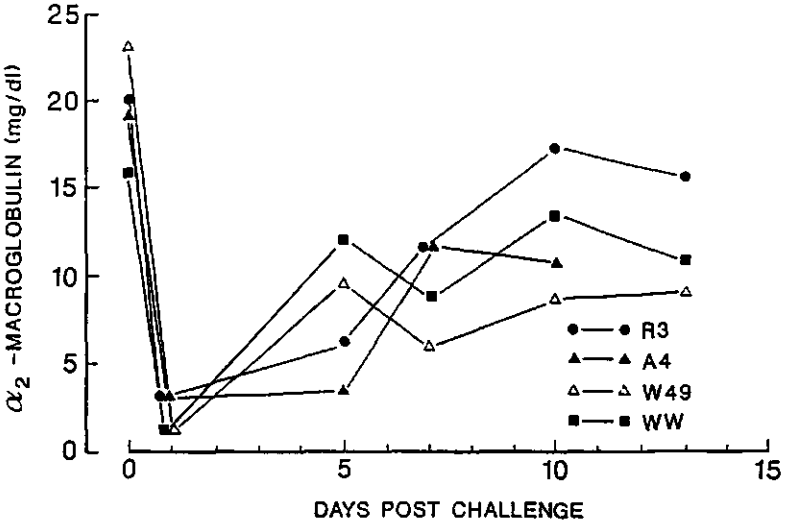

Fig. 2. Serum α_2-macroglobulin levels (mg dl^{-1}) in carp experimentally infected with virulent, atypical A. salmonicida: a Polish 5th generation line R3; A Hungarian, 4th generation line R8; an Israeli, 1st generation, gynogenetic, line A4; a Dutch, 2nd generation, gynogenetic line W49; and a Dutch, 1st generation, gynogenetic line WW.

rise in α^2-M levels in carp W49, these remained at a very low level thereafter (i.e. about 50% baseline). Among the other carp lines, fish surviving the initial drop in α_2-M levels had increasing antiprotease levels during the experimental period of 13 days. This was particularly apparent in the R3 carp. These fish had only a 40% mortality by day 13, whereas all other carp showed ≥ 70% mortality by that time.

Table 2. Percent mortality of carp lethally infected with A.salmonicida.[a]

carp line	day 0	day 5	day 7	day 10	day 13
W49	0	10	40	70	80
WW	0	10	30	50	80
A4	0	20	50	70	70
R3	0	0	0	20	40

[a] initial number of fish for each carp line was 10.

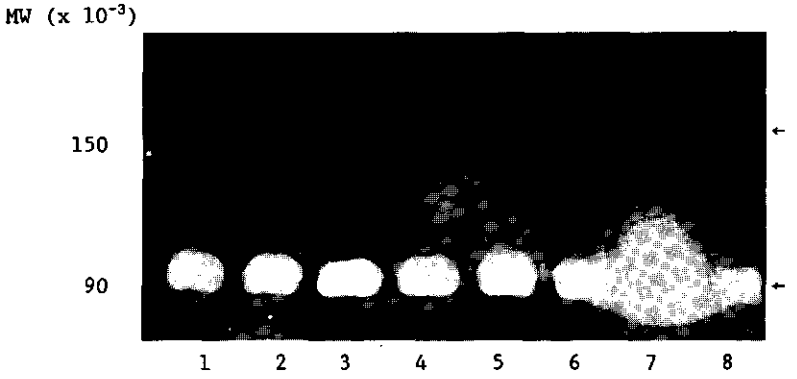

Fig.3. Zymogram of the plasminogen-dependent proteolytic activity of serum of carp at 0 (controls, odd numbers) and 6 days post-lethal challenge (even numbers) with virulent, atypical A. salmonicida. Carp lines were: a Dutch 1st generation gynogenetic line WW, a Dutch 2d generation gynogenetic line W49, an Israeli 1st generation gynogenetic line A4 and a Polish 5th generation line R3, respectively. Molecular weight of the human plasminogen-activator standards (20 mIU) were 68 kD for tissue plasminogen activator (TPA) and 53 kD for urokinase-type plasminogen activator (UPA). Arrows indicate plasmominogen-dependant proteolytic activity at 90 and 150 kD.

Plasminogen-dependent antiproteases in carp serum. The zymography analysis permitted the identification of plasminogen-dependent proteolytic activity, present in serum samples from the different carp lines (Fig. 3). All showed a lytic band in the 90 kD region. However, only the Polish carp line R3 presented, in addition a clear band at 150 kD. This fine band was particularly evident in the serum of carp 6 days post-challenge (lane 8, Fig. 3).

DISCUSSION

In mammals, the role of serum antiprotease in physiological homeostasis and control of proteolytic tissue destruction has been well documented. Antiproteases are found to regulate the inflammatory proteins, secreted locally by PMN cells and activation products of the complement, coagulation and fibrinolytic pathways (Travis and Salvesen, 1983). They also interact with microbial proteases released endogenously (Kueppers and Bearn, 1966; Solovich and Wichner, 1971; Barrett and Starkey, 1973; Morihara et al., 1979; Potempa et al., 1986). Results from this study suggest that carp

serum antiproteases play a similar regulatory role during bacterial infection. In addition to bacterial enzymes, endogenous proteases released during the acute inflammatory response of the host to the bacterium, may contribute to a rapid loss of the neutralizing potential of the serum. Exhaustion of active serum antiproteases may be a key factor influencing the survival of carp infected with A. salmonicida.

Differences in disease susceptibility among carp lines

Carp from 5 genetic lines showed different susceptibilities to A. salmonicida. The maximum survival times varied by at least 10 days between the most susceptible and the more resistant fish. The 50% survival time more then doubled between the more susceptible and resistant carp lines tested (W49 and R3, respectively), the other lines showing intermediate susceptibilities. Most studies to date have reported on susceptibility differences among salmonid species. In particular, reports on intrastrain variability in resistance to A. salmonicida in trout (Embody et al., 1925; Hayford and Embody, 1930; Snieszko, 1958; Ehlinger et al., 1964) and carp (Sövényi et al., 1988) led to planned selection programs for the production of genetically resistant fish lines (for review, see Austin and Austin, 1987). Insufficient information on the mechanisms involved in A. salmonicida pathogenicity and therefore, of specific criteria for selection may have restricted the success of these programs.

Protease production by A. salmonicida in vivo

Previous work showed that our virulent atypical A. salmonicida strain is an active serine protease producer in culture (unpublished results), as are most virulent typical strains (for review, see Austin and Austin, 1987). The characteristic pathological signs seen in carp infected with A. salmonicida suggests that the bacteria also release proteases in vivo. This is in agreement with several authors working with typical strains (Shieh and McLean, 1975; Sheeran and Smith, 1981; Mellergaard, 1983; Sakai, 1985).

Serum α_1-AT antiprotease in infected carp

In this study, we could monitor serum levels of α_1-AT antiprotease, in carp infected with A. salmonicida. Alpha$_1$-AT was specifically monitored because of its important role in elastic tissue repair by neutrophil elastase inactivation in mammals (Gadek and Crystal, 1983; Duswald et al., 1985; Takemura et al., 1985; Carrell, 1986; Ossana et al., 1986). Mammalian plasma α_1-AT can increase 4-fold in response to increases in serum protease

levels (Perlmutter et al., 1989). In certain inflammatory disease states however, antiprotease activity can be downregulated by neutrophil reactive oxygen metabolites (Pabst et al., 1982; Carrell, 1986; Ossanna et al. 1986), or degraded by endogenous inflammatory (Banda et al., 1985; Johnson et al., 1986), or bacterial proteases (Potempa et al., 1986). Ellis (1987) mentions the existence of α_1-AT in the serum of trout, but to our knowledge the inhibitor has not been previously described in carp.

Baseline α_1-AT levels were not significantly different among the carp lines and did not vary throughout the infection period. These results are in agreement with Ellis (1987) who showed indirectly that α_2-M was the only antiprotease involved in the neutralization of A. salmonicida protease in trout. Since neutrophils are known to be activated in the infected carp (unpublished results), down-regulation of α_1-AT by oxygen metabolites could have occurred. Alternatively, the proteases produced by A. salmonicida may be resistant to the action of α_1-AT, as suggested by Ellis in trout (1987).

Serum α_2-M antiprotease in infected carp

Alpha$_2$-M also has a broad spectrum of antiprotease activities in mammals (Dobvich and Vicher, 1971; Barrett and Starkey, 1973). It has previously been described in several fish species (Starkey and Barrett, 1982, a b; Ellis, 1987) but not in carp. Ellis (1987) mentions the existence of 3 antitrypsin factors in rainbow trout serum, one of which is α_2-M. Our results suggest that α_2-M is involved in protease inactivation in carp infected with A. salmonicida. Results obtained in this study showed that the 4 genetic carp lines tested had comparable baseline serum α_2-M levels. Serum α_2-M dropped sharply after bacterial injection and then slowly rose again. Although all carp survived this initial severe depletion of antiprotease, many infected fish died before their α_2-M levels had returned to baseline values. Starkey et al. (1982) described a similar drop in plaice antiprotease when these fish were intravenously injected with the protease thermolysin. This response also occurred very rapidly, but α_2-M levels remained low for only 30 h. The difference in time of antiprotease recovery may be fish species related, or due to proteolytic enzyme differences.

Further, the rapidity of the α_2-M reaction was surprising, if it indeed correlated with the presence of bacterial proteases in carp serum, because our bacterial strain is known to start releasing active serine proteases

after 3 days in culture (data not published). Immediate release of inflammatory proteins by activated granulocytes, or products of complement activation could have contributed to this initial response. In this case, a lowering of the antiprotease levels before the bacteria actively start producing exotoxins would have serious detrimental effects on the fish. Alternatively, the bacteria may release either more or different proteases in vivo than they do in culture. Serum may even enhance protease production as proposed by Ellis and Stapleton in trout (1987).

Differences in the α_2-M response among carp lines

Clear differences were seen in the recovery of serum antiprotease levels among the various carp lines. The line W49 showed low α_2-M levels and an overall poor survival to the bacterial infection. The carp line A4, which is also a susceptible line, showed very low α_2-M levels up to 5 days post-sublethal challenge. Since many fish had died by day 13, the rise in α_2-M probably reflects a selection for carp with higher α_2-M levels at the end of the experimental period. On the other hand, the α_2-M levels of the R3 carp, which showed the highest survival rate in this experiment, rose continuously up to day 13 post-challenge. The carp line WW showed intermediate serum inhibitor kinetics and overall survival. A more rapid initial antiprotease recovery might provide an early protection in these fish. These results indicate that maintaining serum neutralizing capability is critical to the survival of the infected carp. Alpha$_2$-M levels post-challenge correlated with carp survival time. The ultimate death of the animals could not be prevented, probably because an excessive amount of serum proteases exceeds the neutralizing capacity of the serum.

A possible third antiprotease in the serum of the R3 carp line

A. salmonicida has been shown by several authors to secrete in culture, a serine protease of a molecular weight of 90 kD (Mellegaard, 1983; Tajima et al., 1984), that we also identified in our atypical strain of A. salmonicida after 48-72 h of culture (unpublished results). A zymography analysis of serum from all of the carp lines studied showed a serine protease, plasminogen-dependent activity in the same molecular weight region, which probably corresponds to bacterial exotoxin. Ellis and Stapleton (1988) have shown that trout serum was capable of enhancing the proteolytic activity of A. salmonicida. A similar situation in carp may lead to an accelerated production of exotoxin by the bacteria in vivo.

In addition, another lytic band was found at 150 kD, only in the carp line R3. The proteolytic activity in that zone could correspond to a protease-antiprotease complex capable of neutralizing the 90 kD bacterial exotoxin. It is also possible, however, that the bacterium may release in the serum of carp a proteolytic enzyme which is normally not secreted in culture. Since the carp line R3 shows more resistance to A. salmonicida, this result deserves further investigation.

In conclusion, quantitative and possibly also qualitative serum antiprotease measures in the sera of genetically different carp lines correlated with the outcome of an A. salmonicida infection in carp. These traits could potentially be used for selection of more resistant carp lines.

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Chapter 8

SUMMARY AND CONCLUSIONS

The outcome of a bacterial infection depends on the interaction between pathogen and host. The ability of the microbe to survive in the host depends on its invasive potential (i.e. spreading and multiplication), and its ability to obtain essential nutrients and to resist the host's defense system. On the other hand, the host's resistance to a bacterial attack depends on its physiological state, the intensity of the bacterial attack and the efficacy of the defense system to neutralize toxins and eliminate the pathogen.

The objective of this thesis was to better understand the host-pathogen interaction in Carp Erythrodermatitis, an acute disease caused by the bacterial pathogen Aeromonas salmonicida. An overall picture of the disease process is outlined in the conclusions.

In chapter 3, carp sublethally infected with live virulent or avirulent A. salmonicida were tested for their ability to mount a humoral immune response to sheep erythrocytes. Fewer antibody forming cells in the pronephros and lower anti-SRBC titers in serum were found in infected fish, as compared to controls. Both avirulent and virulent bacteria elicited a suppression of the humoral immune system. In the case of virulent bacteria, this was not a delayed response to the antigen, since antibody titers ultimately tapered off. The same was not seen for the avirulent strain.

Virulent bacterial cells, as well as extracellular products (ECP) also had an effect on the cellular immune system of carp, particularly on leucocytes. Indeed, infected fish showed an increase in large peripheral white blood cells, but ultimately became leucopenic. Secondly, rejection of skin allografts was accelerated in infected fish, as compared with non-infected animals, suggestive of polyclonal leucocyte activation. A marked suppression of the inflammatory response to skin allografts was seen in infected animals. It was not possible in this first study to distinguish effects caused by the bacterium from those caused by ECP. An investigation of the separate effects of washed bacterial cells and of bacterial culture

supernatant on carp leucocytes was therefore initiated.

In chapter 4, bacteria-free supernatants from cultures of virulent or avirulent strains of A. salmonicida were obtained after different culture times. Early cultures contained mostly bacterial fragments and free LPS, while active exotoxins were also present in older cultures. The effects of these bacteria-free supernatants were tested on the mitogenic PHA response of carp pronephric leucocytes in vitro. Crude supernatants from virulent cultures modulated the response, whereas avirulent supernatants had no effect. The proliferative response of PHA was enhanced by early (20 hr) virulent supernatant, but severely depressed by supernatant from later (72-96 hr) cultures. Leucocyte proliferation also occurred in the absence of PHA, with early and heat-inactivated supernatants but at a much lower level, suggesting that only stimulated cells (blasts) responded to the mitogenic substance(s) present in the supernatants. Cytokines, such as IL-2 were probably also involved in the proliferative response of these leucocytes.

We then checked if cell-associated material and/or purified LPS, known to be present in the bacterial culture supernatants, were responsible for the stimulation of carp leucocytes. Both were indeed found to be stimulatory. However, LPS-"depleted" supernatants still retained proliferative capacities, suggesting that inactivated bacterial proteins may also be mitogenic.

The modulating effects seen with ECP could explain the blastogenic response of carp leucocytes seen in vivo. Also, active exotoxins, found to be cytolytic to carp leucocytes, could explain the leucopenia seen during later stages of the disease.

In order to further investigate the role played by the non-specific defense system of carp in the disease process, a selection of several carp lines exhibiting a reproducible high (A4 and W49), moderate (WW) and low susceptibility (R3 and R8) to the bacterium was made (Chapter 5). Complement activation in sera from these five carp lines was studied because of its possible role in the neutralization of A. salmonicida ECP and in the killing of the bacteria ("alternative" route). Washed cells, crude or LPS-"depleted" culture supernatants and purified A. salmonicida LPS were found to activate carp complement and deplete serum of active complement factors. A significant difference in complement activation was

seen among the carp lines tested. In particular, an enhanced activation was seen in the more resistant carp line R3. The role of complement as a bactericidal and toxin-neutralizing agent is in agreement with our findings. Fish exhibiting low initial bactericidal potential would be from the start at risk of acute pathology.

In chapter 6, we investigated the role of carp serum transferrin in the resistance of carp to CE. The analysis of the Tf polymorphism in 4 carp lines resulted in several distinctive variants. The DD genotype seemed to correlate with a lower susceptibility to the bacterium, whereas the F'C genotype seemed to correspond with a higher disease susceptibility. Other results on the other hand, could not be interpreted at this point. In particular, the serum Tf genotype GD was found in both highly susceptible and more resistant carp lines. More work, using carp showing these specific genotypes seemed warranted.

Results on the iron-binding capacity of the fish serum showed a higher binding potential for the DD genotype, found only in the more resistant carp. However, the lowest iron-binding capacity was seen for the GG genotype, which was also found in relatively resistant carp. Finally, the relative concentration of Tf in the serum of the various carp lines did not seem to correlate with their susceptibility to the disease. With these preliminary data, several Tf genotypes were identified in our carp lines, some of which may correlate with lower and higher susceptibilities to the bacterium. However, Tf did not seem to be the most important factor in resistance of carp to A. salmonicida.

Without an efficient mechanism to neutralize the proteolytic enzymes present in the blood circulation during bacterial infection, not only the immune system, but also important physiological systems such as the coagulation, fibrinolytic, and kinin pathways are put at risk. Also, irreversible proteolytic degradation of connective and muscle tissues begins to occur, as seen during gross clinical observation of infected carp. In this disease, proteases in the blood of the fish had several origins: bacterial ECP, complement activation products, and other inflammatory products released by activated granulocytes.

In chapter 7, carp were found to possess at least two serum antiproteases, i.e. α_1-antitrypsin (α_1-AT) and α_2-macroglobulin (α_2-M). Using the zymography technique, a third protease/antiprotease complex could be

identified in the serum of the more resistant carp line R3, after infection with A. salmonicida. This complex was not found in the sera of the other more susceptible carp lines. More work is needed to identify this antiprotease and to investigate its significance in disease resistance.

Carp from the 4 selected carp lines were given a lethal challenge with A. salmonicida and monitored at regular time intervals for their serum α_1-AT and α_2-M levels. Serum α_1-AT levels remained at baseline level in all carp lines, throughout the infection. The absence of α_1-AT involvement in ECP neutralization was conspicuous, since the antiprotease has a broad antiproteolytic activity. Oxygen metabolites at the site of inflammation may have inactivated the antiprotease. In contrast, serum α_2-M levels dropped drastically in all carp, shortly after challenge. The recovery of antiprotease levels was slow in all animals. Serum α_2-M of the carp line A4 remained at a critically low level for at least 5 days post-challenge. Maximum α_2-M levels reached after 13 days were highest for the more resistant R3 carp, and lowest for the susceptible W49 carp. The recovery time of serum antiprotease levels could play an important role in the resistance of carp to A. salmonicida, by prolonging the survival time of the fish and by allowing the specific immune system to respond.

CONCLUSIONS

Two main factors account for the acute pathology, in a disease like Carp Erythrodermatitis (CE): 1) the virulence of the bacterium and 2) the ineffectiveness of the defense mechanisms of the fish. Differences seen in the extent of the pathological symptoms and in the outcome of the infection relate to one or both of these factors.

1. Virulence of the bacterium.

- The virulence of Aeromonas salmonicida is based upon its ability to invade the host, to resist the defense mechanisms and to obtain essential nutrients.

- The additional membrane layer (A-layer) of virulent A. salmonicida strains plays a major role in the resistance against the bactericidal activity of carp serum (i.e. hindrance of antibody and complement binding to target cells), as well as against the attack by phagocytes.

- Our virulent atypical strain of A. salmonicida (V234/81) has a low invasive capacity (i.e. slow growth rate, strict nutritional and metabolic requirements for maintaining virulence). It secretes proteolytic enzymes with collagenase and gelatinase activity, which break down host skin, connective tissue and muscle. These enzymes are released in order to facilitate penetration. However, the bacteria remain at the skin surface and are usually not recovered from blood or internal organs of infected carp. A local release of proteases leads to the hemorrhagic and necrotic surface lesions. Variation in the type and amount of extracellular products (ECP) released by A. salmonicida in vivo and the survival time of the fish leads to differences in the extent of the disease symptoms.

- The soluble bacterial factors with proteolytic and hemolytic activities are also means for the pathogen to overcome nutritional deprivation, by digestion of collagen and muscle tissue (providing essential amino acids) and by liberation of hemoglobin from lysed red blood cells (providing essential iron).

2. Defense mechanisms of the carp.

- While bacterial cells do not seem to penetrate into the blood or internal organs of the fish, free LPS and exotoxins are probably responsible for the disturbance in the carp's inflammatory and immune system.

- Polyclonal leucocyte activation is induced by the bacterial LPS and other ECP, but this response is counteracted by the activity of the cytolytic enzymes, leading first to the presence of blasts in the peripheral blood of infected fish and later leucopenia. In both cases, regulation of B and T cell responses is affected.

- A. salmonicida ECP (i.e. LPS and probably also proteases) strongly

activate carp complement and granulocytes. The release of inflammatory products, at a distance from the proliferating bacteria, exhausts the neutralizing activity of carp serum and seems to contribute to toxicity. Uncontrolled proteases result in disturbances in the coagulation system and in changes in vascular permeability, leading to edema and internal hemorrhages.

- The continuous exposure to bacterial and endogenous inflammatory proteases also cause a rapid decrease of the total serum proteins. As some serum antiproteases (i.e. α_2-M), immunoglobulins and complement factors become depleted, carp serum not only loses its neutralizing but also its bactericidal potential.

- In the final stage of the disease, the severe skin ulceration, combined with a general weakening of the defense system, rapidly leads to an invasion by opportunistic pathogens. The fish die of acute respiratory distress and shock due to septicemia.

- In addition to environmental effects (i.e. water temperature and quality, handling stress) and physiological state of the fish (i.e. nutritional and reproductive status), which are known to influence disease resistance, the extent of the disease symptoms and the survival of carp infected with A. salmonicida are also influenced by differences in genetics.

- Genetic differences in the susceptibility to A. salmonicida observed among various carp lines are probably due to the ECP-neutralizing or bacterial killing potential of the carp serum or mucus.

- Carp serum antiproteases clearly play a significant role in the disease outcome. The relatively fast recovery of normal serum α_2-M levels and the possible presence of an additional antiprotease type(s) in resistant carp lines suggest that both quantitative as well as qualitative differences may be important in disease resistance.

- Serum transferrin genotype and iron binding capacity may also contribute to disease resistance, as atypical A. salmonicida show a lower capacity to compete for iron as compared with the typical bacterial strains. However, preliminary results suggest that transferrin may not be a major factor in resistance to CE. Transferrin genotypes were identified, which seemed to correlate with lower or higher resistance to CE. More work using carp with these specific transferrin genotypes and crossbreeding of these carp lines is needed to elucidate the exact role of transferrin in this disease.

- In the future, it will be important to develop carp lines with an efficient first or second line of defense against A. salmonicida, allowing fish more time for specific immune responses and immunological memory. If memory can be obtained, effective vaccination should be one of the promising developments.

SAMENVATTING EN CONCLUSIES

De afloop van een bacteriële infectie is afhankelijk van de interactie tussen ziekteverwekker en gastheer. De mogelijkheid van de microbe om te overleven in een gastheer is afhankelijk van: haar binnendringend vermogen (verspreiding en vermenigvuldiging), de mogelijkheid om essentiële voedingsstoffen te verkrijgen en om de afweer van de gastheer te weerstaan. Daarentegen hangt de afweer van de gastheer tegen de bacteriële aanval af van: haar fysiologische status, de intensiteit van de bacteriële aanval en de doelmatigheid van het afweersysteem om toxinen te neutraliseren en ziekteverwekkers te verwijderen.

Het doel van dit onderzoek was om tot een beter inzicht te komen in de gastheer-ziekteverwekker interactie tijdens de ziekte erythrodermatitis van de karper. Dit is een acute ziekte, die veroorzaakt wordt door de bacterie Aeromonas salmonicida. Een totaalbeeld van deze ziekte is weergegeven in de inleiding en de conclusies.

Bij karpers, die sublethaal waren geïnfecteerd met virulente of niet-virulente A. salmonicida, hebben we in hoofdstuk 3 onderzocht in hoeverre deze dieren nog in staat waren om een humorale immuunrespons te ontwikkelen tegen schape rode bloedcellen. We vonden hierbij minder antilichaam vormende cellen in de kopnier en minder anti-SRBC antilichamen in het serum van geïnfecteerde karpers, in vergelijking met niet-geïnfecteerde vissen. Zowel virulente als niet-virulente A. salmonicida veroorzaakten een onderdrukking van het humorale immuunsysteem. Bij virulente bacteriën is dit niet op te vatten als een uitgestelde respons tegen het antigen, omdat antilichaamtiters nooit de piekwaarde van de controles bereikten. Dit werd niet waargenomen bij de niet-virulente bacteriestam.

Zowel virulente bacteriecellen als extracellulaire produkten (ECP) hadden tevens een effect op het cellulaire immuunsysteem van de karper, in het bijzonder op de witte bloedcellen. Geïnfecteerde vissen vertoonden eerst een toename in grote perifere witte bloedcellen (blasten), maar kregen in een latere fase een gebrek aan witte bloedcellen. In de tweede plaats werd de afstoting van een huidtransplantaat versneld in geïnfecteerde vissen, in vergelijking met niet-geïnfecteerde dieren, hetgeen een polyclonale lymfocytenactivatie suggereert. Er werd een opvallende remming van de ontstekingsreactie als gevolg van een huidtransplantatie waargenomen

bij geïnfecteerde vissen. In dit eerste onderzoek was het niet mogelijk om door de bacterie zèlf veroorzaakte effecten te onderscheiden van effecten van uitgescheiden extracellulaire producten (ECP). In het volgende hoofdstuk werden daarom de afzonderlijke effecten van gewassen bacteriecellen en bacteriecultuur-supernatant op karper witte bloedcellen onderzocht.

Bacterie-vrije supernatantia van kweken van virulente of niet-virulente A. salmonicida stammen werden verkregen na verschillende kweektijden (hoofdstuk 4). Vroege kweken bevatten voornamelijk bacteriële fragmenten en sporen van LPS, terwijl exotoxinen vooral aanwezig waren in supernatantia van oudere kweken. Het effect van deze bacterie-vrije supernatantia werd in vitro getest op de mitogene PHA respons van witte bloedcellen uit de kopnier van de karper. Ongezuiverde supernatantia van virulente kweken beïnvloedden de respons, terwijl niet-virulente supernatantia geen effect hadden. De celdeling werd gestimuleerd door vroege virulente supernatantia, maar sterk onderdrukt door supernatantia van oudere kweken. Deling van witte bloedcellen trad ook op in afwezigheid van PHA na toevoeging van vroege en hitte-geïnactiveerde supernatantia, maar dan wel op een veel lager niveau. Dit suggereert dat alleen reeds geactiveerde cellen (blasten) reageerden op de mitogene substantie, die in het supernatant aanwezig was. Cytokinen, zoals IL-2, waren waarschijnlijk ook betrokken bij de stimulatie van deze witte bloedcellen.

Vervolgens werd nagegaan of cel-geassocieerd materiaal en/of gezuiverd LPS, hetgeen in het bacterie-kweek supernatant was aangetoond, verantwoordelijk was voor de stimulatie van karper witte bloedcellen. Beide factoren bleken inderdaad te stimuleren. Supernatantia, waaruit het LPS was verwijderd, bleken echter nog steeds in staat celdeling te stimuleren, hetgeen aangeeft dat geïnactiveerde bacteriële eiwitten wellicht ook mitogene eigenschappen bezitten.

De modulerende effecten die tijdens proeven met LPS en ECP werden waargenomen zouden een verklaring kunnen zijn voor het verschijnen van blastvormige witte bloedcellen tijdens de vroege fase van de ziekte. De door de bacterie uitgescheiden toxinen veroorzaken wellicht in een latere fase het gebrek aan witte bloedcellen.

Om de rol van de niet-specifieke afweer bij karpers verder te bestuderen, werden verschillende karperlijnen geselekteerd, die een reproduceerbare hoge (A4 en W49), matige (WW) of lage (R3 en R8) gevoeligheid voor

de bacterie vertoonden (hoofstuk 5). Complementactivatie in sera van de vijf karperlijnen werd bestudeerd, omdat het complementsysteem misschien betrokken is bij de neutralisatie van A. salmonicida ECP. Gewassen bacteriecellen, ongezuiverde of LPS-arm kweeksupernatant en gezuiverde A. salmonicida LPS bleken het karper complement te activeren en het serum van actieve complementfactoren te beroven. Een belangrijk verschil in complementactivatie werd waargenomen bij de onderzochte karperlijnen. Met name werd een sterke activatie waargenomen in de resistentere karperlijn R3. De rol van complement als een bacteriedodend en toxine neutraliserend agens wordt door onze bevindingen bevestigd. Vissen met een oorspronkelijk laag bacteriedodend vermogen zouden reeds bij voorbaat een verhoogd risico lopen op een acuut verlopend ziekteproces.

In hoofstuk 6 werd de betekenis van het karperserum-transferrine voor de afweer tegen CE onderzocht. De analyse van de transferrine genotypen van 4 geselekteerde karperlijnen resulteerde in verschillende varianten. Het DD genotype bleek te correleren met een lagere gevoeligheid voor de bacterie, terwijl het F'C genotype bleek te corresponderen met een hogere ziektegevoeligheid. De overige resultaten konden niet op een zelfde manier worden verklaard. In het bijzonder de waarnemingen met het GD transferrine type, dat zowel in zeer gevoelige, als in de meer resistente karperlijnen werd gevonden. Verder onderzoek met deze transferrine genotypen lijkt nodig.

Resultaten betreffende het ijzerbindend vermogen van visseserum toonde een hoger bindend vermogen bij het DD genotype, hetgeen alleen bij de resistentere karpers voorkwam. Het laagste ijzerbindend vermogen werd echter waargenomen bij het GG genotype, hetgeen ook de meer resistente karpers betrof. Verder bleek de relatieve hoeveelheid transferrine in het serum van de verschillende karperlijnen niet overeen te komen met hun ziektegevoeligheid. Bij deze voorlopige waarnemingen werden dus verscheidene transferrine genotypen waargenomen in onze karperlijnen, waarvan sommige zouden kunnen correleren met een hogere, dan wel lagere gevoeligheid voor de bacterie. Transferrine bleek echter niet de belangrijkste factor te zijn bij de afweer van de karper tegen A. salmonicida.

Zonder een efficiënt neutralisatiemechanisme voor de eiwitsplitsende enzymen die aanwezig zijn in de bloedsomloop tijdens de infectie, zouden belangrijke fysiologische mechanismen zoals bloedstolling, fibrinolyse en

kinine metabolisme in de verdrukking kunnen komen. Ook treedt een niet-omkeerbare proteolytische afbraak van bind- en spierweefsel op, hetgeen werd waargenomen bij uitwendig klinisch onderzoek van geïnfecteerde vissen. De proteasen in de circulatie van de zieke vis kunnen van verschillende oorsprong zijn: bacteriëel ECP, complement activatie producten, of ontstekingsproducten die door geactiveerde granulocyten worden afgegeven.

In hoofdstuk 7 wordt beschreven, dat karpers tenminste twee serum anti-proteasen bezitten: α_1-antitrypsyne (α_1-AT) en α_2-macroglobuline (α_2-M). Met behulp van de zymografie techniek kon na injectie van vissen met A. salmonicida een derde protease/antiprotease complex worden geïdentificeerd in het serum van de resistente karperlijn R3. Dit complex werd niet gevonden in de sera van de overige karperlijnen. Identificatie hiervan en van het onbekende antiprotease behoeft nader onderzoek.

Karpers van de vier geselekteerde lijnen werd een lethale injectie gegeven met A. salmonicida en vervolgens werden deze dieren onderzocht op α_1-AT en α_2-M niveaus in het serum. Het niveau van α_1-AT in het serum bleef ongewijzigd in alle karperlijnen gedurende de gehele infectie. Dit suggereert dat α_1-AT niet betrokken is bij het neutraliseren van eiwitsplitsende enzymen tijdens deze ziekte. Daarentegen nam het niveau van α_2-M in het serum bij alle karpers binnen 24 uur na infectie aanzienlijk af. Het herstel van antiprotease niveaus was langzaam. De hoeveelheid α_2-M in het serum van karperlijnen A4 en W49 bleef op een kritisch laag niveau gedurende tenminste vijf dagen na injectie. De hoogste α_2-M niveaus werden na 13 dagen bereikt bij de resistentere karperlijn R3, en de laagste hoeveelheid werd gevonden bij de relatief gevoelige W49 karpers. Serum antiprotease zou een duidelijke rol kunnen spelen in de afweer van de karper tegen A. salmonicida, door de de overlevingstijd te verlengen en daardoor de mogelijkheid te scheppen voor een reactie van het specifieke immuunsysteem.

CONCLUSIES

Twee belangrijke factoren zijn verantwoordelijk voor het verloop van een ziekte als erythrodermatitis bij de karper: 1) de virulentie van de bacterie; 2) de ineffectiviteit van het afweermechanisme van de vis. De

verschillen die worden waargenomen in de ernst van de ziekteverschijnselen en de afloop van de infectie hebben te maken met één of beide bovengenoemde factoren.

1. Virulentie van de bacterie.

- De virulentie van de Aeromonas salmonicida is gebaseerd op het vermogen om de gastheer binnen te dringen, om het afweersysteem te weerstaan, en om essentiële voedingsstoffen te verkrijgen.

- De extra laag (A-laag) aan de buitenzijde van de celmembraan, die virulente A. salmonicida stammen bezitten, speelt een belangrijke rol in de afweer tegen de bacteriedodende werking van karperserum, (b.v. door de werking van antilichaam te remmen, door te voorkomen dat complement factoren aan de bacteriecellen kunnen binden), en tevens in het voorkomen van aanvallen van fagocyterende cellen.

- Onze virulente atypische stam van A. salmonicida (V234/81) heeft een laag binnendringend vermogen, d.w.z. een langzame groei en stricte voedings- en stofwisselingseisen om de virulentie in stand te houden. Ze scheidt proteolytische enzymen uit met collagenase en gelatinase werking, hetgeen afbraak veroorzaakt van de gastheerhuid en van bind- en spierweefsel. Deze enzymen komen vrij om het binnendringen te vergemakkelijken. De bacterie blijft echter op het huidoppervlak en komt meestal niet voor in bloed en organen van geinfecteerde karpers. Dit plaatselijk vrijkomen van protease leidt tot de bloederige en necrotische zweren. Variatie in het type en de hoeveelheid van de door A. salmonicida uitgescheiden extracellulaire producten (ECP) leidt tot verschillen in de ernst van de ziekteverschijnselen.

- De oplosbare bacteriële factoren met eiwitsplitsende en bloedafbrekende activiteit zijn bovendien een hulpmiddel van de ziekteverwekker om aan voedingsstoffen te komen door vertering van bind- en spierweefsel, hetgeen essentiële aminozuren oplevert, en door het vrijmaken van haemoglobine uit gelyseerde rode bloedcellen, hetgeen ijzer oplevert.

2. Afweermechanisme van de karper.

- Aangezien de bacteriecellen over het algemeen niet in de interne organen of het bloed van de gastheer binnendringen, zijn het vrije LPS en de exotoxinen waarschijnlijk verantwoordelijk voor de verstoring van het ontstekingsmechanisme en het immuunsysteem van de karper.

- Polyclonale witte bloedcelstimulatie wordt bevorderd door het bacteriële LPS en andere ECP, maar deze reactie wordt weer tegengewerkt door de activiteit van celafbrekende enzymen. Dit leidt aan de ene kant tot het verschijnen van blasten in het perifere bloed van geïnfecteerde vissen, maar uiteindelijk tot een gebrek aan witte bloedcellen. In beide gevallen is de regulatie van B- en T-cel reacties verstoord.

- A. salmonicida (ECP), d.w.z. LPS en waarschijnlijk ook proteasen, activeren karper complement en granulocyten. Het vrijkomen van onstekingsproducten, op enige afstand van de zich vermenigvuldigende bacteriën, leidt tot uitputting van de neutraliserende activiteit van karperserum en lijkt bij te dragen aan de toxiciteit. Niet gereguleerde proteasen leiden tot verstoringen van de bloedstolling, en tot veranderingen in de bloedvatdoorlaatbaarheid, hetgeen kan leiden tot vochtophoping en interne bloedingen.

- De continue blootstelling aan bacteriële en endogene onstekings proteasen veroorzaken ook een snelle afname van het totale eiwitgehalte in serum. Omdat de concentratie van sommige serum antiproteasen (o.a. α_2-M), immunoglobulinen en complement factoren sterk daalt, verliest het karperserum niet alleen haar neutraliserende werking, maar ook haar bacteriedodend vermogen.

- In de laatste fase van het ziekteproces gaat een ernstige vorming van zweren in de huid samen met een algehele verzwakking van het afweersysteem. Dit leidt tot een snelle invasie van andere opportunistische ziekteverwekkers. De vis sterft uiteindelijk aan acute ademhalingsmoeilijkheden en shock.

- Behalve milieu-effecten zoals watertemperatuur en -kwaliteit, stress als gevolg van menselijke handelingen, en de fysiologische status van de vis zoals voedingstoestand en voortplantingsfase, waarvan de werking op het afweersysteem bekend zijn, kunnen ook genetische factoren een rol spelen bij het verloop van de ziekte.

- Genetische verschillen in gevoeligheid voor A. salmonicida, zoals die tussen verschillende karperlijnen werden waargenomen, zijn waarschijnlijk het gevolg van het ECP-neutraliserende en bacteriedodende vermogen van het karperserum, slijm of andere lichaamsvloeistoffen.

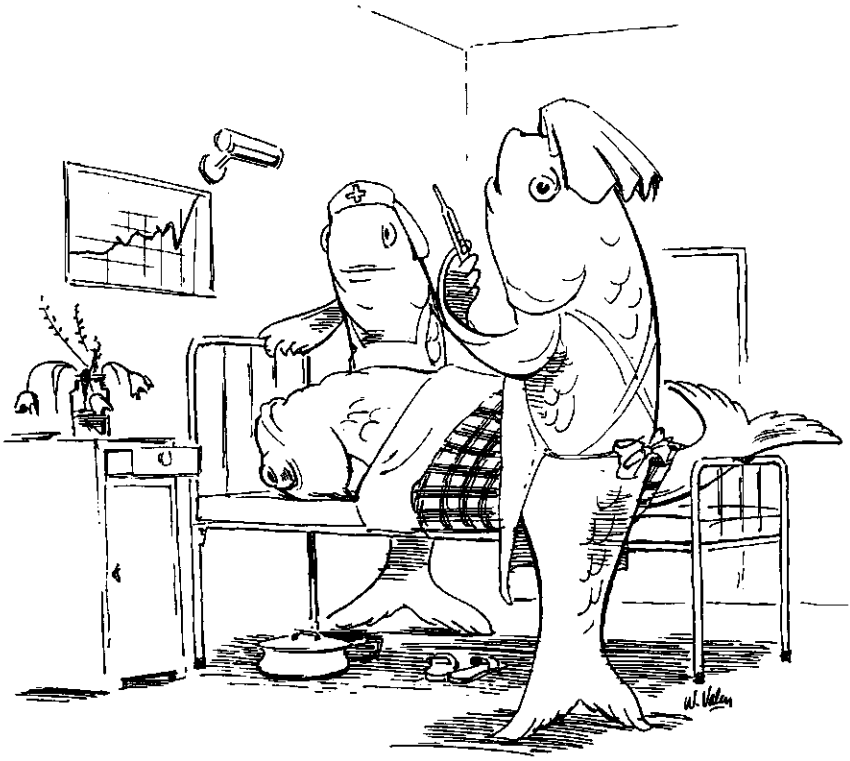
- Antiproteasen in het karperserum spelen duidelijk een rol in het verloop van de ziekte. Het vermogen om α_2-M niveaus na een infectie relatief snel op normale waarden terug te laten keren, en de mogelijke aanwezigheid van één of meer extra anti-proteasetypen in de resistente karperlijn R3, suggereert dat zowel kwantitatieve als kwalitatieve verschillen van belang kunnen zijn.

- Serumtransferrine genotypen en ijzerbindend vermogen zouden ook een bijdrage kunnen leveren aan de ziekteresistentie, aangezien bij atypische A. salmonicida het vermogen om ijzer te binden relatief slecht ontwikkeld is in vergelijking met typische bacteriestammen. Voorlopige resultaten suggereren echter dat transferrine geen belangrijke rol speelt in de resistentie tegen erythrodermatitis. Transferrine genotypen werden geïdentificeerd die misschien iets te maken hebben met hoge of lage resistentie. Nader onderzoek met karpers, die verschillende transferrine genotypen vertonen is nodig om de rol van transferrine bij deze ziekte op te helderen.

- In de toekomst zal het belangrijk zijn om karperlijnen te ontwikkelen met een efficiënte eerste of tweede lijns weerstand tegen A. salmonicida. In dat geval krijgen deze dieren meer tijd om een specifieke immuunrespons en immunologisch geheugen op te bouwen. De ontwikkeling van een afdoende vaccinatiemethode mag vervolgens verwacht worden, wanneer inderdaad specifiek immunologisch geheugen kan worden opgewekt.

Curriculum Vitae

Catherine Nancy Pourreau was born on April 30th 1958 in Neuilly sur Seine (France). After her secondary school education in Massy-Palaiseau, she studied at the College of Wooster, Ohio (USA) and obtained her Bachelor's degree in Biology in 1980. In 1984, she obtained her Master's degree in Oceanography at the College of William and Mary, School of Marine Sciences, in Williamsburg, Virginia, USA. In the fall of 1984, she accepted a Research Associate position at the department of Experimental Animal Morphology and Cell Biology of the Wageningen Agricultural University. Since January 1988, she works in the Medical Department of Eurocetus b.v., in Amsterdam, where she now holds a position of Clinical Research Associate.



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