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DEVELOPMENT OF A VACCINE FOR BACTERIAL KIDNEY DISEASE IN SALMON

Final Report

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EXECUTIVE SUMMARY

The approach toward developing and evaluating potential vaccine candidates has required a thorough examination of candidate antigens, methods of immune response evaluation, the application of possible modulatory substances, as well as the evaluation of the efficacy of the final products. Considering these aspects of the experimental design, the manuscripts comprising this report have been arranged in a sequence which should facilitate an understanding by the reader-at-large of the progress of this study.

The initial phase of this project was dedicated to the identification of candidate antigens for use in vaccine development. During this phase, examination of the growth characteristics of Renibacterium salmoninarum revealed that the bacteria were producing large quantities of soluble protein antigens (MS-5, 1984 Annual Report). In moribund experimentally infected fish this antigenic load can be found to approach concentrations of 1 mg/ml of serum. This high level of soluble antigen production by a pathogen alerted us to the possibility that it may be of considerable biological importance in the disease process. Studies were thus undertaken to characterize the physicochemical nature (MS-l) of these proteins and the antigenicity (MS-2,3,4,5,11). These studies were essential to the future isolation and purification of unique, and potentially protective antigens, and also provided us with the assays required to monitor antigenicity during vaccine synthesis. These antigenic analyses have been a major benefit for diagnosis of BKD-positive fish (MS-2,5). The development of monoclonal reagents were also extremely useful because

they demonstrated the presence of common antigens in a variety of bacterial isolates of a worldwide distribution (MS-3,4).

Acquisition of this panel of monoclonal reagents, which is still under the process of expansion, allows for more sophisticated methods of vaccine design to be employed. Basically, a process of epitope mapping (i.e. fine structure analysis) of important antigens can be conducted with these highly specific antibody reagents. Through 'the use of this methodology, specific antigenic and/or toxigenic sites on the antigen can be identified. Thus, important structures leading to immunogenicity or virulence can be isolated, studied, and eventually be used in the development of highly specific vaccines via molecular cloning or chemical modification techniques.

Assessment of potential toxicity or virulence of an isolated antigen is important to vaccine design since toxins can be factors in pathogenesis. Indeed, many diseases have been controlled by the development of important toxoids, or chemically inactivated toxins. Such inactivation must only affect the toxigenic determinants, while leaving the antigenic determinants intact. Progress in this area of research with bacterial kidney disease has been relatively recent. Although a number of suspect activities have been associated with R. salmoninarum or its secreted products, thus far no one activity has been directly demonstrated to be responsible for the virulence observed in vivo. One plausible explanation may be that the toxigenic materials are not as potent as the toxins isolated from other pathogens. This seems likely since BKD is a chronic disease, thus, these toxins are probably acting in a protracted fashion. In our screening for potential toxigenic activity, it was deemed necessary to develop in vitro assays that could assess the capacity of lymphocytes to perform normal functions such as proliferation or antibody synthesis.

These assays could then be used to detect the presence of agress in-like toxins by relatively rapid screening rather than the use and maintenance of large populations of experimental fish. Agressins are basically toxins which act to destroy or impair the host immune system. The elaboration of such molecules would not only limit the efficacy of any BKD vaccine, but would also result in the increased susceptibility of any infected fish to resist infection by other pathogens. Further, these assays permit an investigator to determine the effectiveness of various detoxification procedures used in the generation of toxoid vaccines. Our use of these assays revealed the existence of such agressin-like activity in R. salmoninarum soluble antigens (MS-8). Employment of the plaque-forming cell assay revealed that an endogenous protease activity could spontaneously detoxify preparations of soluble antigen (MS-10). A potential mechanism by which aggressin activity was manifested was through leukoagglutinating or via effects on the macrophage (MS-9).

An important, albeit fortuitous, observation that was made during the generation of toxoids, was that detoxification by formalin treatment was effected not by the formalin, but rather by the elevation of the temperature required for the reaction (MS-10). This detoxification was discovered to be due to the enhancement of the endogenous protease activity, which exhibits optimal activity in temperatures in excess of 37°C. Whether the cleaved products of the parent molecule(s) could serve as toxoids is not certain at this time, however, prolonged incubation at these elevated temperatues results in the total digestion of all detectable antigens. Therefore, if detoxification by proteolysis is desired, close monitoring of the incubation time and/or incorporation of protease

inhibitors will be required to insure that all immunogenic fragments are not totally destroyed.

Another practical concern related to the protease activity is its effect on antigens used in diagnostic assays. Antigens used in immunoassays must not be prepared, incubated, or inadvertantly kept at temperatues in excess of 17°C for extended periods of time without risking the partial or total loss of all antigens.

Essential to the development of a vaccine is a firm knowledge of the immune capabilities of the animals which are to be vaccinated. This is of particular importance with bacterial kidney disease and with the response of fish species in general. It has been the contention of some investigators that there may be intrinsic differences beween Pacific salmon (Oncorhynchus kisutch and tshawytscha) and Salmo species (Salmo salar and gairdneri - now O. mykiss). The most successful attempts at immunoprophylaxis have thus far only been forthcoming in Salmo or Salmo-like species of Oncorhynchus (O. mykiss). Furthermore, with respect to the vaccination of fish species in general it is imperative that one become fully cognizant of the potential antibody or immune response repertoire to pathogen associated antigens. Many studies indicate that fish may be much more restricted in their abilities to produce antibodies with a wide variety of specificities, as opposed to what is observed in mammals (reviewed in MS-14). Our studies have demonstrated that the three species, O. kisutch, tshawytschaw, and mykiss all primarily recognize the major 57 kd protein, which has been attributed to have a variety of toxigenic properties. Examination of the various western blots performed with sera from immunized individuals of these species demonstrate some

differences, however they do not appear to be of major consequence (MS-l 1).

Vaccine trials primarily focused on the use of immunomodulatory agents to enhance responsiveness and protection to R. salmoninarum. First attempts screened the use of unconjugated antigens with various forms of immunomodulating agents, such as muramyl-dipeptide (MDP), Escherichia coli lipopolysaccharide, and Vibrio anguillarum extract (85 AR). 'The use of Vibrio anguillarum was of particular interest because Vibrio bacterins are the most efficacious vaccines that are commercially available for salmonids. Furthermore, our own studies (MS-6 and -7) indicate that Vibrio anguillarum lipopolysacccharide is an extremely potent polyclonal activator of salmonid lymphocytes. Thus, it was felt that conjugation or admixture with R. salmoninarum may serve to boost or enhance salmonid resistance to BKD. Survival data, however, indicated that the greatest differences occurred among fish vaccinated with Freund's complete adjuvant (MS-12), which contains the highly immunostimulatory, Mycobacterium tuberculosis (killed). These bacteria are known to enhance immune responsiveness by non-specifically activating macrophage function. Thus, since R. salmoninarum is a facultative intracellular parasite, this form of immunoenhancement should be particularly protective. Furthermore, the final challenge study (MS-13), which incorporated Mycobacterium chelonii (a Mycobacterium of salmonid origin), either alone or with soluble antigen, demonstrated a statistically significant degree of protection over controls or R. salmoninarum cell wall polysaccharide. No difference in survival was observed between those fish which received M. chelonii alone or with soluble antigen. There are at least three possible explanations of phenomenon:

1. The primary effective mechanism of immunoprophylaxis is the non-specific induction of cellular immunity.
2. Preparation of soluble antigen requires specific detoxification to be' fully protective.
3. A state of specific immunity may exist in the M. clonei + soluble vaccinated fish, but the specific anti-soluble antigen immunity cannot be assessed until the contribution of non-specific immunity has diminished.

Considering the lack of induction of any noticeable difference in agglutinating antibody titers and the relative lack of correlation between ELISA antibody titers and relative degrees of protection (MS-13, 87AR), it is possible that humoral immunity may not be a significant factor for the induction of protective immunity.

Finally, in futher consideration of the role of humoral antibodies, it now appears that the formation of immune complexes may have serious consequences for the infected fish (MS-12). These studies demonstrate the existence of immune complexes of soluble antigens and endogenous antibody in infected animals. This finding is of considerable importance since it may explain the lack of detectable serum antibodies in fish with BKD. Such a situation should arise if a high antigenic load is generated during the infection and, consequently, this antigen saturates most, or all, of the available antibody. Further, a more serious consequence of immune complex generation is the possibility of a Type III hypersensitivity reaction. The immunopathology of type III hypersensitivity is strikingly similar to the pathology seen in BKD infected kidney tissue, consisting of antigen and antibody deposition, thickening of glomerular membranes, granulomatous lesions, and the presence of immune complexes. The

occurrence of these symptoms may indicate that some amount of the observed pathology of BKD is due to an aberrant immune response.

LITERATURE REVIEW

History

The first published case of BKD was described in 1930 (1). This case occurred in Atlantic salmon (S a 1 m o s alar) in Aberdeenshire Dee (and hence occasionally termed Dee disease) and the river Spey in Scotland . The first report of its occurrence in the United States was by Belding and Merril (3) in brook trout (Salvelinus fontinalis), brown trout (Salmo trutta) and rainbow trout (Salmo eairdneri) from a hatchery in Massachusetts.

The disease occurs in a variety of species of the Salmonidae family including: coho salmon (Oncorhvnchus kisutch), chinook salmon (Oncorhvnchus tshawvtscha), sockeye salmon (Oncorhvnchus nerka) (4), lake trout (Salvelinus namavcush) (5), pink salmon (Oncorhvnchus eorbuscha) (6), yamabe (Oncorhvnchus masou) and cutthroat trout (Salmo clarkii) (1).

The organism has also been isolated from non-salmonid fish also. The bacterium was isolated from Pacific herring (Clupea harengus pallasi L.) present in cages in which coho salmon with clinical BKD were being held (7). Hicks et al., (8) have experimentally infected fathead minnows (Pimep h ale s promelas) and the common shiner (Notropis cornutus). Bell working with Pacific lamprey (Lamnetra tridentata) attempted to infect this non-salmonid host without success (9). Lamprey were chosen for these studies because these cyclostomes are among the species that live in relatively close association with

salmon in fresh water and prey upon salmon in fresh and salt water. In other studies, Bell et al. (72) demonstrated that sablefish (Anoplopoma fimbria) can also be infected by intraperitoneal injection of Renibacterium salmoninarum resulting in death within 50-71 days. Bacterial kidney disease is now routinely reported in hatchery salmonid populations from the United States, Canada, Spain, Italy, France, Iceland, England, Scotland, Yugoslavia, Japan and Chile (10). The foregoing information indicates that salmonid fishes are the primary natural host among cultured fish for the bacterium and that other species of fish may only be infected artificially.

Characteristics of Renibacterium salmoninarum

Renibacterium salmoninarum is a small (0.3-1.5 µm by 0. l-l .O µm), non-motile, non-encapsulated, strongly gram-positive rod that usually occurs in pairs (1). The organism is fastidious in its requirement for L-cysteine and possesses a slow growth rate on even the best of defined media, Kidney Disease Medium-2 (KDM-2) (11). Approximately 10-14 days are required for maximal growth of the bacteria and up to 3-4 weeks for primary isolation of the bacterium from infected fish. The optimum temperature for growth of the bacterium ranges from 15 - 18°C (13). The bacterium is aerobic and the culture becomes more basic during the growth (1). KDM-2 medium contains cysteine and 20% serum along- with peptone and yeast extract. Evelyn (11) indicated that serum is an essential

ingredient for culture of the bacterium. In addition to KDM-2, charcoal agar (KDM-C) (12), cysteine blood agar (13), cysteine serum agar (CSA) (1) and Mueller Hinton medium supplemented with cysteine (14) have been used to culture R. salmoninarum. In charcoal agar, the addition of 0.1 % activated charcoal replaces the requirement for serum supplementation. Daly and Stevenson (12), who developed this medium postulated that both serum and charcoal may serve to adsorb toxic substances from the medium. An advantage of using KDM-C in a broth culture is the elimination of the possibility of serum protein contamination in the culture supernatant. This is of particular importance when the supernatant is processed for production of extracellular products/antigens derived from the bacterium. Regardless of the medium used, optimum growth of the bacterium requires cysteine supplementation in conjunction with the addition of serum. Another medium of use is cysteine blood agar (CBA) which is a modification of Dorset's egg medium that contained cysteine and human blood (20%). Fryer and Sanders (1) have found that the human blood requirement in CBA can be replaced with either fetal bovine or calf serum.

Ordal and Earp isolated the bacterium directly from a lesion of an infected fish onto CBA and thereby were able to complete Koch's postulates, thus demonstrating R. salmoninarum to be the causative agent of this disease (13). Selective isolation of R. salmoninarum from an infected animal was enhanced by the development of a selective KDM-2 (SKDM-2) medium which incorporates the antibiotics such as D-cycloserine, polymyxin B

sulfate and oxolinic acid (15). These compounds were found to be antibacterial for most of the common contaminating organisms, while R. salmoninarum was insensitive. The development of a selective medium has been of particular interest since the slow growth of R. salmoninarum allows other, contaminating bacteria to overgrow the culture quite readily. Another means of overcoming this problem is the drop plate method as developed by Evelyn (11). This method was recommended over the spread plate method because the later technique allows the contaminating bacteria to overgrow R. salmoninarum. Of all the media that are available, the commonly used ones are KDM-2, KDM-C and Mueller Hinton medium with cysteine supplementation.

The G+C content of the DNA of R. salmoninarum ranges from 52.47 to 53.55, averaging 53 (18). Studies have been conducted on the cell wall composition and reveal interesting, and as yet controversial observations. Fryer and Sanders (1) have reported that the major amino acids found in the peptidoglycan were lysine, glutamic acid, glycine and alanine and the sugars were rhamnose, mannose, glucose and arabinose; glucose being the principal sugar. Kusser and Fiedler (19) and Fiedler and Draxl (20) confirmed the amino acid composition reported by Fryer and Sanders, but differed in their analysis of the polysaccharide composition. They reported the constituents of the cell wall polysaccharide as being galactose, rhamnose (6-deoxy-galactose), N-acetyl-fucosamine (2-acetamido-2,6-dideoxy-galactose) and N-acetyl-glucosamine; galactose being

the principal sugar. One interesting feature is the presence of N-acetyl fucosamine, which is rarely found in the cell walls of gram-positive bacteria. They also showed that the cell walls of R. salmoninarum do not possess teichoic acids, contrary to the observations of Fryer and Sanders, who suggested the presence of low amounts of teichoic acids (1). The cell wall polysaccharide was reported to comprise 60-70% of the dry weight of the cell walls (20).

Goodfellow et al. (21) and Fryer and Sanders (1), both reported the absence of mycolic acids in the cell walls. Embley et al. (22) demonstrated that the fatty acid profiles consisted of methyl-branched fatty acids and the polar lipid pattern was that of diphosphatidyl glycerol with two major, six or seven minor glycolipids, and two unidentified minor phospholipids. They also reported that there are menaquinone components consisting of unsaturated menaquinones with nine isoprene units.

Taxonomy

Originally Ordal and Earp (13) and Smith (16) suggested that the bacterium be placed in the genus Corvnebacterium, based on morphological studies. Bullock et al. (23) felt that the resemblance between the bacterium and members of the genus, Lis teria should place it within this genus. Sanders and Fryer (18) placed the bacterium, based on the G+C content and cell wall composition, within the Coryneform group as a new genus and species. In more recent studies, Stackebrandt et al. (24)

have utilized a 16S ribosomal RNA cataloguing approach to classify R. salmoninarum. Evaluation of more than 165 gram-positive organisms from 50 genera revealed that R. salmoninarum is a member of the actinomycetes subdivision, having closest similarities to Arthrobacter and Micrococcus. Confirmatory studies, using reverse transcript sequencing of 16S ribosomal RNA, were also performed which, again, demonstrated similarities between R. salmoninarum and Arthrobacter (S. Gutenberger and Dr. S. Giovannoni, Dept. of Microbiology, Oregon State University, personal communication). They performed studies, as described above, with Bacillus subtilis, Listeria monocytogenes, Brochothrix thermosphactum, Bacillus brevis, Clostridium innocuum, Arthrobacter globiformis and Streptococcus violaceoruber, and were convinced that the bacterium has the closest similarity with Arthrobacter globiformis.

Pathology

Belding and Merril (3) were the first to describe the pathology associated with BKD. Externally it is characterized by exopthalmia, a distended abdomen, and occassionally pustules on the skin. The infection is systemic and has a marked affinity for the kidney tissue. Internally, the infected animal presents extensive petechial hemorrhages, splenomegaly, a swollen kidney covered with a grayish white membrane, accumulation of peritoneal fluid and pustular lesions on the kidney. The lesions

characteristically contain a thick white fluid consisting of leucocytes, bacteria and cellular debris (1,6,16,30). Smith (16) described the nature of the grayish white 'false' membrane histologically and showed that there are three distinct layers. The outermost layer appears to consist of fibrin and nucleated cells, the next contains leucocytes with visible nuclear fragmentation and are in the process of being phagocytized by histiocytes, and the innermost layer is comprised of fibroblasts and histiocytes. Bruno and Munro (39) found that the infection often resulted in a significant decrease in the hematocrit, red cell diameter, hemoglobin and an increase in the number of monocytes, thrombocytes and neutrophils. A significant increase in bilirubin, blood urea nitrogen, and potassium were also found in infected fish, along with decreases in total serum protein, cholesterol and sodium (40). Bruno (40) has also demonstrated that the fast-migrating serum proteins (albumins) were primarily affected in infected fish. He proposed that death of infected fish was due to the obliteration of normal kidney and liver structure by the dissemination of large granulomatous lesions. Impaired renal function and heart failure was also considered to be a contributing factor to mortality due to invasion of myocardium by phagocytic cells containing the bacterium (38).

Epizootiology

Disease Transmission: Mitchum and Sherman (25) demonstrated that natural, horizontal transmission of BKD from infected wild brook trout (*Salvelinus fontinalis*) to newly stocked hatchery brook trout, brown trout (*Salmo trutta*) and rainbow trout (*Salmo gairdneri*) can occur within a small lake or stream system. The stocked trout, thus exposed to this infected population, died in nine months or less. Ordal and Earp (13), however, felt that BKD was less severe in trout than salmon. *Renibacterium salmoninarum* is known to be transmitted horizontally (23,25), however there is increasing evidence that it is vertically transmitted (27,28,29).

Currently there is no experimental evidence that infection occurs by water-borne transmission of the bacteria, but it is likely that the primary reservoir for the bacteria is infected or carrier fish. Austin and Rayment (26) studied the survival of *R. salmoninarum* in water and sediment of 56 freshwater fish farms in England and Wales, which had a history of BKD, and could not demonstrate the presence of the organisms using a fluorescent antibody technique (26). In the same study they demonstrated the presence of the bacteria in fecal matter of experimentally infected fish and found that they survived for only 21 days after all the experimental fish had succumbed to the disease. Published reports on water-borne challenge of fish with *R. salmoninarum* are not available except for the attempts reported by McCarthy et al. (66). In these experiments

undiluted broth cultures of the bacteria were used to challenge juvenile rainbow trout (Salmo gairdneri) both by intraperitoneal (i.p) injection and by immersion. The i.p. injection resulted in 75% mortality (within 35 days) and the immersion challenge produced 14% mortality (within 45 days), by which time there was complete mortality in the i.p. group. Belding and Merril (3) artificially infected fish by injecting purulent material from kidney abscesses of infected fish. Experimentally infecting fish by feeding infected fish tissues has yielded varying results. One hundred percent transmission of BKD was accomplished by feeding infected flesh and viscera of adult chinook salmon to young chinook salmon (30). Smith (16), however, found that fish could not be infected, if the infected fish tissues were stored at -17 to -200C for more than four months. This finding suggested that the bacterium retains its virulence for less than four months after freezing. Parasites have been suggested as being vectors in the transmission of R. salmoninarum (23,31). Bullock et al., (23) artificially infected brook trout by introducing the bacteria by first abrading or pricking the skin. They, thus, proposed that parasites which cause skin lesions may be involved in the transmission of the bacterium. These observations were concurred by Cusack and Cone (31) who suggested that skin-penetrating ectoparasites might be involved in the transmission of R. salmoninarum.

Effects of Environmental and Genetic Factors on Bacterial Kidney Disease: A number of environmental factors have been found to exert an influence on the severity of BKD including

water temperature, diet, and entrance into salt water. Belding and Merril (3) noted the seasonal occurrence of the disease and its association with warm water. They found that high mortalities were confined to spring and early summer, and tends to parallel the rise in water temperature. They also observed that hatchery epizootics routinely occurred at water temperatures above 11°C, which is also associated with an earlier onset of the disease. Fryer and Sanders (1) reported that the mean day to death (MTD) was approximately 25 at water temperatures between 15-20.5°C, while it was 70 days at 4°C, indicating an inverse correlation of mortality with water temperature.

The affect of dietary factors on BKD has been of interest to researchers (Ref. 1). Diets high in fluorine and iodine have been observed to be associated with a lower prevalence of BKD compared to diets high in calcium, magnesium, zinc, iron, copper and cobalt (32,33). Paterson et al., (32) conducted a nutritional study for two consecutive years with Atlantic salmon reared under natural conditions at Margaree Hatchery (Canada), where a high incidence of natural BKD exists. They concluded that incorporating high concentrations of fluorine and iodine was important in the control of clinical BKD. They also felt that the dietary requirements and metabolism of the trace elements are influenced by the mineral concentration in the feed, surrounding water, as well as the environmental conditions and physiological stage of the animal.

Suzumoto et al., have demonstrated that the genetic make up of juvenile coho salmon may be responsible for their resistance to BKD (34). They demonstrated that among the three transferrin genotypes of coho salmon (e.g., AA, AC and CC), the AA genotype was the most susceptible while the CC genotype was the most resistant to experimental infection. Although the sample size used in this study (six per group) was quite small, the implication of this observed phenomenon is of interest. It suggests that certain stocks of fish may, to some degree, be genetically resistant to BKD.

Studies were undertaken to investigate the occurrence of BKD during the ocean phase of the salmon life cycle. Banner et al. demonstrated that BKD is an important factor effecting survival in experimental saltwater conditions and in the ocean (35, 36). It may be that the fish are infected in the hatchery and harbor the bacteria internally during their migration into the ocean. Banner et al.,(35) have collected salmon exhibiting clinical signs of BKD from the coastal waters of Washington and Oregon, and have confirmed the presence of the bacterium using a fluorescent antibody technique. Their earlier work (36) confirmed that mortalities due to BKD can increase in a saltwater environment.

Mechanisms of Pathogenicity

Possible Virulence Factors: There are an increasing number of recent reports describing the existence of

extracellular factors produced by R. salmoninarum and their possible roles in pathogenicity. Some of these observed activities were the ability to agglutinate rabbit erythrocytes (41), leukoagglutinating and macrophage inducing properties (42), hemolytic activity against rabbit erythrocytes (17), DNAase and protease activities (17). None of the above-mentioned factors, however, have been shown to be responsible for any pathological manifestations of the disease. This is in direct contrast to other salmonid pathogens such as Aeromonas salmonicida, which have proteolytic (78), leukolytic (79) and hemolytic (80) activities that can reproduce the characteristic histopathological effects when injected into rainbow trout .

Bruno and Munro (39) have postulated that the decreased hematocrit values seen during BKD may be due to the sequestering of erythrocytes in the spleen. This sequestering, they postulated, may be precipitated by the elaboration of soluble factor(s) derived from R. salmoninarum possessing affinity for erythrocytes. Although these researchers did not identify the soluble factor(s), they felt that such a cell-bound factor operating by this mechanism could explain the splenomegaly and the decreased hematocrit values observed in infected animals. Bruno (38) also proposed that direct damage to soft tissues may be due to products liberated from the bacterium, or from disrupted macrophages, as well as the release of hydrolytic and oxidizing enzymes of macrophage origin. Although many properties were observed associated with the bacterium, none of them was definitively demonstrated to act as

virulence factors in vivo, with the possible exception of Bruno's studies. In these studies it was demonstrated that there was a direct relationship between auto-agglutination, hydrophobicity and virulence among various isolates of R. salmoninarum (43). The strains that were virulent in test animals were sticky, auto-agglutinating and possessed a hydrophobic cell surface compared to non-adherent, non-agglutinating low-virulent strains.

Hypersensitivity Reactions: A number of researchers have studied the structural and histopathological aspects of this disease (37,38). Young and Chapman (37) proposed that the pathological changes in the fine structure of the glomeruli and renal tubules during experimental and natural infections resembled those of mammalian renal diseases, glomerulonephritis and nephrotic syndrome. Thickening due to subendothelial proteinaceous deposits and irregularities found in the basement membrane of the glomeruli indicated that these structures were damaged. The renal tubule cells were also seen to have ruptured mitochondria and multiple vacuolar spaces adjacent to the endoplasmic reticulum, indicating irreversible cellular injury. The induction of a host immune and/or inflammatory response appeared evident during both these experimental and natural infections. Host cells resembling macrophages or polymorphonuclear leucocytes with phagocytosed bacterial cells often appeared in lesions. Electron microscopy of these cells showed the morphological integrity of the phagocytosed bacterial cells and evidence of their active division, indicating intracellular survival. They proposed that

the observed subendothelial deposits resembled antigen-antibody complexes that accumulated during nephrotic serum nephritis, thus indicating a type III hypersensitivity reaction. Bruno (38) also defined BKD as a diffuse, chronic granulomatous inflammatory reaction. He conducted studies with rainbow trout and Atlantic salmon in which he performed histopathological examination of various organs from infected fish and confirmed the observations of Young and Chapman. In more recent histological examinations of kidney tissues from naturally infected chinook salmon, revealed protein deposits which resulted in a thickening of the endothelium of glomeruli, indicating possible antigen-antibody complex formation (personal communication, Dr. R. Hedrick, University of California, Davis).

Detection and diagnosis

Early studies devoted to the detection and diagnosis of *R. salmoninarum* were based on gram-staining (44), culturing of the tissue isolates on selective media, and observation of the actual clinical signs of the disease (46). Lillies Allochrome was also used for the detection of the bacterial cells which. resulted in better clarity when compared to the gram stain (50). Subsequently, sensitive immunological methods were developed, incorporating specific antisera in various types of assays. The first such assays was a method of immunodiffusion (53). In this test specific precipitin lines were formed when rabbit antiserum

was reacted with tissue homogenates of infected fish. Bullock and Stuckey (48) were the first to develop the fluorescent antibody technique (FAT) for the detection of R. salmoninarum. They used both goat and rabbit antisera on kidney smears from infected fish and showed that the technique was more sensitive than the gram stain. Laidler (45) has also confirmed the results of Bullock and Stuckey. Paterson et al. (46) have also used an indirect FAT analysis for detection of R. salmoninarum in the wild salmonid populations of the Marjaree River system in Canada. Elliott and Barila (47) have also used IFAT method to detect and quantify the bacterium in the coelomic fluid of spring chinook salmon by first concentrating the bacteria onto polycarbonate filters.

A staphylococcal co-agglutination test was developed that employed rabbit antisera coupled to staphylococcal protein A. This reagent was used to produce antigen-specific agglutination with heat extracted antigens from infected fish kidney tissues (51). This method proved to be highly specific and sensitive as seen when 758 fish from 23 farms were tested. The rate of detection in this study was found to be as high or higher than that seen with gram staining or clinical examinations. Counter immunoelectrophoresis (52,58) has also been employed to detect heat extracted antigen from infected fish kidney tissues. The modified peroxidase and anti-peroxidase procedure developed by Sakai et al., (54) basically followed the form an enzyme linked immunosorbent assay (ELISA) method. In this method the antigen is coated on cellulose ester membranes and

incubated with rabbit antiserum, followed by goat anti-rabbit serum, and, finally, horseradish-peroxidase coupled anti-goat IgG. This assay was tested in the field and appeared to be superior to FAT analysis without crossreactions with other fish pathogens. ELISAs (56,57) represent the latest development in diagnostic strategies for the detection of BKD. Pascho and Mulcahy (56), in particular, have studied various parameters of the method such as selection of suitable assay plates, incubation temperatures, times of incubation for each reagent, and different dilution buffers. They have shown that the method can be highly specific, without crossreactions with heat extracted antigens of selected species of bacteria. The sensitivity of the assay ranged from 2-20 ng of soluble antigens. A summary of the various diagnostic procedures have been reviewed by Cipriano et al.,(57) and Pascho et. al. (58).

Increased specificity and sensitivity can also be afforded by the development of monoclonal antibodies to R. salmoninarum antigens (59,60). Wiens and Kaattari (59) have demonstrated the efficacy of monoclonal antibodies in identifying soluble protein antigens from infected fish sera, both by ELISA and Western blot analysis. They also tested the reactivity of the monoclonal antibodies against ten different isolates of the bacteria and found that the monoclonal antibodies reacted similarly with all ten isolates. It was further demonstrated that the monoclonals reacted consistently with a 57-58 kd protein doublet presence on the cell surface of all the isolates tested. The two monoclonal antibodies did not crossreact

with other species of gram-positive or gram-negative fish pathogens.

Control of Bacterial Kidney Disease

Bacterial Kidney Disease is one of the most difficult bacterial fish diseases to control. One plausible reason for this may be that the bacterium is a facultative intracellular parasite (37) which can survive and multiply within phagocytic cells. This intracellular nature could result in the protection of the bacterium from humoral antibody and antibiotic treatment.

Initial attempts at prophylaxis were reported by Belding and Merril (3). In this first attempt they tried a form of nutritional prophylaxis. They felt that the disease was of a metabolic nature and, thus, they incorporated cod-liver oil, iodine, clam meal, and other vitamin supplements in the form of green vegetation in the diet for extended periods of time. This treatment met with no success.

Attempts at chemoprophylaxis and therapy have been more rigourously pursued. Initially, incorporation of sulfadiazine in the diet, resulted in decreased mortality during the administration period and for a short time thereafter (61). A few weeks after the discontinuance of the treatment, the mortality rate, once again, increased. Although there have been reports on the use of other sulfa drugs, none were found to be capable of eliminating the infectious agent from the host. In one detailed study, Wolf and Dunbar (62) tested 34 therapeutic

agents on 16 strains of R. salmoninarum in vitro and, based on antibiotic feeding trials, concluded that erythromycin fed for 21 days gave the best protection. The application of erythromycin is also supported by studies (63). Intraperitoneal injection of antibiotics such as erythromycin and oxytetracycline have yielded variable results (1). The most current practice is to inject mature females with erythromycin prior to spawning, and then to feed the offspring erythromycin for 21 days (Tony Amandi, personal communication). Use of erythromycin in preventing vertical transmission also has been studied by Evelyn et al. (70). They have reported that when brood fish were injected with the antibiotic, 30 to 56 days prior to spawning, bactericidal concentrations of the antibiotic were found in the eggs which protected them from a subsequent artificial infection. It is of importance to note that all the above-mentioned trials indicate that certain antibiotic treatments can be helpful in breaking the infection cycle.

Immunology and Protective Immunity

The development of antisera to various isolates of R. salmoninarum has revealed that there is serological homogeneity among different isolates from a worldwide distribution (20,59,64). This may be of practical benefit in the preparation of vaccine(s), in that one preparation may be able to cross-protect animals infected by other isolates. At present, however, there is no immunoprophylactic agent available for use with hatchery-

reared fish. A likely reason for this is related to the bacterium's intracellular nature. This property could facilitate the escape of the bacterium from both antibiotics and antibody. However, even though this may be occurring, no reports of infected fish possessing circulating antibody subsequent to a clinical episode of BKD have been forthcoming (65). Therefore, at this point, several attempts to stimulate a specific humoral immune response to protect fish against R. salmoninarum have been reported using different forms of antigen and adjuvants (32,66,67,68). These trials did not yield a protective preparation, although some of the tests did demonstrate a reduction of the incidence of lesions of BKD and the number of bacteria. McCarthy et al., (66) have used formalin-killed bacteria (bacterin) without adjuvant to immunize rainbow trout and found that the intraperitoneal injection of pH-lysed bacterial cell preparation was protective. No protection was observed when the fish were vaccinated by immersion or by hyperosmotic infiltration (69). Paterson et al., (67) used formalin-killed R. salmoninarum in Freund's complete adjuvant (FCA) to immunize Atlantic salmon post-yearling parr and demonstrated a reduced prevalence of BKD lesions compared to control animals smolts were examined 1 year after vaccination. Two species of Pacific salmon, coho and sockeye, were used to test anti-BKD vaccines (74), and were not found to be protected following natural or experimental (injected) challenge with live pathogen. Evelyn et al., (74) concluded that Oncorhynchus species may not benefit from vaccination to the degree seen with Salmo species (66,67).

All the preparations studied thus far have been effective in reducing lesions of BKD, but have not been found to be effective in completely eradicating the bacteria. Although the published attempts at vaccination of fish against BKD are not numerous, there are some factors that must be addressed in the evaluation of vaccine efficacy. These include the appropriate dosage per fish depending on size and/or age, route of immunization (intraperitoneal injection or immersion or hyperosmotic infiltration or feeding), establishment of suitable challenge procedures (intraperitoneal injection or immersion), and determination of the length of time needed before challenge after vaccination.

Immunopotentiating Agents and Vaccine Development

Certain substances, when administrated along with an antigen will enhance the immune response to that particular antigen. These substances are commonly termed adjuvants or immunopotentiating agents. There are reports on the use of such substances in treating fish diseases. Freund's complete adjuvant (FCA) is the most commonly used adjuvant and is composed of a killed preparation of Mycobacteria suspended in mineral oil. It has been shown that FCA stimulates antibody production against an antigen when injected in the form of an emulsion. This adjuvant also stimulates delayed type hypersensitivity (DTH), which can play an important role in the production of protective immunity against intracellular microbial and parasitic infections

(77). Studies have demonstrated that FCA (modified by incorporating M. butvricum) alone can protect fish from infection due to Aeromonas salmonicida (70). Other adjuvants tested in the same study were levamisole and MDP (N-acetyl-muramyl-L-alanyl-D-isoglutamine). MDP is the smallest component of the Mycobacteria capable of replacing the whole cell in FCA (78). Derivatives of MDP, such as FK-156 and FK 565 have found use as adjuvants in some prophylactic preparations used for fish diseases (76).

The intracellular nature of R. salmoninarum and the lack of a correlation of circulating antibody with protection, suggests that stimulation of cell-mediated immunity may be the prophylactic method of choice for BKD. Induction of cell-mediated immune (CMI) responses results in the activation of phagocytic cells and digestion of the pathogens residing within (77). It is possible to enhance the CMI response with adjuvants such as FCA, although such immunization procedures would be impractical for large scale vaccine programs. In such instances, it may be possible to physically link antigens from Renibacterium salmoninarum to the active component of FCA, MDP, and use this material in bath immunizations or hyperosmotic infiltration methods. Future research towards developing an effective prophylactic treatment for BKD, may require the effective generation of specific CMI.

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MANUSCRIPT #1

CHARACTERIZATION OF SOLUBLE PROTEIN ANTIGEN(S) PRODUCED BY THE FISH PATHOGEN, RENIBACTERIUM SALMONINARUM

To be submitted

CHARACTERIZATION OF SOLUBLE PROTEIN ANTIGEN(S) PRODUCED BY THE FISH PATHOGEN, *RENIBACTERIUM SALMONINARUM*

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ABSTRACT

We report the physicochemical characterization of soluble protein antigen(s) (SA) produced by *Renibacterium salmoninarum.* Supernatants from the bacterial cultures were processed for the preparation of SA and characterized by the use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting, molecular exclusion chromatography, saturated ammonium sulfate (SAS) precipitation, and isoelectric focusing (IEF). Approximately 14 molecular weight species were routinely observed in the SA preparation which were capable of reacting with rabbit-anti SA. A prominent 57 kd protein fraction could be isolated by gel filtration and SAS precipitation. Isoelectric focusing indicated that the pI of all proteins was below 5.2.

INTRODUCTION

Renibacterium salmoninarum is the causative agent of bacterial kidney disease (BKD), one of the most devastating diseases to salmonid culture (Sanders and Fryer, 1980). It

causes a chronic, systemic infection with granulomatous lesions primarily occuring on the kidney, spleen, liver (Fryer and Sanders, (1981). One of the more interesting characteristics of the bacterium is its ability to survive and multiply within the in phagocytic cells of the host (Young and Chapman, 1978; Bruno, 1986; Gutenberger, S., personal communication).

R. salmoninarum is a Gram positive, diplobacillus, requiring cysteine for its growth (Evelyn, 1977). There has been relatively little work performed on the characterizaton of the extracellular products elaborated by this bacterium. The work performed in this laboratory and that of others (Getchell et al., 1985; Daly and Stevenson, 1987) have yielded information on the properties of these extracellular products. These studies have indicated that the products possess hydrophobic and hemagglutinating (Daly and Stevenson, 1987), leukoagglutinating (Wiens and Kaat tari, in preparation), immunosuppressive (Turaga et al. 1987b) and protease (Turaga and Kaattari, in preparation) activities and have been associated with decreasing hematocrit values in infected fish (Turaga et al. 1987b).

These foregoing studies have indicated that the 57 kd protein may be responsible for most of these activities. It has also been shown that the 57 kd protein is a common antigen found in all isolates tested (Getchell et al. 1985). Although the 57 kd protein has been implicated as being responsible for these, except for protease activity, biological activities there are no published reports that characterize the soluble proteins produced by the bacterium.

This study therefore employed a variety of biochemical methods to isolate and characterize the soluble product(s) and/or antigen(s) (SA). To date, no single factor has been identified as being sufficient to produce the disease *in vivo*, suggesting virulence may be multifactorial. The studies performed here are essential in that, they may help delineate the activities associated with different species of the SA.

MATERIALS AND METHODS

Growth of Renibacterium salmoninarum

Renibacterium salmoninarum (ATCC 33209) was grown in modified KDM-2 medium (Evelyn, 1977) without the serum supplementation. Cultures were grown in 250 ml shake flasks at 170C until the late log phase of the culture (about 10-12 days). The culture stock was then aliquoted in sterile snap cap tubes (Falcon) and stored at -700C.

Buffers

Phosphate buffered saline (PBS), pH 7.2 and 0.01 M, was prepared by dissolving 7.5 g of NaCl, 0.245 g of KH_2PO_4 and 0.809 g of Na_2HPO_4 in 1 liter of distilled water. Tris-buffered saline (TBS), pH 8.2 was prepared by dissolving Tris base 6.07 g, EDTA 0.3 g and NaCl 8.7 g in 1 liter of distilled water.

Preparation of Soluble Antigen (SA) of Renibacterium salmonianrum

The preparation of soluble antigen was basically that of Turaga et al. (1987a). Briefly, five hundred ml of the sterile KDM-2 medium in 2.8 L Erlenmeyer flasks were inoculated with a 5% (v/v) inoculum of the bacteria which was in late log stage of growth. The culture flask was then incubated on a reciprocating shaker at 17°C for 10-14 days. As these cultures grew to late log phase they were then centrifuged at 4000 x g for 30 min. The supernatant was removed and concentrated by passage through a PTGC 10000 NWML filter packet in a Minitan Ultrafiltration apparatus (Millipore Corp., Bedford, MA). Typically a ten-fold reduction of the original volume was achieved. This supematant was further concentrated by ammonium sulphate (Sigma, MO) precipitation. A 50% precipitation was performed by addition of 3 13 g/1000 ml of the concentrated supernatant. The salt was added gradually over a period of one hour with constant stirring while holding the supernatant in an ice bath. This solution was then stirred for an additional 3 hours in the ice bath. The precipitate was removed by centrifugation at 400 x g for 30 minutes at 4°C, and resuspended in 10 ml of PBS. Using a saturated ammonium sulfate (SAS) solution (Campbell et al, 1970), two additional 50% precipitations were performed. The final precipitate was then resuspended into 10 ml of PBS and dialyzed at 4oC against three changes of 3 liters of PBS over a period of 16 hours. The preparation was then filter-sterilized

using a 0.45µm filter (Corning, Corning, NY). The protein content of the preparation was determined by the method of Lowry et al. (1951).

Rabbit-anti Soluble Antigen Antibody Preparation

Female New Zealand white rabbits were injected subcutaneously between the scapulae (1.4 ml) and in the footpads (0.4 ml each) with a 1 :1 emulsion of SA and Freund's complete adjuvant (FCA) (Difco, MI). The rabbits were rested 30 days then bled weekly. Seven weeks post-immunization the rabbits were boosted using the same protocol. The serum was aliquoted and stored at -700C.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electronhoresis (SDS-PAGE)

The method followed was basically that of Laemmli (1970). Separating gels of 10% polyacrylamide and a stacking gels of 3% polyacrylamide were used in all the experiments. The apparatus used was a mini gel electrophoresis unit (Idea Scientific, Corvallis, OR) and the power source was an EC 600 (E-C apparatus Corporation, St. Petersburg, FL). The samples were mixed with the sample buffer at a 1:1 ratio and placed in boiling water bath for 3 minutes prior to electrophoresis. The samples were usually loaded in a duplicate fashion so that one half could be stained for protein while the other half was used for Western

blotting. Samples containing 5 μg of protein were applied to each lane. During the electrophoresis a constant current of 10 mA was applied during migration through the stacking gel and 20 mA during migration through the separating gel.

Transblotting

After the electrophoresis, -the protein bands were transferred from the gel onto nitrocellulose (NC) paper (Bio Rad, Richmond, CA), using a transblot apparatus (Bio Rad, Richmond, C A) (Towbin et al. 1983; manufacturers instructions). The protein transfer was done in transblotting buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol) and by applying 100 V for one hour (with cooling of the buffer by placing a unit with ice in the transfer chamber). After transblotting the nitrocellulose paper was divided in half for protein staining and for Western blotting.

Protein Staining

After transblotting, the NC paper was incubated in 0.3% tween 20 in PBS for 30 minutes at 30°C. The NC paper was then washed in the same buffer with three changes of the buffer over 15 minutes, while under constant agitation at room temperature. After brief rinsing with distilled water, Aurodye forte (Janssen Life Sciences Products, Olen, Belgium) protein staining reagent was added to the NC paper with constant shaking at room

temperature till dark purple bands became visible. This usually occured in about 2-3 hours. The blot was then briefly rinsed with distilled water and blotted dry.

Western Blotting

The transblotted proteins were alternatively examined for the presence of antigens by Western blot analysis and the method was basically that of Towbin et. al. (1979). The NC paper was incubated in 3% bovine serum albumin (BSA) in T-TBS (0.1% tween-20 in TBS) solution at 370C for one hour. The paper was then washed with three changes of T-TBS for a period of 15 minutes, while under constant shaking. Biotinylated rabbit anti-SA (Turaga et. al., 1987a) was used at a concentration of 5 μg in 10 ml of T-TBS, was incubated with the NC paper for one hour at room temperature. The NC paper was then washed as described above. A 1/100 dilution of streptavidin-peroxidase (Sigma, St. Louis, MO) in T-TBS was incubated with the NC paper for 30 minutes at room temperature. After washing the NC paper as above with T-TBS, the antigen bands were stained by incubating the NC paper with a substrate solution at 370C until the bands were darkly stained. The substrate solution consisted of 10 ml of PBS, 2 ml of 4-chloronapthol (3 mg/ml in methanol; Bio Rad, Richmond, CA) and 10 μl of hydrogen peroxide.

Molecular Exclusion Chromatography of Soluble Antigen

Soluble antigen was fractionated, with respect to the molecular weight, by the use of gel filtration. Briefly, one ml (3 mg/ml) of soluble antigen was chromatographed on a P-150 column (polyacrylamide gel column, Bio-Rad, Richmond, CA) using PBS as the elution buffer. The flow rate was 7 ml/hour and 2.0 ml fractions were collected the 0. D. of the fractions was measured (Spectronic 21) at a wavelength of 280 nm. The peak fractions were subjected to SDS-PAGE analysis.

Saturated Ammonium Sulfate (SAS) Precipitation of Soluble Antigen

Soluble antigen was subjected to differential SAS precipitations (10%, 20%, 30%, 40%, 50% and 80%). Each precipitation was performed by adding the necessary quantity of SAS to attain a concentration of 10% in the soluble antigen solution. This mixture was then incubated for 30 minutes on an ice bath with constant stirring. The preparation was then centrifuged at 4000 x g for 15 minutes at 4oC to pellet the precipitate and the supernatant was subjected to similar treatment upon addition of a sufficient quantity of SAS to yield the next higher SAS concentration. This process was continued until1 all the precipitations were performed. Thus, each precipitation contained only proteins capable of being precipitated within a range of SA concentration (i.e., 50-80%; 40-

50%; 30-40% etc.). The pellet from each precipitation was resuspended in 0.5 ml of PBS and dialysed against PBS with three changes of the buffer at 4oC. **SDS-PAGE** analysis was performed on these different SAS precipitated preparations.

RESULTS

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis of Soluble Antigen (SA)

SDS-PAGE, a technique which separates proteins depending on their mass and charge, revealed the existence of approximately 14 different molecular weight species within the soluble antigen preparation. The molecular weights of these species ranged from 22 kilodaltons to 72 kilodaltons. The prominent bands were of 58, 57, 49, 39, 37, 30 and 27 kd molecular weight. The total number of bands varied slightly between different batches of SA, but the prominent band pattern remained the same. In duplicate gels, which were processed for western blotting, all the 14 protein bands reacted antigenically with the rabbit anti-SA (Fig 1.1).

Molecular Exclusion Chromatography of Soluble Antigen

In these studies a polyacrylamide gel matrix was employed to further characterize the different fractions of soluble antigens produced by chromatographic separation. Fig.

1.2 demonstrates the distribution of proteins as they are eluted from the column. Fractions from the peak shown in Fig. 1.2.a. were analysed by SDS-PAGE to determine the separation of different molecular weight fractions. The fractions tested were 11 through 16, 18, 20, 24 and 30 (Fig. 1.2 b). The prominent 57 kd protein band appears in fraction in 11 and trace amounts of it appear in later fractions where the lower molecular weight fractions become more prominent. The chromatography thus proved to be a useful tool in characterizing different molecular weight fractions of the soluble antigen.

Sequential S A S Precipitation of Soluble Antigen

Analysis of differential SAS precipitates by SDS-PAGE (Fig. 1.3) indicated that the different molecular weight antigenic species can be resolved by this method. Isolation of the prominent 57 kd band occurs optimally at 30% SAS precipitation, while 50-80% pricipitation for isolation of smaller molecular weight fractions of the soluble antigen.

Isoelectric Focusing

Isoelectric focusing was performed on the soluble antigen preparations from cultures of R. salmoninarum grown in KDM-2 and ultrafiltered KDM-2 medium. The gel shown in the figure 1.4 has a pH range of 3-10. The standards have a pI range of 5-10.5, the top-most being ß-lactoblobulin (pI 5.2). Antigen

preparations from both KDM-2 and ultrafiltered KDM-2 were observed to have pIs below the 5.2. The gel suggests that both preparations contain eight distinguishable proteins, two major and six minor and no bands in the KDM-2 medium control.

DISCUSSION

There are an increasing number of recent reports describing the extracellular factors expressed by R. salmoninarum and their putative functions (Daly and Stevenson, 1987; Turaga et al., 1987a & b; Turaga and Kaattri, in preparation; Wiens and Kaattari, in preparation). None these studies utilized purified molecular weight species of soluble antigen (SA). In addition, there are no reports of physically characterizing these extracellular products.

This report, therefore, describes the physicochemical characteristics of SA produced by R. salmoninarum. The molecular weight analysis performed by SDS-PAGE (Fig. 1.1) indicated that there were approximately 14 bands of which 7 were distinctly prominent. In other experiments, differences in the number of bands obtained with different batches of SA has been observed, but the prominent bands remained the same. One possible reason for this variability appears to be the existence of a protease activity associated with the SA preparation (Turaga and Kaattari, in preparation). Proteolytic cleavage of the major protein(s) may likely result in a number of breakdown products as lower molecular weight species. Western

blot analysis indicates that all the protein bands were antigenic when reacted with rabbit-anti SA. Monoclonal antibodies developed against SA (Wiens and Kaattari, 1989) have also shown to recognise the same major protein bands as are seen here. They also demonstrated that the 58 & 57 kd proteins were also associated with R. salmoninarum cells.

Molecular exclusion chromatography (MEC) using polyacrylamide gel column facilitates the separation of different molecular weight species of SA (Fig. 1.2) The 57 kd molecular weight molecule and others in this weight range were effectively isolated by this procedure.

Another biochemical approach which was effective in separating the different molecular weight species of SA was saturated ammonium sulfate (SAS) precipitation. Isoelectric focusing (IEF) demonstrated that the proteins found in SA have a pI below 5.2, indicating that they are acidic proteins. The power of this technique is that it separates proteins on the basis of their isoelectric points (pIs). Again, the variation in number of bands observed between IEF and SDS-PAGE may be due to the breakdown proteins because of the protease activity. An explanation for the restricted pI pattern for all the protein bands in SA may be that they may be the products of a single parent molecule which was partially digested. They, thus, may possess some core region 'which contained the bulk of the functional groups that contribute to generation of the pI of the molecule.

The foregoing methods were designed to evaluate methods by which different molecular weight species of SA may be

isolated and used in future experiments to assess their biological activity. Such purified moities could also be used to generate specific antisera and possible candidates for vaccine. Purified bacterial products have been used in identification of their biological activities such as, the role of toxins of Vibrio in molluscs (Nottage and Birkbeck, 1987); isolation of immunosuppressor substance produced by Strantococcus mutans (Santarem et al., 1987), purification of hemolytic toxin from a fish pathogen, Aeromonas salmonicida (Nomura et al., 1988) and purification of an extracellular-protease from Pseudomas cepacia (McKevitt et al., 1988).

ACKNOWLEDGEMENTS

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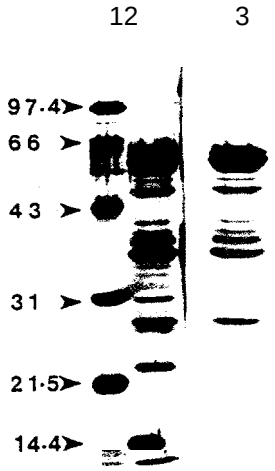
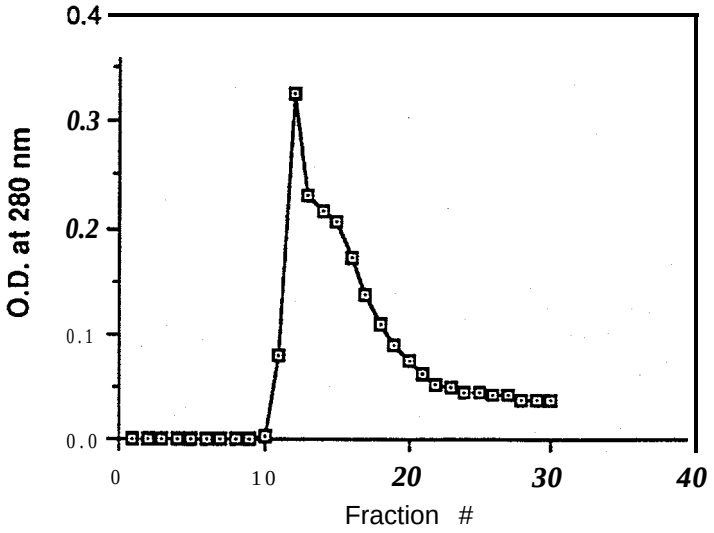


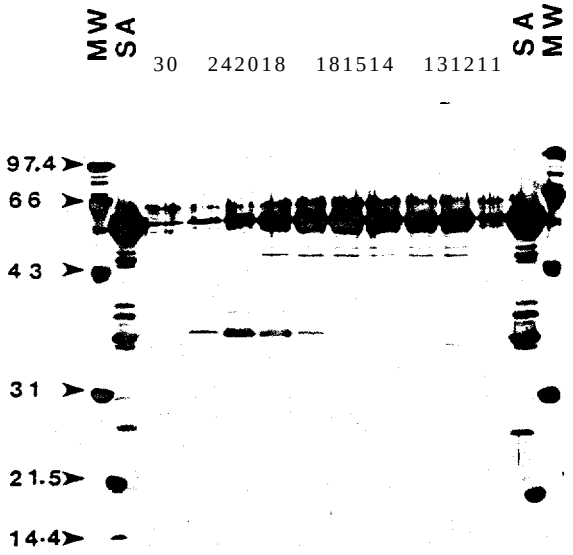
Fig. 1.1. SDS-PAGE and western blot analysis of soluble antigen: A. Protein staining (Aurodye[R] forte) of electrophoretically separated protein bands of molecular weight markers (lane 1) and SA (lane 2). B. Western blotting of soluble antigen indicating the antigenic nature of all the bands (lane 3) stained for protein.

Fig. 1.2. Molecular exclusion chromatography of SA. A. 0. D. values of 2 ml fraction of a P-150 column fractionated preparation of soluble antigens are depicted. B. SDS-PAGE analysis of chromatographic fractions 11 through 16, 18, 20, 24 and 30. Lanes containing unfractionated soluble antigen (SA) and molecular weight (M. W.) are also shown.

A


0.4
0.3
0.2
0.1
0.0
O.D. at 280 nm
0
10
20
30
40
Fraction #

B


MW
SA
30
24
20
18
18
15
14
13
12
11
SA
MW
97.4
66
43
31
21.5
14.4

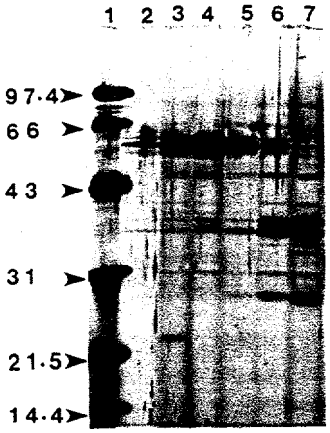


Fig. 1.3. SDS-PAGE analysis of the preparations obtained by sequential SAS precipitation of soluble antigen. Lane 1 (mol. wt. markers), lane 2 (10%), lane 3 (20%), lane 4 (30%), lane 5 (40%), lane 6 (50%) and lane 7 (80%) precipitations.

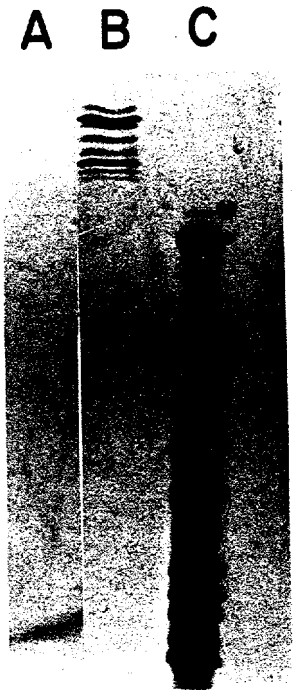


Fig. 1.4. Isoelectric focusing of soluble antigen. Lane A, KDM-2 medium control; lane B soluble antigen; lane C, pI markers. The pI marker of ß-lactoglubulin (pI value of 5.2) is identified.

MANUSCRIPT #2

WESTERN BLOT ANALYSIS OF A FISH PATHOGEN

In press; in Techniques in Fish Immunology

Western Blot Analysis of a Fish Pathogen

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Western blotting is a widely used procedure by which a mixture of molecules can be simultaneously characterized electrophorectically and antigenically. This is accomplished by coupling the technique of electrophoresis with that of the enzyme immunoassay.

Initially, a sample is subjected to electrophoresis for separation of antigens according to their charge and size, or size alone. A second electrophoretic step is conducted to transfer the antigens from the gel to an immobilizing surface, such as niuocellulose paper as originally described by Towbin et al. (1983). After this transfer the antigen components can easily be probed with a specific enzymeconjugated antibody. A chromogenic substrate is then added to determine which electrophoretic band is bound by the antibody. Western blotting is useful for a number of purposes including characterization of unknown antigens or antibody specificities (Campbell, 1984), confirmation of the presence of bacterial antigens in sera or tissues (Turaga etal.,1987), and detection of seropositive individuals which have been exposed to a pathogen (Sarngandhan et al. 1984). While protein antigens are typically analysed in Western blots, a number of other macromolecules can be separated and transferred to nitrocellulose including glycoproteins, lipoproteins, glycolipids, and lipopolysaccharides (Bers and Garfin, 1985). The primary advantage of Western blotting, as opposed to other immunoassays, is the high degree of specificity in resolving distinct antigens. Clinically, this technique has been useful in the identification of false positives, when used in conjunction with other immunoassays such as an ELISA (Weiss et al. 1985). There are, however, two disadvantages associated with Western blot analysis. First, the Western blot is mainly a qualitative assay and quantification of antibody or ***antigen*** is difficult (Heegaard and Bjerrum, 1988b). Second, if the antigen sample must be denatured (such as in SDS-PAGE), antigenic activity may be reduced or destroyed (Bers and Garfin, 1985; Dunbar, 1987).

As the investigator begins to develop a Western blot protocol for detection of a new antigen, a number of parameters will require optimization. An initial consideration for sucessful Western blotting should be the determination of the optimal electrophoretic parameters for resolution of the antigens of interest. This primarily requires the identification of the percentage of acrylamide that yields optimal separation of the electrophoretic bands gable 1). Next, it must be empirically determined whether the antigens are capable of binding to the nitrocellulose. Finally, the antigen detection procedure must be highly specific and sensitive (Oberfelder, 1989). For more detailed information a number of reviews on the theoretical and technical aspects of polyacrylamide gel electrophoresis (Hames et al., 1981;Dunbar, 1987), and Western blotting (Gershioni and Palade, 1983; Bers and Garfin, 1985: Oberfelder, 1989, and Heegaard and Bjerrum,1988a,b) have been published.

In the following protocol, a specific procedure is described which has been used to detect the presence of a ***57*** kd protein produced by ***Renibacterium salmoninarum*** (a gram positive salmonid fish

pathogen) during an infection. Additionally, Western blotting has been useful for characterizing the specificities of polyclonal antisera (rabbit and salmonid) and murine monoclonal antibodies to extracellular and *cell* surface antigens of *R. salmoninarum.*

Table 1. Relationship of optimal molecular weight separation in relation to the percent polyacrylamide concentration in the gel.

Mol. Weight Range of Proteins	% Acrylamide in Separating Gel
70,000 — 200,000	5.0
40,000 — 150,000	7.5
20,000 — 100,000	10.0
10,000 — 70,000	12.5
8,000 — 50,000	15.0

MATERIALS:

Equipment

1. Electrophoretic gel apparatus (Mini Protean System, Bio-Rad)
2. Power supply (EC 600, EC Apparatus Corporation)
3. Microliter syringes, 50 µl capacity (Hamilton Company)
4. Shaker 5. Water bath 6. 37°C incubator
7. Gloves
8. Disposable, polystyrene petri dishes, 100 x 15 mm style

Reagents and Stock Solutions

1. Antigen: Soluble protein (SP) from concentrated culture supernatant of *Renibacterium salmoninarum* (Turaga et al., 1987)
2. Antibodies:
   a) Monoclonal antibody 4D3 (Wiens and Kaattari, 1989)
   b) Goat anti-mouse antibody (polyvalent Igs) peroxidase labeled (Hyclone)
   c) Biotinylated rabbit anti-Soluble Protein antibody (Turaga etal., 1987)
3. Nitrocellulose paper (Bio-Rad)
4. Aurodye protein stain (Jansen Chemicals)
5. Substrate, 4-chloro-naphthol (Bio-Rad)
6. Nanopure water
7. Pre-stained molecular weight markers (Sigma)
8. Whatman #1 filter paper
9. Hydrogen peroxide 30%
10. Sample Dye (2) Stock
    0.15 gm tris base
    0.4 gm Sodium Dodecyl Sulfate (SDS) (Electrophoresis grade, Bio-Rad)
    1.0 ml 2-Mercaptoethanol
    2.0 ml Glycerol
    7.0 ml nanopure water
    0.02 gm bromophenol Blue
11. **Gel Buffer, pH 8.1-8.4**
    14.4 gm Glycine
    3.03 gm Tris Base
    1.0 gm SDS
    Bring to one liter with nanopure water. Do not adjust the pH with acid or base.
12. **Transblot** Buffer, pH 8.1-8.4
    14.4 gm Glycine
    3.03 gm Tris Base
    200.0 ml Methanol
    Bring to one liter with nanopure water.
    Do not adjust the pH with acid or base.
13. Phosphate Buffered Saline 10 mM, pH 73
    8.0 gm NaCl 0.2gm KCl
    1.44 gm Na_2HPO_4 $2H_20$
    0.2 gm KH_2PO_4
    Bring to one liter with nanopure water.

14. Stock Substrate Solution
    0.15 gm 4-cloro-naphthol
    50.0 ml methanol
    Store in the dark at -20°C
15. Water Saturated Isobutanol
    30.0 ml dd H_2O
    10.0 mlisobutanol
    16. Tris Buffered Saline (TBS)
    pH 806.07 gm Tris base
    0.4 1 gm Disodium EDTA (Fw 372)
    8.7 gm NaCl
    Bring to 1 liter with ddH_2O
17. Tween - Tris Buffered Saline (TTBS), pH 8.0
    1 .Oml Polyoxyethylene Sorb&an Monolaurate (Tween-20)
    Bring to 1 liter with TBS.
18. Separating gel recipe for a 12% gel (Two mini-gels, modified from Lammeli et al., 1970)
    4.6 ml nanopure H2O
    1.25ml 3 M Tris HCL pH 8.8
    4.0 ml 30% acrylamide
    0.8% bisacrylamide deaerate 10 mm.
    0.1 ml 10% SDS in dd H2O
    0.05ml Ammonium per-sulfate (AMPS), 0.1 gm/ml in ddH2O, (made just prior to use)
    0.005 ml N,N,N',N'-Tetramethylethylenediamine (TEMED)
19. Stacking gel recipe
    8.2 ml nanopure H2O
    0.4 ml 3 M Tris HCL pH 6.8
    1.25ml 20% acrylamide
    0.8% bisacrylamide deaerate 10 min.
    0.1 ml 10% SDS
    0.08ml AMPS
    0.008 ml TEMED

METHODS

The following Western blot protocol is designed for use with the Mini- Protean II electrophoresis system supplied by Bio-Bad. The gel assembly, quantity of acrylamide used per gel, power settings, time of electrophoresis, and conditions for transblotting may vary if equipment from other manufacturers is used.

1. Assemble the gel holder by sandwiching two spacers between the outer edge of two pre-cleaned glass plates (102 x 83 cm glass plates and 0.75mm spacers). Tightly clamp the plates together along the edges so that no leaks are possible. It is important to wear gloves at all times to prevent protein contamination of the glass plates, and contact with acrylamide, which is a potent neurotoxin.

2. Prepare the separating gel mixture and deaerate under a vacuum for 10 minutes. Insert a well forming comb in between the glass plates, and using a marker pen, make a mark 1 cm below the bottom teeth of the fully inserted comb. Remove the comb. Add the SDS, AMPS and TEMED to the gel mixture, gently swirl, and use a pasteur pipette to quickly fill the gel holder to the premade mark. Pour the solution along one of the side spacers with a constant flow so as not to introduce bubbles into the gel. Approximately 200 µl of water saturated isobutanol should then be slowly layered on top of the liquid gel. The isobutanol serves to isolate the gel from atmospheric oxygen which impedes polymerization, and also eliminates irregularities on the surface of the gel. Allow the gel to polymerize for 1 h before rinsing off the isobutanol with nanopure water. At this point the gel can be stored for up to two weeks if it is kept refrigerated (4°C) in a moist, air tight chamber.

3. Prepare stacking gel and deaerate for 10 minutes. Set the well-forming comb into the top portion of the glass sandwich and pour the stacking gel solution until the teeth of the comb are covered. Allow polymerization to occur for 30-40 min.

4. Insert the gel sandwich into the gel apparatus according to the manufacturer's sug-

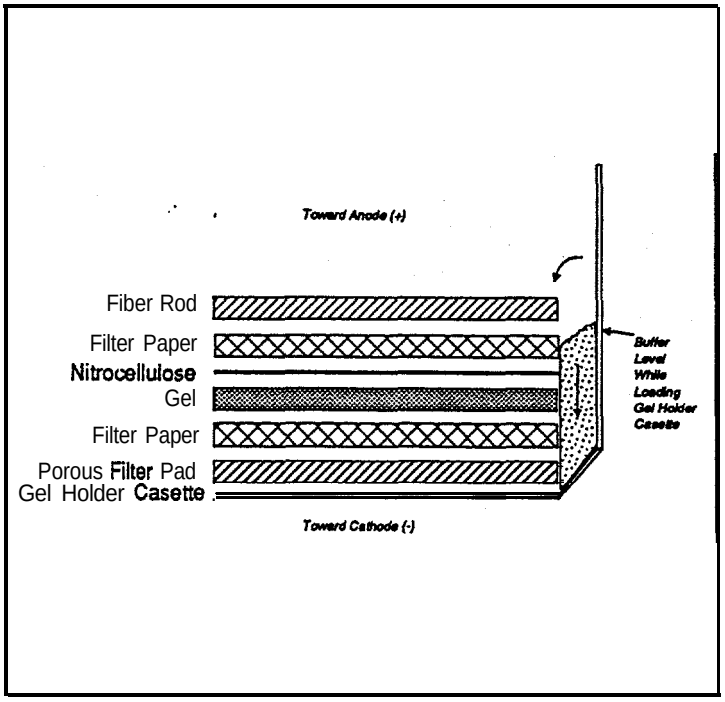

Figure 1. Organization of materials inside of the gel casette holder for transfer of proteins from the gel to nitrocellulose paper.

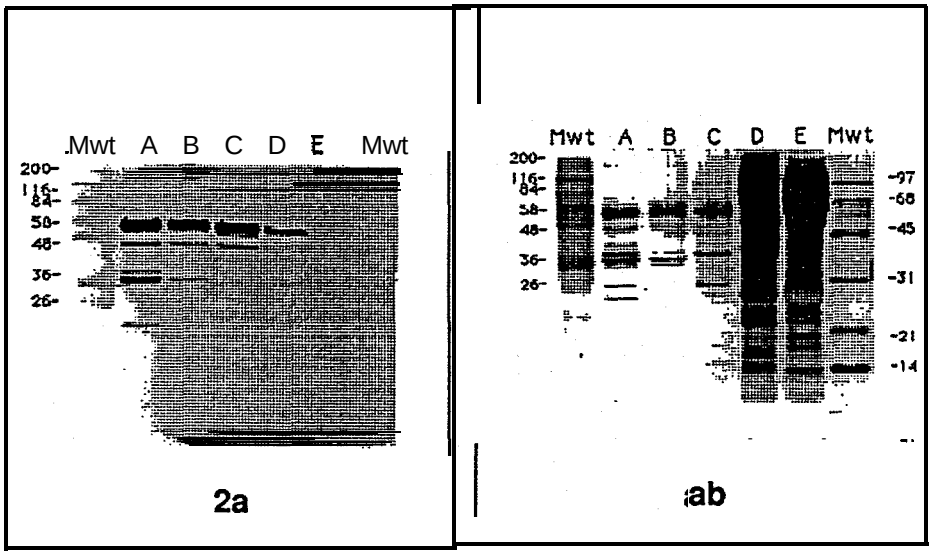

Figure 2. An example of a Western blot (Fig 2a) and a total protein stain (Fig2b) of two replica 12% SDS poiyacryfamide gels.The nitrocellulose in Figure 2A was probed with an anti R. salmoninarum monoclonal antibody and a goat anti-mouse horseradish peroxidase linked second antibody.The nitroceliulose in Figure 2B was stained for total protein with colloidal gold. Lanes are as follows, (Mwt) Sigma pre-stained molecular weight markers, A) R. saimoninarum soluble protein, B) Double distilled water wash of R. saimoninarum ceils, C) R. salmoninarum whole ceils, D)Coho salmon sera from three experimentally infected fish, E) Control coho salmon sera from three fish, Mwt) Bio-Rad low molecular weight markers.

staining etc.) to assess whether complete transfer of the proteins has occurred.

3. After blocking, wash the NC paper with three, 5 min TTBS changes while continually shaking, on a reciprocating shaker.

4. Place the NC paper in the primary antibody (either the described monoclonal antibody, or rabbit anti-SP biotinylated conjugate) solution (about 5 µg/ml in T-TBS). The blot should be completely covered with the solution and at no point should it be allowed to dry. Incubate for 1 h at room temperature, with gentle shaking.

5. Repeat step 3.

6. Add a sufficient volume of the secondary antibody reagent (goat anti- mouse peroxidase labeled and diluted 1/500 in T-TBS for the monoclonal antibody system, or streptavidin-peroxidase conjugate at a 1/200 dilution in T-TBS for the biotinylated rabbit anti-SP system) at room temperature with gentle shaking. Incubate the goat anti-mouse for 1 hour and the streptavidin-peroxidase for 0.5 hr.

7. Repeat step 3. Rinse two times for 1 min with TBS alone to remove the tween.

8. Prepare the substrate solution immediately prior to the addition to the NC paper. Mix 2 mls of 4-chloro-naphthol stock substrate solution, with 10 mls of PBS, and 10 ul of hydrogen peroxide. Add the substrate to the NC paper and incubate at 25-37oC until dark purple bands appear.Stop the development of the blot by rinsing the NC paper in distilled water for a minimum of 5 min. Blot the NC paper dry and store in the dark. If 4-chloro-naphthol is used the blot will begin to fade after several weeks. The blot should be photographed soon after development if a precise record is required for later analysis or publication.

PROTEIN STAINING OF THE BLOT

1. Block the NC paper by incubating in PBS, containing 0.3% tween-20, at 37oC for 1.0 h. The blocking solution used in the immunostaining cannot be used here, since it contains the protein BSA.

2. Wash the NC paper with three, 10 min. rinses with the same blocking buffer while continuously shaking.

3. Rinse the NC paper with nanopure water for 1 min, and add approximately 10 ml of Aurodye staining reagent. Shake for approximately 3 h and dark red bands should appear. There is no over-staining problem associated with this reagent.

DISCUSSION

This protocol describes the technical aspects of performing a Western blot. Figure 2a shows a Western blot which was developed with a mouse monoclonal antibody (4D3) and a goat anti-mouse horseradish peroxidase second antibody. The monoclonal antibody recognizes a 57 — 58 kd protein present in concentrated culture supernatant (lane A), and a number of breakdown products. The 57—58 kd protein is also associated with the bacterial cell (lanes B and C) and present in experimentally infected coho salmon sera (lane D).No antigen was detected in control coho salmon sera (lane E).A duplicate of this blot was stained for total protein and illustrates the specificity of the monoclonal antibody.

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gestions. Add sufficient gel buffer to the upper and lower chambers to submerge the top and bottom edges of the gel to a depth of approximately 1 cm. Remove the well forming comb and use a Pasture pipette to gently rinse out each well.

5. Mix the samples 1:1 with the 2X sample buffer and heat in a boiling water bath for 2-3 minutes. Typically, a total of 0.5 to 5 µg of protein in a total volume of 5 ul is loaded into a 0.5 wide by 1 cm deep well. Samples are loaded most accurately with a microliter syringe. The syringe should be rinsed with gel buffer at least ten times before a different sample is loaded. Introduce pre-stained molecular weight markers into wells at either end of the gel for subsequent comparison with test samples. These markers are also useful for monitoring the progress of electrophoresis and the efficiency of transblotting.

6. Electrophorese with a constant voltage (100 V), until the dye front reaches the bottom edge of the gel (approx. 1.5 h).

7. After electrophoresis, disconnect the power supply, and remove the gel sandwich. Gently pry the glass plates apart by twisting on the gel spacers. The gel should stick to one of the glass plates.

8. Carefully slide the gel off the glass plate and into transfer buffer and incubate approximately 10 min. Equilibrate the nitrocellulose paper, fiber pads, and filter papers in the transfer buffer at this time. The nitrocellulose paper should be slowly wetted from one corner to prevent air entrapment.

9. Partially submerge the gel holder casette in transblot buffer and layer the blotting materials in the order shown in Figure 1. First, layone presoaked fiber pad and at least three filter papers onto the cassette which will be closest to the cathode (- or black lead). Remove trapped air bubbles after each step by rolling a pastuer pipette or a test tube over each layer. If the air bubbles are not completely removed the electrophoretic transfer will not be uniform. Next,carefully lay the equilibrated gel on the filter paper. Place a piece of nitrocellulose paper (onto which the protein bands will betransferred) on top of the gel, making sure that there is even contact throughout. Place another set of filter papers on top of the nitrocellulose, followed by the other fiber pad, to complete the sandwich. Clamp the cassette together.

11. Place the cassette into the transblot module with the gel side oriented toward the cathode (- or black lead).

12.Fill the chamber with the transblot buffer, connect the leads to the power supply and select an appropriate voltage for the length of the transfer. Either 100 volts for 1.5 h, or 30 volts for 14 h are suggested. Both operations should be performed in a cold room with constant buffer mixing using a stir bar and stir plate. If the transferis conducted at 100 volts, the chamber should also be cooled with an additional cooling unit (usually supplied with the apparatus).

13. After the transfer, turn off the power supply and remove the cassette from the buffer chamber. Tweezers are used to remove the nitrocellulose paper and place it in an appropriate blocking solution.

IMMUNOSTAINING OF THE BLOT

1. Block the remaining free protein binding sites on the nitrocellulose paper with a 1% BSA-TTBS solution at 37°C for 1 h.

2. It is advised to also process the gel for protein staining(Coomassie blue, silver

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MANUSCRIPT #3

MONOCLONAL ANTIBODY ANALYSIS OF COMMON SURFACE PROTEINS OF *RENIBACTERIUM SALMONINARUM*

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MONOCLONAL ANTIBODY ANALYSIS OF COMMON SURFACE PROTEIN(S) OF *RENIBACTERIUM SALMONINARUM*

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We are reporting the development of two monoclonal antibodies which recognize ten separate isolates of Renibacterium salmoninarum. Both monoclonal antibodies recognize a 57 kDa protein doublet and several lower molecular weight antigens present in bacterial lysates. The 57 kDa antigen appears to correlate with a 57 kDa surface protein named antigen F by Getchell et al. (1985). Antibody capture ELISA analysis indicates that the antibodies recognize different epitopes of the same protein(s). Cross-reactions were not observed with control bacteria or with normal chinook salmon serum proteins.

INTRODUCTION

Renibacterium salmoninarum, the causative agent of bacterial kidney disease (BKD), causes significant mortality in salmonid populations throughout the world (Fryer and Sanders, 198 1). BKD has historically been a difficult disease to control, due to its chronic nature (Fryer and Sanders, 1981), the possibility of vertical transmission (Evelyn et al., 1984), and the lack of a commercial vaccine. A wide variety of serological techniques have been developed for detection of the pathogen, including those employing immunodiffusion (Chen et al., 1974), indirect fluorescent antibody (Bullock and Stuckey 1975), coagglutination (Kimura and Yoshimizu, 198 1), counterimmunoelectrophoresis (Cipriano et al., 1985), peroxidase-antiperoxidase (Sakai et al. 1987), and antibody capture enzyme-linked immunosorbent assays

(ELISA), (Turaga et al. 1987, and Pascho and Mulchay, 1987). Monoclonal antibody technology provides the ability to enhance immunological or serological analysis by providing a uniform reagent of potentially unlimited quantity. Arakawa et al. (1987) have developed thirteen different monoclonal antibodies to R. salmoninarum cells, however, they found that only one antibody recognized all tested isolates. This antibody also cross-reacted in an ELISA with three other species of gram-positive bacteria.

We are reporting the development of two monoclonal antibodies which recognize different epitopes of antigen F (Getchell et al., 1985). These monoclonal antibodies appear to be specific for R. salmoninarum and do not cross-react with other gram-positive and gram-negative bacteria tested.

MATERIALS AND METHODS

Bacterial Strains

R. salmoninarum isolates were a generous gift from Dr. J.S. Rohovec and C.R. Banner, Dept. of Microbiology, Oregon State University. The origins of the 10 isolates are listed in Table 1. The bacteria were cultured for 7-12 days in Kidney Disease Medium (KDM-II) with 10% calf serum (Gibco, Buffalo N.Y.) as described by Evelyn (1977). Streptococcus facium, S. *fecalis*, and *Lactobacillus piscicola* were grown in Muller-Hinton medium (Difco, Detroit, MI), while Vibrio anguillarum and Aeromonas salmonicida were grown in tryptic soy broth (Difco).

Soluble Protein Preparation

R. salmoninarum isolates ATCC 33209 and D6 were grown for 10 days in two, one liter flasks containing KDM-II without calf serum. The soluble proteins were harvested from the supernatant as described by Turaga et al. (1987).

Briefly, cells were removed by centrifugation (6000 x g for 30 min.) and the supematant was concentrated to 100 mls (20x) by ultrafiltration with a PTGC-10,000 NMWL filter packet (Millipore, Bedford, MA). The proteins were -further concentrated by two 50% saturated ammonium sulfate (SAS) precipitations and dialyzed against three 1 liter changes of 10 mM phosphate buffered saline (PBS), pH 7.2. The solution was filter sterilized with a 0.45 µm filter (Millipore, MA) and stored at 4oC.

Production of Monoclonal Antibodies (McAbs)

Balb/c mice were immunized with 0.1 ml of a mixture of 1 O.D. (500 nm) formalin-killed R. *salmoninarum* and 50 µg of soluble protein (ATCC 33209) in Freund's Complete Adjuvant (Difco). After 4 weeks, mice were boosted with a mixture of 25 µg of soluble protein and 10 ug of E. *coli* LPS serotype 026:B6, (Difco). Three days later SP2 myeloma cells were fused with the primed splenocytes after which wells containing antibody secreting hybridomas (4D3 and 2G5) were cloned twice by limiting dilution and injected into Balb/c mice for preparation of ascitic fluid. Ascitic fluid was harvested after 8-12 days, SAS precipitated, and resuspended in 10 mM PBS, diluted 1 :1 with glycerol, and stored at -200C. Both monoclonal antibodies were of the IgG_1 isotype as determined by the ICN Immunobiologicals (Lisle, IL) isotyping kit.

Biotinylation of Antibody

Antibody was dialyzed in 0.1 M NaHC03 and reacted with a ratio of 1 .0 mg of protein to 5.7 µl of a 0.1 M solution of BNHS (Biotin-N Hydroxysuccinimide, Calbiochem, CA) dissolved in distilled dimethyl formamide (Kendall et al. 1983). The mixture was reacted for one hour, and dialyzed against three changes of PBS during a 24 hour period. The biotinylated antibody was diluted

1: 1 in glycerol and stored at -200C.

Preparation of Cells for Electrophoresis and Western Blotting

Bacterial cells were centrifuged at 6000 x g, for 0.5 hours; 200 µl of the pellet was subsequently washed three times with 1 ml of 10 mM PBS, resuspended in an equal weight to volume of PBS, and frozen at -200C. Two microliters of cells were resuspended in 48 µl of double distilled H_2O and 50 µl of sample buffer consisting of 120 mM tris base, 4% w/v SDS, 10% v/v 2-mercaptoethanol, 20% v/v glycerol, and 3 mM bromophenol blue. The samples were immediately immersed in boiling water for 3 minutes and were electrophoresed in a 12% SDS-polyacrylamide gel (83 x 103 mm mini-slab gel, Idea Scientific, Corvallis OR) with a constant current of 20 mAmps for approximately 1.5 hours. The proteins were transferred from the gel to nitrocellulose paper by electrophoresis at 60 V for 3.5 hrs., at 4oC (Bio-Rad Trans-Blot apparatus), according to a modified method of Towbin et al. (1979). The nitrocellulose was subsequently blocked for 1 hr., at 37°C, with 3% bovine serum albumin (BSA, Sigma) diluted tris buffered saline (TBS), pH 8.0. McAb 4D3 was diluted 1/500 in TBS and allowed to react with the blots for 1 hour at room temperature. Excess antibody was removed by three, ten minute rinses with 0.1% Tween(20)-TBS (T-TBS). Peroxidase-conjugated, goat anti-mouse Ig (Hyclone, Logan UT) was diluted 1/200 in T-TBS and applied to the blot for 1 hr after which it was rinsed 3x as described above. Visualization of the protein bands, was achieved using a substrate solution consisting of 2 ml of 4-chloro-napthol (Bio-Rad)(3 mg/ml in methanol), 10 µl of 30% H202 (Fisher Scientific, NJ), and 10 mls of 10 mM PBS. The other half of the blot was stained for total protein with 10 mls of Aurodyecollodial gold solution used as recommended by the manufacturer (Janssen, Belgium).

Collection of Sera from Experimentally Infected Salmon

Sera was collected from three 20-30 gm chinook salmon which had been experimentally infected with 0.1 ml of the D6 isolate of R. salmoninarum for twenty days. Equal amounts of sera from the three infected fish or three noninfected fish were pooled and diluted 1/5 in double distilled water and mixed with an equal amount of sample buffer, and subjected to electrophoresis and western blotting as described above.

Indirect Fluorescent Antibody Techniques (iFAT)

Kidney smears of fish infected with the D6 isolate of R. salmoninarum were made on pre-cleaned micro slides (VWR, CA) and fixed for 15 seconds with a 1:1 acetone:xylene solution. McAb 4D3 or 2G5 ascites was diluted to 1.5 µg/ml in 10 mM PBS, and 100 µl of the solution was added to the slide for 30 minutes. After a 5 minute wash with PBS, 50 µl of biotinylated goat anti-mouse (Hyclone) diluted 1/100 in PBS was added for 15 minutes, after which 50 µl of Av-FITC (HyClone) diluted 1/250 was added for 15 minutes and washed for 5 minutes. A 1% methylene green solution was used as a counter stain. Slides were observed under a standard microscope (Zeiss) utilizing an IV F1 epi-fluorescent condenser and a 12 V, 100 watt halogen tungsten light source. All ten of the R. salmoninarum isolates and three gram-positve control bacteria were also analyzed by indirect FAT. Bacterial colonies from 15 day plate cultures were picked and dried on 3% gelatin coated slides which were subsequently heat fixed. Fluorescent staining was performed as described above.

Epitope Analysis Utilizing 4D3 and 2G5 Antibodies

Epitope analysis was performed using a double antibody capture ELISA as described by Berzofsky (1984), and Kohno et al. (1982). Briefly, one hundred µl of a 5 µg/ml solution of either monoclonal antibody, or soluble protein (in carbonate-bicarbonate coating buffer, pH 9.6) was coated overnight at 170C (Voller et al. 1976) onto EIA flatbottom plates (Costar, Cambridge, MA). The plates were inverted, with shaking, to remove excess protein. All wells were subsequently blocked for 1 hr with 150 µl of 1% BSA (Fraction V, Sigma) diluted in T-TBS. After washing three times with T-TBS and TBS, one hundred µl of a 5 µg/ml solution of soluble protein diluted in T-TBS or T-TBS alone (negative control) were added to appropriate wells for one hour. After rinsing, 100 µl of 5 µg/ml biotinylated antibody was added for 1.5 hours. A 1/250 dilution of streptavidin-horseradish peroxidase (Sigma) was added for 45 minutes. After the last rinse, 100 µl of substrate solution was added and color development was monitored spectrophotometrically at 405 nm on an EIA autoreader (Model 310, Biotek Instruments, Burlington, VT, USA). The substrate solution was a mixture of 10 ml of citrate buffer (pH 4.0), 10 µl of H_2O 2, and 75 µl of a 10 mg/ml solution of 2,2'-Azinobis, 3-ethyl benzathiazoline sulfonic acid (ABTS) in distilled water.

RESULTS

R. salmoninarum Isolate Characterization by Western Blotting

Cellular proteins of ten geographically distinct isolates (Table 1) were electrophoresed and tested by Western blotting with McAb 4D3. The antibody reacted with one major protein (Mr 57 kd) and four minor proteins (Mr 58, 50, 45, 43 kd) present in all isolates (Figure 1). No cross-reactive proteins were detected in the gram-positive control bacteria (Figure 3.1). Additionally, no cross-reactive proteins were detected in Western blots of the

gram-negative salmonid pathogens Vibrio anguillarum or Aeromonas salmonicida (data not shown). Protein profiles of the ten isolates of bacteria also suggest that the isolates are very homogeneous (Figures 2).

Assessment of *R.* salmoninarum Proteins in Infected Fish Sera

R. salmoninarum 57 and 58 kd proteins were detected in experimentally infected fish sera by western blot analysis using McAb 4D3 (Figure 4, lane 2). There was no cross-reactivity with normal serum proteins (lane 1).

Indirect Fluorescent Antibody Technique

An indirect fluorescent antibody assay was used to detect unique surface epitopes on the R. *salmoninarum* cells. Experimentally infected chinook salmon kidney smears and colony smears of the ten isolates all tested positive, while none of the other gram-positive control bacteria fluoresced.

ELISA Epitope Analysis

To determine if the two monoclonal antibodies recognize different epitopes, a double antibody capture ELISA was used. In this assay antibody was bound to the wells, soluble protein added, and .a biotinylated second antibody added to determine if any epitopes were still available. No increase in optical density was observed when either 4D3 or 2G5 were coated onto the plate and the homologous biotinylated second McAb was used (Figure 4A and B). Use of a heterologous monoclonal or polyclonal antibody indicated that epitopes were available (80-l 00% of the maximal OD). As a positive control, to determine that the biotinylated antibodies were equally functional, soluble protein was coated directly onto the ELISA well and the binding of each biotinylated antibody assessed. Figure 5C shows that the optical densities of the wells

were all within 10% of one another.

DISCUSSION

This study demonstrates the presence of at least two common epitopes on the cell surface of ten separate isolates of R. *salmoninarum*. These epitopes were defined by two murine monoclonal antibodies 4D3 and 2G5. Both monoclonal antibodies recognize a 57-58 kd protein doublet and several lower molecular weight antigens present in bacterial preparations. ELISA epitope analysis suggests that each monoclonal antibody recognizes a single epitope. In addition, the two recognized epitopes are different (Figure 5, A and B).

Within the complex of antigens recognized by the two monoclonal antibodies is a 57 kDa band which correlates with a 57 kd surface protein named antigen F by Getchell et al. (1987). Turaga et al. (1987) have also observed that this is the predominant protein found in experimentally infected fish sera and is also seen in Figure 4. The electrophoretic profiles of Aurodye stained nitrocellulose blots of the 10 isolates appear homogenous, supporting the observed uniformity in other characteristics such as biochemical properties, cell wall carbohydrates, and hydrophobicity (Bruno and Munro, 1986, Fiedler and Draxl, 1986, Daly and Stevenson 1987).

Arakawa et al. (1987) have produced monoclonal antibodies to a heat stable antigen and found antigenic diversity among isolates. They speculate that the antibodies might recognize antigen F epitopes which are isolate specific. Antigenic variability of epitopes on one protein is not uncommon; Buchanan et al. (1987) characterized 14 separate epitopes on a 65 kd Mycobacterium Zeprae protein. Thirteen epitopes were common to other Mycobacterial species while one monoclonal antibody recognized an epitope which was species specific.

Monoclonal antibodies 4D3 and 2G5 bind two separate epitopes which are common to all tested isolates of *R. salmoninarum* and yet are species specific. The apparent pan-specificity of the monoclonal antibodies and the non cross-reactivity with other bacterial species and normal chinook serum proteins indicates that the two antibodies maybe useful diagnostic reagents.

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Table 1. Designation and source of R. salmoninarum isolates.

1. Lea-l-74 (ATCC 33209)	Leaburg Hatchery, McKenzie River, OR; chinook salmon.
2. 33739 (ATCC)	Brook trout
3. D6	Oregon; from coho salmon held in salt water
4. Little Goose	Little Goose Dam, Idaho; chinook salmon
5. K-28	France; from the eye of pen reared coho salmon
6. K-70	England rainbow trout
7. 684	Norway
8. K50	Norway, net pen cultured Alantic salmon
9. Kvilan	Kvilan, Iceland; Alantic salmon
10. Grindavik	Grindavik, Iceland; Alantic salmon

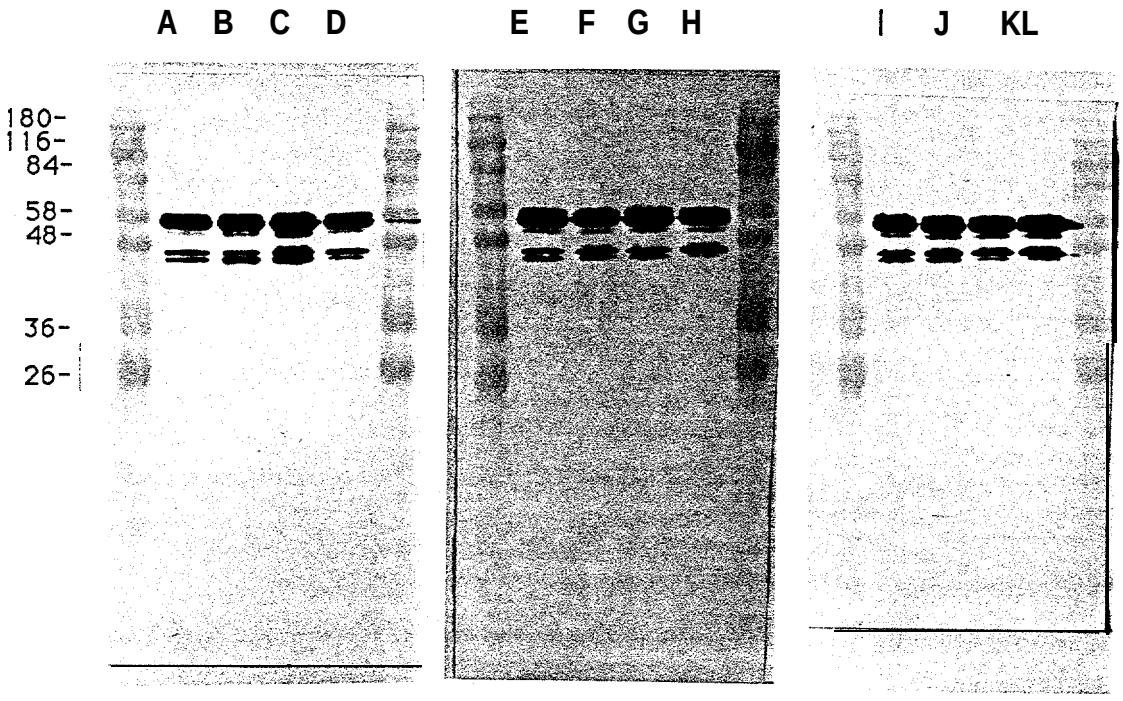

Figure 1. Western blot analysis of ten isolates of Renibacterium salmoninarum using monoclonal antibody 4D3. Lanes are as follows: A, E, and I) ATCC 33209, B) ATCC 33739, C) D6 isolate, D)Little Goose isolate, F) K-28 isolate, G) K-70 isolate, H) 684 isolate, J) K-50 isolate, K) Kvilan isolate, L) Grindavik isolate. The weights of Sigma pre-stained molecular weight markers are listed on the left.

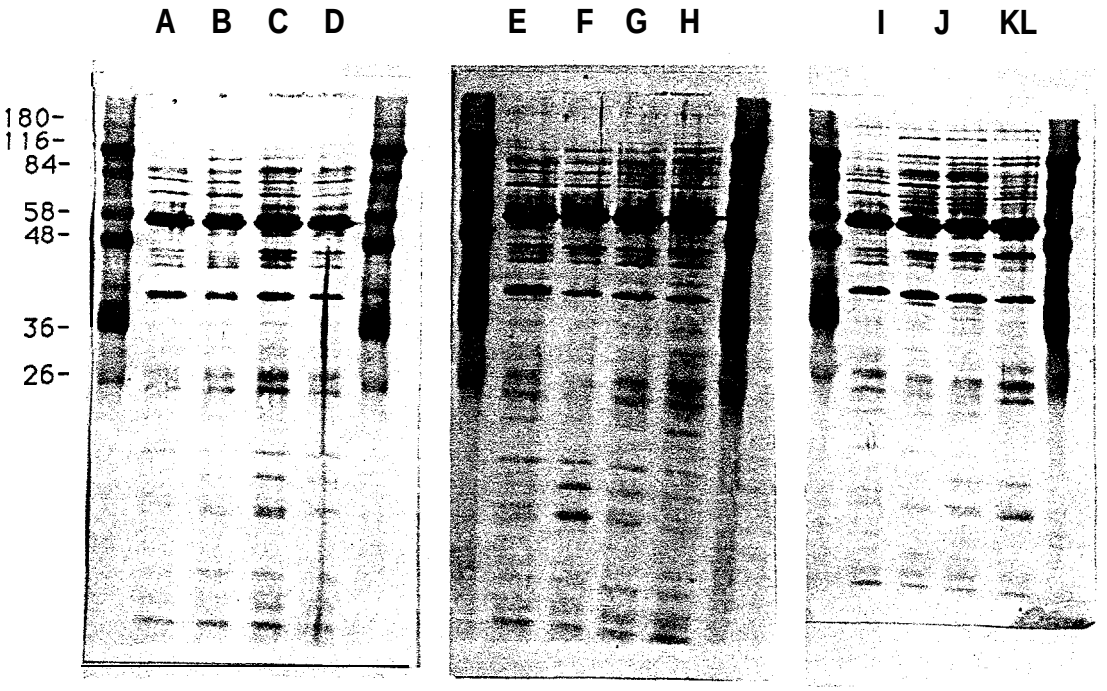

Figure 2. Total protein profile as detected by Aurodye colloidal gold staining of duplicate nitrocellulose strips from Fig. 1.

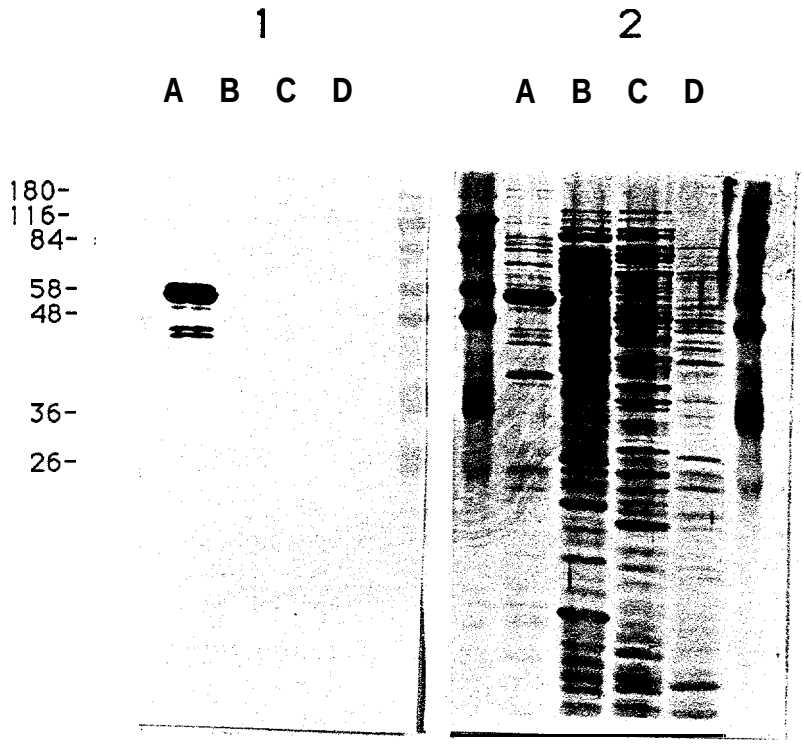

Figure 3. Western blot analysis of cellular antigens of Renibacterium salmoninarum and Gram positive control bacteria using monoclonal antibody 4D3 (panel 1), and total protein profile as detected by colloidal gold staining (panel 2). Lanes are as follows; A) ATCC 33209, B) Lactobacillus pisicocola, C) Streptococcus facium, and D) Streptococcus fecalis.

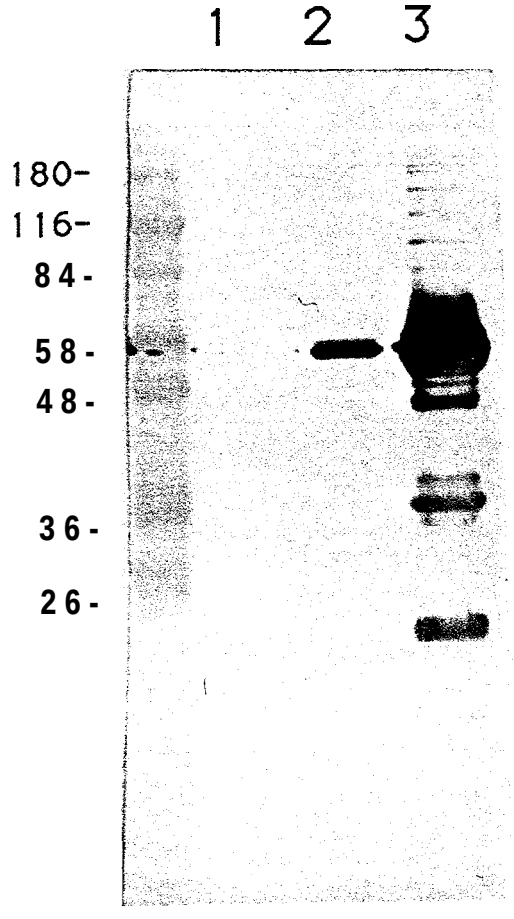

Figure 4. Western blot of infected chinook sera using McAb 4D3. Lanes are as follows: 1) Pooled sera from three control fish (5 µl), 2) Pooled sera from three experimentally infected fish (5 µl) and, 3) R. salmoninarum soluble proteins (3 µg).

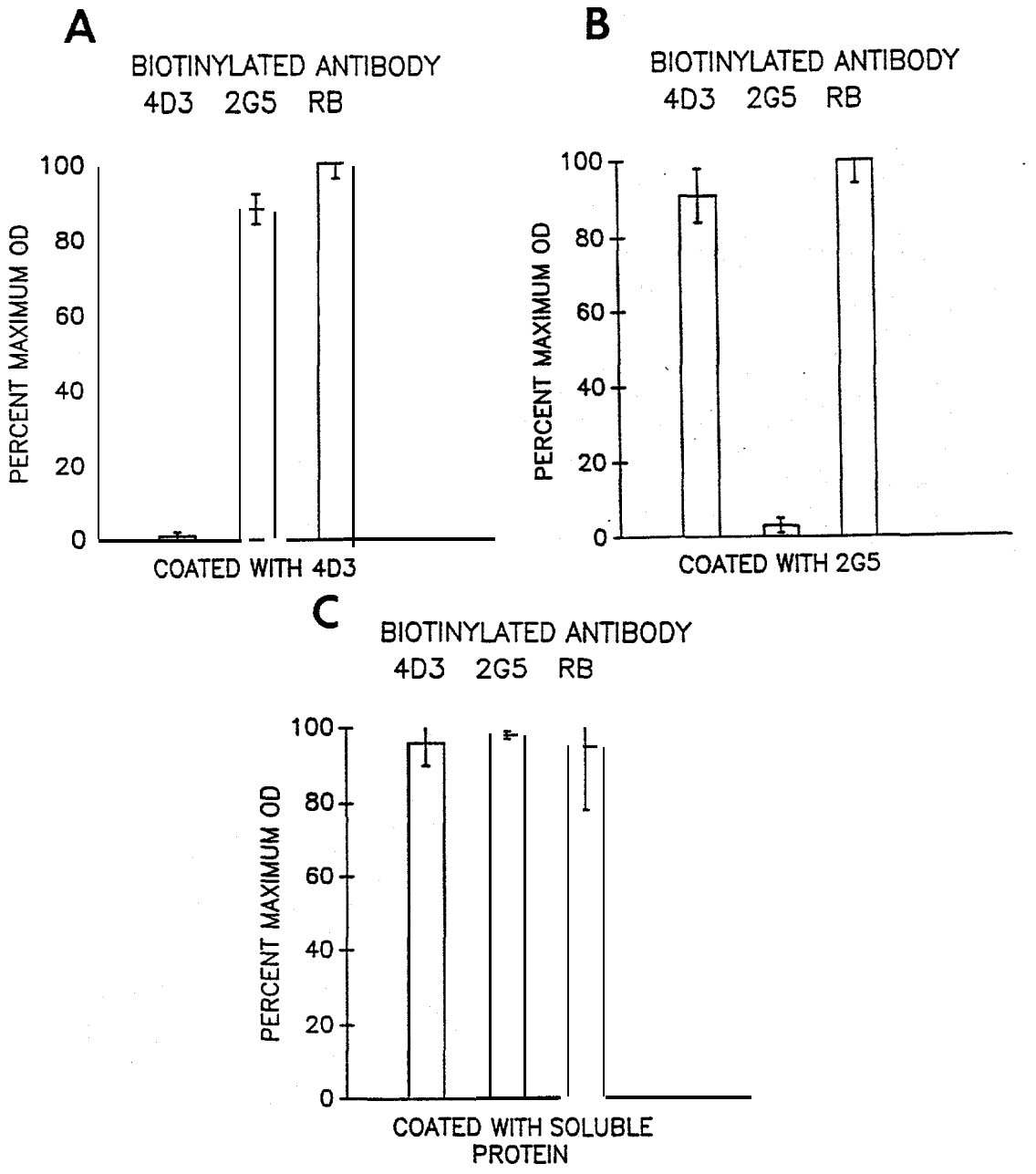

Figure 5. Epitope analysis by indirect ELISA. Triplicate wells were coated with either monoclonal antibody 4D3 (panel A), 2G5 (panel B), or R. salmoninarum soluble proteins (panel C) used as a positive control. Soluble protein was then added, incubated and removed. Biotinylated antibody (4D3, 2G5, or polyvalent rabbit anti-soluble protein (RB)) was used to assay apparent soluble antigen in each well. Data is expressed as a percentage of the maximum OD (panel A=.713, B=.319, C=.740), after subtraction of the background.

MANUSCRIPT #4

EPITOPE MAPPING OF A 57-58 kD *RENIBACTERIUM SALMONINARUM* PROTEIN USING FOUR MONOCLONAL ANTIBODIES

To be submitted

EPITOPE MAPPING OF A 57-58 KD *RENIBACTERIUM SALMONINARUM* PROTEIN USING FOUR M-URINE MONOCLONAL ANTIBODIES

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ABSTRACT

Six murine monoclonal antibodies have been produced which recognize Renibacterium salmoninarum cells. Four of the monoclonal antibodies recognize a bacterial 57-58 kD protein, which has been associated with an aggression and adhesion activities toward fish leukocytes. A competitive ELISA and Western blot techniques have been used to preliminarily determine epitopes on the 57-58 kD protein recognized by four monoclonal antibodies (4D3, 2G5, 3H1, and 1D2).

INTRODUCTION

Renibacterium salmoninarum, a gram-positive fish pathogen, produces a large amount of a 57-58 kD protein which is common to all tested isolates of the bacterium (11,27). This protein can be isolated from concentrated culture supernatants and detected in experimentally, or naturally infected, fish tissues and sera. Evidence suggests that this protein may serve as an aggression or adhesion molecule for the bacterium and may play an important role in the pathogenesis of bacterial kidney disease (9,19, Wiens and Kaattari, in preparation).

Elucidation of the role of this protein in disease pathology, has been facilitated by the production of monoclonal antibodies (27). Monoclonal antibodies are useful tools for elucidating the antigenic structure and function of a number of important proteins (6,21). The first step in characterizing the antigenic structure of a protein is the generation of a panel of antibodies which recognize distinct epitopes of the antigen under study. A number of methods have been published describing the technique of epitope mapping using monoclonal antibodies (1,10,17,18,24). In this paper, we have used four monoclonal antibodies in a competitive

ELISA and a Western blot for the preliminarily determination of epitope locations on a R. salmoninarum 57-58 kD protein.

MATERIALS AND METHODS

Bacterial strains and soluble protein production.

R. salmoninarum 33209 was a generous gift from Dr. J. S. Rohovec, Department of Microbiology, Oregon State University. The bacteria were cultured for 10 days at 17°C in KDM-II (8) without a calf serum supplement. After 10 days growth, which generated an O.D. greater than 1.0 (500 nm), soluble protein was harvested by the method of Turaga et al. (20). Briefly, bacterial cells were removed by centrifugation at 6000 x g for 30 min and the supernatant was concentrated 10 x by ultrafiltration. The retentate was further concentrated by two 50% saturated ammonium sulfate precipitations. To remove the ammonium sulfate, the soluble protein was dialyzed against 3 one liter changes of 10 mM phosphate buffered saline (pH 7.4) (PBS). The soluble protein was filter sterilized (0.45 µm) and stored at 4°C. Soluble protein prepared in this manner contains a number of breakdown products produced by an endogenous high molecular weight protease (Turaga and Kaattari, in preparation).

Cells were also saved and washed 3 times in PBS and resuspended to a final stock concentration of 20 mg (wet weight) of cells/ml of PBS for use in the R. salmoninarum cell ELISA.

Protease K treatment of bacterial cells.

Seventy mgs of cells were mixed with 70 µl of PBS and an equal volume of 20 mg/ml solution of proteinase K (Boehringer Mannheim) and

incubated at 37°C for one hour. Cells were washed 3 x with 1 ml of PBS, resuspended to 20 mg/ml, and frozen at -20°C.

Production of monoclonal antibodies

Balb/c mice were injected intraperitoneally with 10 mg of PBS-washed cells mixed 1:1 with Freund's Complete Adjuvant (Gibco, Grand Island, NY). Two months post-immunization, the mice were injected with 1 mg of wet packed cells mixed with 10 µg of E. coli LPS Serotype 026:B6 (Difco, Detroit, MI). Mice were sacrificed three days post-challenge and the spleens removed aseptically. Splenocytes were then fused with SP2/0 cells using the polyethylene glycol method (12). Hybridomas were screened by three types of ELISA using as a coating antigen either whole cells, proteinase K (PK) treated whole cells or soluble protein (SP). Four wells were found to be positive (1D2, 3H1, 3D5, and 1F6) and were each cloned twice by limiting dilution. Isotyping was performed using the ICN Immunobiologicals kit (Lisle, IL).

The production of monoclonal antibodies 2G5 and 4D3 have been described previously (27). Monclonal antibodies 2G5 and 4D3 were purified from ascites fluid using a protein A column chromatography according to the manufacturers instructions (Bio-Rad, Richmond, CA).

Cell ELISA

EIA flat bottom plates (Costar, Cambridge, MA) were coated for one hour with 2.5 µg/ml of poly-L-lysine diluted in coating buffer (24). Either 500 µg of PBS washed R. salmoninarum cells or protease K treated cells were then incubated for one hour on the coated wells. Plates were then washed 3x with 0.3% Tween 20 (Sigma, St. Louis, MO) in tris buffered

saline (50 mM tris, 150 mM EDTA, pH 8,.0)(T-TBS) followed by a 3x wash with TBS. Plates were blocked with a 3 percent solution of bovine serum albumin (BSA) in TTBS (Sigma) at 37°C for one hour. Test supernatant was incubated for one hour followed by washing as described above. A 1:4000 dilution of goat anti-mouse conjugated to horse-radish peroxidase (Hyclone, Logan, UT) diluted in 3% BSA-TTBS was added to detect the presence of the primary murine monoclonal antibody. After the last rinse, 100 µl of the substrate solution was added and color development was monitored spectrophotometrically at 405 µm on an EIA autoreader (Model 310, Biotek Instruments, Burlington, VT, USA). The substrate solution was a mixture of 10 ml of citrate buffer (pH 4.0), 10 µl og H202, and 75 µl of a 10 mg/ml solution of 2,2'-Azinobis, 3-ethylbenzathiazoline sulfonic acid (ABTS) in distilled water.

Competitive ELISA

A solid-phase competitive enzyme immunoassay, modified from the method of Wagner et al. (25) was used to compare recognized epitopes of monoclonal antibodies 1D2 and 3H1 with those of 4D3 and 2G5. One hundred µl of soluble protein (1 µg) was coated by overnight incubation onto EIA plates at 17°C. One percent BSA-TBS was used as a blocking agent (200 µl) for 1 hr at 37°C. One hundred µl of a various concentrations of non-biotinylated monoclonal antibody (inhibitor) was then added for 1 hr at room temperature. One hundred µl of a biotinylated monoclonal antibody was then added to probe the available epitopes. After washing, a 1:250 dilution of streptavidin in 1% BSA-TBS was applied for 30 min followed by a final washing and the addition of substrate.

Western blotting

Soluble protein samples were mixed with sample buffer and boiled as described in Turaga et al. (21). A 12% SDS-PAGE gel was used to separate the proteins using a mini-protein II electrophoresis unit (Bio-Rad). Transblotting was performed for 1.25 hr at 100 V (20). Pre-stained molecular weight markers (Sigma) were used to calculate the relative molecular weights of the soluble proteins.

Indirect Fluorescent Antibody Technique

Antibodies were tested with an indirect fluorescent antibody technique as described in Wiens and Kaattari (27).

RESULTS

Six murine monoclonal antibodies have been produced which recognize antigens on the Renibacterium cell surface as assayed by the cell ELISA (Table 1). Four of these hybridomas (1D2, 3H1, 4D3, and 2G5) were also found to specifically recognize the 57-58 kD protein as determined by the soluble protein ELISA and by Western blotting. Hybridoma 1F6 recognizes a protease-sensitive molecule, while 3D5 recognizes a protease-insensitive molecule both of which reside only on the bacterial cell surface, thus both antigens are distinct from the 57-58 kD protein and have not been further characterized.

A competitive ELISA was used to determine whether the four anti-57-58 kD protein McAbs recognize similar or different epitopes. Biotinylated 2G5 was co-incubated with each of the three non-biotinylated monoclonal antibodies (4D3, 1D2, 3H1) to determine if the same epitopes were shared (Figure 1). A non-biotinylated 2G5 was used as a positive control for inhibition. Culture supernatant from 3H1 was found to strongly

inhibit 2G5 binding while 1D2 supernatant and protein A purified 4D3 had no effect. None of the three antibodies were able to block biotinylated 4D3 (Figure 2). The relationship between 1D2 and 3H1 has not yet been determined by the competitive ELISA technique.

Electrophoresis and subsequent Western blotting of the soluble protein revealed a number of immunoreactive bands with estimated molecular weights of 58, 57, 43-45, 40, 38, 36, 21, and 20 kD (Figure 3). All of the predominant proteins present in the soluble protein preparation (Fig. 3a) are recognized by one or more of these monoclonal antibodies. Other than monoclonal antibody 1D2, which faintly recognizes all of the breakdown products, the other three monoclonal antibodies recognized specific bands. Monoclonal antibodies 3H1 and 2G5 are very similar in their recognition pattern except 3H1 weakly recognized a 21 kD band which 2G5 does not. Monoclonal antibody 4D3 does not bind a 40 kD band recognized by the three other antibodies.

DISCUSSION

A number of proteins have been analyzed to determine the number, and spatial arrangement of antigenic sites on the molecules (2,5,22). Antigenic regions of proteins have been found to consist of both continuous epitopes and assembled topographic epitopes. Continuous epitopes consist of continuous sequences of amino acids, while assembled topographic epitopes are composed of non-contiguous sequences brought in close proximity by the folded polypeptide chain (23). Most epitopes consist of between 5-7 amino acids (3,4), and are correlated with the exterior portion of the native protein and are highly mobile segments (19,26). The information gained from epitope mapping and functional studies has

revealed important information regarding the immunogenicity of proteins for vaccine design **(15).**

The precise location of protein epitopes can only be determined if the primary amino acid sequence and three dimensional structure of a protein is known. However, epitopes can be mapped to general regions of a protein using ELISA and Western blot techniques (17,25). Based on the ELISA and Western blot data, a preliminary epitope map of the 57-58 kD protein has been proposed (Figure 4). It was assumed that all recognized bands are due to proteolytic degredation of the 57-58 kD protein. Since monoclonal antibody 1D2 recognized all the bands it probably binds to a interal portion of the protein. Monoclonal antibody 4D3 does not recognize a 40 kD band, therefore, it probably binds closer to one of the termini of the protein. It cannot be determined with this analysis if 4D3 binds nearer to the amino or carboxy termini. The competitive ELISA indicated that McAbs 2G5 and 3H1 recognized a similar antigenic determinant, but by Western blot analysis, 3H1 recognized a 21 kD breakdown product not apparently bound by 2G5. Therefore, the epitopes recognized by the two antibodies are not identical but possibly overlapping. A second possibility, however, is that 2G5 has a lower affinity than 3H1 and the antibody concentration was not high enough for binding. Further studies are being conducted to distinguish these two possibilities. The molecular weights of the soluble proteins were calculated based on pre-stained molecular weight standards. Using a non pre-stained molecular weight markers Turaga and Kaattari (in preparation) have determined slightly different molecular weights for some of the bands.

The 57-58 kD protein is present on the bacterial cell surface and epitopes defined by McAbs 3H1, 4D3, and 2G5 are directly accessible to antibody while associated with the bacterium. Future research will attempt to correlate these defined epitopes with immunosuppression and bacterial adherence. The identification of toxigenic determinants will aid in future vaccine preparation and design.

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Table 1. Monoclonal antibody characterization of cell-associated and soluble proteins produced by R. salmoninarum.

Designation	Isotype	Cell ELISA	PK ELISA	SP ELISA	IFAT
4D3	IgG1k	+		+	+
2G5	IgG1k	+		+	+
3H1	IgG1k	+		+	+
1D2	IgMk	+		+	ND
1F6	IgM	+			ND
3D5	ND	+	+		ND

ND= not determined.

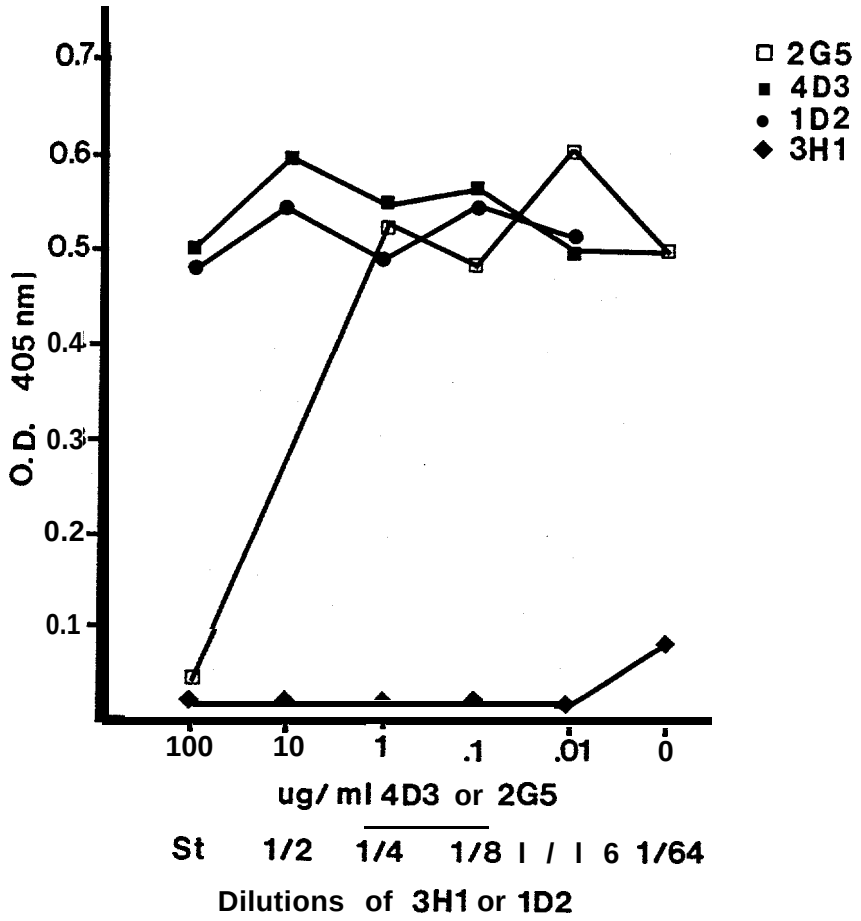


Figure 1. Epitope analysis using a competitive ELISA. Wells were coated with soluble protein, and subsequently incubated with one of the four unlabled anti-soluble protein monoclonal antibodies. Dilutions of tissue culture supematant (3H1, 1D2) or protein A purified (4D3, 2G5) monoclonal antibody were used as an inhibitor. Five µg of biotinylated 2G5 was then added to assay apparent soluble protein in each well.

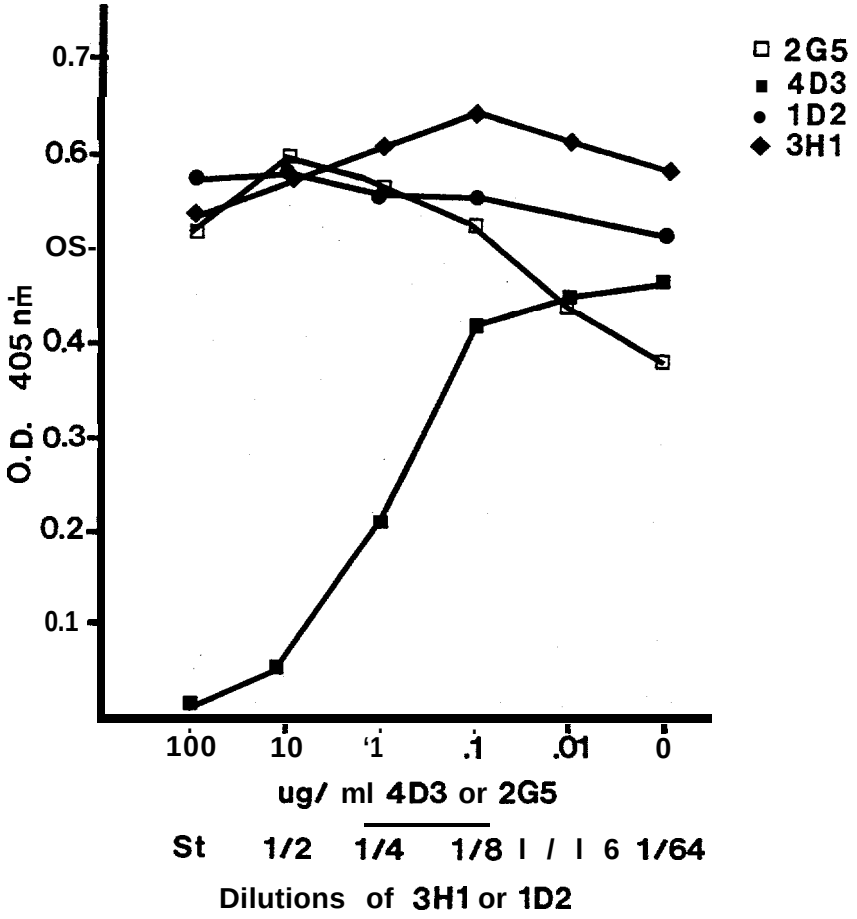


Figure 2. Epitope analysis using a competitive ELISA as described in Fig. 1, except five µg of biotinylated 4D3 was added to assay apparent soluble antigen in each well.

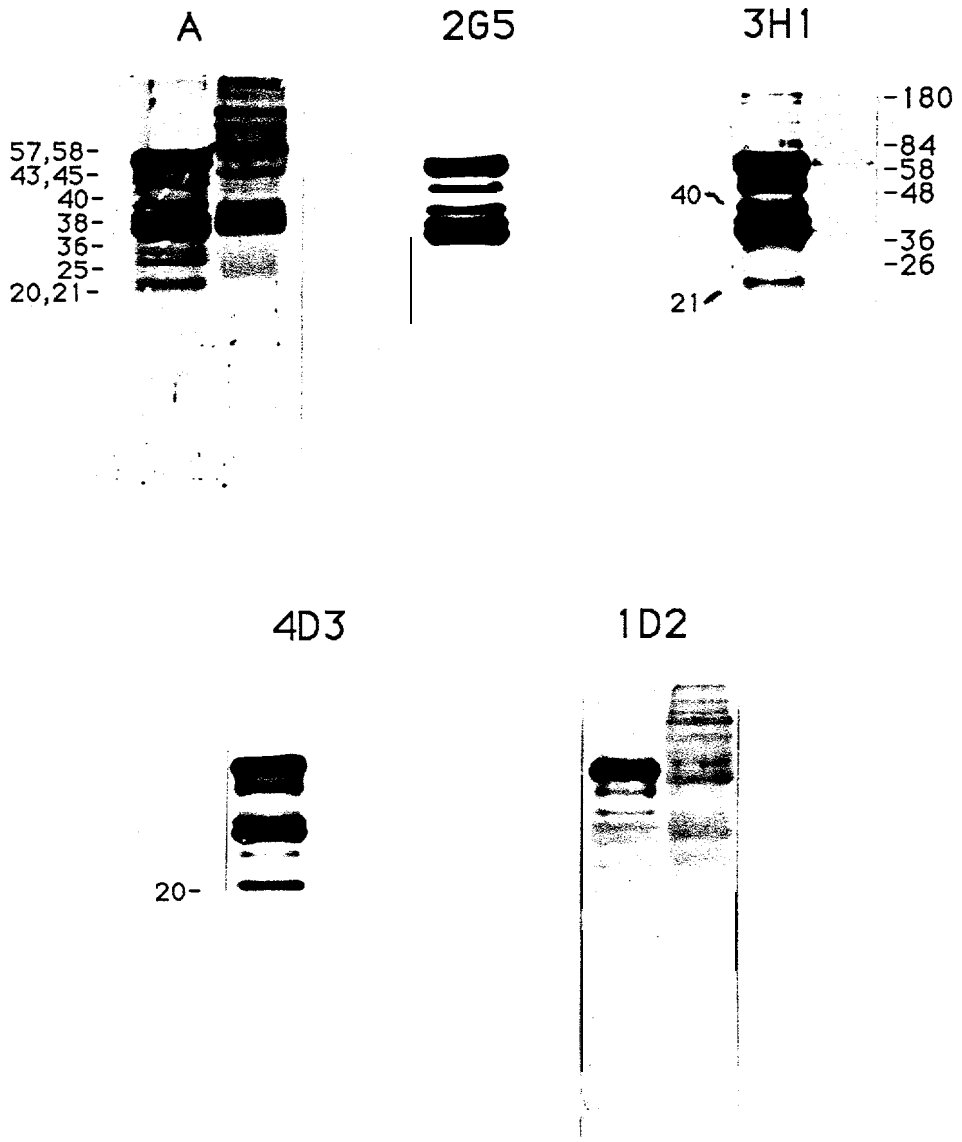


Figure 3. Western blot and total protein stain of replica1 lanes of soluble protein. Three μg of soluble protein per lane was electrophoresed in a 12% SDS polyacrylamide gel and transfered to nitrocellulose. Blots were separately incubated with either tissue culture supernatant (3H1, 1D2), or 5 mg/ml of protein A purified (2G5, 4D3) monclonal antibody. A 1:500 dilution of goat anti-mouse horseradish peroxidase was used as a second antibody. The relative molecular weights of the soluble proteins are indicated to the left of the blot stained for total protein (A), and the molecular weights of the prestained markers are indicated on the right.

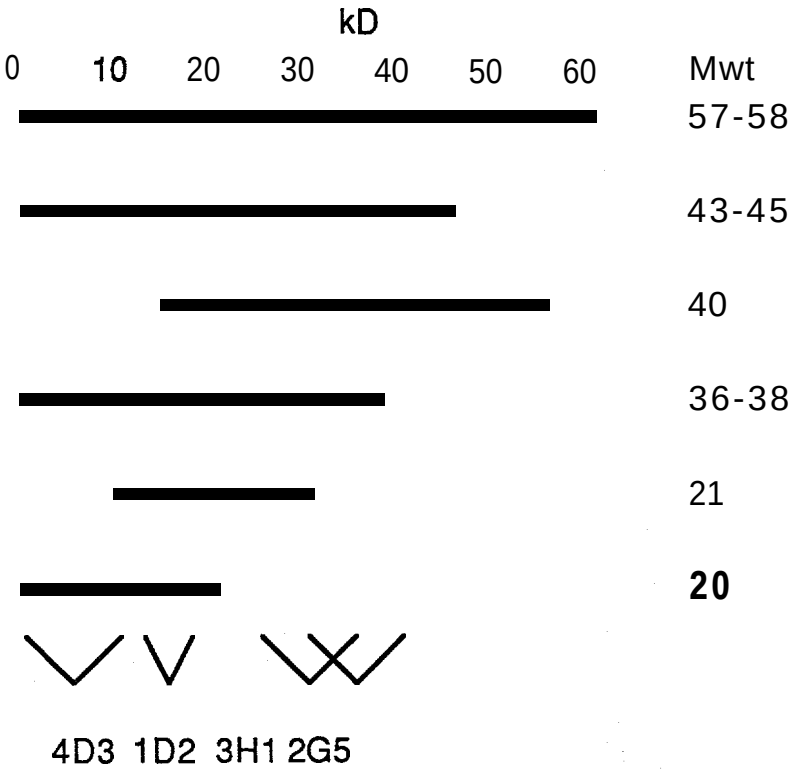
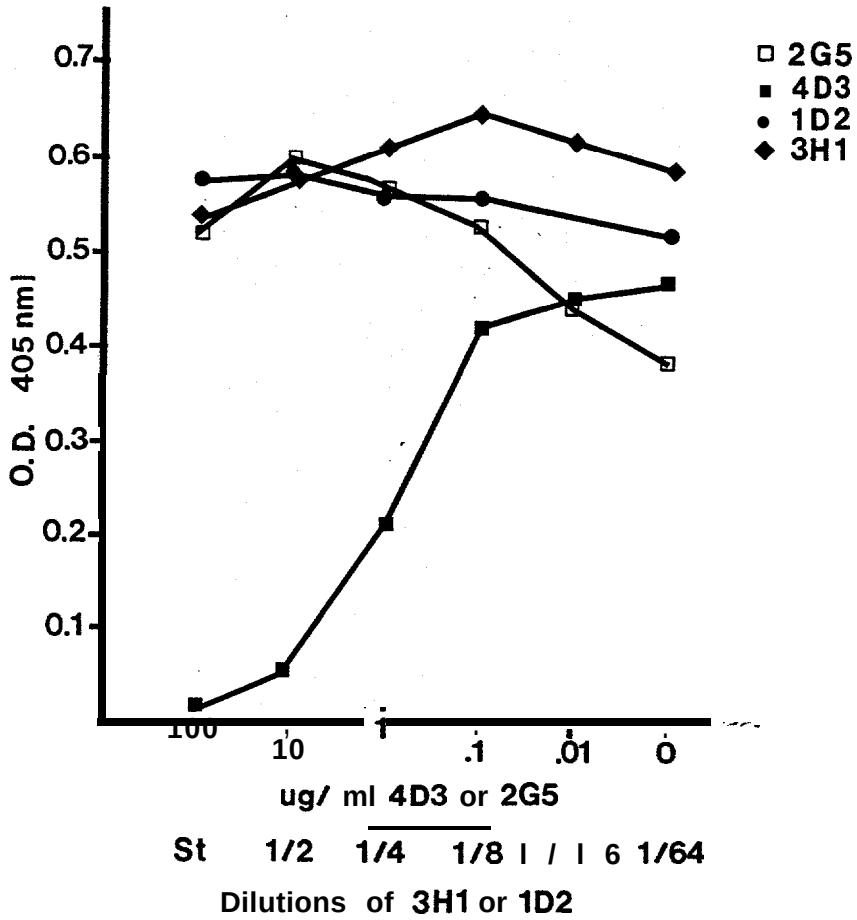


Figure 4. Preliminary map of four epitopes located on the R. salmoninarum 57-58 kD protein. This map was constructed with data from both Western blots and the competitive ELISA. A linearized 57-58 kD protein with putative proteolytic fragments is depicted.


0.7
0.6
0.5
0.4
0.3
0.2
0.1
O.D. 405 nm
2G5
4D3
1D2
3H1
100
10
1
.1
.01
0
ug/ ml 4D3 or 2G5
St 1/2 1/4 1/8 1 / 1 6 1/64
Dilutions of 3H1 or 1D2

MANUSCRIPT #5

ANALYSIS OF *RENIBACTERIUM SALMONINARUM* ANTIGEN PRODUCTION *IN SITU*

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ANALYSIS OF *RENIBACTERIUM SALMONINARUM* ANTIGEN PRODUCTION *IN SITU*

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ABSTRACT

This study represents the first report of the development and use of an enzyme-linked immunosorbent assay (ELISA) and western blot analysis to monitor the production of *Renibacterium salmoninarum* soluble antigens (SA) in infected salmon. The sensitivity of the ELISA permitted detection of soluble antigens in sera samples to concentrations of 0.1 ug/ml. This assay demonstrates a clear resolution between infected and non-infected fish in experimental infections. The ELISA and western blot systems were used to assess the temporal progress of the disease in a quantitative and qualitative manner.

INTRODUCTION

Bacterial kidney disease (BKD) is one of the most widespread and devastating salmonid diseases in North America, Europe and Asia (Fryer and Sanders, 1981). The causative agent of this disease, *Renibacterium salmoninarum*, is a gram-positive, pleomorphic, diplobacillus which has been classified as a unique species as well as genus (Sanders and Fryer, 1980).

Furthermore, this disease is not only a serious problem for hatchery reared-salmonids, but it has also been demonstrated to occur in wild populations (Mitchurn, et al., 1979). Currently, no commercial vaccine exists for BKD, and diagnostic procedures are primarily limited to detection using gram stain (Pippy, 1969) or fluorescent antibody techniques (Bullock and Stuckey, 1975; Laidler, 1980).

An alternate means that may be used to follow the progress of this disease would be the monitoring of soluble antigens, secreted by R. salmoninarum in the host. Previous studies have identified specific molecular weight antigens produced in broth culture (Getchell et al., 1985), and we have now found that these specific molecular weight antigens are also produced in infected salmon. ELISA and western blot techniques have been employed here to quantitatively and qualitatively monitor the serum levels of the soluble antigen (SA) during the disease process. This form of in situ antigen analysis can facilitate our understanding of the pathogenic process of BKD and could be adapted for use in diagnostic procedures.

MATERIALS AND METHODS

Animals

Coho salmon (Oncorhynchus kisutch), aged 6-12 months, were maintained at the Oregon State University Fish Disease

Laboratory. The fish were held in 12°C, pathogen-free well water and fed Oregon Moist Pellet.

Adult, female New Zealand white (NZW) rabbits were maintained by the Laboratory Animal Resource Center at Oregon State University.

Bacterial strain

R. salmoninarum, isolate Lea-l-74 (ATCC 33209), was obtained from Dr. J.S. ROHOVEC, Dept. of Microbiology, Oregon State University and used throughout this study. The bacteria were grown in a modified Kidney Disease Medium (M-KDM-2) without ferric sulphate supplementation as described by Getchell et al. (1985).

Fish were injected, intraperitoneally, with 0.1 ml of a 1 .O O.D. (500 nm) broth culture in the exponential phase of growth.

Preparation o f soluble antigen

SA of *R. salmoninarum* were prepared from the supematants of spent cultures. After growth of the organisms to an O.D. of approximately 1.0 (500 nm), the cells were removed by centrifugation at 6000 x g for 30 min at 4oC. The supernatant fluid was filtered through a "minitan" ultrafiltration apparatus with a PTGC-10,000 NMWL filter packet (Millipore, Bedford, MA), held at 4oC. This filtration removed low molecular weight

molecules (<10 kd), and reduced the volume lo-fold. The retentate, or high molecular weight fraction, was concentrated by 50% saturated ammonium sulfate (SAS) precipitation. This mixture was stirred for 3-4 h at 4°C, and the precipitate was retrieved by centrifugation at 6000 x g for 15 min at 4oC. This precipitate was dissolved in a volume of 0.01 M, phosphate buffered saline (PBS), pH 7.2, equivalent to 10% the original retentate volume. The solution was then reprecipitated twice as described above, and dialysed extensively against PBS at 4oC. The solution was then filter sterilized (0.45 urn) and the protein concentration was measured by the method of Lowry et al. (1951).

Antibody preparation

The anti-SA serum was prepared by injecting a 1:1 mixture of SA (1 mg/ml PBS) and Freund's complete adjuvant (Difco, Detroit, MI, USA) subcutaneously into rabbits, between the scapulae (1.4 ml) and in the hind footpads (0.4 ml each). The rabbits were allowed to rest for 30 days, then bled weekly. Seven weeks post-immunization, the rabbits were boosted and subsequently bled using the same protocol. The serum was aliquoted and stored at -700C.

Biotinylation of antibody

Immunoglobulin was isolated from an aliquot (l-2 ml) of the anti-SA serum by 50% saturated ammonium sulfate precipitation. The pellet was resuspended in 0.01 M PBS and extensively dialysed against the same buffer at 4oC. The protein content was estimated by the method of Lowry et al. (1951). Biotinylation of this antibody fraction was performed as described by Kendall et al. (1983). Biotin-N-hydroxysuccinimide (BNHS) (Calbiochem, La Jolla, CA, USA) was used to covalently bind biotin to the antibody. The reaction mixture was prepared by adding 1 ml of a 10 mg/ml solution of antibody in 0.1 M NaHC03 to 57 µl of a 0.1 M solution of BNHS dissolved in distilled dimethyl formamide (Sigma, St. Louis, MO, USA). After incubation for one hour at 22°C, the reaction mixture was dialysed for 24 h at 4oC against three changes of PBS. After dialysis an equal volume of glycerol was added and the biotinylated antibody was stored at -200C.

ELISA procedure

The detection of SA in the serum of infected fish was accomplished utilizing an antibody capture technique. The method and buffers are modifications of that described by Voller et al. (1976). Individual wells of a 96-well EIA flat-bottom plate (Costar, Cambridge, MA, USA) were coated with 0.1 ml of unconjugated anti-SA antibody (5 µg/ml in carbonate-

bicarbonate coating buffer, pH 9.6) and incubated overnight at 17°C. Following three rinses of the plates with 0.1% Tween-20 in tris-buffered saline, pH 8.2, (TTBS) and three rinses with tris-buffered saline (TBS) alone, the well surfaces were then blocked by incubation with 3% bovine serum albumin (BSA) in TBS. At this point all incubations were performed at room temperature in a humid chamber and the same wash procedure followed each incubation. After rinsing, 100 µl of test serum dilutions were incubated, in parallel, with standards consisting of 5, 1, 0.5, 0.125, 0.1 µg/ml SA in a diluent consisting of normal salmon serum diluted 1/20 in TTBS. The samples were incubated for one hour at room temperature. The plates were then washed and 100 µl of the appropriate dilution of the biotinylated antibody in 1% BSA in TBS was added to each well and allowed to incubate for another hour. After rinsing, 100 µl of an appropriate concentration streptavidin-horseradish peroxidase (S-HRPO) (Sigma) was incubated in each well for 30 min. After the last rinse, 100 µl of substrate solution was added and color development was monitored spectrophotometrically at 405 nm on an EIA autoreader (Model EL 310, Biotek Instruments, Burlington, VT, USA). The substrate solution was a mixture of : 10 ml of citrate buffer (pH 4.0), 10 µl of hydrogen peroxide and 75 µl of a 10 mg/ml solution 2,2'-Azinobis, 3-ethyl benzthiazoline sulfonic acid (ABTS) in distilled water. A standard curve was then plotted and used to determine the concentration of SA in the test sera (Fig. 1).

Sodium dodecpl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Serum samples from infected and normal coho salmon were stored at -200C. Immediately prior to use, samples were mixed 1: 1 in sample buffer (120 mM tris base, 4% w/v SDS, 10% v/v 2-mercaptoethanol, 20% w/v glycerol, and 3mM bromophenol blue), then placed in a boiling water bath for 2-3 min. Samples were then immediately run on 83x103 mm mini-slab gels (Idea Scientific, Corvallis, OR, USA) under conditions similar to those of Laemmli (1970), using a 12% acrylamide separating gel and a 3.7% acrylamide stacking gel, for approximately 1.5 hours with a constant current (20 mAmps).

Western blotting

Protein transfer from gel to nitrocellulose paper (Bio-Rad) was performed according to a modified method of Towbin et al. (1979). Briefly, proteins were transferred by overnight electrophoresis at 30 V and 4oC in a transblot apparatus (Bio-Rad). The nitrocellulose was blocked for one hour with 3% BSA in TBS at 370C. The nitrocellulose bound kproteins were probed with biotinylated rabbit anti-SA diluted 1/1000 in 0.1% tween-TBS for one hour. After a 30 min wash in 0.1% tween-TBS, a 1/200 dilution of S-HRPO in 0.1% tween-TBS was applied for 25 min. Visualization of the protein bands, was achieved using a substrate solution consisting of 2 ml of 4-chloro-naphthol (Bio-

Rad) (3 mg/ml in methanol stock), 10 ml of 0.1 M phosphate buffered saline and 10 µl of hydrogen peroxide incubated for 15-30 min. Blots were rinsed in double distilled water for approximately 3 min and dried.

RESULTS

Quantitative Analysis of Soluble Antig n (SA) Production in Experimental Infections of Salmon

Quantitative assessment of soluble antigen levels in the serum of normal and infected salmon was accomplished by using the ELISA to compare dilutions of various test sera with a standard curve (Fig. 3.1). As can be seen in figure 1 the sensitivity of our assay is such that concentrations as low as 0.1 µg SA/ml of serum can be detected.

To determine if the progress of this disease could be monitored by quantification of, antigen levels using the ELISA, salmon were injected with a standard concentration of live *R. salmoninarum* and the sera from these fish were tested at five day intervals. Fig. 3.2. demonstrates a gradual increase in SA over the course of the disease. Mortalities routinely occurred between day 20 and 40 with this form of experimental infection.

Western Blot Analysis of SA Production in Experimentally Infected Fish

Serum samples from the experimentally infected fish (described above) were also qualitatively analysed by western blotting (Fig. 3.3). Three distinct observations were made using this form of analysis : 1) crossreactive antigens were found in control salmon serum that reacted with the rabbit anti-SA. However; these antigens are readily distinguishable from the SA on the basis of molecular weight. 2) Four major SA bands were identified; I=70 kd, II=60 kd, III=33-37 kd, IV=26 kd (Fig. 3.3, lane G). A 95 kd antigen appears in infected serum (Fig. 3.3, lanes B-E) which is not found in purified SA preparation (Fig. 3.3, lane G). 3) A gradual increase in band density of major SA (I, II, ,111) was observed over the time of the infection. The most prominent band was antigen II.

DISCUSSION

These studies demonstrate the value of an antibody capture ELISA based on the detection of soluble antigens secreted by Renibacterium salmoninarum; This assay was used to trace the progress of experimental BKD infections by quantifying the increase of antigen in the serum (Fig. 3.2). Furthermore, utilization of western blot 'analysis for the detection of R. salmoninarum antigens in infected animals demonstrated a high degree of specificity which easily resolved

many antigens of *R. salmoninarum* as well as false positive reactions.

Control of bacterial kidney disease is a problem of primary concern wherever salmon culture exists. This disease can easily become a chronic problem within a hatchery, since the bacteria are slow growing and infected animals may harbor the organism for long periods before manifesting gross pathology (Fryer and Sanders, 1981). Furthermore, infected brood stock may serve as a reservoir, infecting gametes and thus fry via vertical trasmission (Evelyn et al., 1984). Unfortunately, control cannot be achieved through immunization at this time, since no commercial vaccine is available. The rapid diagnosis of this disease could aid tremendously in its control by the identification of infected brood stock or gametes, and their subsequent culling or treatment.

Diagnosis of this disease thus far, however, has primarily been through the identification of organisms by the Gram stain (Pippy, 1969), fluorescent antibody techniques (Bullock and Stuckey, 1975; Laidler, 1980) and coagglutination (Kimura and Yoshimizu, 1981; Yoshimizu and Kimura, 1985; Cipriano et al., 1985). Pascho and Mulcahy (1987) have developed a procedure to detect a heat-stable soluble antigen of *R. salmoninarum* using an ELISA, but as yet this technique has not been applied to the analysis of infected fish.

ELISAs, such as described here and by Pascho and Mulcahy (1987), are not capable of distinguishing between the various R. salmoninarum antigens that may be present, nor can false positive reactions be identified. Therefore, use of a western blot assay, as we describe, is extremely useful in resolution of these potential shortcomings. The western blot (Fig. 3.3) demonstrates at least four different R. salmoninarum antigens with molecular weights of 70 kd (I), 60 kd (II), 33-37 kd (III), and 26 kd (IV). Control salmon serum reveals two cross reactive antigens (lane I), which are easily distinguished from the soluble antigens (lane G). Therefore, R. salmoninarum specific antigens can be distinguished from the cross-reactive antigens.

During the course of the infection an increase in the soluble antigens are not only seen in the ELISA (Fig. 3.2) but also in the western blot (Fig3.3). Antigen II was detectable in the serum from day 5, while I and III became detectable at later times. Antigen IV, however, did not appear in the serum. Other investigators have identified soluble antigens in infected fish samples by alternate means of detection such as coagglutination (Kimura and Yoshimizu, 1981; Yozhimizu and Kimura, 1985), counterimmunoelectrophoresis (Cipriano et al., 1985) and peroxidase-antiperoxidase systems (Sakai et al., 1987). However, these previous studies relied on antiserum directed toward whole R. salmoninarum cells. This investigation was conducted with antibodies specifically directed to the soluble products released by R. salmoninarum, which reacted only with distinct molecular weight species (Fig. 3.3).

Investigators have previously identified *R. salmoninarum* antigens of distinct molecular weights. Getchell, et al. (1985) described an antigen F (57 kd) as a heat stable major surface antigen, common to several isolates of *R. salmoninarum*. Fiedler and Draxl (1986), however, have reported the major surface antigen has a molecular weight of 70 kd. At present, the biochemical relationship of the soluble antigens reported here to other reported antigens is the object of further analysis.

ACKNOWLEDGEMENTS

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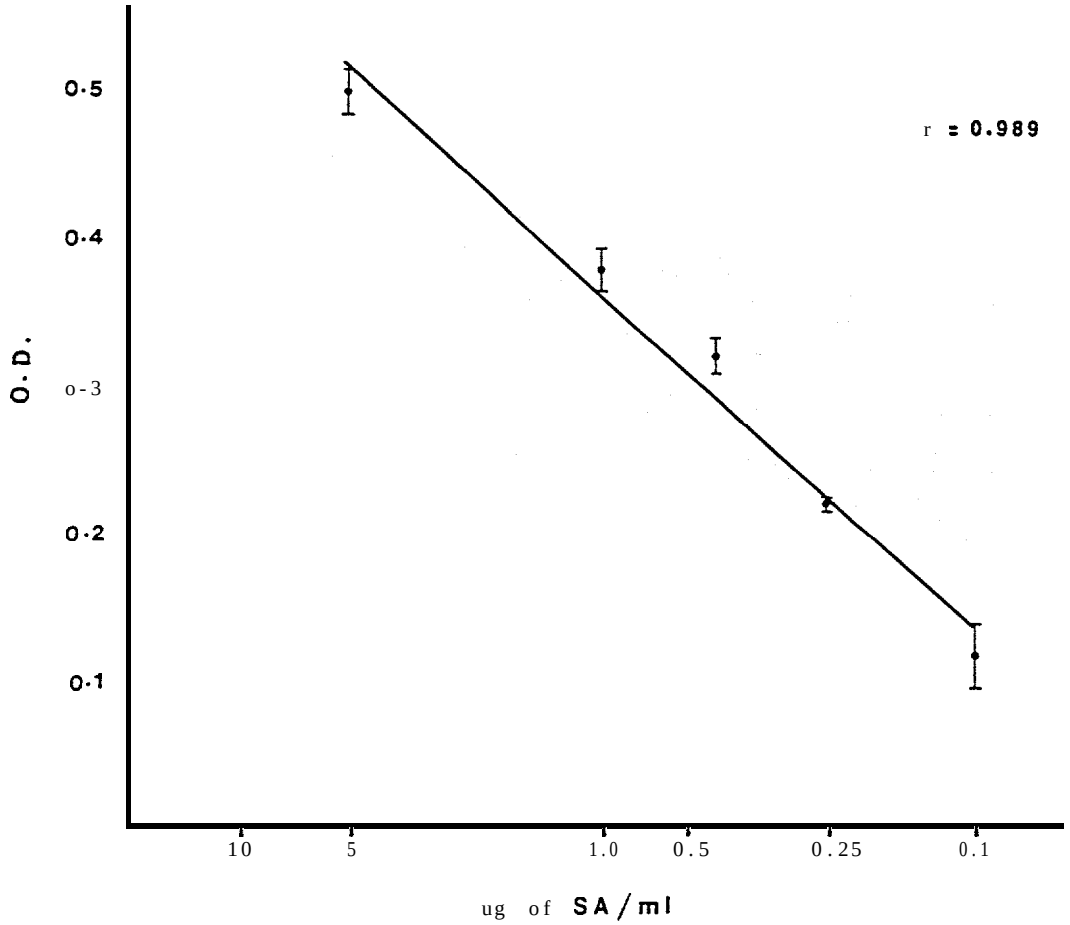

Fig 3.1. ELISA standard curve: an antibody capture method was employed by coating rabbit anti - R. salmoninarum antibody onto the wells of an ELISA plate. Soluble antigen dilutions were made in normal, non-infected control serum to produce concentrations of 5, 1, 0.5, 0.125, 0.1 μg/ml of serum. Each point represents an average of triplicates ± one standard error.

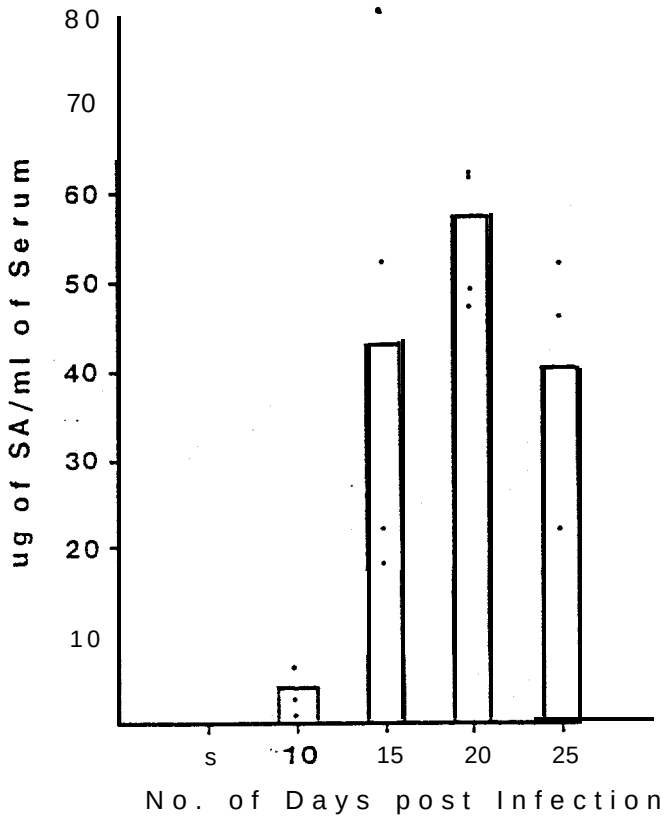

Fig. 3.2. Quantification of soluble antigen during experimental infection. Each point represents the value of the soluble antigen concentration in the serum of one infected animal, sacrificed on day 5, 10, 15, 20, or 25 following injection of live R. salmoninarum on day 0. Each histogram represents the mean of all the points at that concentration.

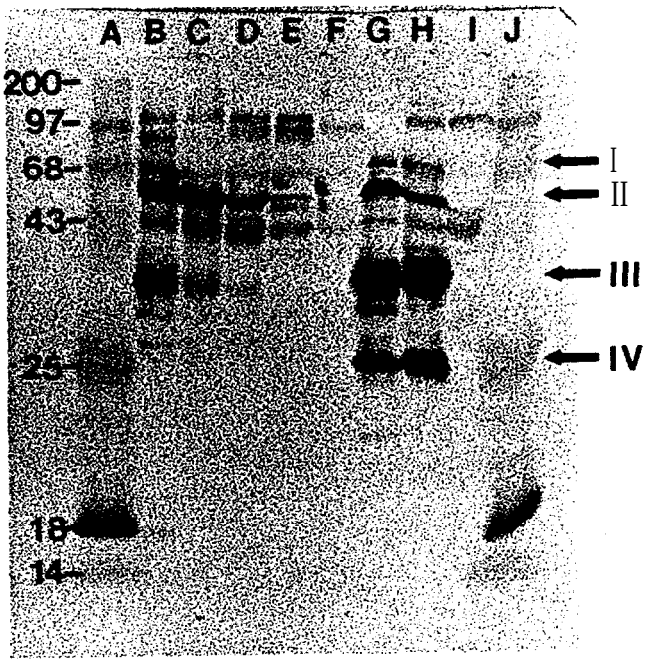

Fig. 3.3 Western blot of infected fish sera. Protein concentration in the lanes are as follows; A) Prestained molecular weight markers (9 µl), B) Day 25 infected coho sera (1.5 µl), C) Day 20 infected coho sera (1.5 µl), D) Day 15 infected coho sera (1.5 µl), F) Day 5 infected coho sera (1.5 µl), G) BKD soluble antigen 1.25 µg, I) Control coho sera, 1.5 µl, J) Prestained molecular weight markers (Bethesda Research Laboratories,. Bethesda, MD, USA), 9 µl. Arrows indicate the four major BKD soluble antigens.

MANUSCRIPT #6

POLYCLONAL ACTIVATION OF SALMONID B LYMPHOCYTES

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POLYCLONAL ACTIVATION OF SALMONID B LYMPHOCYTES

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ABSTRACT. The process of in vitro polyclonal activation of coho salmon (Oncorhynchus kisutch) lymphocytes was examined with respect to the induction of mitogenesis, total immunoglobulin production, and the production of specific antibodies or plaque forming cells. These studies demonstrate that antigen specific stimulation of antibody production is not linked to mitogenic activity, or total immunoglobulin production, while the polyclonal activation of specific antibody production is closely linked to these functions. Stimulation of immunoglobulin production by phytohemagglutinin suggests that this mitogen may not be limited to T cell activation in salmonids or, alternatively, it may induce the production of lymphokines capable of polyclonally activating B cells. Further, fetal calf serum was found to cause production of large amounts of immunoglobulin in vitro without antigenic stimulation.

INTRODUCTION

Polyclonal B cell activators (PBCAs) are substances which possess the ability to stimulate many, if not all, lymphocytes without the requirement for antigenic specificity (1,2). These substances include polysaccharides (3,4), lipopolysaccharides (5) and proteins (6-8). This form of stimulation can be detected by increased proliferation and the complete differentiation of B cells to antibody secretion (1,9). PBCAs have thus been, used to elucidate the various mechanisms of B cell differentiation because of their ability to stimulate larger populations of lymphocytes than could be activated simply by antigen specific stimulation.

Past work in the salmonid (10,11) and other piscine systems (12,13) has primarily focused on the ability of mitogens to stimulate proliferation. This study compares the ability of two of these mitogens, lipopolysaccharide (LPS) and phytohemagglutinin (PHA), to induce specific antibody and general immunoglobulin production. The role that commonly used serum supplements may play in the non-specific generation of total immunoglobulins or specific antibodies is also examined.

MATERIALS AND METHODS

Animals

Yearling coho salmon (50-100 g) were kept in ambient (12°C) pathogen-free well water in 220 gallon circular tanks at the Oregon State University Fish Disease Laboratory. The fish were fed the Oregon Moist Pellet diet. Adult female BALB/c mice (Simonsen Laboratories, Gilroy, CA) were maintained by Laboratory Animal Resource Center at Oregon State University,

Culture media

Media components were purchased from Whittaker M. A. Bioproducts (Walkersville, MD) unless otherwise noted. Holding medium consisted of 100 µg/ml gentamicin (Sigma, St. Louis, MO) and 10% fetal calf serum in RPMI 1640 (Gibco;Grand Island, NY). Tissue culture medium (TCM) was a modification of the medium described by Mishell and Dutton (14). Briefly, RPMI 1640 containing L-glutamine and sodium bicarbonate was supplemented with 10% fetal calf serum, 50 µg/ml gentamicin, 50 µM 2-mercaptoethanol (MCB, Cincinnati, OH), and 10 µg/ml each of adenosine, uracil, cytosine, and guanine (Sigma). A nutritional cocktail for culture supplementation was also prepared as described by Tittle and Rittenberg (15) and fed on alternate days to the cultures as described below.

Mitogens and antigens

Stock solutions lipopolysaccharide W (LPS) from E. coli 055:B5 (Difco, Detroit, MI) were pasteurized in distilled water for 30 minutes at 70°C. Stock solutions of phytohemagglutinin P (Difco, Detroit, MI) were prepared in TCM and sterilized by filtration through a 0.45 µm filter. Trinitiophenylated-LPS (TNP-LPS) was prepared by the method of Jacobs and Morrison (16).

Cell cultures

Fish were sacrificed by cerebral concussion and their pronephros tissue placed in holding medium (HM) on ice. A single cell suspension of each organ was obtained by aspiration through a 1 ml syringe. The resulting cell suspension was incubated on ice to allow organ fragments to settle. The supernatant, a single cell suspension, was then washed once in HM by centrifugation at 500 x g for 10 minutes at 4°C. The final washed cell pellet was resuspended in TCM. Lymphocytes were enumerated by the use of a hemacytometer. Cell viability was determined by trypan blue exclusion. The antibody, plaque-forming cells (PFC), or total immunoglobulin assays were performed on triplicate cultures of 4 x 10^6 lymphocytes in 200 µl TCM added to the wells of a 24-well, flat-bottomed tissue culture plate (Corning, Corning, NY) containing 200 µl of antigen. Mitogen assays employed triplicate cultures of 5 x 10^5 lymphocytes in 50 µl TCM added to the wells of a 96-well, flat bottomed cultures (Corning) containing mitogen. All tissue culture plates were then incubated in plastic culture boxes (C.B.S. Scientific, Del Mar, CA) in an atmosphere of 10% CO_2 at 17°C. Cultures used in the production of antibody, PFCs, and immunoglobulin were maintained by adding 50 µl of nutritional cocktail daily. Previous studies revealed that daily feedings

or feedings on alternate days of the 50 µl volume of cocktail produce optimal responses, Single feedings of any quantity are not sufficient for optimal responses. The cells and supernatants were harvested on day 9. Mitogen cultures were not supplemented with cocktail.

Mitogen assay

Twenty-four hours prior to harvest on day 5, each well was pulsed with one µCi of tritiated thymidine (methyl-^{3}H, ICN Biomedicals, Irvine, CA) prepared in 50 µl of TCM. Cells were harvested with distilled water onto glass fiber filters, with a Skatron cell harvester (Sterling, VA). The filters were then dried, placed in vials with scintillation cocktail (10) and counted on a Beckman liquid scintillation counter (EC 3800).

Plaque forming cell assay

Cells were harvested from individual wells and washed once as described above. Cell pellets were resuspended in 0.5 ml HM, and the lymphocytes enumerated with a Model ZM Coulter counter (Coulter, Hileah, FL). A modification of the Cunningham plaque forming cell assay (17) was performed using 100 µl of the lymphocyte suspension, 25 µl of a 10% suspension of trinitrophenylated sheep red blood cells in modified barbital buffer (MBB), and 25 µl of steelhead trout (Salmo gairdneri) serum, diluted in MBB, as previously described (18). The Cunningham chambers were incubated for l-3 h at 17°C. Plaques were enumerated under low power with the aid of a dissecting microscope.

Preparation of biotinylated anti-salmonid immunoglobulin

The hybridoma cell line (1-14), which produces a monoclonal antibody to trout immunoglobulin (generous gift of Dr. G. Warr, Dept. of Biochemistry, Univ. of South Carolina), was injected intraperitoneally, into BALB/c mice. After two weeks, the ascites fluid was aspirated from the cavity and the supernatant fluid was precipitated three times with 50% saturated ammonium sulfate (SAS). This preparation was then extensively dialyzed against 0.1 M phosphate buffered saline, pH 7.2 (PBS), filter sterilized, and tested for anti-coho immunoglobulin activity. The specificity and affinity of this monoclonal for coho immunoglobulin was demonstrated by the competitive inhibition of 1-14 binding to trout immunoglobulin. Equivalent concentrations of immunopurified (see below) coho immunoglobulin or trout immunoglobulin were found to equally inhibit the binding of 1-14 to solid phase bound trout immunoglobulin in an ELISA.

Biotinylated 1-14 was prepared using the method described by Kendall et al. (19). Briefly, 3 mg of the SAS cut 1-14 antibody was dialyzed extensively against 0.1 M carbonate buffer. Following dialysis, 20 µl of 0.1 M biotinyl-n-hydroxysuccinimide ester (Cal-Biochem, La Jolla, CA) in dimethyl formamide was mixed with the 1-14 preparation for one hour at room temperature. The mixture was dialyzed against PBS, then mixed 1:1 with glycerol and stored at -20°C.

Preparation of coho anti-TNP immunoglobulin. Anti-TNP coho immunoglobulin was purified from the serum of coho salmon which had been hyperimmunized with TNP-keyhole limpet hemocyanin (20). The serum antibodies were immunopurified by passage over a TNP-lysyl-sepharose affinity column. Anti-TNP antibodies were eluted with 3 M KSCN after extensive washes with

PBS. The antibody preparation was then dialyzed against PBS. This antibody preparation was used as a source for the coho immunoglobulin and anti-TNP antibody standard's in the ELISA analyses.

ELISA procedure for quantification of total immunoglobulin

Wells of a 96-well ELISA plate (Costar, Cambridge, MA) were coated overnight with 0.1 ml of a 5 µg/ml solution of the 1-14 monoclonal antibody in coating buffer (21) at 17°C in a covered plate. Following the coating procedure, the surface of the wells were blocked by an incubation with 1% bovine serum albumin in tris buffered saline, TBS, (21). The blocking solution was incubated for one hour at room temperature, removed and followed by the incubation of coho immunoglobulin standards (see above) or culture supernatants for three hours. All samples and other reagents were prepared in TBS with 0.1% Tween 20 added (TTBS). After rinsing, 0.1 ml of a 1/500 dilution of biotinylated 1-14 in TTBS was added and incubated for two hours. This incubation was followed by rinses with TTBS and TBS, and an incubation with 0.1 ml of 1/100 dilution of streptavidin-horseradish peroxidase (S-HRPO) for 20 min. Following this incubation and subsequent rinses, 0.1 ml of the substrate (75 µl ABTS, 5 µl H_2O_2, and 10 ml of 0.2% (w/v) citrate buffer, pH 4.0) was added. Optical densities were read at 405 nm on a Biotek EL310 ELISA reader (Burlington, VT).

Quantification of anti-TNP antibody

The procedures and reagents for the quantification of TNP-specific antibodies were the same as described above, except that the wells were first coated with 0.1 ml of a 0.5 µg/ml solution of TNP-BSA in coating buffer. Immunopurified anti-TNP standards. or culture supernatants containing anti-TNP were then added to the wells. Following this incubation, the biotinylated 1-14 and S-HBPO system described above was employed for quantification of bound antibody.

RESULTS

Kinetics and characterization of the in vitro and TNP PFC response

Anterior kidney lymphocytes were stimulated with 0.4 µg/ml TNP-LPS, which was determined to be the optimal dose for the generation of TNP-PFC at all time points. Lymphocytes were harvested and the number of PFC determined for days 5, 7, 9 and 11 of culture. Figure 1 depicts a typical kinetics assay, however, it is not unusual to find an equivalent optimal response on day 7 and/or 8. The maximal response produced in this assay was 3186 PFC/10^6 lymphocytes. Maximal responses can range from approximately 1000 - 10000 PFC/10^6 lymphocytes. The specificity of these PFC was demonstrated by the complete inhibition of PFC when assayed in the presence of 10^{-3} M TNP-lysine. No inhibition was observed with the same concentration of unhaptenated lysine. Furthermore, these lymphocytes did not produce PFCs to unhaptenated SRBC. Cells cultured without antigen demonstrated a neglible response which did not exceed 155/10^6 lymphocytes at any time period.

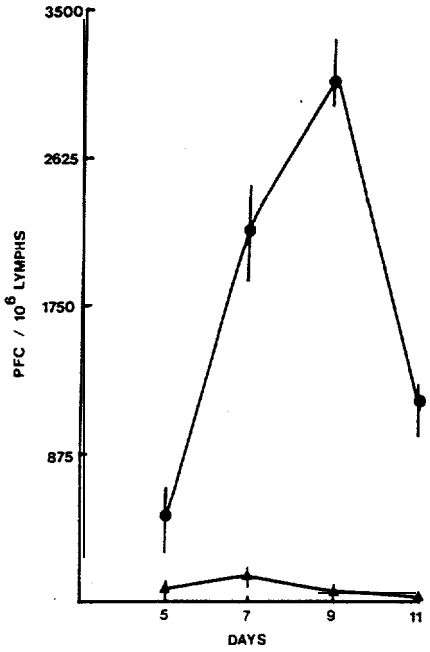

FIG. 1

Kinetics of the in vitro plaque forming cell response for lymphocytes stimulated with TNP-LPS (●), or without antigen (▲). Each point represents the average of triplicate cultures and the error bars represent two standard errors about the mean.

Comparison of antigen specific and polyclonal responses

Escherichia coli lipopolysaccharide (LPS) was used to demonstrate the activity of a typical polyclonal B cell activator (PBCA), as described in various mammalian cell culture systems (1,5). Figure 2A demonstrates that LPS functions in a manner typical of PBCAs, in that the mitogenic dose response closely parallels the non-specific generation of plaque-forming cells (PFC) to the trinitrophenyl (TNP) hapten. In contrast, the trinitrophenylation of LPS (TNP-LPS) produces a specific antigen, which induces a specific PFC dose response that is not linked to the mitogenic dose response for TNP-LPS (Fig. 2B).

Polyclonal activation of B cells by a T cell mitogen

Figure 3 depicts the dose response for phytohemagglutinin (PHA), with respect to tritiated thymidine uptake and the production of anti-TNP PFC. The dose response curves for both functions closely parallel one another, as was found with LPS stimulation. The production of PFC indicates that PHA is either directly or indirectly responsible for antibody production.

Alternate method for the assessment of polyclonal activation

Although anti-TNP PFC generation demonstrates the induction of antibody producing cells by PBCAs, the comparison of the amount of anti-TNP antibody to the total immunoglobulin (Ig) produced in culture allows for a more accurate indication of polyclonal activation. Figure 4 illustrates the utility of this form of analysis. The specific antigen TNP-LPS (4A), LPS (4B), and PHA (4C) produce total Ig levels that closely parallel the mitogenic dose responses of Figures 2 and 3 (data not shown), which would be expected with polyclonal activation. However, the specific antigen, TNP-LPS, produces a dramatic increase (Fig. 4A) in the percent of total Ig that are anti-TNP antibodies at the optimal antigenic dose (0.4 μg/ml). The percent anti-TNP antibody generated by unhaptenated LPS (Fig. 4B) and PHA (Fig. 4C) remains at a low and constant value at all doses, which would be expected in the non-specific stimulation of antibody production.

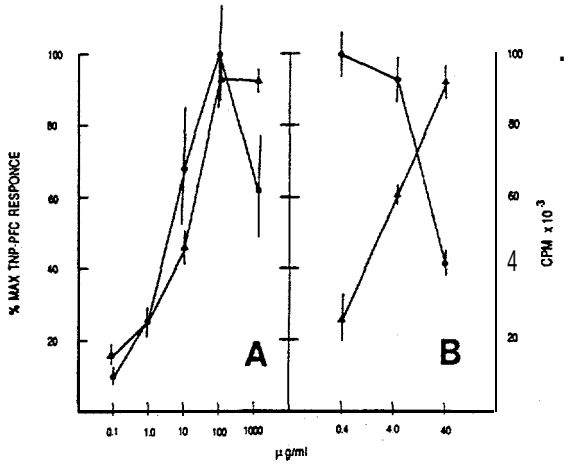

FIG. 2

A) Mitogenic (A) and plaque forming cell (●) dose responses for E. coli lipopolysaccharide. B) Mitogenic (▲) and plaque forming cell (●) dose response for TNP-LPS. Each point represents the average of triplicate cultures and the error bars represent two standard errors about the mean. The maximium PFC response for TNP-LPS was 4072 PFC 10^6 lymphocytes and for LPS alone was 830 PFC/10^6 lymphocytes.

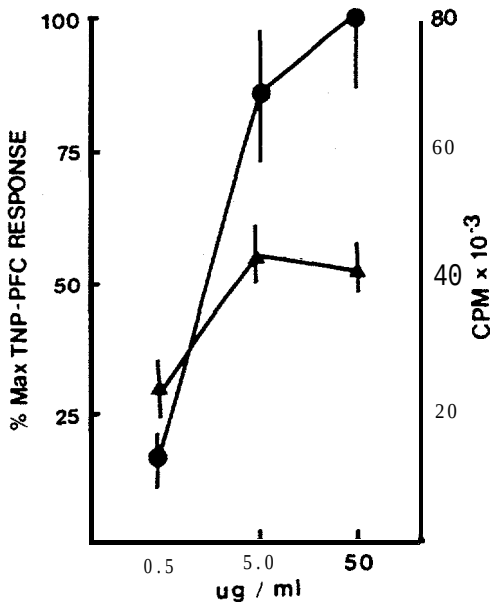

FIG. 3

Mitogenic (A) and plaque forming cell (●) dose responses for phytohemagglutinin. Each point represents the average of triplicate cultures and the error bars represent two standard errors about the mean, The maximum PFC response was 540 PFC/10^6 lymphocytes

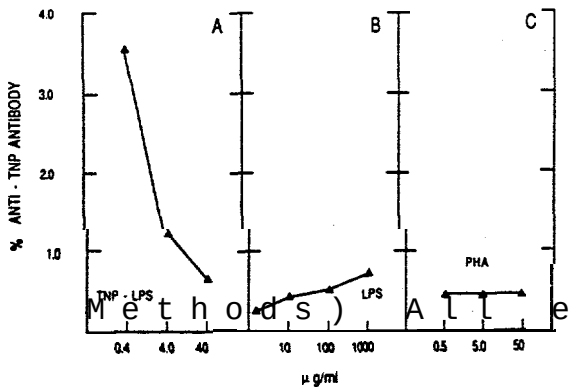

FIG. 4

Amount of anti-TNP antibodies expressed as a percentage of the total immunoglobulin produced in culture. Each point (A) represents the average concentration of anti-TNP antibodies divided by the average concentration of salmonid Ig found in pooled culture supernatants. Individual concentrations were interpolated from standard curves utilizing a sandwich ELISA (Materfals and Methods) All estimations of mean concentrations were associated with 0.9 confidence intervals that ranged not more than 20% about each mean.

Role of fetal calf serum in immunoglobulin production

In our in vitro culture system, it is not uncommon to observe some anti-TNP PFCs in the absence of antigen (Fig. 5). It was felt, therefore, that the addition of fetal calf serum (FCS) alone may induce some or all of these PFC. To examine this possibility, cultures were supplemented with 10%, 1%, and 0% FCS. This reduction in FCS reduced the number of antigen-induced as well as background PFC in cultures.

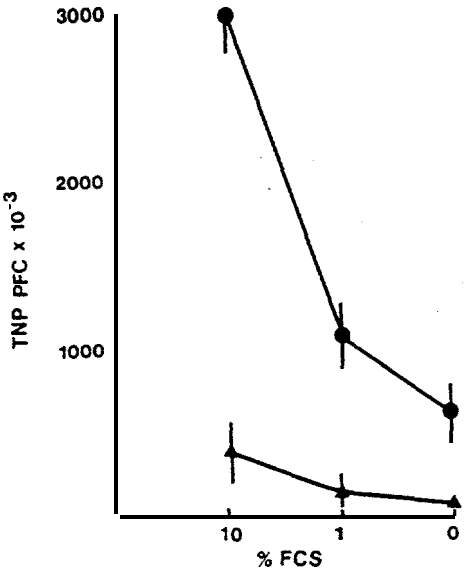

FIG. 5

Anti-TNP PFC responses elicited by TNP-LPS (●) and no antigen (A) are depicted from cultures supplemented with 10%, 1% and 0% fetal calf serum. Each point represents the average values of triplicate cultures and the lines represent two standard errors about the mean,

The supernatants from TNP-LPS stimulated cultures were also assayed for the amount of anti-TNP and total Ig produced. As can be seen from Table 1, a decrease in the concentration of fetal calf serum from 10% to 0%, led to an increase in the percent anti-TNP from 2% to 35% of the total Ig. In all cultures the amount of anti-TNP antibodies was relatively constant. The change in the percent anti-TNP was primarily due to a decrease in total Ig production.

TABLE I

Effect of Fetal Calf Serum on Anti-TNP and Immunoglobulin Production

% FCS IN CULTURE[a]	Total Ig (μg/ml)	Anti-TNP (μg/ml)	% ANTI-TNP Ig[b]
10	37.5	0.77	2.0
1	3.8	0.49	12.9
0	1.4	0.49	35.0

[a]Supernatants were derived from cultures stimulated with 0.4 μg/ml TNP/LPS.

[b]$\% \text{ Anti-TNP Ig} = \frac{\mu g \text{ Anti-TNP}}{\mu g \text{ Total Ig}} \times 100$

DISCUSSION

In this study we have found that the mitogens, LPS and PHA, cause proliferation of coho salmon lymphocytes, as has been reported for rainbow trout (10,11), carp (13), and channel catfish (12). In addition, we demonstrate that these substances are also excellent polyclonal B cell activators (PBCAs) of salmonid lymphocytes, as shown by the production of hapten specific plaque forming cells and non-specific immunoglobulin. Examination of the production of TNP-specific antibodies as a percentage of the total immunoglobulin (Fig. 3) reveals that the antigen, TNP-LPS, produces a sharp elevation of this percentage at the optimal concentration for antigenic stimulation. This is not the case for mitogen stimulated cultures which produce a constant, low percentage of TNP-specific antibodies. Such a concentration-independent function would be expected if the non-specific stimulation of lymphocytes were occurring,

It is not surprising that LPS serves as a PBCA, as LPS is a potent stimulator of mammalian B lymphocyte proliferation and differentiation (5). However, PHA has routinely been considered to be strictly a T cell mitogen (22,23) in mammalian systems. Recent work in piscine systems (24-27) has demonstrated morphological, functional, and antigenic differences among fish lymphocytes which would suggest that PHA/concanavalin A (conA) and LPS responslve lymphocytes may be functionally equivalent to T and B cells, respectively. In our system, PHA was shown to stimulate PFC and immunoglobulin production. Thus, either PHA must directly stimulate salmonid B cells, or stimulate T cells to elaborate factors which can induce B cells to secrete immunoglobulin. It is of interest that Dosch et al. (28) have noted that PHA, in the presence of irradiated human T cells, can act as a polyclonal activator of B cells. They conclude that irradiation most likely abrogates T suppressor activity which would prevent the polyclonal response. In the salmonid system, removal of suppressor function by irradiation was not required to produce a polyclonal B cell response.

It has been found necessary to employ fetal calf serum (FCS) rather than salmonid serum as a tissue culture supplement for three primary reasons: 1) Salmonid serum sources (coho, chinook, trout) have been quite variable in their ability to support cell culture. 2) Procurement of sufficient serum pools for routine culture is not possible. 3) The use of homologous serum in vitro would interfere with the detection of in vitro induced salmonid immunoglobulin or antibody. Fetal calf serum supports our tissue cultures, with background (in the absence of antigen) levels of PFCs comparable to that found with homologous serum sources. To determine if background levels of PFCs,'antibody, or total immunoglobulin are induced by the FCS, the tissue culture supernatants were analyzed for the percentage of TNP-specific antibodies when the FCS was reduced or eliminated. Reduction of FCS to 1% or total elimination reduced the background levels of plaques and immunoglobulin. Although overall responses to PCAs and antigen are generally reduced, a relative increase in the percentage of TNP-specific antibody occurred (Table I). Therefore, FCS acts to increase the amount of non-TNP antibody produced. This could occur by polyclonal activation, activation of B cells specific for bovine serum proteins, or by the enhanced survival of lymphocytes cultured in higher concentrations of FCS. The later explanation would appear to be the least likely since: 1) There was no detectable difference in survival of lymphocytes cultured on 1% or 10% FCS, and yet there was a ten-fold d&crease in the total Ig produced of 1% FCS. Cells cultured in 0% FCS

demonstrate a 50% reduction in survival, but Ig production is decreased by 96% in the 0% FCS cultures. 2) There is a marked preferential reduction of total Ig, but not anti-TNP-specific antibody. This would indicate that decrease Ig production was not simple due to a reduction in general lymphocyte viability. Such a non-specific stimulation by serum supplementation has been observed in mammalian culture systems (29). It is strongly suggested that if investigators wish to examine antigen-specific responses without any contribution due to polyclonal activation or increased immunoglobulin production. The effects of varying concentrations of FCS within their cultures should be monitored.

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VIBRIO ANGUILLARUM ANTIGEN STIMULATES MITOGENESIS AND POLYCLONAL ACTIVATION OF SALMONID LYMPHOCYTES

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Vibrio anguillarum Antigen Stimulates Mitogenesis and Polyclonal Activation of Salmonid Lymphocytes

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ABSTRACT. An antigen preparation of Vibrio auguillarum, a salmonid pathogen, acts as a potent in vitro mitogenic stimulator of splenic and pronephric (anterior kidney) lymphocytes from coho salmon (Oncorhynchus kisutch), chinook salmon (0. tshawytscha) and rainbow trout (Salmo gairdneri). This antigen (VA) is comparable in its mitogenic activity to Concanavalin A (Con A), Escherichia coli lipopolysaccharide (LPS), and phytohemagglutinin (PHA). VA gives peak mitogenic responses in coho five days after initiation of cell culture. VA also appears to be a nonspecific polyclonal activator as determined by the generation of plaque forming cells to trinitrophenyl (TNP) and fluorescein (FI) haptenic determinants. Chemical characterization is limited, but it appears that Vibrio LPS could be responsible for these activities.

INTRODUCTION

Mitogens, substances which stimulate in vitro lymphocyte proliferation, have proved useful as probes of immune responsiveness (1,2). Mitogens have been found to induce differentiation of lymphocyte populations by the selective stimulation of B lymphocytes by lipopolysaccharide (3), purified protein derivative of tuberculin (4), and dextran sulfate (5), or of T lymphocytes by Concanavalin A (6), and phytohemagglutinin (6), or by stimulation of both B and T lymphocytes by pokeweed mitogen (7). Although initially used with mammalian lymphocytes, fish lymphocytes have also been found to respond to these mitogens in vitro. Mitogen responses have been studied in rainbow trout (8,9,10), carp (11), and channel catfish (12). Whether the same functional distinctions between B and T cells as is found with mammalian lymphocytes can be made with fish lymphocytes is not, as of yet, clear, although recent evidence suggests that this may be possible (12).

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Numerous substances derived from bacteria and metazoans have been found to be polyclonal activators of mammalian B cells in that they stimulate both host B cell proliferation and antibody secretion. Some best characterized B cell polyclonal activators include lipopolysaccharides from gram negative bacteria (3), the purified protein derivative of tuberculin (4), protein A from Staphylococcus aureus (13), and polymerized flagellin from Norcardia brasiliensis (14). These substances have proven to be useful immunological tools for delineating the ontogeny of B cell responsiveness and dissecting the mechanism of B cell activation (15).

Past work (16,17) has shown that an antigen extract of Vibrio anguillarum (VA) stimulates a specific anti-Vibrio anguillarum antibody response in vivo. It is possible, however, that this response maybe due in part to non-specific mitogenic and/or polyclonal activating components, working in concert with specific V. anguillarum antigens. Thus, in order to determine the mitogenic and polyclonal activating properties of VA, we have utilized an in vitro culture system, developed for the mitogenic stimulation (18) and induction of antibody responses (19) of salmonid lymphocytes. Since V. anguillarum is a fish pathogen in a wide variety of marine fish including salmonids (20,21), the in vitro analysis of its effects on their lymphocytes may assist in understanding mechanisms of both the pathogenesis of this bacteria and the immune responsiveness of the host fish.

MATERIALS AND METHODS

Animals and Facilities

All fish were supplied by the Oregon Department of Fish and Wildlife and maintained in 460 liter circular fiberglass tanks supplied with 12°C well water at the Oregon State University Fish Disease Laboratory (OSUFDL), Corvallis, Oregon. The fish were fed Oregon Moist Pellets daily. Seventy to one hundred gram fish were used for these studies.

Culture media

Media components were purchased from Whittaker M.A. Bioproducts, Walkersville, MD, unless otherwise noted. Cell suspensions were made in holding medium which consisted of 100 ug/ml gentamicin and 10% fetal calf serum in RPMI 1640 with L-glutamine (Gibco, Grand Island, NY), supplemented with 0.2% (w/v) sodium bicarbonate. Mishell-Dutton modified tissue culture medium (TCM) was used for tissue culture and consisted of RPMI 1640 with L-glutamine and bicarbonate supplemented with: 10 mM L-glutamine, 10% fetal calf serum (hybridoma screened), 50 ug/ml gentamicin, 50 uM 2-mercaptoethanol (MCB, Cincinnati, OH), and the nucleosides; adenosine, uracil, cytosine, and guanosine (10 ug/ml, Sigma, St. Louis, MO). A media supplement (nutritional cocktail) was also prepared as previously described (22) and fed on alternate days to the cultures as described below.

Mitogens

Stock solutions of lipopolysaccharide B (30 mg/ml) from E. coli 055:B5 (Difco, Detroit, MI) were pasteurized for 30 minutes at 70°C in distilled water. Further dilutions for tissue culture were made in TCM. Stock solutions of PHA (Difco, Detroit, MI), and Con A (Sigma, St. Louis, MO) were made in TCM (1 mg/ml) and sterilized by filtration through a 0.45 um filter.

Vibrio anguillarum O-antigen extract and lipopolysaccharide

The *Vibrio* extract was prepared from V. *anguillarum* strain LS-174 as described in Sakai et al. (16) with minor modifications. Fifty ml of thawed packed cells which had been formalin killed and stored frozen were suspended in 10 volumes of 2% saline and placed in a boiling water bath for 2 hours. Cells were washed 3 times in 2% saline by centrifugation at 10,000 x g for 10 minutes at 4°C. The final pellet was resuspended in 95% ethanol, and incubated 48 hours at 37°C. The cells were then washed 2 times in acetone by centrifugation at 3,000 x g for 10 minutes and dried by an overnight incubation 37°C. The cells were then ground to a fine powder with mortar and pestle and stored at 4°C. The soluble *Vibrio* extract used for these studies was prepared by boiling the powder in 0.1 M phosphate-buffered saline, pH 7.2 (PBS) at 10 mg/ml in a boiling water bath for one hour with frequent agitation. This suspension was then centrifuged at 1,000 x g to remove particulates and filter sterilized (0.45 um). The protein concentrations of the filtered extract was determined by the method of Lowry et al. (24).

Vibrio anguillarum lipopolysaccharide was prepared as described by Westphal et al. (25) and resuspended to the appropriate dilution in TCM. Concentrations of *V. anguillarum* lipopolysaccharide were expressed as ug/ml carbohydrate as determined by the phenol/sulfuric acid method (26).

Lymphocyte preparation

Fish were euthanized by cerebral concussion. The pronephric and splenic tissues were then dissociated in tissue culture medium as described by Kaattari, et al. (19). Briefly, single cell suspensions of each organ were obtained by repeated aspiration and expulsion of the tissues through a 1 ml syringe. The resulting cell suspension was incubated on ice to allow organ fragments to settle. The supernatant, a single cell suspension, was then washed once in tissue culture medium (TCM) by centrifugation at 500 x g for 10 min at 4°C. The cell pellets were resuspended in TCM to a concentration of 2 x 10^7 viable lymphocytes per ml, as determined by trypan blue exclusion.

Mitogen assay

Fifty ul (10^6 cells) of the lymphocyte suspension were added to individual wells of a 96-well flat-bottom microculture plate (Corning Glass Works, Corning, NY). Fifty ul of the mitogen were then added to these suspensions. The cultures were incubated in an airtight gasbox, (Model 624, C.B.S. Scientific Co., Del Mar, CA) at 17°C, in a blood-gas environment containing 10% CO_2. The cells were fed 10 ul of the media supplement every other day for the duration of the culture period. Twenty-four hours before harvest, cells were pulsed with 1.0 uCi of tritiated thymidine (NEN, Wilmington, DL) in 10 ul of TCM. Cells were harvested with distilled water onto glass fiber filters, with a Skatron cell harvester (Sterling, VA). The filters were then dried, placed in scintillation vials with cocktail (6g PPO, Sigma, 5 mg POPOP, Amersham, Arlington Heights, IL, in 1 L toluene, after Etlinger et al. (9)), and counted on a Beckman liquid scintillation counter, (Model EL 3800, Fullerton, CA).

Plaque forming cell assay

Two hundred ul of the lymphocyte suspension were added to each well of a 24-well flat-bottom microculture plates (Corning Glass Works, Corning, NY). To this suspension 200 ul of the appropriate dilutions of antigen were added. The cultures were incubated in an airtight gasbox at 17°C, in a blood-gas environment containing 10% CO_2. The cells were fed 50 ul of feeding cocktail (22) every other day for the duration of the culture period. After 9 d of incubation, cell cultures from individual wells were removed via gentle aspiration using a Pasteur pipette, and deposited into separate plastic radioimmunoassay tubes (VWR Scientific, Seattle, WA) and held on ice. The tubes were centrifuged at 500 x g for 10 min at 4°C. The supernatants were removed, and the cell pellets resuspended in RPMI 1640 to an appropriate lymphocyte concentration for plaque forming cell (PFC) enumeration. Cells secreting anti-TNP antibody were then detected by the Cunningham modification of the Jerne plaque assay (27). One hundred ul of the lymphocyte suspension, 25 ul of TSRBC (28) or Fluoresceinated-SRBC (29), and 25 ul of an appropriate dilution of steelhead serum in MBB, were mixed in individual wells of a 96-well round-bottom microtiter plate (Falcon, Oxnard, CA). This mixture was deposited in a Cunningham slide chamber, sealed with paraffin and incubated for 2 h at 17°C. Plaques were enumerated using a low power dissecting microscope. Lymphocyte numbers were determined for each lymphocyte suspension, using a Coulter Counter, (Model ZM, Coulter, Hialeah, FL) with 0.08 M PBS as a diluent. Plaque forming responses were expressed as PFC/culture.

RESULTS

Mitogenic studies

Coho salmon (*Oncorhynchus kisutch*) anterior kidney and splenic lymphocytes undergo mitogenesis in response to a range of VA concentrations (Fig. 1). The data are a summary of results from 7 to 15 individual fish, there is considerable variability in cpm between individuals, however, lymphocytes from all fish tested exhibited mitogenesis to VA. The optimal dose for stimulation of lymphocytes was variable, between species or individuals within a species, usually occurring between 5 and 500 ug/ml for a particular mitogen preparation.

VA is also mitogenic for lymphocytes from the other two salmonid species tested, chinook (*O. tshawytscha*) and 'rainbow trout (*Salmo gairdneri*)(Fig. 2). The data presented are from representative experiments with cells from a pool of organs from 5 fish. As with coho, all the fish tested respond mitogenically to the extract.

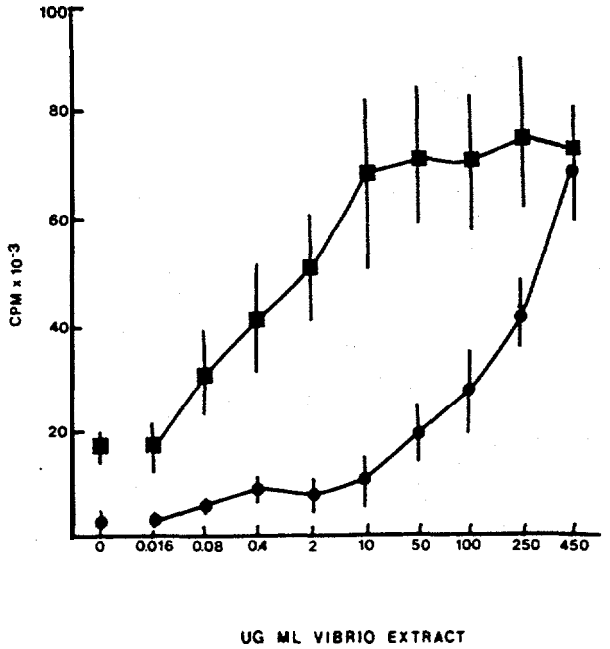
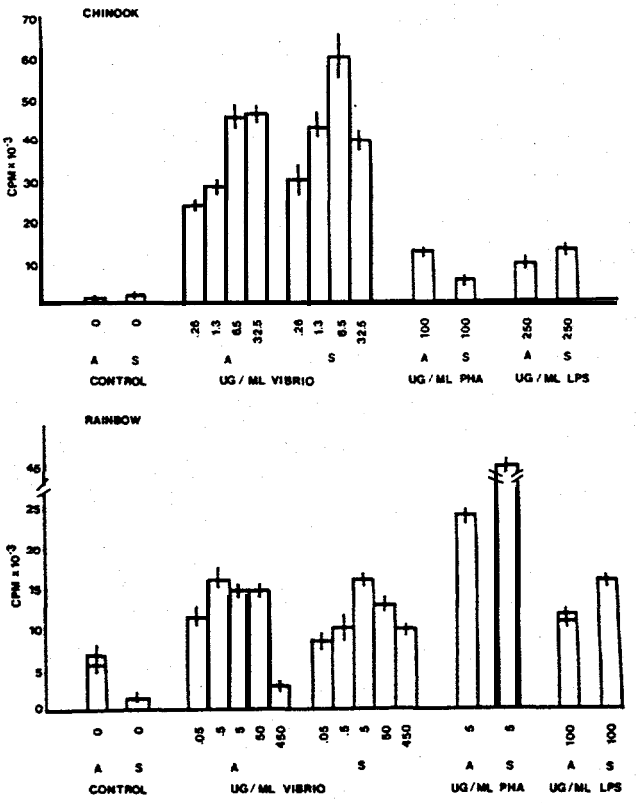


FIG. 1

Mitogenic responses of coho lymphocytes to Vibrio anguillarum extract. Splenic (●) and anterior kidney (■) lymphocytes were cultured with various doses of Vibrio extract and mitogenesis determined by the uptake of tritiated thymidine. Data given represent the mean CPM ± one standard error. Seven fish were used at each dose for the splenic responses, except for the 450 ug/ml dose which is represented by five replicates. Fifteen fish were used at each dose for the anterior kidney responses, except for the 100 ug/ml and 450 ug/ml doses which are represented by 14 and 10 replicates, respectively.

FIG. 2

Mitogenic responses of chinook salmon and rainbow trout lymphocytes to Vibrio anguillarum extract. Anterior kidney (A) and splenic (S) lymphocytes from chinoook salmon and rainbow trout were cultured with various doses of Vibrio extract and optimal doses of PHA and LPS. Mitogenesis determined by the uptake of tritiated thymidine (H_3T). Data are presented as the mean CPM + one standard error of trip&ate cultures.



Kinetics of VA mitogenesis with coho lymphocytes

VA demonstrates similar kinetics of tritiated thymidine uptake as is seen with other mitogens such as E. coli LPS and PHA (Fig. 3). Maximal proliferation appears to occur between day 4 and 5 for all mitogens in the anterior kidney, maximal proliferation for VA and PHA also appears to occur during this time period with splenic lymphocytes, whereas peak proliferation in response to LPS continues through day 6. There was no significant stimulation of cpm in the absence of antigen in the anterior kidney, however, a significant increase occurred between day 4 and 9 with the splenic lymphocytes.

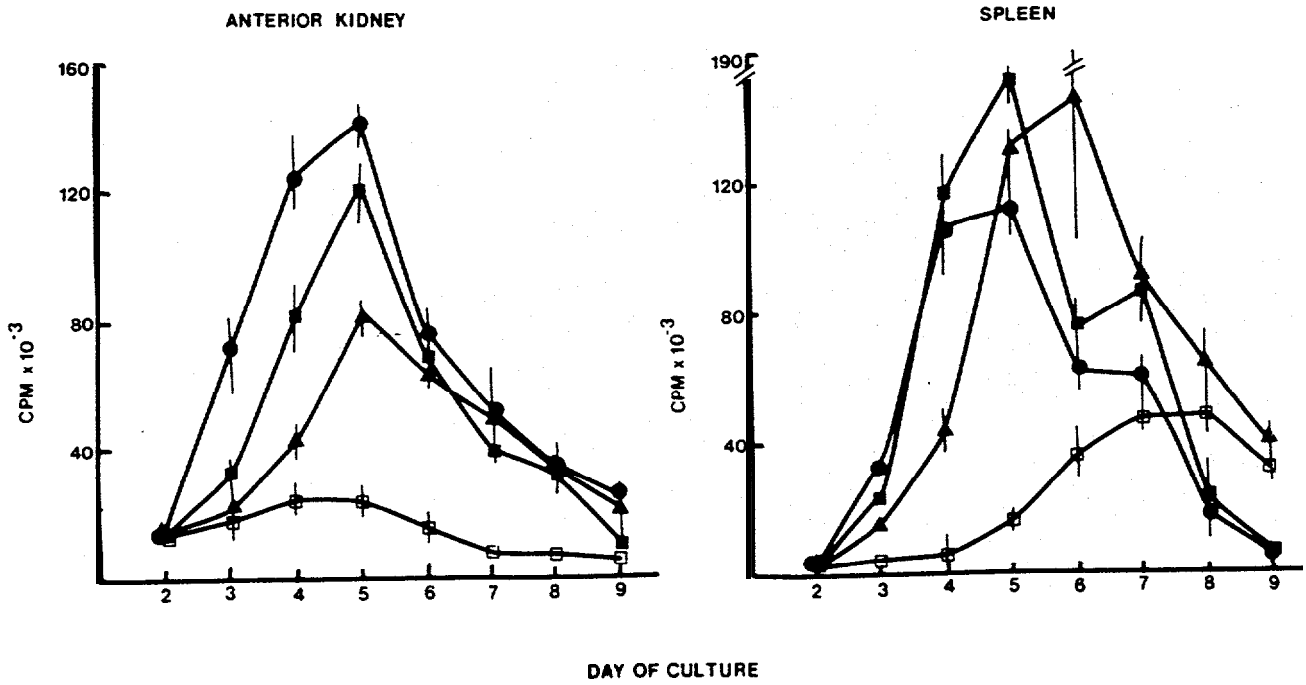


FIG. 3

Kinetics of mitogenesis. Anterior kidney and splenic lymphocytes were cultured with optimal concentrations of the Vibrio extract (■ ,250 ug/ml), LPS (▲ ,100 ug/ml), PHA (● ,5ug/ml), or without mitogen (□). Each day represents the CPM of cultures harvested after a 24 hour pulse of tritiated thymidine. Data are presented as the mean CPM ± one standard error of triplicate cultures.

Polyclonal activation of coho B cells by Vibrio extract as assessed by PFC production

Vibrio extract was able to stimulate PFC responses in anterior kidney and splenic lymphocytes to TNP and fluorescein haptenic determinants (Fig. 4). These responses were comparable to those elicited by PHA or LPS.

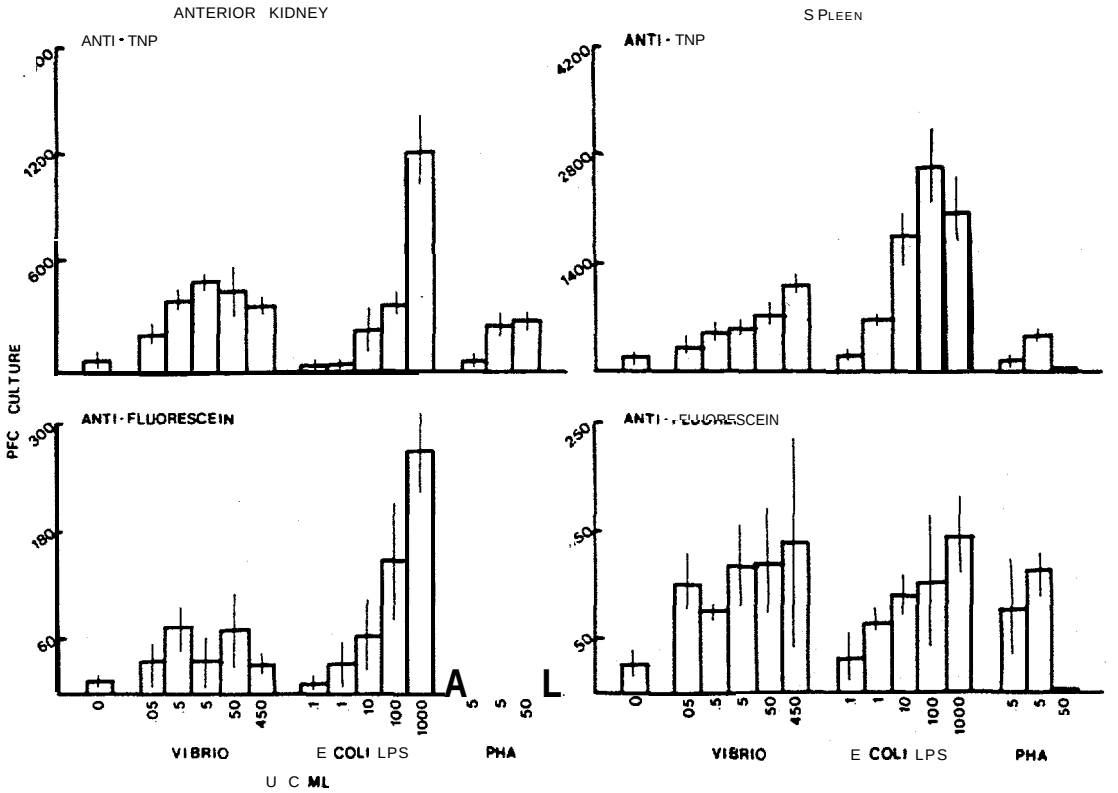


FIG. 4

Polyclonal activation of coho lymphocytes with Vibrio extract. The ability of optimal concentrations of Vibrio extract, LPS, and PHA to induce anti-TNP and anti-fluorescein plaque-forming cell is depicted. No PFCs were detected against unhaptenated SRBC. Data are presented as the mean PFC/culture ± one standard error of triplicate cultures.

Mitogenesis and polyclonal activation of coho lymphocytes by Vibrio anguillarum lipopolysaccharide (Westphal)

One of the most likely products of a gram negative organism that would induce lymphocyte proliferation and antibody secretion would be lipopolysaccharide. Thus to determine if V. anguillarum lipopolysaccharide can stimulate such responses we cultured a Westphal extract of V. anguillarum (Table I) with coho splenic lymphocytes.

Table I. Mitogenesis and Polyclonal Activation by *V. anguillarum* Lipopolysaccharide (Westphal)

V. anguillarum LPS (ug/ml)	PFC/culture[1]	CPM[2]
40	247 ± 17	26443 ± 1822
4	209 ± 28	16091 ± 1791
.4	209 ± 40	11502 ± 779
0	90 ± 10	8845 ± 403

1. Data on day nine is presented as the mean number of TNP plaque-forming cells/culture ± one standard error of triplicate cultures. No PFC were detected against unhaptenated SRBC.
2. Data on day five is presented as mean CPM ± one standard error of triplicate cultures on day five.

The data demonstrate that *V. anguillarum* lipopolysaccharide is capable of inducing TNP plaque-forming cells as well as lymphocyte proliferation.

DISCUSSION

A commonly used somatic antigen extract of *Vibrio anguillarum*, a pathogen of marine fish including salmonids, induces a high level of specific immunity *in vivo* (16,17). A possible reason for the immunogenicity of this extract, as well as for killed cells (30-32) and other *V. anguillarum* extracts (33) may be the existence of substance(s) that are mitogenic or polyclonal activators for fish lymphocytes.

Utilizing a culture system for the expression of lymphocyte proliferation and antibody secretion (18,19) we demonstrate that *Vibrio anguillarum* somatic antigen extract (VA) contains a.mitogenic substance(s) which stimulates anterior kidney and splenic lymphocytes (Fig. 1 and 2) of all three salmonid species tested (coho salmon, chinook salmon and rainbow trout). The mitogenic substance(s) appears to stimulate nonspecific lymphocyte proliferation and B cell differentiation in coho salmon lymphocytes, as determined by incorporation of tritiated thymidine and non-specific antibody production (Fig. 4). These responses suggest that the *Vibrio* extract acts as a polyclonal activator, in a similar manner to LPS and PHA (18).

The kinetics of mitogenic stimulation is also quite similar to other mitogens PHA and *E. coli* LPS tested suggesting a similar mode of stimulation. A likely candidate for such non-specific stimulation would be the lipopolysaccharide (LPS) of *V. anguillarum*. Previous work with mouse lymphocytes (34) has demonstrated the mitogenicity and adjuvant-like effects of *V. anguillarum* LPS. Using the same extraction procedures (25) we have isolated *V. anguillarum* lipopolysaccharide and cultured comparable concentrations (on the basis of carbohydrate content) to mitogenic concentrations of the somatic antigen. It can be seen from Table I that this LPS extract of *V. anguillarum* also possesses mitogenic and polyclonal activating properties for salmon lymphocytes.

The fact that Vibrio anguillarum is an important fish pathogen (20,21), and the ability of this extract to cause such potent in vitro immunological responses, suggests that this material may have some role in a immunity from this disease. Thus, it may have some practical application, in either crude or purified form, through conjugation to other non-immunogenic antigens to produce immunogenic vaccines.

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MANUSCRIPT #8

BACTERIAL KIDNEY DISEASE: THE POTENTIAL ROLE OF SOLUBLE PROTEIN ANTIGEN(S)

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BACTERIAL KIDNEY DISEASE : THE POTENTIAL ROLE OF SOLUBLE PROTEIN ANTIGEN(S)

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ABSTRACT

Soluble protein antigens, isolated from the supernatants of Renibacterium salmoninarum cultures, have been found to suppress the in vitro antibody responses of coho salmon, Oncorhynchus kisutch, lymphocytes and to be associated with decreasing haematocrit values in vivo.

INTRODUCTION

Renibacterium salmoninarum is responsible for one of the most devastating salmonid bacterial diseases in the world (Fryer & Sanders,1 98 1), bacterial kidney disease (BKD). Of major epidemiologic importance is the fact that this disease is not only a big problem in hatchery-reared salmonids, but also in wild populations (Mitchum et al., 1979).

Unfortunately, little information has been forthcoming as to the possible molecular mechanisms of the pathogenesis of BKD. This study, therefore, focuses on the possible toxigenic role(s) that R. salmoninarum soluble proteins (SP) may have in BKD pathology.

MATERIALS AND METHODS

Renibacterium salmoninarum soluble antigens were prepared as described by Getchell et al. (1985). The effects of SP on the *in vitro* antibody responses of normal and infected coho salmon to trinitrophenylated-lipopolysaccharide (TNP-LPS; Jacobs & Morrison, 1975) were examined using *in vitro* tissue culture medium (TCM) and techniques (Kaattari & Yui, 1987). Prior to culture addition, SP was diluted in tissue culture medium and filter sterilized (0.45 urn). Haematocrit values were assessed using heparinized blood samples taken from 5 fish at 10 intervals after intraperitoneal injection of 0.1 ml of 1 O.D. (500 nm) live *R. salmoninarum*. The plasma portions of these samples were analyzed for the concentration of SP by use of an ELISA procedure (P. Turaga et al., in prep.).

RESULTS

In vitro cultures of anterior kidney lymphocytes, stimulated with an optimal concentration of TNP-LPS, were suppressed by 10 and 100 $\mu g\ ml^{-1}$ SP (Fig. 4.1). Comparable concentrations of the control protein, chicken ovalbumin, were not suppressive. This suppression was not due to a toxic effect,

since control and suppressed cultures expressed equivalent cellular viability as assessed by trypan blue exclusion staining.

Anterior kidney lymphocytes from normal and infected. fish were cultured with TNP-LPS (Fig. 4.2). Cultures of lymphocytes from infected fish (possessing 30-80 μg ml- 1 serum SP) demonstrated a marked suppression as compared to lymphocytes from normal fish. This suppression was comparable to the suppression seen when normal lymphocytes are co-cultured with 100 ug ml- 1 SP.

Examination of experimentally infected salmon at various times post-injection revealed a distinct association of decreasing haematocrit with increasing levels of SP antigen in the serum (Fig. 4.3).

DISCUSSION

These studies demonstrated that soluble antigens produced by R. salmoninarum are capable of suppressing the in vitro antibody response, and are associated in vivo with decreasing haematocrit values.

Studies concerning the mechanisms of pathogenesis for R. salmoninarum have primarily been limited to the analysis of the histopathology (Wood & Yasutake, 1956; Hendricks & Leek, 1975; Lester & Budd, 1979; Young & Chapman, 1978) and the appearance of abnormal clinical indices (Hunn, 1964). Although the initial focus of the infection appears to be the kidney (Wood

& Yasutake, 1956) with subsequent haematological dysfunction (Hunn, 1964), the disease eventually becomes systemic, with lesions occurring in many organs and tissues.

The identification of R. salmoninarum toxins has not been forthcoming, except for the detection of an haemolysin-like activity found in formalinized cells (Bruno & Munro, 1986). Those authors have suggested that a putative toxin may be responsible for the decreases in haematocrit values and for indices of splenomegaly associated with the disease. Our observations of increasing serum SP levels associated with decreasing haematocrits lend support for that pathogenic mechanism.

The in vitro antibody assay revealed that a non-cytotoxic antigen(s) was capable of suppressing the production of the antibody response. Of particular interest were the observations in the present study of a decrease in the number of adherent (e.g. macrophage) cells upon culture with SP. Recent studies with catfish (Miller et al., 1985) and with coho salmon lymphocytes (Tripp & Kaattari, in prep.) reveal that antibody responses to TNP-LPS require adherent cell function. Since antibody responses from lymphocytes from infected fish appear suppressed (Fig. 2), it may be possible that immune dysfunction in vivo could be mediated by the elaboration of these antigens. Further studies, however, will be required to determine the role of alternative in vivo mechanisms (e.g. bacterial destruction of tissues, or contamination due to live R. salmoninarum) involved in the reduction of antibody-producing cells from infected fish.

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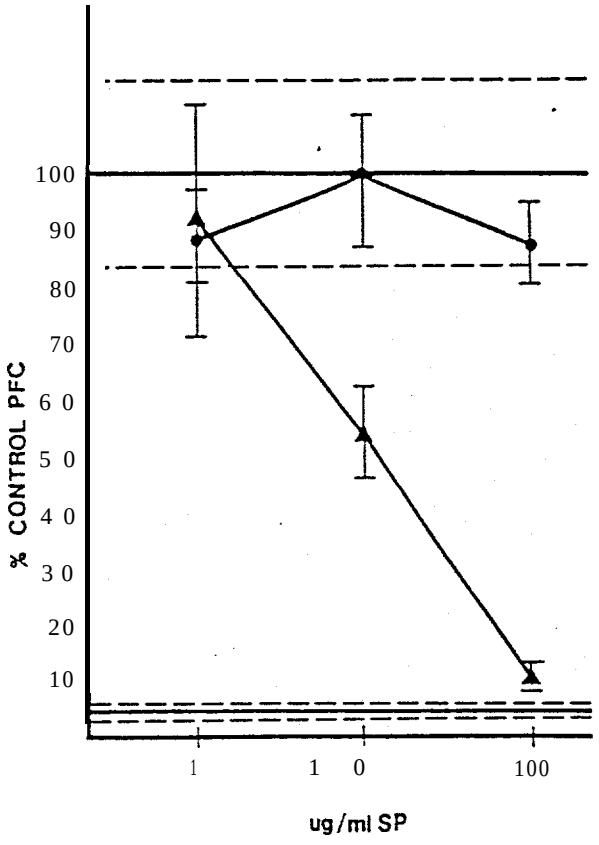

Fig. 4.1. Lymphocytes from anterior kidney were cultured with TNP-LPS in the presence of 1, 10, 100 ug SP (▲) or ovalbumin (•). The control response (without protein addition) was equal to 431 + 21 antibody-producing cells per 10^6 lymphocytes. Each point represents the mean of triplicate cultures and bars + 1 S.E.

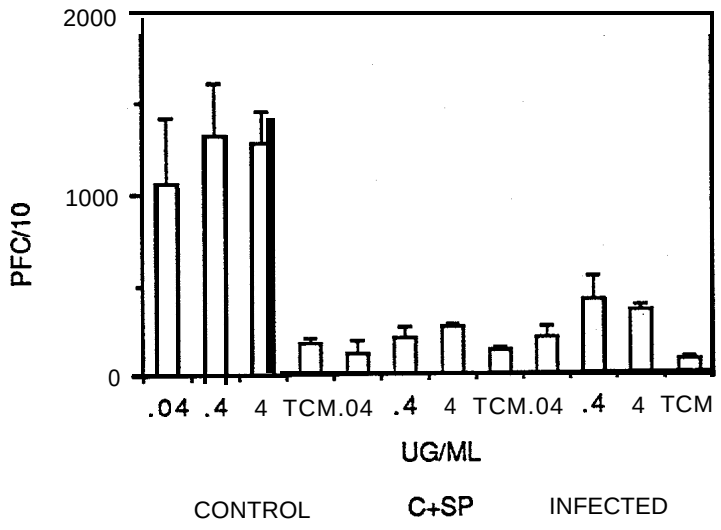

Fig. 4. 2. Lymphocytes from the anterior kidneys of normal and infected salmon were cultured with 0.04, 0.4, and 4.0 ug ml^{-1} of TNP-LPS concentrations. A portion of the normal lymphocytes were also cultured with 100 ug ml^{-1} SP (a concentration comparable to that seen in infected fish). Each histogram represents the mean of triplicate cultures and + 1 S.E. are indicated.

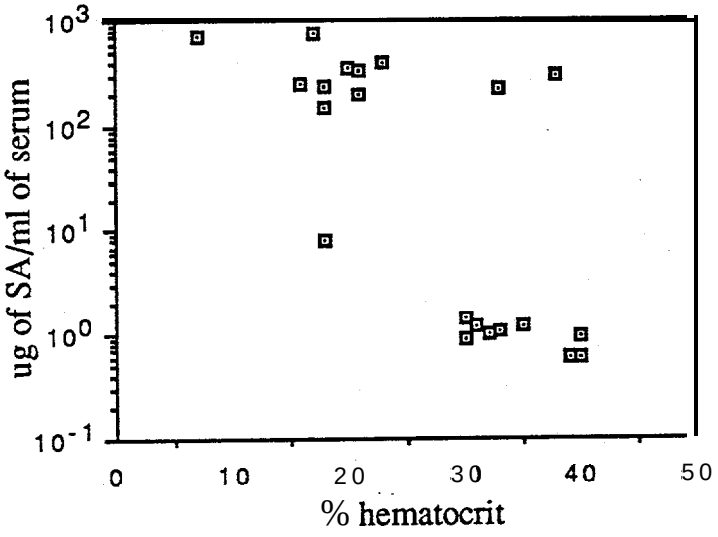

Fig. 4. 3. Haematocrit values, expressed as percentage packed red cell volume, plotted versus the corresponding SP serum concentration for individuals sampled at various stages of infection.

MANUSCRIPT #9

CHARACTERIZATION OF A LEUKOAGGLUTINATING AND MACROPHAGE INDUCING PROTEIN PRODUCED BY RENIBACTERIUM SALMONINARM

To be submitted

Characterization of a Leukoagglutinating and Macrophage Inducing Protein Produced by Renibacterium salmoninarum

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To Be Submitted to Diseases of Aquatic Animals

Abstract

Renibacterium salmoninarum, a gram-positive fish pathogen, produces large quantities of a 57-58 kD protein which can be isolated from infected fish tissue, the surface of the bacteria, or bacterial culture supernatants. Getchell et al., (1985) identified this protein as antigen F, which is common to all tested isolates of R. salmoninarum. We have previously shown that concentrated culture supernatant inhibits the in vitro antibody response to an unrelated antigen, trinitrophenylated-lipopolysaccharide. Correlated with the inhibition of the antibody response was the in vitro agglutination of both surface Ig-positive and negative leukocytes and the induction of large, esterase-positive cells. Coho salmon (Oncorhynchus kisutch) leukocytes from the anterior kidney, spleen and peripheral blood were agglutinated, however, erythrocytes were not. Peripheral blood leukocytes from other salmonids were also found to be agglutinated by this protein. Preliminary evidence suggests that the 57-58 kd protein directly binds fish leukocytes.

Introduction

Renibacterium salmoninarum is a small gram-positive bacterium responsible for Bacterial Kidney Disease (Fryer and Sanders, 1981). The bacterium causes chronic mortality within salmonid populations, and can be vertically transmitted from adult to fry through the egg (Evelyn et al. 1984). Limited information exists concerning possible virulence factors produced by R. **salmoninarum** which may account for the pathogenesis of infection. Bruno and Munro (1983)

have described catalase, DNAse, casein and gelatin protease, and hemolysin activities common to 25 isolates, however, these properties have not been correlated with virulence. However, auto-agglutination and cell surface hydrophobicity has been correlated with increased mortality and with a decrease in time until death (Bruno 1988). It has been suggested that the hydrophobic cell surface may aid in phagocytosis (Daly and Stevenson, 1978; Bruno, 1988) where the bacterium can reportedly survive intracellularly (Young and Chapman, 1978; Bruno, 1986). Cell surface hydrophobicity is decreased after protease treatment suggesting a protein component may be responsible for this property (Daly and Stevenson, 1987).

Getchell et al. (1985) described a predominant 57 kD protein antigen, common to ten isolates of the bacterium, named antigen F. Turaga et al. (1987b) have shown that this protein is found in sera from experimentally infected fish. We have found that a 57-58 kD protein doublet is a major component of a crude soluble protein fraction from spent bacterial culture supernatants. Turaga et al. 1987a) have shown that soluble protein(s) are able to suppress the in vitro antibody response to an unrelated antigen, trinitrophenylated-lipopolysaccharide. Similarly, experimentally infected coho salmon have a decreased in vitro ability to respond to TNP-LPS. The immunosuppressive factor has been recently shown to be inactivated by an endogenous serine protease after preincubation at 370C for more than 12 hr (Turaga and Kaattari in preparation). In this manuscript we describe protease sensitive, leukoagglutinating, and macrophage inducing activities of the soluble protein(s) which may contribute to the virulence of the bacterium.

METHODS

Soluble Protein Preparation

Renibacterium salmoninarum ATCC 33209 was grown in 1 liter flasks with constant shaking at 170C. KDM-II medium was prepared according to Evelyn, (1977) except no serum was added, and the medium was ultrafiltered using a PTGC-10,000 NMWL filter packet (Millipore, Bedford, MA) to remove molecules larger than 10 kD. After 10 days growth, which generated an O.D. greater than 1.0 (500 nm), soluble protein was harvested by the method of Turaga et al. (1987b). Briefly, bacterial cells were removed by centrifugation at 6000 x g for 30 min and the supematant was concentrated 10x by ultrafiltration. The retentate was further concentrated by two 50 % saturated ammonium sulfate precipitations. To remove ammonium sulfate, the soluble protein was dialyzed against 3 one liter changes of10 mM phosphate buffered saline (pH 7.4)(PBS), and one 1 liter change of RPMI 1640 (Gibco, Grand Island, NY). The soluble protein was filter sterilized (0.45 µm) and stored at 4oC.

Peripheral Blood Leukocyte Separation

Two to eight ml of peripheral blood was collected in sterile heparin-coated tubes (Venoject, Terumo Medical, MD) from the caudal vein of 400-600 gm coho salmon (Oncorhynchus kisutch), chinook salmon (Oncorhynchus tshawytscha), cutthroat trout (Oncorhynchus clarkii) or rainbow trout (Oncorhynchus *mykiss*). Three ml of blood was diluted four-fold in RPMI 1640 and centrifuged at 500 x g for 10 min to remove plasma and heparin. The cells were resuspended to the

original volume in RPMI and 10 ml of this suspension was carefully layered on top of 10 ml of histopaque 1077 (Sigma, St. Louis, MO). The layered preparation was then centrifuged at 800 x g for 30 min which pelleted the erythrocytes while the leukocytes remained at the interface. The cells were gently removed with a pasteur pipette and centrifuged for 13 min at 500 x g, and resuspended in complete RPMI (Yui and Kaattari, 1987).

Leukoagglutination Assay

Fifty ul of anterior kidney, spleen or peripheral blood leukocytes at a concentration of 2 x 107 cells/ml were co-incubated with 50 µl of either soluble protein, or a control protein (hen egg ovalbumin), or concentrated KDM-II medium without sera. These cultures were performed in 96 well plates (Corning, Coming, New York), incubated at 17°C in at 10% blood gas mixture. Wells were visually examined for leukocyte agglutination and photographed after one or four days.

Immunofluorescence Staining

Leukocytes were removed from culture after one or four days by gentle aspiration with a pasteur pipette. Smears were made on pre-cleaned glass slides which were fixed with acetone for 5 min and allowed to air dry for 30 min. After a 5 min wash in PBS the slides were incubated for 1 hr with a 1:1000 dilution of McAb l-14 ascites (G. Warr). Excess antibody was removed by three 5 min washes with PBS and then incubated for 30 min with a 1:200 dilution of goat anti-mouse FITC conjugate (Sigma). After three,10 min rinses the slides

were mounted and observed under a standard microscope (Zeiss) utilizing a iV F1 epi-fluorescent condenser and a 12 V, 100 watt halogen tungsten light source.

Enumeration of Large, Vacuolated Cells (LVC)

Cells were removed after 5 days in culture and diluted 1:2 in 0.4% trypan blue in PBS. Viable cells were identified by having one or several large, clear vacuoles and were photographed at a 1000x magnification.

Non-Specific Esterase Staining of LVC

A non-specific alpha naphthyl acetate esterase stain was performed on coho anterior kidney and peripheral blood leukocytes according to the method of Pearse (1972).

Leukocyte Protein Extraction

Peripheral blood leukocytes (2 x 106) were mixed with either 40 µg of soluble protein, a control protein (monoclonal antibody 2G5) or tissue culture medium alone in a final volume of 200 µl. The cells were incubated at 170C for 3 hrs with shaking every 15 min. Free soluble protein was removed by five (500 x g) 1 ml washes with RPMI 1640. Leukocyte proteins were extracted by the modified method of Jones (1980). Ten ul of extraction buffer (10 mM Tris-HCl, pH 7.2, 0.15 M NaCl, 0.02% NaN3, 0.5% (w/v) Nonidet P40) was incubated with the cells for 15 min on ice and subsequently microfuged for 10 min to remove the cell nuclei. The supernatant was removed and frozen at -700C. Electrophoresis was performed using 0.75 mm, 12 %

SDS-polyacrylamide gel (Laemmli et al. 1970) and the mini-protean II (Bio-Rad). The proteins were transblotted onto nitrocellulose overnight with 30 V according to the method of Towbin et al. (1979).

Separation and Electroelution of Biotinylated Soluble Proteins

Assessment of protease activity was accomplished by the use of a purified and biotinylated 57 kD protein. Soluble protein was biotinylated according to the method of Kendall et al. (1983). Removal of endogenous protease in the soluble protein preparation was achieved by the electrophoresis of 700 µg of biotinylated soluble protein on a 10% SDS-PAGE preparative gel (Hoefer Scientific Instruments, San Francisco, CA). A current of 20 mAmps was applied for 3 hr while the sample dye was migrating in the stacking gel, and 30 mAmps during migration through the separating gel. Strips containing protein from the end of the gel and were stained with coomassie blue to identify sections containing the 57 kD protein. The corresponding sections of the unstained gel were excised with a razor blade and the proteins were electroeluted from the slices (Bio-Rad apparatus) for 12 hr with a current of 8-10 amps. The samples were subsequently dialysed in PBS and filter sterilized. The protein- concentration determined by the method of Lowry et al. (1951).

Determination of Protease Activity in 370C Treated Samples of Soluble Protein.

Two ug of heat-treated soluble protein was mixed with 0.8 µg of biotinylated and gel-purified 57 kD protein substrate for 18 hrs.

Samples were then electrophoresed in a 12% SDS polyacrylamide gel and assayed by Western blotting. The presence of biotinylated 57 kD protein was detected by probing for 1 hr with a 1:200 dilution of streptavidin horseradish peroxidase conjugate (Hyclone, Logan, UT) followed by the addition of substrate (4-chloro-naphthol).

RESULTS

Two novel biological activities were discovered after the addition of soluble protein to in vitro cultured salmon leukocytes. First, after several hours in culture leukocytes were observed to be agglutinated (Figure la) as opposed to control cultures (Figure lb). In addition, leukocytes from the coho anterior kidney, spleen and peripheral blood demonstrated the same agglutination as well as peripheral blood leukocytes from chinook salmon, rainbow, and cutthroat trout. Soluble protein concentrations above 5 μg/ml agglutinated leukocytes. High concentrations of bacterial cells (500 μg/ml wet weight of cells) were also found to agglutinate leukocytes. These large clumps of agglutinated cells contained both surface Ig+ and Ig- cells as determined by immunofluorescent staining with monoclonal antibody 1-14. Secondly, after two days in culture, large, vacuolated cells (LVC) appeared (Figure 2a,b). These viable cells varied in size and number of vacuoles. No LVC were observed in control cultures with ovalbumin or KDM-II. The number of LVC induced by the soluble protein was dose dependent, and always less than 5 % of the total number of leukocytes added to the initial culture (Figure 3). These cells were not adherent and stained positive with a non-specific esterase stain after removal

from culture and fixation on glass slides.

The protease sensitivity of this biological activity was ascertained by incubation at 370C for 18 hrs. Both the leukoagglutinating activity and the induction of large, vacuolated cells (LVC) was completely inhibited by 370C pre-treatment of the soluble protein. Concomitantly, SDS-polyacrylamide gel electrophoresis revealed the degradation of the 57-58 kD protein doublet due to the activity of a high molecular weight protease (Figure 4). The protease in the soluble protein preparation was still active against a endogenous substrate (biotinylated, electroeluted 57 kD protein) even after 370C treatment (Figure 5, lane D). Therefore, the protease does not appear to be responsible for the leukoagglutinating activity or the induction of large, vacuolated cells. In addition, the biological activity of the soluble protein is retained after 370C treatment if alpha-2 Macroglobulin (a biologically relevant protease inhibitor) is present.

The ability of soluble protein to bind to leukocytes was assayed by preparing leukocyte membrane extracts after incubation with soluble protein or a control protein. The 57-58 kD protein bound to leukocytes while none of the lower molecular weight proteins found in the soluble protein preperation were present (Figure 6A, lane D). Binding was deemed specific because the control protein was not detected in the membrane extracts (Figure 6A, lane B). Equal amounts of leukocyte extracts were run in each lane as determined by the total protein stain (Figure 6B).

DISCUSSION

Renibacterium salmoninarum produces a large quantity of a 57-58 kD protein which can be identified on the cell surface, in culture supernatant, and in infected fish tissue and serum (Wiens and Kaattari, 1989). Previous studies have demonstrated a protease sensitive immunosuppressive factor present in concentrated bacterial culture supernatants (Turaga et al. 1987a; Turaga and Kaattari, in preparation). In order to further elucidate the role of this protein in the pathogenesis of BKD we have cultured leukocytes with R. salmoninarum soluble protein. In this manuscript we describe leukoagglutinating and macrophage inducing activities of this soluble protein.

The extent of leukoagglutination was dependent on the concentration of soluble protein, with a lower threshold of 5 ug/ml. These tested concentrations of soluble protein are present in the sera of experimentally infected fish (Turaga et al. 1987b). A 57-58 kD protein was present in agglutinated leukocyte membrane extracts suggesting that this protein may cause the observed agglutination. Daly and Stevenson, (1987) have identified a rabbit hemagglutinin which has a molecular weight of 57 kD.

The leukoagglutinating and hemagglutinating activity of the 57-58 kD protein resembles a number of non-fimbrial proteins which function as adhesion molecules for bacteria (Jones and Isaacson, 1983; Arp, 1988). Daly and Stevenson, (1987) and Bruno, (1988) have proposed that the hydrophobic cell surface may enhance attachment of bacterial cells to phagocytes. The 57-58 kD protein may contribute to the hydrophobicity and/or serve as a specific receptor which

recognizes a ligand on the leukocyte cell surface resulting in attachment and intracellular invasion. The non-specific esterase staining suggests that the vacuolated cells are monocytes/macrophages, or a subset thereof. Possibly, these cells have endocytosed large quantities of the 57-58 kD protein causing the appearance of the large vacuoles. Chang and Andersen, (1974) have described bone marrow macrophages with a similar mophology when the cells are cultured with high concentrations of serum.

Since the soluble protein has been found to produce in vitro suppression of leukocyte function (i.e. production of PFC), the observation of this proteins adherence to leukocyte membranes suggests that it may be a likely candidate for the induction of this suppression. Further *in* vivo functional studies are required to determine the biological role of the 57-58 kD protein. Determination of in vivo activities will be important for future development of potential toxoid vaccines.

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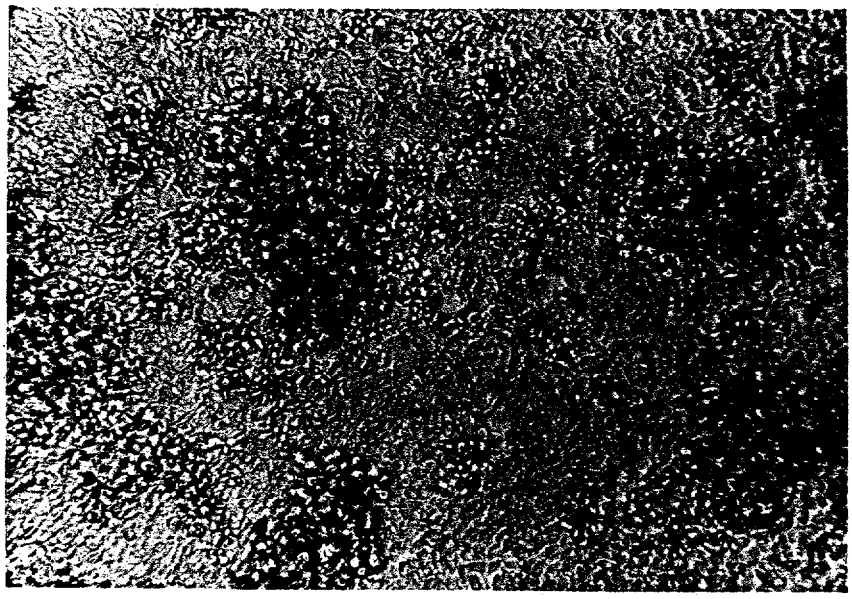

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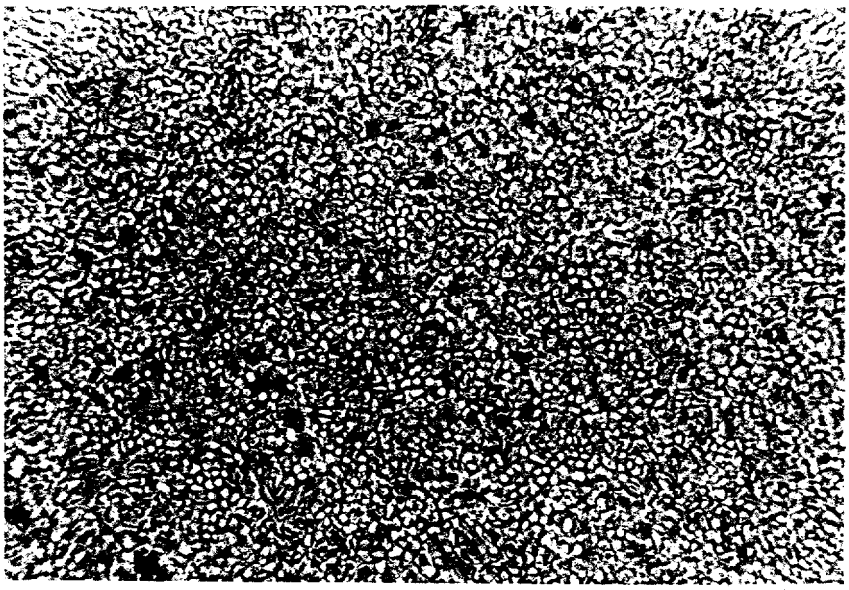
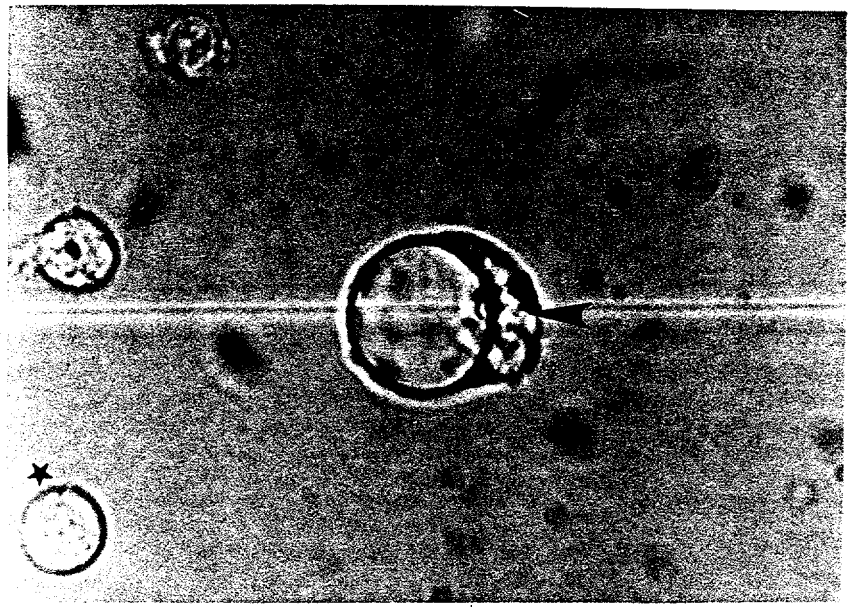


Figure 1. Incubation of coho anterior kidney cells with 100 ug/ml of soluble protein (A), or without soluble. protein (B) after 3 days in culture. Arrows identify agglutinated clumps of leukocytes while erythrocytes appear non-agglutinated (160x).

A



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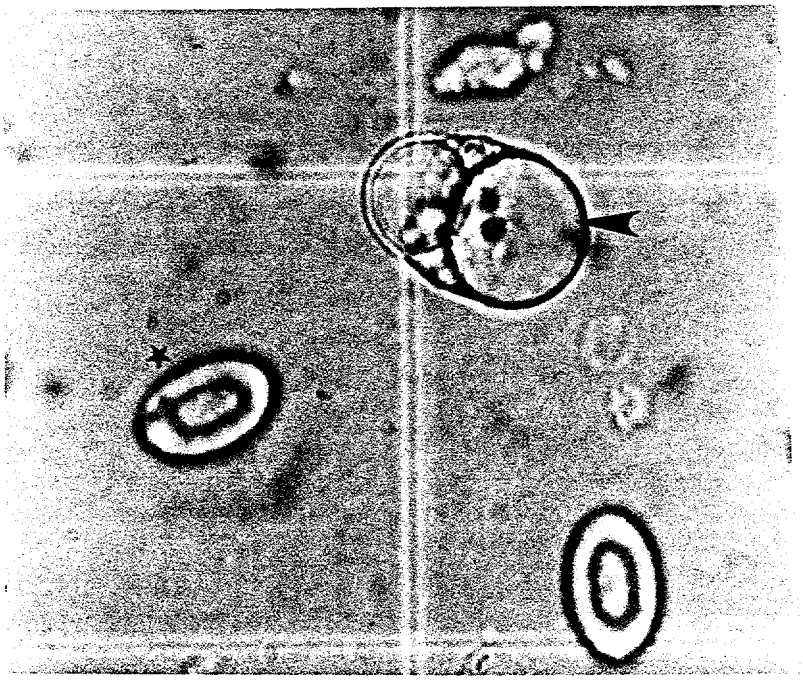

Figure 2. Large vacuolated cells (arrows) induced after incubation with 100 ug/ml soluble protein for 2 days. A star indicates a lymphocyte (A) and an erythrocyte (B) for size comparison (1000x).

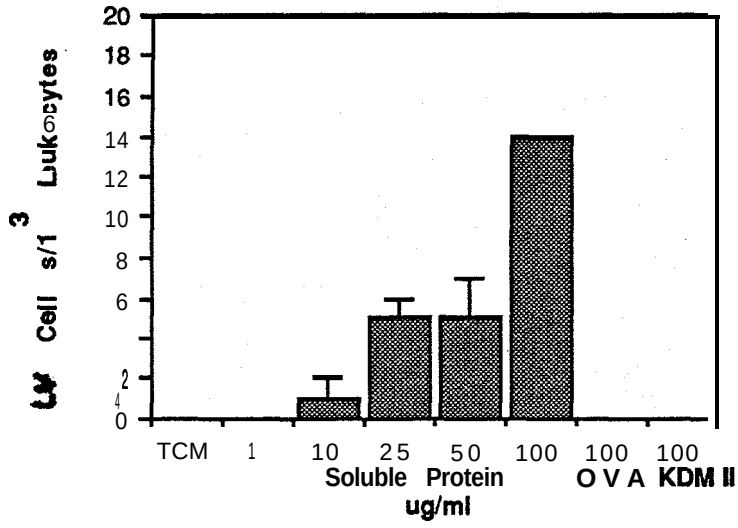

Figure 3. Enumeration of viable, large, vacuolated cells (LVC) after 5 days incubation at 17°C. Anterior kidney leukocytes were counted using a hemocytometer and graphically represented as a fraction of the total number of leukocytes added to culture. No LVC were observed upon the addition of 100 μg/ml control protein, hen egg ovalbumin, or KDM-II.

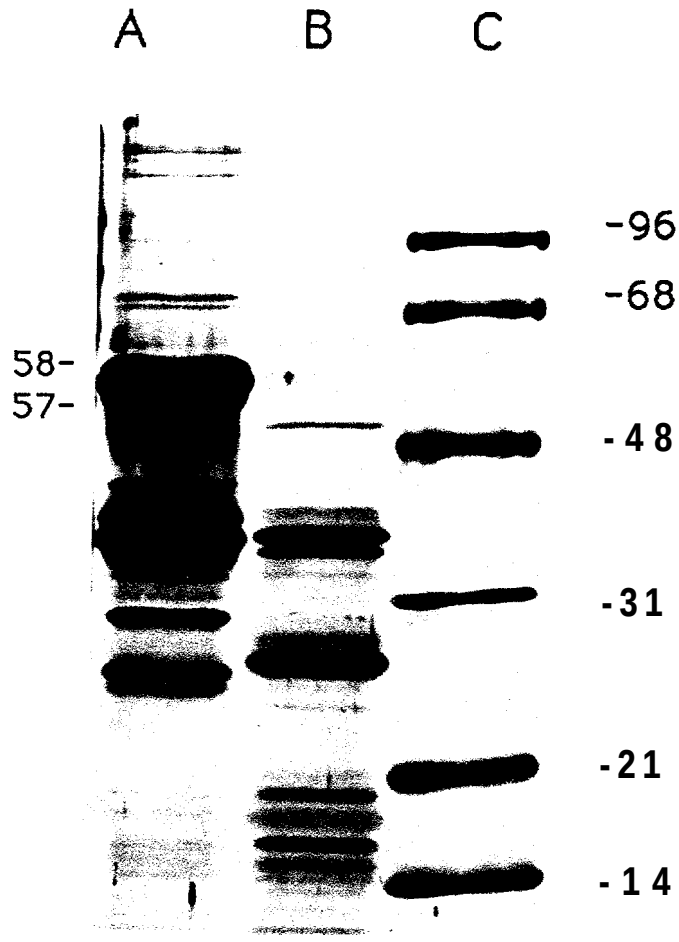

Figure 4. Effects of 18 hr, 37°C pretreatment (lane B) or 4°C pretreatment (lane A) on soluble proteins. Four µg of protien/lane was electrophoresed and transferred to nitrocellulose and stained for total protein. Lane C contains Bio-Rad low molecular weight markers.

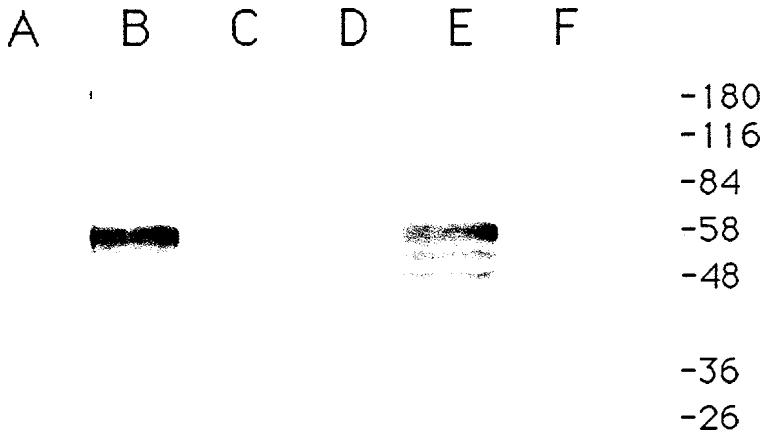

Figure 5. Western blot of purified and biotinylated 57 kD protein (B-57) used as a substrate to detect the presence of proteolytic activity. Lanes are as follows: A) Pre-stained molecular weight markers, B) B-57 kD alone, C) B-57 + soluble protein, D) B-57 + 37°C treated soluble protein, E) B-57 + pre-boiled soluble protein. The presence of biotinylated substrate was detected by the addition of avadin horseradish peroxidase and substrate.

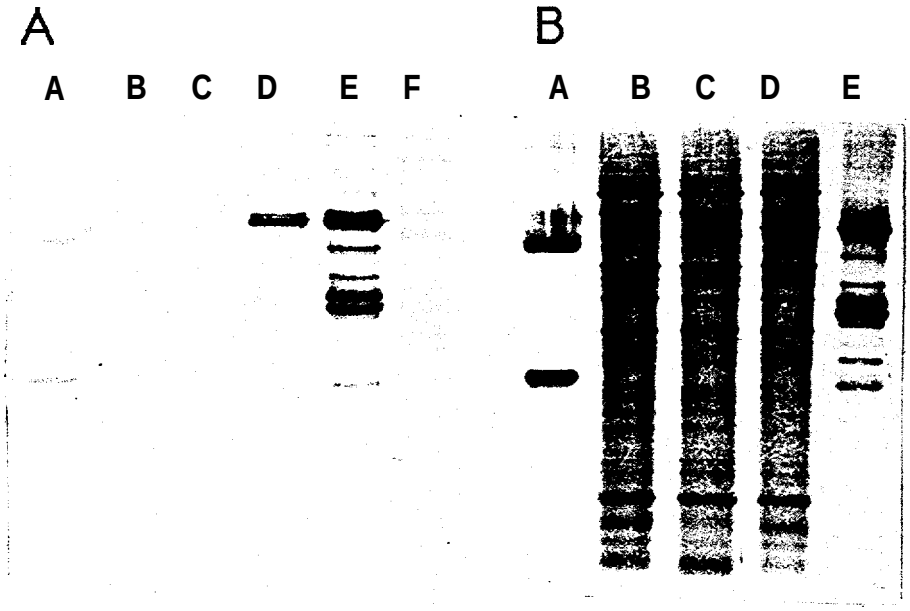

Figure 6. Western blot (A) and total protein stain (B) of coho salmon peripheral blood leukocytes incubated with or without soluble protein or with a control protein (protein A purified monoclonal antibody 2G5). The Western blot was developed with 5 μg/ml monoclonal antibody 4D3 as a primary antibody and a 1:500 dilution of goat anti-mouse horseradish peroxidase as the secondary antibody. The lanes are as follows: A) 2 μg control protein 2G5, B) Leukocytes + control protein, C) Leukocytes alone, D) Soluble protein + leukocytes, E) 2 μg of soluble protein, and F) Pre-stained molecular weight markers (Sigma).

MANUSCRIPT #10

IDENTIFICATION AND CHARACTERIZATION OF A PROTEASE ACTIVITY ASSOCIATED WITH A SOLUBLE ANTIGEN PREPARATION OF *RENIBACTERIUM SALMONINARUM*

To be submitted

IDENTIFICATION AND CHARACTERIZATION OF PROTEASE ACTIVITY ASSOCIATED WITH A SOLUBLE ANTIGEN PREPARATION OF RENIBACTERIUM SALMONINARUM

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ABSTRACT

A protease activity associated with a soluble antigen preparation of Renibacterium salmoninarum was identified and partially characterized. It was shown that the protease activity against the soluble antigen was more pronounced at a temperature of 370C and above and not at 4°C, 170C and 230C. Phenyl methane sulfonyl fluoride (PMSF), L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butane (TPCK), alpha 2-macroglobulin (alpha-2M) and 50%, but not 10%, ethanol and methanol were found to be inhibitors of the protease activity. Phosphorylase B, bovine serum albunin and soybean trypsin inhibitor were also found capable of serving as substrates. We also demonstrate that this protease activity can act to neutralize the immunosuppression induced by the soluble antigen.

INTRODUCTION

Renibacterium salmoninarum is the causative agent of one of the most devastating diseases of salmonid fish, bacterial kidney disease (BKD) (Fryer and Sanders, 1981). The bacterium

is a gram positive, pleomorphic, diplobacillus and is classified as a unique species and genus (Sanders and Fryer, 1980). It requires cysteine for growth (Evelyn, 1977) and is a slow growing bacterium even on the best of the defined media. It has been shown that there is uniformity of biochemical properties among the different isolates tested (Bruno and Munro, 1985; Fiedler and Draxl, 1986). A unique peptidoglycan structure as well as an unusual cell wall polysaccharide incorporating N-acetyl fucosamine as one of the constituents has been identified (Fiedler and Draxl, 1986). Smith (1961) has shown that protease and catalase activities have been associated with the bacterium and Bruno and Munro (1986) have demonstrated both DNAase and hemolysin activities. In more recent studies, hydrophobic and hemagglutinating properties have been demonstrated (Daly and Stevenson, 1987) as well as a relationship between auto-agglutination, cell surface hydrophobicity and virulence of the bacterium (Bruno, 1988). Except for the studies of Bruno (1988), none of the biochemical factors or activities described for the bacterium have been shown to be associated with the virulence of the bacterium.

This study represents the first report on the identification and characterization of protease activity associated with a soluble antigen preparation of Renibacterium salmoninarum. We also show the relationship of protease activity and its biological activity, in vitro.

MATERIALS AND METHODS

Prenaration of Soluble Antigen

Renibacterium salmoninarum was cultured in KDM-2 (Evelyn, 1977) medium without serum supplementation, for 10-14 days in shake flasks at 17° C. The culture was then centrifuged at 4000 x g for 30 minutes at 4oC and the supernatant was processed for the isolation of soluble antigen (SA) according to the method of Turaga et al. (1987a).

Buffers

Phosphate buffered saline (PBS), pH 7.2 and 0.01 M, was prepared by dissolving 7.5 g of NaCl, 0.245 g of KH_2PO_4 and 0.809 g of Na_2HPO_4 in 1 liter of distilled water. The PBS used in protein staining was prepared by dissolving 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.2 g of KH_2PO_4 in 1 liter of distilled water. Tris base 6.07 g, EDTA 0.3 g and NaCl 8.7 g were dissolved in distilled water to prepare tris buffered saline (TBS), pH 8.2.

Lymnhocyte Culture Media

Different media preparations used in lymphocyte culture and plaque assay were prepared as described by Kaattari and

Yui (1987). Tissue culture medium (TCM) consisted of RPMI 1640 containing L-glutamine and sodium bicarbonate was supplemented with 10% fetal calf serum, 50 μg/ml gentamicin, 50 μM 2-mercaptoethanol and 10 μg/ml each of adenosine, uracil, cytosine and guanine (Sigma, St. Louis, MO). Holding medium consisted of 100 μg/ml gentamicin, 10% fetal calf serum (Whittaker M.A. Bioproducts) in RPMI 1640 (Gibco, Grand Island, NY) with bicarbonate supplementation. All the components were purchased from Whittaker M. A. Bioproducts, unless otherwise noted.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

The method followed was basically that of Laemmli (1970). Separating gels of 10% polyacrylamide (Sigma, St. Louis, MO) and stacking gels of 3% polyacrylamide were used in all the experiments. The apparatus used was a mini gel electrophoresis unit (Idea Scientific, Corvallis, OR) and the power source was an EC 600 (E-C apparatus Corporation, St. Petersburg, FL). The samples were mixed with the sample buffer (120 mM tris base, 4% w/v SDS, 10% v/v 2-mercaptoethanol, 20% w/v glycerol, and 3mM bromophenol blue) at a 1:1 ratio and placed in boiling water bath for 3 minutes prior to electrophoresis. The samples were usually loaded in a duplicate fashion so that one half of the gel could be stained for protein while the other half was used for Western blotting. Samples containing about 5 μg of protein were

applied to each lane. During the electrophoresis a constant current of 10 mA was applied during migration through the stacking gel and 20 mA during migration through the separating gel.

Transblotting

After the electrophoresis, the protein bands were transferred from the gel onto nitrocellulose (NC) paper (Bio Rad, Richmond, CA), using transblot apparatus (Bio Rad, Richmond, CA) (manufacturers instructions; Towbin et al., 1979). The transblotting was done in transblotting buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol) and by applying 100 V for one hour. The transblot buffer was continuously cooled by placing a cooling unit in the transblot chamber and constantly stirring the buffer. After transblotting the nitrocellulose paper was processed for protein staining and/or Western blotting.

Protein Staining

After transblotting, the NC paper was incubated in 0.3% tween 20 in PBS for 30 minutes at 300C. The NC paper was washed in the same buffer for 15 minutes with three changes of the buffer, while under constant agitation at room temperature. After a brief rinsing with distilled water, Aurodye^{R} forte (Janssen Life Sciences Products, Olen, Belgium) protein staining reagent was applied to the NC paper with constant shaking at

room temperature until dark purple bands became visible. This usually occurs in about 2-3 hours. The blot was then briefly rinsed with distilled water and blotted dry.

Western Blotting

The transblotted proteins (as described above) were alternatively examined for the presence of antigens by Western Blot analysis and the method was basically that of Towbin et. al. (1979). The NC paper was incubated in 3% bovine serum albumin (BSA) in T-TBS (0.1% tween-20 in TBS) solution at 370C for one hour. The paper was then washed with three changes of T-TBS during a period of 15 minutes, while under constant shaking. Rabbit anti-SA-biotinylated (Turaga et. al., 1987a) was used at a concentration of 5 μg in 10 ml of T-TBS, was incubated with the NC paper for one hour at room temperature. The NC paper was washed as above. A 1/100 dilution of streptavidin-peroxidase (Sigma, St. Louis, MO) in T-TBS was incubated with the NC paper for 30 minutes at room temperature. After washing the NC paper as above with T-TBS, the antigen bands were stained by incubating the NC paper with the substrate solution at 37° C till the bands were darkly stained. The substrate solution consisted of 10 ml of PBS, 2 ml of 4-chloronapthol (3 mg/ml in methanol; Bio Rad, Richmond, CA) and 10 μl of hydrogen peroxide.

Demonstration of Protease Activity Associated with Soluble Antigen

The presence of protease activity was demonstrated to affect soluble antigen (SA) by incubating it at 370C overnight and loss of prominent bands compared to original preparation on SDS-PAGE analysis.

Identification of alternate substrates for the protease was performed by incubating soluble antigen with both high and low molecular weight markers for SDS-PAGE analysis (Bio-Rad, Richmond, CA) at 370C. This facilitated the testing of a number of proteins as potential substrates. The proteins included among the high molecular weight markers were: myosin (200,000 d), ß galactosidase (116,250 d), phosphorylase B (97,400 d), bovine serum albumin (BSA) (66,200 d) and ovalbumin (43,000 d). · The low molecular weight markers were: phosphorylase B, BSA, ovalbumin, carbonic anhydrase (31,000 d), soybean trypsin inhibitor (21,500 d) and lysozyme (14,400 d). After overnight incubation of the soluble antigen with these markers at 37°C, the samples were subjected to SDS-PAGE analysis (Laemmli, 1970). Molecular weight markers were prepared by diluting the concentrate (1/30) in nanopure water. Soluble antigen (1 mg/ml in PBS) was mixed 1:1 with the prepared molecular weight markers and incubated at 370C .

Characterization of the Nature of the Protease Activity

The characterization was accomplished by using the protease inhibitors: phenyl methane sulfonyl fluoride (PMSF), L-1-chloro-3-[4-tosylamidol-4-phenyl-2-butane (TPCK), alpha-2 macroglobulin (alpha-2 M). Different starting concentrations of inhibitors and soluble antigen were: PMSF at 100 mM in methanol, TPCK at 20 mg/ml in absolute ethanol, alpha-2 M at 25 units/ml in sterile distilled water and soluble antigen at 1 mg/ml in PBS. For the incubation, 1 µl each of PMSF, TPCK and alpha-2 M was added to 10 µl of soluble antigen along with methanol and ethanol controls. These samples were then subjected to SDS-PAGE analysis.

Lymphocyte Cell Culture

Fish were sacrificed by an anesthetic overdose in a benzocaine bath. The benzocaine bath was prepared by diluting 2 ml of a stock solution [10% (w/v) of benzocaine (Sigma, MO) in methanol] into 4 gallons of water. After the fish were fully sedated the caudal peduncle was severed and the fish were exsanguinated (this procedure reduces the number of red blood cells during the subsequent isolation of leukocytes from the anterior kidney). Anterior kidney tissue was aseptically removed, placed in a sterile tube with holding medium and held on ice. A single cell suspension of the tissue was prepared by gently passing the tissue repeatedly through a one ml syringe.

Tissue aggregates were then allowed to settle. The supernatant, containing single cells, was then washed twice by centrifugation at 500 x g for 10 minutes at 4oC using the holding medium. The cellular pellet was resuspended in TCM. Viable cell counts were performed utilizing the trypan blue exclusion procedure (Phillips, 1973). The cell suspension was then adjusted to a final concentration of 2 x 107 cells/ml TCM. From this cell suspension, 0.1 ml was then aliquoted into each well of a 24 well, flatbottom, tissue culture plate (Corning, NY) containing appropriate quantities of antigen (TNP-LPS) (Jacobs and Morrison) or TNP-LPS plus soluble antigen from Renibacterium salmoninarum. Prior to culture addition, the soluble antigen was dialysed in RPMI 1640 (with bicarbonate) overnight at 4oC (2 ml of 3 mg/ml soluble antigen was dialysed in one liter of the buffer). The plates were then incubated at 170C in Incubator Culture Chamber (C.B.S. Scientific, Del Mar, CA, model #624) in an atmosphere of 10% C02, 10% 02 and the balance N2. The cultures were maintained by adding 50 µl of nutritional supplement (cocktail medium) (Tittle and Rittenberg, 1978) on alternate days.

Plaque Forming Cell (PFC) Assay

Cells secreting anti-trinitrophenyl (TNP) antibodies upon in vitro antigenic stimulation were detected by a modification of the Cunningham plaque assay (Cunningham and Szenberg, 1968). Cells from each well were harvested on day nine of the culture

and washed twice with RPMI. The pellet from the final wash was resuspended into 0.2 ml of RPMI 1640 and were held on ice until plated. One hundred µl of the cell suspension, 20 µl of a 10% suspension of TNP-sheep red blood cells (TNP-SRBC) (Rittenberg and Pratt, 1969) in modified barbital buffer (MBB) and 20 ul steelhead serum (diluted in MBB) as the source of complement were mixed in individual wells of a 96 well microtiter plate (Linbro, McLean, VA). The contents of each well was then pipetted into a slide chamber, sealed with paraffin and incubated for l-2 hours at 17°C. Plaques (the clear zones of lysis within the TNP-SRBC lawn) were then enumerated under low power using a dissecting microscope.

RESULTS

Identification of Protease Activity

The normal storage condition of soluble antigen was at 4oC and demonstrated a consistent band pattern on SDS-polyacrylamide gel electrophoresis. It was a fortuitous observation that when SA was incubated at 37°C, there was a disappearance of most of the protein bands when tested on SDS-PAGE (Fig. 2.1).

Effect of Temperature on Protease Activity

Soluble antigen was incubated at different temperatures and was observed for any change of band pattern when SDS-PAGE analysis was performed. The temperatures used were 4°C, 17°C, 23°C, 370C and 56°C. The presence of protease activity was demonstrated by loss of prominent bands on a SDS-PAGE gel compared to the original preparation. Fig. 2.2 indicates that both at 370C and 560C temperatures the protease activity was demonstrated. For all subsequent protease assays, incubation of soluble antigen at 370C overnight was used.

Kinetics of Proteolysis

The kinetics associated with the proteolysis of soluble antigen was studied by incubating the SA at 370C and aliquots were withdrawn at 0, 2, 4, 8, 12 and 24 hours. The aliquots were mixed with an equal volume of sample buffer and placed in boiling water bath for 3 minutes. These aliquots were processed immediately for SDS-PAGE analysis or stored at -200C until the analysis was performed. Most of the proteolysis occured within the initial 2 hours of incubation (Fig.2.3). This proteolytic activity had particularly pronounced effect on the 57 kd protein band. Most of the proteolysis occurred within 24 hours of incubation, except that the band at 52 kd remained throughout the incubation period and the bands that appeared at lower

molecular weights are probably due to the products of the proteolysis.

Charaterization of the Protease

Soluble antigen was incubated at 370C with proteolytic inhibitors such as PMSF, TPCK and alpha-2 M and it was found that the proteolysis was inhibited (Fig. 2.4). It was also found that ethanol and methanol at a concentration of 50%, but not 10%, also protected soluble antigen from proteolysis. Western blot analysis of these samples indicated that, inhibition of proteolysis, protected the antigenic nature of the soluble antigen.

Identification of Substrate for the Protease

In an attempt to identify alternate substrates for the protease, soluble antigen preparation was incubated with the molecular weight markers, both high and low (Bio-Rad, Richmond, CA). These marker preparations possess the following proteins: myosin, ß galactosidase, phosphorylase B, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme. SDS-PAGE analysis of these samples after incubation with the soluble antigen preparation (Fig.2.5) demonstrated that phosphorylase B (97,000 d), BSA (66,000 d) and soybean trypsin inhibitor (21,500 d) were digested and thus can serve as substrates for the protease activity.

Biological activity of soluble antigen

In our previous studies we demonstrated that SA had a' suppressive effect on the in vitro antibody responses of coho salmon lymphocytes to a defined antigen TNP-LPS (Turaga et al., 1987). Figure 2.6 demonstrates the dose-dependent suppressive effect of SA when co-cultured with TNP-LPS and lymphycytes.

Biological Activity of Temperature-Treated Soluble Antigen

Soluble antigen (0.5 mg/ml in RPMI 1640) was incubated at 370C and periodic samples were withdrawn as before. These aliquots were tested in the in vitro PFC assay. Disappearance of the protein bands correlated with the loss of immunosuppressive nature of SA, when it was incubated at 370C (Fig. 2.7) .

DISCUSSION

Prior to this study the only proteolytic activity described for R. salmoninarum was gelatin liquifaction (Smith, 1964) and no subsequent studies have characterized this protease activity. This work represents the first report on the identification and characterization of protease activity associated with a soluble antigen (SA) preparation of Renibacterium salmoninarum.

Initial studies indicated that the substrate for the protease(s) was soluble antigen itself. In an attempt to identify other more defined substrates, soluble antigen was incubated with molecular weight markers used in SDS-PAGE analysis. This facilitated the search by allowing a number of proteins to be studied in a single digestion. It was revealed that phosphorylase B, BSA and soybean trypsin inhibitor were susceptible to the proteolytic activity of the soluble antigen. It must be cautioned, however, that commercial kits of molecular weight markers incorporate glycerol along with a denaturing agent such as dithiothriotol. This is of importance since if the substrate assay is performed with native protein substrates, no proteolysis will be evident (data not shown). This indicates that these particular substrates must be in atleast a partially denatured condition in order to facilitate the proteolysis.

The protease activity was inhibited by PMSF, alpha-2 M, TPCK and in concentrations of 50% ethanol and methanol, while the inhibitors such as zinc sulfate, EDTA did not inhibit the proteolysis (data not shown). These studies demonstrate that the protease associated with soluble antigen may be a serine protease and possibly chymotrypsin-like protease. Other studies in our laboratory have indicated that the protease may have molecular weight of > 200,000 d (Rockey, D.D. and Kaattari, S.L., manuscript in preparation).

Examination of the protein blots indicated that the protease activity has pronounced effect on the 57 kd protein (Figs. 2.1 and 2.2). Most of the 57 kd protein was degraded within two hours

of incubation of SA at 370C and was almost completely digested by 12 hours of incubation. The protease activity associated with SA indicates that its storage conditions should have a dramatic influence in its antigenic quality and situations where the SA is incubated at 370C for ELISA may result in a significant loss of antigen.

We have previously demonstrated that soluble antigen suppressed in vitro antibody responses to TNP-LPS as assessed by the plaque forming cell (PFC) assay (Turaga et al., 1987b). It is of interest to note that this suppressive effect was almost completely neutralized when the preparation of SA that was pre-incubated at 370C for 24 hours (Fig. 2.6). The kinetics of the degradation of the 57 kd protein band paralleled the progressive decrease in suppression by the soluble antigen (Fig. 2.6). This may indicate that the 57 kd protein is responsible for the in vitro toxicity of the soluble antigen.

Daly and Stevenson (1987) have proposed that the 57 kd protein may be a hemagglutinin for rabbit erythrocytes and Getchell et al. (1985) have shown that the 57 kd protein is the major, common surface antigen of Renibacterium salmoninarum. among seven different isolates of the pathogen. Other studies conducted in our laboratory have demonstrated that incubation at 370C also neutralized another biologic activity of SA, namely a leukocoagglutinating activity (Wiens and Kaattari, manuscript in preparation).

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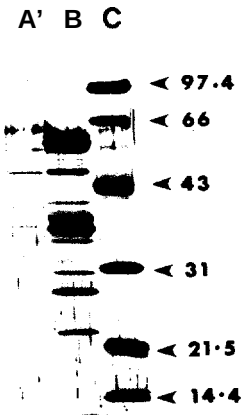


Fig. 2.1. SDS-PAGE analysis of soluble antigen (5 μg) incubated overnight at different temperatures. Lane A (370C) , lane B (40C) and lane C (mol. wt. markers). The nitrocellulose paper was stained for protein with Aurodye[R] forte.

A B C D E

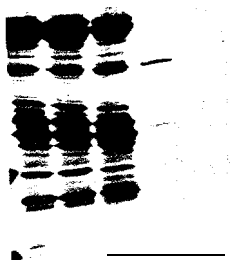


Fig. 2.2. SDS-PAGE analysis of soluble antigen (5 μg) incubated overnight at different temperatures: Lane A (40C) , lane B (17°C) , lane C (230C) , lane D (370C) and lane E (56oC) . The nitrocellulose paper was stained for protein with Aurodye[R] forte.

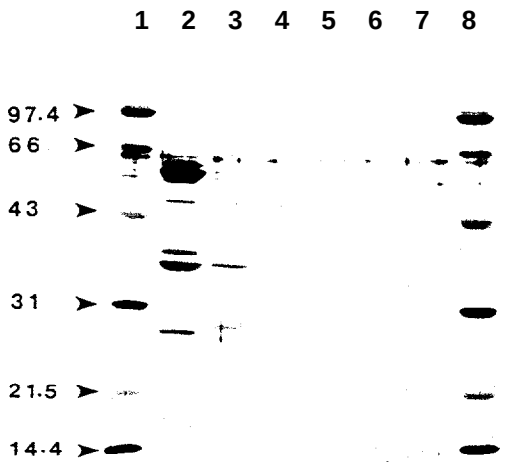
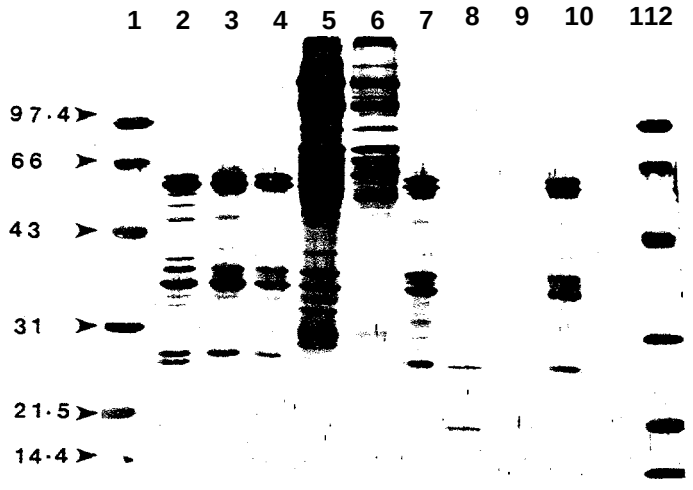


Fig. 2.3. SDS-PAGE analysis of soluble antigen incubated for various lengths of time at 370C. Each lane was loaded with 5 μg of SA. lane 1: mol. wt. markers, lane 2: 0 hr, lane 3: 2 hr, lane 4: 4 hr, lane 5: 8 hr, lane 6: 12 hr, lane 7: 24 hr incubation and lane 8: mol. wt. markers. The nitrocellulose paper was stained for protein by Aurodye^{R} forte.

Fig. 2.4. Effect of protease inhibitors on SA protease activity. SDS-PAGE analysis of preparations of SA incubated with the inhibitors at 370C. Lane 1: mol. wt. markers, lane 2: SA alone, not incubated at 37°C, lane 3: with PMSF, lane 4: with TPCK, lane 5: with alpha-2 macroglobulin, lane 6: alpha-2 macroglobulin alone, lane 7: 50% ethanol, lane 8: 10% ethanol, lane 9: 10% methanol, lane 10: 50% methanol, lane 11: PBS and lane 12: mol. wt. markers. A. protein staining of the nitrocellulose paper with Aurodye^{R} forte and B. immuno-staining with rabbit-anti SA.

A


1 2 3 4 5 6 7 8 9 10 112
97·4
66
43
31
21·5
14·4

B

1 2 3 4 5 6 7 8 9 10 1112
180
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84
58
48·5
36·5
26·6

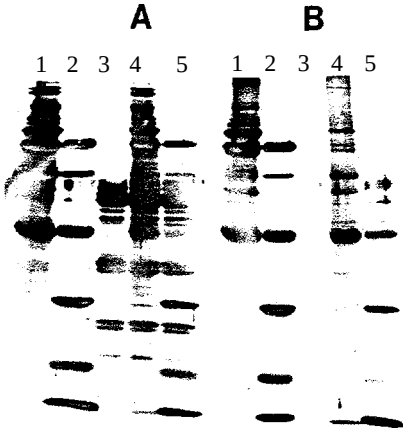

Fig. 2.5. Substrate characterization of the protease. Protein staining of SDS-PAGE of the preparations. lane 1: high molecular weight markers HMW (1), lane 2: low molecular weight markers LMW (2), lane 3: soluble antigen SA alone (3), lane 4: (l)+(3) and lane 5: (2)+(3). A. the samples at 0 hr and B. the samples incubated at 370C overnight.

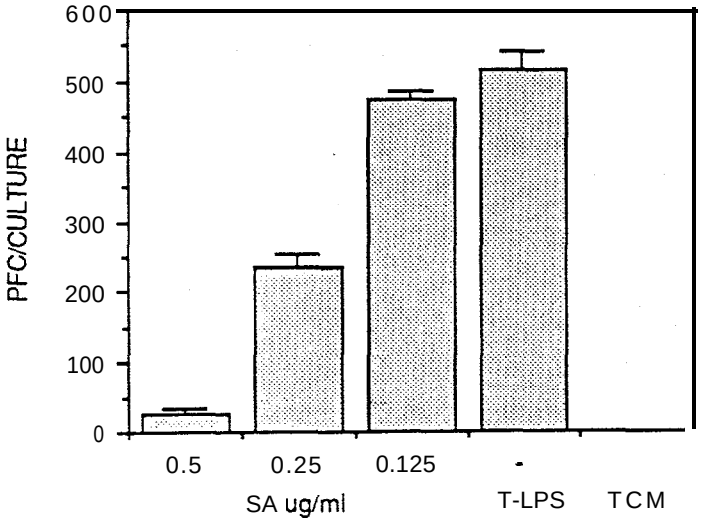

Fig. 2.6. Effect of soluble antigen on the plaque-forming cell response to TNP-LPS. Anterior kidney leukocytes were either co-cultured with TNP-LPS in the presence of various concentrations of soluble antigen (125, 62.5 and 31.25 μg/ml). A control consisting of leukocytes in tissue culture medium (TCM) only is depicted. Each histogram represents the mean of 4 cultures and the error bar represents one S.E.

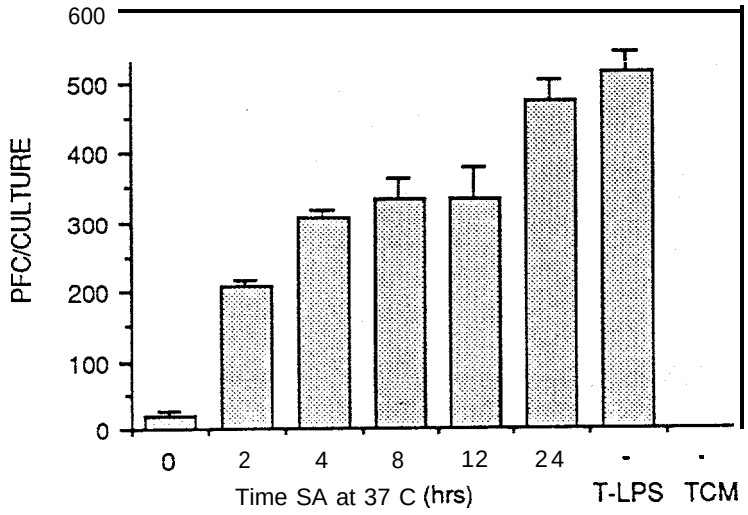

Fig. 2.7. The effect of preincubated soluble antigen on the in vitro PFC assay. Soluble antigen was preincubated for 0, 2, 4, 8, 12 and 24 hours at 370C before co-culture with anterior kidney leukocytes. The bar graphs shows the number of PFCs/culture when the soluble antigen preparations were co-cultured with TNP-LPS, leukocytes cultured with TNP-LPS alone and TCM alone. Each histogram represents the mean of 4 cultures and the error bar represents one S.E.

MANUSCRIPT #11

CHARACTERISTICS OF THE ANTIBODY RESPONSE OF THREE SALMONID SPECIES TO SOLUBLE ANTIGEN SECRETED BY RENIBACTERUM SALMONINARM

To be submitted

CHARACTERISTICS OF THE ANTIBODY RESPONSE OF THREE SALMONID SPECIES TO SOLUBLE ANTIGEN(S) SECRETED BY *RENIBACTERIUM SALMONINARUM*

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ABSTRACT

Antibody responses of coho salmon (*Oncorhynchus kisutch*), chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout (*Oncorhynchus mykiss*) to soluble antigen from *Renibacterium salmoninarum* were examined. A protocol was developed for the comparison of antiserum specificities from different species and individuals of the genus *Oncorhynchus*, using both ELISA and Western blot analysis. Variable titers of antibody were found both within and between the three species tested. The ELISA titers of the sera and their specificities for soluble antigen as assessed by Western blot analysis, mostly demonstrated a strong recognition of the 57 kd protein of soluble antigen, however some variation in overall specificity was observed.

INTRODUCTION

Bacterial kidney disease (BKD) of salmonids is caused by *Renibacterium salmoninarum* (Sanders and Fryer, 1980). The disease is responsible for devastating losses both of hatchery-reared (Fryer and Sanders, 1981) and wild salmon populations

(Mitchum et al., 1979). The organism is a slow-growing, Gram positive, diplobacillus which has been classified into a unique genus and species (Sanders and Fryer, 1980). The bacterium is dependent on cysteine for its growth which can survive and multiply intracellularly within phagocytic cells (Young and Chapman, 1979; Bruno, 1986; Daly and Stevenson, 1987). A number of methods have been developed to detect the organism in diseased fish, they include: the use of selective medium for solation (Pippy, 1969), fluorescent antibody techniques (Bullock and Stuckey, 1975; Laidler, 1980; Elliott and Barila, 1987), counter immunoelectrophoresis (Groman, 1975), co-agglutination (Kimura and Yoshimizu, 1981), enzyme linked immunosorbent assays (ELISAs) (Pascho and Mulcahy, 1987; Dixon, 1987; Turaga et al., 1987) and Western blot analysis (Turaga et al., 1987). Estimation of agglutination titers and precipitin titers (Evelyn, 197 1; Banowetz, 1974; Bruno, 1987) have also been used to detect antibody in infected fish. However, detection of antibody levels has not correlated well with survival of infected fish. It has also been suggested that there can be false-positive reactions in agglutination assays, due to serum components such as lectins or crossreacting antibodies (Bruno, 1987).

Most of the studies, thus far utilized either rabbit antisera or mouse monoclonal antibodies, to characterize R. salmoninarum antigens. Antigens characterized by these antibodies may not represent those that may be recognized in fish, which is the host system. Such serological characterization utilizing fish serum will aid in developing better vaccine candidates for prophylactic

treatment of the disease. Therefore, one should examine the specificities of immune responses to salmonid pathogen in salmonid host. We demonstrate, in this study, a protocol to characterize the specificity of antibodies to the soluble products of R. s al monin arum which are found in fish serum. This characterization first requires the standardization of the individual serum samples by an ELISA titration protocol. Specificity analysis can then be performed on equivalent concentrations of each individual serum through Western blot analysis.

MATERIALS AND METHODS

Animals

Yearling coho salmon (Oncorhvnchus kisutch), chinook salmon (Oncorhvnchus tshawvtscha) and rainbow trout (Oncorhvnchus mvkiss) weighing 500-600 g, were used in this study. The salmon were maintained in 220 gallon circular tanks at Oregon State University-Fish Disease Laboratory (OSU-FDL) in ambient (120C) pathogen-free well water. Rainbow trout were maintained under identical conditions at Environmental Protection Agency, Goodnight facility, Corvallis, OR. All fish were fed Oregon. moist pellets (OMP).

Buffers

Phosphate buffered saline (PBS), pH 7.2 and 0.01 M, was prepared by dissolving 7.5 g of NaCl, 0.245 g of KH_2PO_4 and 0.809 g of Na_2HPO_4 in 1 liter of distilled water. Tris-buffered saline (TBS), pH 8.2 was prepared by dissolving Tris base 6.07 g, EDTA 0.3 g and NaCl 8.7 g in 1 liter of distilled water.

Immunization and collection of antisera

Soluble antigen (SA) (2 mg/ml in PBS), prepared according to the method of Turaga et al. (Turaga et al., 1987a), was emulsified in Freund's complete adjuvant (FCA) (Difco, Detroit, MI) at a 1:1 ratio (v/v). Each fish was immunized intraperitoneally with 0.1 ml of this preparation. The fish were bled one month post immunization through the caudal vein. Sera was prepared and stored at -200C until use.

Determination of Serum Antibody Titers

The ELISA method and the buffers were modified version of Voller et al. (1975). Briefly, one hundred µl of soluble antigen (5 µg/ml in carbonate/ bicarbonate buffer, pH 9.6) was coated on the wells of Enzyme Immuno Assay (EIA) plate by overnight incubation at 170C. After blocking the wells with 1% bovine serum albumin (BSA) in 0.1% tween 20 in tris-buffered saline, (TTBS) (100 µl/well) for one hour, wells were washed three times with TTBS followed by TBS. One hundred µl of

hyperimmunized coho anti-SA serum dilutions prepared in TTBS (1/100, 1/1000, 1/5000, 1/10000 and 1/20000) were incubated for one hour in the wells along with dilutions of the experimental fish sera (1/30, 1/150, 1/300 and 1/900). The dilutions of hyperimmune serum were used to standardize values obtained from the various ELISA tests. The plate was washed as described above and then an appropriate dilution of biotinylated (Kendall et al., 1983) anti-fish immunoglobulin monoclonal antibody, l-14 (DeLuca et al., 1983) in TTBS (100 @/well) was added and incubated for one hour at room temperature. After washing the plate, 100 µl of a 1/100 dilution of streptavidin-horseradish peroxidase (Sigma, St. Louis, MO) was added and incubated for 45 minutes. The wells were washed again and 100 µl of the substrate soluution was added, the color development was measured spectrophotometrically at 405 nm on an EIA autoreader (model EL 310, Biotek Instruments, Burlington, VT). The substrate solution was a mixture of: 10 ml of citrate buffer (pH 4.0), 10 µl of hydrogen peroxide and 75 µl of a 10 mg/ml solution 2,2'-Azinobis, 3-ethyl benzthiazoline sulfonic acid (ABTS) in distilled water. The amount of antibody activity in each test serum was determined by comparison of the volume required for 50% of the maximum 0. D. (1 unit of antibody activity) with the volume required for the same 50% response. Thus according to Arkoosh and Kaattari (1989),

$$\text{Units of activity (test serum)} = \text{Units of activity(standard)} \frac{\text{Volume of standard reauired for 50\%}}{\text{Volume of test ser. required for 50\%}}$$

Preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The method followed was basically that of Laemmli (1970). Separating gels of 10% polyacrylamide and a stacking gels of 3% polyacrylamide were used. The apparatus used was a mini gel electrophoresis unit (Idea Scientific, Corvallis, OR) and the power supply was an EC 600 (E-C apparatus Corporation, St. Peters burg, FL). Instead of using a toothed comb in the stacking gel, a preparative gel comb was used. Twenty µl containing 20 µg of SA was mixed with equal volume of sample buffer and placed in boiling water bath for 3 minutes before electrophoresis. During the electrophoresis a constant current of 10 mA was applied during migration through the stacking gel and 20 mA during migration through the separating gel.

Transblotting

After the electrophoresis, the protein bands were transferred from the gel onto nitrocellulose (NC) paper (Bio Rad, Richmond, CA), using transblot apparatus (Bio Rad, Richmond, CA) (Towbin et al.; manufacturers instructions). The transblotting was conducted in transblotting buffer (25 mM Tris, 192 mM

glycine, 20% v/v methanol) by applying100 V for one hour. The buffer was cooled by placing a cooling unit with ice in the chamber. After transblotting the, nitrocellulose paper was processed for Western blotting.

Western Blot analysis

Western blot analysis was done basically after the method of Towbin et al. (1979). Upon transfer of SA proteins, the nitrocellulose (NC) paper was placed in a blocking solution consisting of 3% BSA in TTBS for one hour at 370C. The NC paper was then washed with three changes of TTBS over a period of 15 minutes. The NC paper was then placed in a miniblotter apparatus (Immunetics, Boston, MA), which consists of multiple slots, each with a capacity of 100 µl. The fluid placed in these slots comes into direct contact with the NC paper. The different dilutions of the test sera were applied to the slots and incubated for one hour in a humid chamber at room temperature. The NC paper was then washed with TTBS and an appropriate dilution of biotinylated anti-fish immunoglobulin (1- 14) was applied and incubated for one hour. The paper was washed and horseperoxidase labelled streptavidin was added to the NC paper and incubated for 45 minutes. The paper was washed again and the bands of soluble antigen which were bound by salmonid antibody were stained by placing the NC paper in the substrate solution. The substrate solution consisted of 2 ml of 4-chloro-napthol (Bio-Rad) (3 mg/ml in absolute methanol),10 ml of 0.1 M

phosphate buffered saline (pH 7.2) and 10 µl of hydrogen peroxide.

RESULTS

Standardization of the Sera for Western Blot Analysis

A. Determination of Hyperimmune Serum antibody activity

The titer determination of the standard (hyperimmune) serum was performed to allow for determination of the amounts of each test serum which would yield comparable concentrations. The titration was first performed on a pool of hyperimmune serum, using the ELISA method and a standard curve was generated as shown in Fig. 6.1. The number of anti-SA antibody units of the hyperimmune serum was calculated to be 5500 units/100 µl from the standard curve (i.e., 50% (1 unit) of the maximum O. D. is produced by 0.018 ul of the serum, thus the number of units in 100 ul is 5500).

Determination of minimal number of these antibody units required for production of a positive reaction on Western blot was performed by using different dilutions of the titered hyperimmune serum. An adequate amount of antiserum needed to produce a detectable reaction was found to be the equivalent of 67 units, or 100 µl of a 1/81 dilution of hyperimmune serum (Fig 6.3). The determination of this quantity of activity was essential for the comparison of specificities of the different sera by Western blot.

B. Standardization of the Sera from Different Species

Each individual antiserum was titrated and the amount of antibody activity and dilution volumes required to produce 67 units of activity were determined (Table 6.1). These dilutions of the sera were used in a Western blot analysis to compare their soluble antigen recognition specificity.

Additionally it is of interest to note the large variation of the titers within and between the species of fish tested. Chinook salmon, especially showed no or low titers of anti-soluble antigen antibody compared to coho salmon or rainbow trout.

Western Blot analysis of different test sera

The dilution volumes as shown in table 6.1 were used in Western blot analysis. All the antisera recognized the 57 kd protein band of SA (Figs. 6.2 A-C), and to different degrees of intensities and most of the sera also recognized the 58 kd protein band. Other lower molecular weight bands of soluble antigen were not detected by the sera at the dilutions used.

DISCUSSION

Attempts to generate immune responses to *R. salmoninarum* antigens and correlate the response to protection from bacterial kidney disease (BKD) has been of interest to many researchers (McCarthy et al., 1984; Paterson et al., 1985; Evelyn et al., 1988). The production of agglutination titers to various

immunogens was routinely used to monitor the efficacy of immunization. At the same time, detection of antibody levels were found not to correlate well with the survival of fish infected with R. salmoninarum. One confounding aspect of such studies was the use of unpurified antigen preparations, such as heat or formalin killed , sonicated or lysed bacterial suspensions.

Earlier studies incorporated polyclonal or monoclonal antibodies (Getchell et al., 1985; Turaga et al., 1987a; Wiens and Kaattari, 1989) in characterizing antigens produced by R. salmoninarum. It is, however, also important to identify the antigens recognized by salmonid host if vaccination is to be attempted. Hastings and Ellis (1988) performed such studies with Aeromonas salmonicida, another fish pathogen, to determine the antigenicity of its extracellular products (ECPs) in rabbits versus rainbow trout. They demonstrated that rabbits developed antibody titers to the virulence factors, hemolysin and protease, whereas the trout did not. When the fish were passively immunized with the rabbit antiserum and subsequently challenged with the bacteria, they were found to be protected. This finding suggests that certain fish vaccines may not be efficacious because the fish are not capable of producing antibodies to crucial antigens. One alternative to this dilemma may be through modification of the extracellular products to improve their immunogenicity in fish.

This report demonstrates a means of characterizing the antibody response of three species of salmonid fish to extracellular soluble antigen(s) produced by R. salmoninarum,

The procedure (ELISA) to estimate the antibody activity is to determine comparable quantities of test sera which can be qualitatively compared as to their specificity. The antibody titration here utilized the anti-fish Ig monoclonal antibody (l-14), thus it was necessary to develop a means whereby only comparable amounts of 1-14 recognizable antibodies were being detected. If this is not done different reactivity patterns could be due to simply to different amounts of antibody activity within each sample. The development of such a method was facilitated by the ELISA procedure and calibrating the test sera using a standard preparation of hyperimmune serum. Thus, comparably calibrated volumes of test sera were used in the qualitative analysis by Western blotting. Using this technique it was found that there are some variations in specificities of the sera in recognizing the SA. Such analyses may aid in identification of potentially susceptible fish species or stocks if they are found to lack the ability to recognize different antigens of the bacterium.

In other studies, two species of Pacific salmon, coho (Oncorhynchus kisutch) and sockeye (Oncorhynchus nerka) were used to test anti-BKD vaccines (killed bacterial preparations in Freund's complete adjuvant) and were not found to be protected following natural or experimental (injected) challenge with live pathogen (Evelyn et al. 1988). Evelyn et al., concluded that Oncorhynchus species may not benefit from similar vaccination procedure to the degree seen with Salmo species. It would appear from these studies that any difference in susceptibility to BKD among these species may not be dependent on differential

recognition of soluble antigen. Thus it would be important to examine specificities of different species to the bacterial antigen and determine if a lack of recognition may be contributory to any difference in susceptibility. Knowledge the antigens that are recognized by the fish species should aid in the development of methods to assess the effectiveness of antigen preparation procedures or the incorporation of various immunomodulating substances in various vaccines.

ACKNOWLEDGEMENTS

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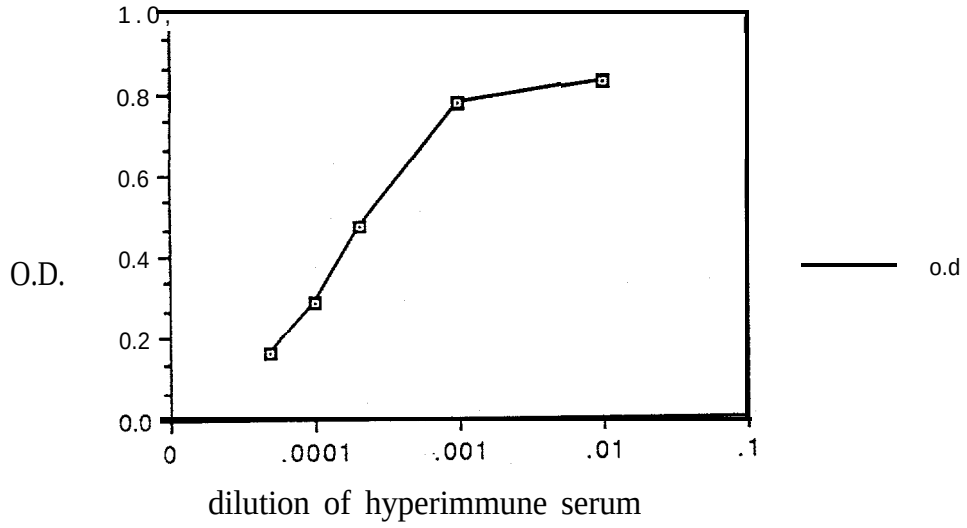

Fig 6.1. S tandardazition of hyperimmune serum. The ELISA values are plotted as 0. D. vs the dilution of the serum.

Table 6.1. A. Antibody titration of sera from Raibow trout.

Fish Number	# of Units[1]	Dilution[2]
1	NT[3]	____
2	71	1.05
3	102	1.52
4	NT	42
5	NT	____
6	NT	2.3
7	66	____
8	27	DNR[4]
9	37	2.3
10	3571	16.9

1 = No. of units/0.1 ml of the serum
2 = Dilution of serum required to produce 67 units of activity
3 = no titer
4 = dilution not required

Table 6.1. B. Antibody titration of sera from Coho salmon.

Fish Number	# of Units[1]	Dilution[2]
1	NT[3]	———
2	370	5.5
3	NT	———
4	114	1.7
5	55	DNR[4]
6	55	DNR
7	270	4.0
8	370	5.5
9	43	DNR
10	143	2.1

1 = No. of units/0.1 ml of the serum
2 = Dilution of serum required to produce 67 units of activity
3 = no titer
4 = dilution not required

Table 6.1. C. Antibody titration of sera from Chinook salmon.

Fish Number	# of Units[1]	Dilution[2]
1	277	4.1
2	16	DNR[4]
3	19	DNR
4	NT[3]	____
5	NT	____
6	NT	____
7	66	DNR
8	27	DNR
9	37	DNR
10	3571	53

1 = No. of units/0.1 ml of the serum
2 = Dilution of serum required to produce 67 units of activity
3 = no titer
4 = dilution not required

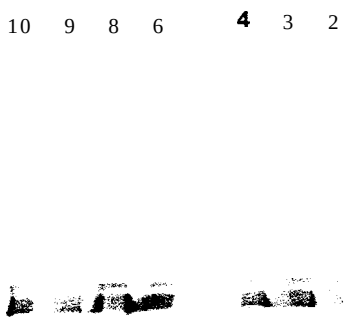

Fig. 6.2. A. Western blot analysis of SA using immune sera from Rainbow trout. The sera were diluted according to the values from table 6.1 A. and the sera from fish number 2, 3, 4, 6, 8, 9 and 10 were employed. All the sera recognize the 57 kd protein band of SA with different intensities.

Fig. 6.2. B. Western blot analysis of SA using immune sera from Coho salmon. The sera were diluted according to the values from table 6.1 B. and the sera from fish number 2, 4 through 10 were employed. All the sera recognize the 57 kd protein band of SA with different intensities.

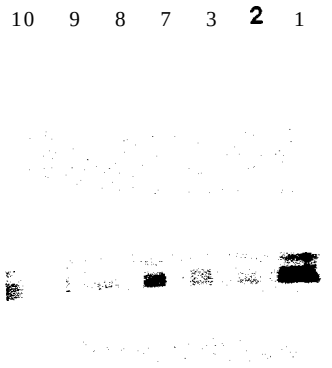

Fig. 6.2. C. Western blot analysis of SA using immune sera from Chinook salmon. The sera were diluted according to the values from table 6.1 C. and the sera from fish number 1, 2, 3, 7, 8, 9 and 10 were employed. All the sera recognize the 57 kd protein band of SA with different intensities.

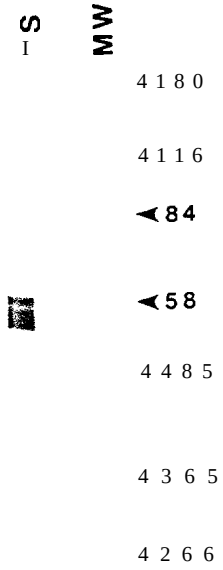

Fig. 6.3. Western blotting of SA using hyperimmune serum (HS). The serum was diluted so as to give 67 units of activity. Mol. wt. markers (MW) are also depicted.

MANUSCRIPT #12

PRESENCE OF SERUM IMMUNE COMPLEXES IN SALMON WITH BACTERIAL DISEASE

To be submitted

PRESENCE OF SERUM IMMUNE COMPLEXES IN SALMONWXTH BACTERIAL KIDNEY DISEASE

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ABSTRACT

This study represents the first report on the demonstration of soluble immune complexes (ICs) in fish that are infected with Renibacterium salmoninarum. An antibody capture ELISA method was employed to detect the presence of immune complexes. An indirect and corroborating evidence of this phenomenon was demonstrated by immunohistochemical staining of kidney tissue from infected fish, for both antigen and antibody.

INTRODUCTION

Renibacterium salmoninarum, is the causative agent of bacterial kidney disease (BKD) in salmonid fish. The bacterium is a Gram positive, pleomorphic, diplobacillus (Sanders and Fryer, 1980), which can survive and multiply intracellularly in phagocytic cells (Young and Chapman, 1978). Bacterial kidney disease is one of the devastating diseases in hatchery-reared

(Fryer and Sanders, 198 1) and also in wild salmonid populations (Mitchum et al., 1979).

The disease produced is the result of a chronic, systemic and granulomatous infection. External signs of the disease are exopthalmia, distended abdomen and pustules. Internally the disease is characterised by petichial hemorrhage, splenomegaly and a swollen kidney covered with a greyish white membrane (Smith, 1964). In advanced stages of the disease, the peritoneal cavity is filled with serous fluid and pustular lesions can be found on the kidney. In earlier studies, we have demonstrated that the bacterium elaborates large quantities of soluble antigen (SA), reaching concentrations of 1 mg/ml in serum (Turaga et al 1987a). It has been observed that in naturally infected fish showing clinical signs of the disease, there were no detectable antibody titers (Banowetz, 1974). A possible explanation for this phenomenon may be that the quantity of SA produced *in vivo* is large enough to mask any antibody that is produced and thereby making it unavailable for subsequent detection. It is reasonable to assume that if such a situation were occurring, the resultant antigen-antibody complexes would be soluble and saturated with antigen.

An ELISA method is described which detects the presence of antibody within immune complexes (ICs) formed to a specific antigen of *Renibacterium salmoninarum*. This study demonstrates the existence of immune complexes in serum from infected fish.

MATERIALS AND METHODS

Animals

Fingerling coho salmon (50-100 g) were kept in ambient (12°C) pathogen-free well water in 220 gallon circular tanks at the Oregon State University Fish Disease Laboratory (OSU-FDL), Corvallis, Oregon. Experimental infections were performed using 30 gallon tanks with the same water source. All the fish were fed Oregon Moist Pellet (OMP).

Growth of Renibacterium salmoninarum

Renibacterium salmoninarum was grown in modified KDM-2 medium (Evelyn, 1977) without the serum supplementation. Cultures were grown in 250 ml shake flasks at 17°C until the late log phase of the culture (about 10-12 days). The culture stock was then aliquoted in sterile snap cap tubes (Falcon) and stored at -70°C.

Prenaration of Soluble Antigen o f Renibacterium salmoninarum

Five hundred ml of the sterile .KDM-2 medium in 2.8 L Erlenmeyer flasks were inoculated with 5% (v/v) of an inoculum of the bacteria which was in late log phase of growth. The culture flask was then incubated on a reciprocating shaker at 17°C for 10-14 days. As these cultures grew to late log phase they were then centrifuged at 4000 x g for 30 min. The supernatant was removed and concentrated by passage through

a PTGC 10000 NWML filter packet in a Minitan Ultrafiltration apparatus (Millipore Corp., Bedford, MA). Typically ten-fold reductions of the original volume were achieved. This supernatant was further concentrated by ammonium sulphate (Sigma, MO) precipitation (3 13 g of ammonium sulphate/1000 ml of concentrated supernatant). The salt was added gradually over a period of one hour with constant stirring, while holding the supernatant in an ice bath. This solution was stirred for an additional 3 hours at 4°C. The precipitate was removed by centrifugation at 400 x g for 30 minutes at 4°C, and resuspended in 10 ml of PBS [pH 7.2, 0.01 M (7.5 g of NaCl, 0.245 g of KH_2PO_4 and 0.809 g of Na_2HPO_4 dissolved in one liter of distilled water)]. Using a saturated ammonium sulfate (SAS) solution, two additional 50% precipitations were performed. The final precipitate was then resuspended in 10 ml of PBS and dialyzed extensively at 4°C against 3 liters of PBS with three changes of the buffer over a period of 16 hours. The preparation was then filter sterilized using a 0.45mm filter (Corning, Corning, NY). The protein content of the preparation was determined by the method of Lowry et al. (1951).

Artificial Infection

The D6 isolate of *Renibacterium salmoninarum* was grown in KDM-2 with 10% calf serum (Hyclone) in shake flasks at 17°C for about 10 days. The culture suspension was then adjusted to specific concentration (1 O.D., 0.5 O.D. and 0.01 O.D. at 500 nm)

with sterile PBS (pH 7.2, 0.01 M). One hundred µl of this suspension was injected into each fish intraperitoneally, after they were anesthesized with Benzocaine (Sigma) (10% stock in alcohol, 1 ml/gallon of water). Five fish from each group, which had survived 7 weeks post-challenge, were sacrificed and their sera were collected, pooled within the same group, stored at -2opc. These pools were designated as # 1 for the pool of sera from five fish from the group of fish injected with 0.01 O.D. bacterial suspension, #2 for 0.5 O.D. injected group and #3 for 1.0 O.D. injected group.

Immune Complex Immunoassay

Soluble immune complexes (ICs) in the serum samples were detected using an antibody capture ELISA method (Fig 5.1). In this method, EIA plates were coated with 5 µ g/ml of a monoclonal antibody against the soluble antigen of R. salmoninarum, 4D3 (Wiens and Kaattari, 1989) in coating buffer and incubated at 17°C overnight. The plates were then washed with three rinses of 0.1% Tween-20 in tris-buffered saline, pH 8.2, (TTBS) and three rinses with tris-buffered saline (TBS) alone (6.07 g of Tris base, 0.3 g of EDTA and 8.7 g of NaCl dissolved in 1 liter of distilled water). the well surfaces were then blocked by incubation with 3% bovine serum albumin (BSA) in TBS. The wells were washed as above and the test and control sera samples were added at a 1 : 4 dilution in T-TBS to the wells and incubated for one hour at room temperature. After washing the wells, as above, biotinylated (Kaattari and Yui, 1987) anti-fish Ig

antibody, l-14 was added at an appropriate dilution in T-TBS to the wells and incubated for an additional hour. After washing the wells, horseradish peroxidase labeled streptavidin (Sigma, MO), suitably diluted in T-TBS was added to the wells and incubated for 30 minutes. The wells were washed and the substrate solution (100 µl/well) was added and spectrophotometric readings at 405 nm on EIA autoreader (Model 310, Biotek Instruments, Burlington, VT, USA) were monitored. The substrate solution was a mixture of 10 ml of citrate buffer (pH 4.0), 10 µl of hydrogen peroxide and 75 µl of a 10 mg/ml solution 2,2'-Azinobis, 3-ethyl benzthiazoline sulfonic acid (ABTS) (Sigma, St. Louis, MO) in distilled water.

Prenaration of Svnthetic Immune Complexes

In order to test the efficacy of the ELISA to detect immune complexes, synthetic complexes were prepared *in vitro*. Soluble antigen (0.2 ml) diluted in PBS at concentrations of 2, 1, 0.5, 0.25 and 0.125 mg/ml were mixed with 0.2 ml of a 1/50 dilution of hyperimmune coho anti-SA in PBS. The tubes containing these mixtures were incubated for one hour at room temperature and then for 48 hours at 17°C. The tubes were then centrifuged at 2000 x g for 10 minutes. The supernatants were carefully removed and tested in the ELISA as described.

Immunoneroxidase Staininf

The method followed was basically that of Ridley and Ridley (1986). Cryostat sections of pronephros and mesonephros

were prepared (7-8 microns in thickness) from infected fish and were allowed to dry for at least one hour at room temperature. The sections were fixed in acetone for five minutes and allowed to dry for half hour to one hour. The endogenous peroxidase was inhibited by incubation of the sections in methanol with 0.3% hydrogen peroxide for 10 minutes at room temperature. The sections were given three, five minute washes with phosphate buffered saline, pH 7.2, 0.01 M. An appropriate dilution of the primary antibody was prepared in PBS (either 3H1, an anti-SA monoclonal or l-14, an anti-fish Ig monoclonal antibody), overlayed onto the sections and incubated for one hour at room temperature in a humid chamber. The sections were washed with PBS as described above. The secondary antibody (goat anti-mouse IgG-peroxidase was diluted at 1:1000 in PBS and incubated with the sections for 30 minutes at room temperature in a humid chamber. The sections were again washed as above. The sections were incubated with the developing solution for approximately 20 minutes at room temperature and the staining intensity was periodically checked by observation at 25 x using a light microscope. The developing solution was prepared as follows: DAB (3,3' diaminobenzidine; Sigma, MO.) was prepared at a 0.5% concentration in Tris-Cl buffer (0.05 M, pH 7.6) and 0.5 ml aliquots were stored at -2ooc. Immediately prior to use, one 0.5 ml aliquot of the DAB solution was added to 4.5 ml of the Tris-Cl buffer, to this solution a mixture of 1 ml of nanopure water and 30 µl of hydrogen peroxide was added. After staining, the slides were dried in ethanol by immersion in

increasing concentrations of 70, 95 and 99%, dipped in xylene twice and mounted.

RESULTS

Detection of Synthetic Immune Complexes:

Using the ELISA, it was possible to demonstrate antigen-antibody complexes, generated *in vitro* (Fig. 5.2). It is evident from the figure that the *in vitro* complexes exhibit significant titers compared to the controls which include soluble antigen, hyperimmune coho salmon serum and normal coho salmon serum.

Determination of Immune Complexes in Infected Fish Serum

The results depicted in Fig. 5.3 clearly demonstrate the presence of immune complexes in infected coho sera, especially in pool #3, the most heavily infected. This indicates that there are immune complexes present in infected fish serum and while there is no reactivity in the control serum.

Immunoperoxidase Staining

Tissue sections of both pronephros and mesonephros stained with anti-soluble antigen and anti-fish Ig antibody, clearly indicate that a uniform deposition of antigen and antibody is occurring. As can be seen in the Figs. 5.4 - 5.7, the tissue sections from infected fish show diffused staining pattern for both the antibody and the soluble antigen. In the section of

mesonephros stained for soluble antigen (Fig. 5.4), the endothelial staining of the glomerulus is clearly seen. When stained for antibody with anti-fish-Ig there was diffused staining indicating deposition of antibody. In comparison, tissue sections from non-infected fish showed no staining reaction.

DISCUSSION

The involvement of immune system in the pathogenesis of bacterial kidney disease (BKD) has been proposed by Smith (1964), based on his observation of an inflammatory response. Belding and Merril (1935) were the first to observe extensive destruction of glomeruli and the supporting structures suggesting the possibility of glomerulonephritis. Bruno (1986) has proposed that direct damage to soft tissues occurs from products liberated from free bacteria, or from infected and disrupted macrophages, as well as the release of hydrolytic and oxidizing enzymes of macrophage origin. There have been only two detailed studies devoted to the histopathology of BKD (Young and Chapman, 1978; Bruno, 1986), which indicated that the infection appeared to be granulomatous, resulting in inflammatory reactions leading to the observed lesions. They also found that a large accumulation of macrophages, fibroblasts and polymorphonuclear leukocytes (PMNs) occurs around the lesions.

The presence of granulomatous lesions, subendothelial deposits of protein in the kidneys (Young and Chapman, 1978),

large quantities of soluble antigen (Turaga et al., 1987) and apparent absence of antibody during BKD (Banowetz, 1974) has prompted us to investigate the possibillity of the presence of immune complexes (ICs) in infected fish.

This study demonstrates the presence of soluble immune complexes in serum of fish which were artificially infected with Renibacterium salmoninarum. Using two different antibodies, one directed against the antigen and the other against the antibody involved in the antigen-antibody immune complex, facilitated precise identification of such complexes. The ELISA method described is a very useful tool which can be used to detect antibodies, the binding sites of which could be blocked by large quantities of SA, and thus would otherwise not be detected. The anti-SA monoclonal coated onto the plate binds the soluble antigen from the immune complex. When the anti-fish Ig monoclonal antibody is added it recognizes the antibody bound to the plate via the soluble antigen. Thus the method proved to be highly sensitive in recognizing immune complexes. Previous studies associated with viral antibody and the associated immune complexes had utilized a polyclonal antibody against the antigen to coat the ELISA plaate and a monoclonal antibody to recognize the antigen associated antibody (Izui and Lange, 1988). There were problems of cross-reactivity, between the polyclonal and monoclonal antibodies, in their ELISA and such complication was avoided here by using two different monoclonals and biotinylating the later one.

The immunoperoxidase staining also lends indirect support for the existence of immune complexes in infected fish, in that it demonstrates the uniform deposition of both antigen and antibody in the infected kidney tissues. Histochemical staining of tubercular lesions from patients, indicated similar diffused patterns of deposition of antigen (Ridley and Ridley, 1986). They also proposed that antigen excess would make the antibody undetectable in circulation. Whether these deposits of antigen and antibody are in the form of immune complexes cannot be deduced from these studies, however the concurrent occurrence of both antigen and antibody is a necessary condition if such a phenomenon was to occur.

In a normal immune response, the immune complexes are processed by phagocytosis. Some of the ICs escape this process and eventually are deposited in intravascular spaces and tissues such as kidney (Williams, 198 1; Dick and Kirkwood, 1984). If these complexes were to act as seen in human infections, they would release chemotactic factors and induce local inflammatory changes with vessel wall destruction, vasculitis and edema (Dick and Kirkwood, 1984). Sites such as the glomeruli would be damaged by these aggregates and the tissues would be infiltrated by polymorphonuclear leukocytes (PMNs), eosinophils and neutrophils leading to granuloma formation (Cochrane and Koffler, 1973). All the phenomenon mentioned above constitute type III hypersensitivity reaction.

Electron microscopy studies by Young and Chapman (1978) indicated that the pathological changes in the fine

structure of glomerulus and renal tubules found during bacterial kidney disease were similar to mammalian renal diseases as glomerulonephritis and nephrotic syndrome. Recent observations on kidney tissues from naturally infected chinook salmon, Oncorhynchus tshawytscha, (Dr. R. P. Hedrick, University of California at Davis, personal communication) indicated that there is thickening of basement membrane of glomeruli with protein in the kidney tissue sections that were stained with hematoxylin-eosin stain. Demonstration of soluble immune complexes in this study lends support to the electron microscopy studies and to the histochemical studies of Dr. Hedrick and others that there are immune complexes which may be involved in the pathology of the BKD.

ACKNOWLEDGEMENTS

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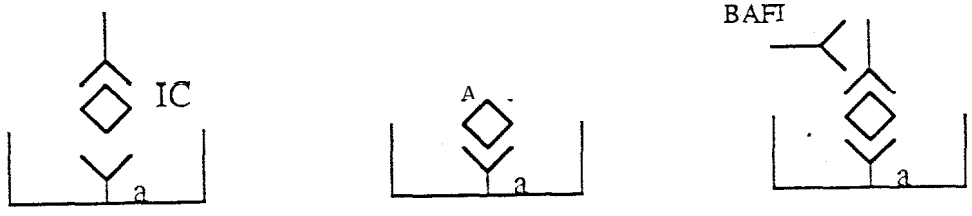


Fig. 5.1 Estimation of immune complexes by antibody capture ELISA. A. anti-soluble antigen monoclonal antibody (a) was coated on to the plate, B. the test sera containing immune complexes (IC) were added and C. biotinylated anti fish-Ig monoclonal antibody (BAFI) was then added.

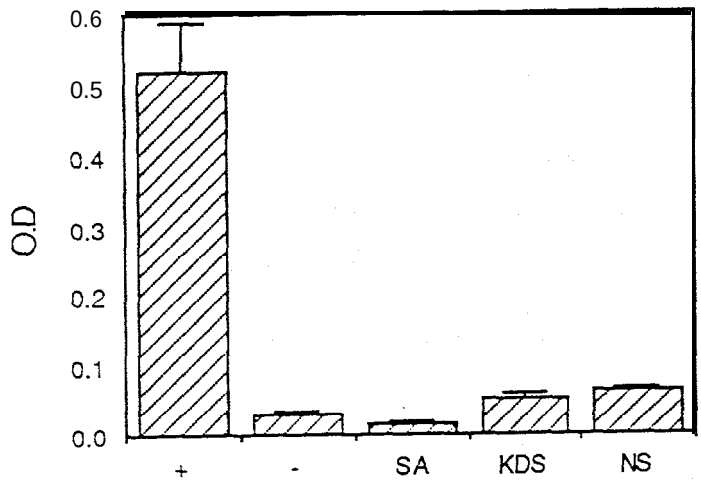


Fig. 5.2. Determination of immune complexes prepared *in vitro*. ELISA titers are expressed in terms of O.D. at 405 nm. + is the value obtained by mixing SA and hyperimmune serum, - is with by mixing SA and normal serum and the controls include soluble antigen alone (SA), hyperimmune coho serum alone (KDS) and normal serum alone (NS). Histograms represent the mean of 3 wells in the ELISA with 1 S.E.

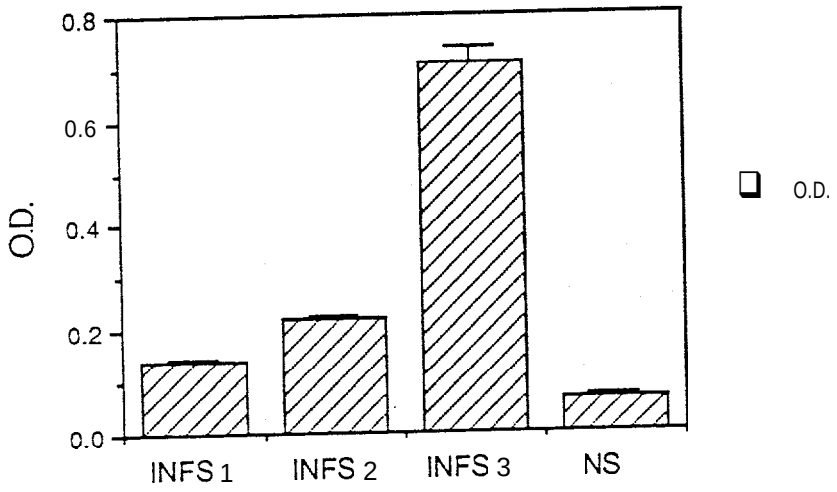
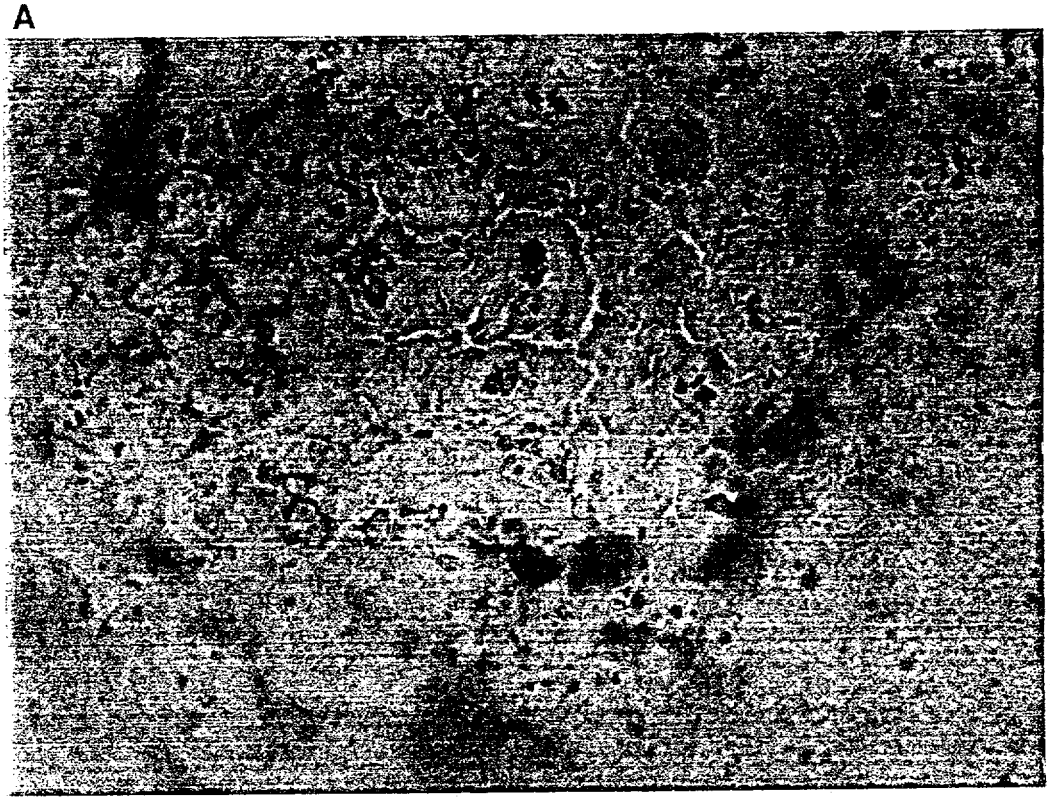


Fig. 5.3. Determination of immune complexes in sera from infected fish. ELISA titers are expresssed in terms of O.D. at 405 nm. Infected fish sera (INFS) samples were derived from fish injected with different concentrations of bacterial suspension [(#1 0.01 O.D.; #2 0.5 O.D.; and #3 1.0 O.D. (500 nm)]. Each histogram represents the mean of 3 wells in the ELISA with 1 SE.-

Fig. 5.4. Immunoperoxidase staining of mesonephros with anti-soluble antigen monoclonal antibody. Tissue section of infected fish (A). The diffused staining pattern around glomerulus is significant compared to tissue section from normal fish (B).

A



B

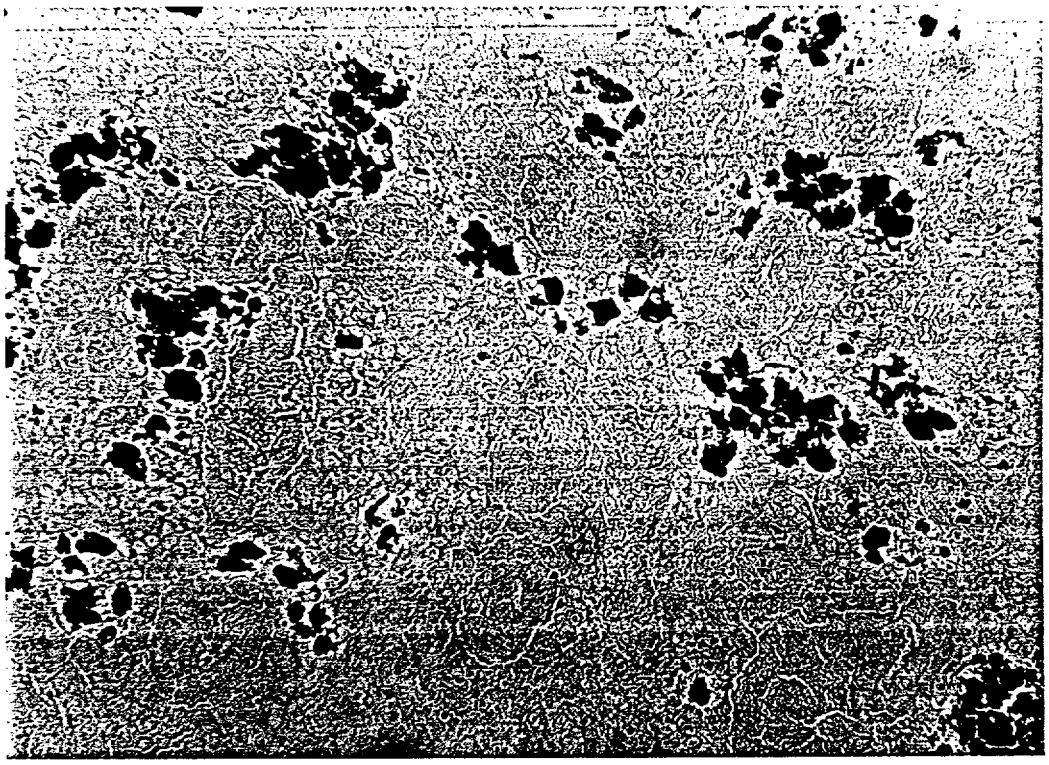


Fig. 5.5. Immunoperoxidase staining of mesonephros with anti fish-Ig monoclonal antibody. Tissue section of the infected fish (A). Along with the diffused staining pattern of antibody, some antibody producing cells are- also stained. Staining of tissue from normal fish (B). The black cells are the melanomacrophages.

A

B

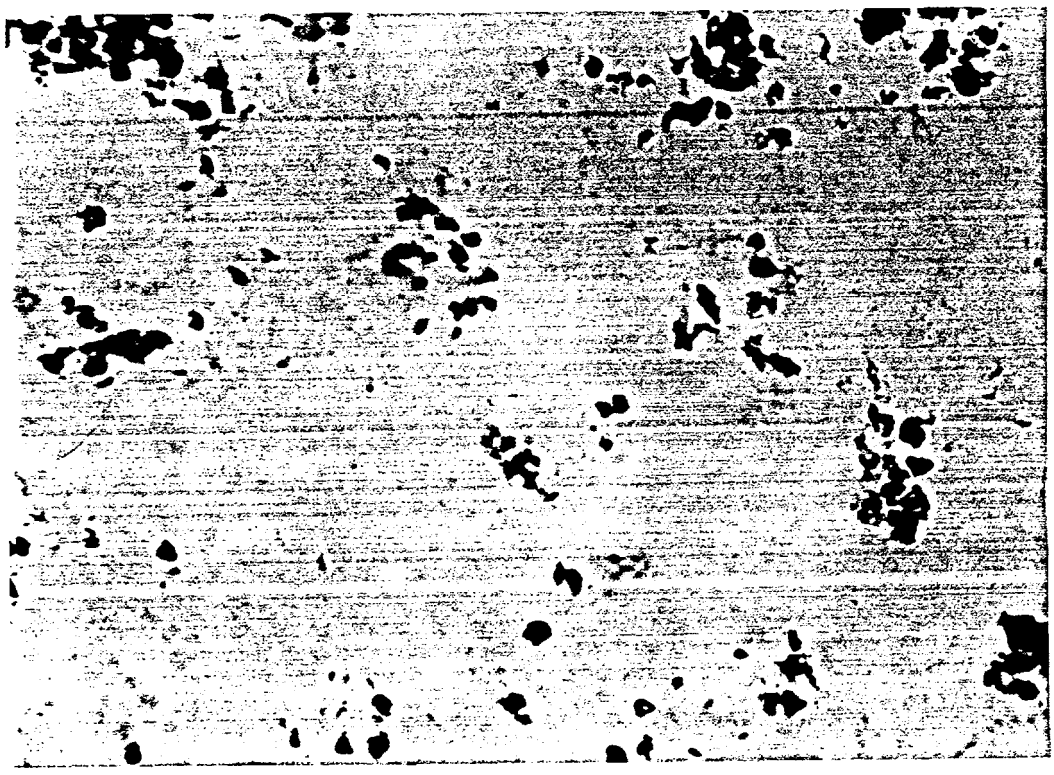
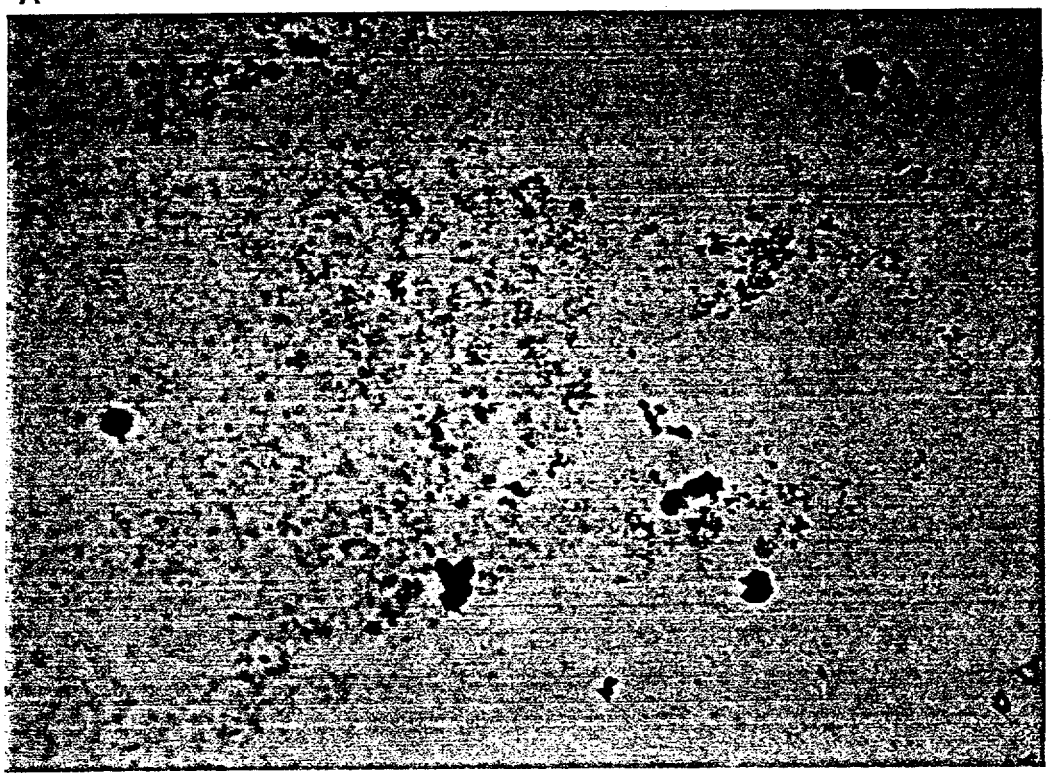


Fig. 5.6. Immunoperoxidase Staining of pronephros with anti-soluble antigen monoclonal antibody. The staining pattern is similar to that of mesonephros. Infected fish tissue section (A) and normal fish tissue section (B).

A



B

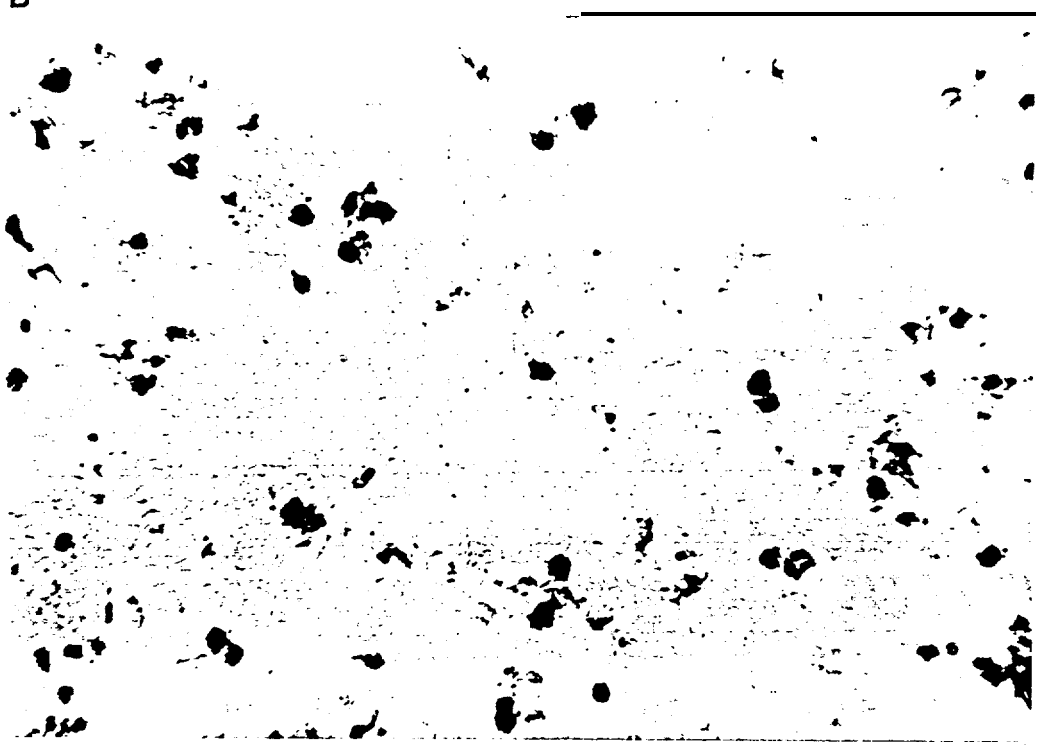
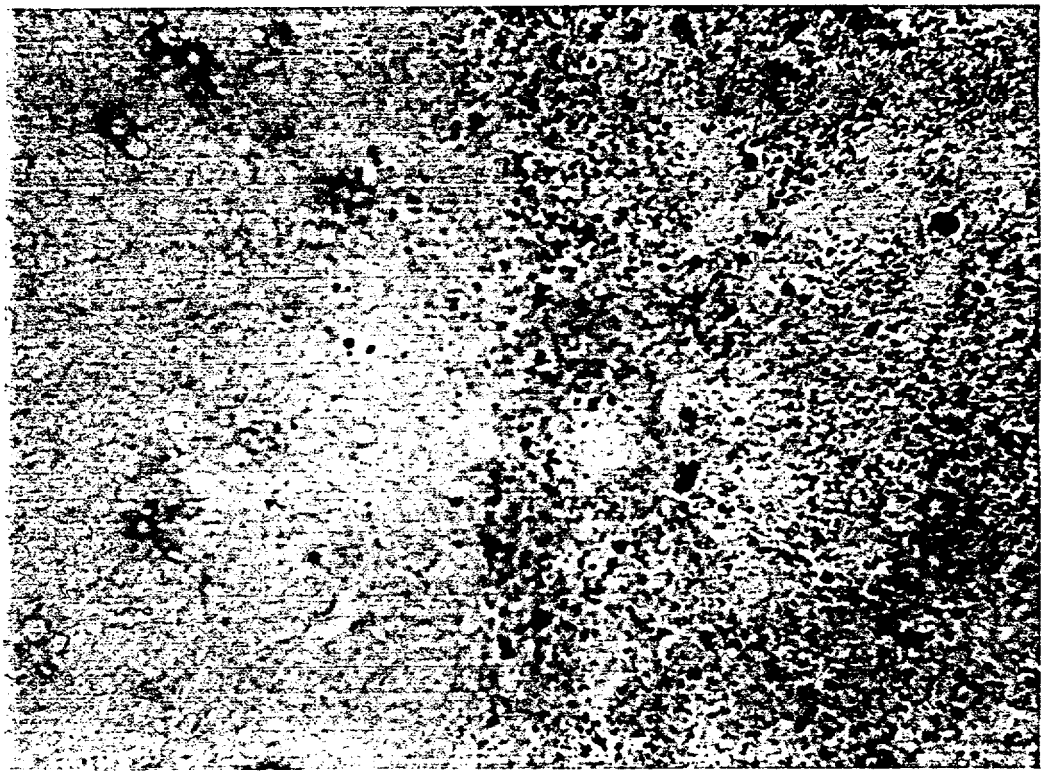
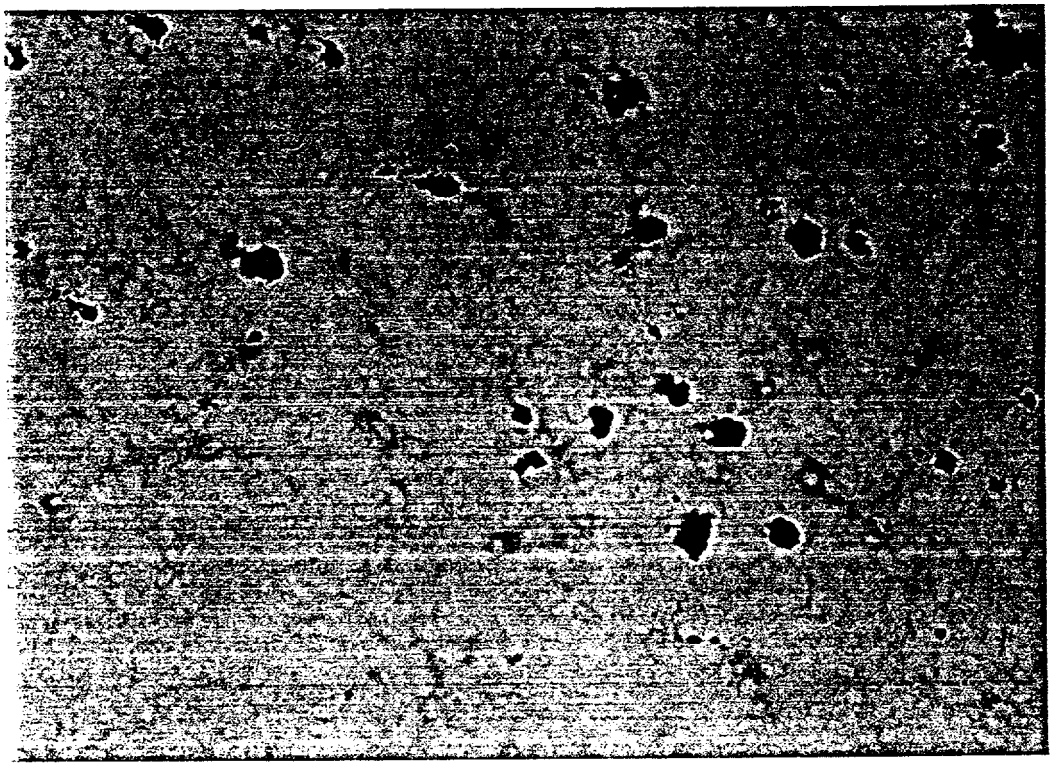


Fig. 5.7. Immunoperoxidase staining of pronephros with anti fish-Ig monoclonal antibody. The staining pattern is similar to that of mesonephros. Infected fish tissue section (A) and normal fish tissue section (B).

A



B



MANUSCRIPT #13

EXAMINATION OF PROPHYLAXIS INDUCED BY VARIOUS IMMUNOGENS AGAINST BACTERIAL KIDNEY DISEASE

To be submitted

EXAMINATION OF PROPHYLAXIS INDUCED BY VARIOUS IMMUNOGENS AGAINST BACTERIAL KIDNEY DISEASE

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ABSTSTRACT

Studies were performed with the intent of developing a prophylactic preparation for the protection of salmon (Oncorhynchus) species against bacterial kidney disease (BKD). The R. salmoninarum antigens used were heat killed bacteria, soluble antigen(s) (SA) produced by the bacteria and a polysaccharide extracted from the bacterial cell walls. These antigens were adminstered to the fish along with immunomodulating agents such as Vibrio an guillarum extract, Freund's complete adjuvant (FCA), formalin killed Vi b r i o anguillarum and heat killed Mycobacterium chelonii. Before challenge with the live pathogen, agglutinin titers and enzyme linked immunosorbent assay (ELISA) titers were performed on the sera from vaccinated fish. The percent survival of the fish indicated that FCA alone or a component thereof (i.e., Mycobacteria) may confer protection. This report represents preliminary studies on the prophylactic effectiveness of various forms of the immnogen.

INTRODUCTION

Bacterial kidney disease of salmonid fish is caused by Renibacterium salmoninarum (Sanders and Fryer, 1980). The disease produces devastating losses in hatchery-reared (Fryer and Sanders, 1981) and wild salmon populations (Mitchum, 1979). The disease is characterized by exophthalmia, a distended abdomen, blisters on the skin, and, internally by petechial hemorrhages, splenomegaly, swollen kidney and granulomatous lesions on various internal organs (Belding and Merril, 1935; Wood and Wallis, 1955; Smith, 1964; Young and Chapman, 1978). The bacterium has been demonstrated to survive and multiply within phagocytic cells (Young and Chapman, 1978; Bruno, 1986; Daly and Stevenson, 1987; S. Gutenberger, Oregon State University, personal communication). This facultative, intracellular nature of the bacterium may protect it from antibiotics and antibodies.

Immunoprophylaxis and chemotherapy, dietary modification and vaccination have been used in attempts to prevent and/or treat this disease. In terms of antibiotic therapy the most current practice is to inject spawning females with erythromycin and then to feed the juveniles with erythromycin for 21 days (Wolf and Dunbar, 1959; T. Amandi, Oregon State University, personal communication). Incorporating high amounts of fluorine and iodine in diets has resulted in lower prevalences of BKD (Paterson et al. 1985; Lall et al. 1985).

Other studies utilized heat killed or formalin killed R. salmoninarum in attempts to vaccinate (Atlantic salmon, Salmo salar, and Rainbow trout, Salmo gairdneri) against BKD (Paterson et al. 1981; McCarthy et al. 1984). Both studies resulted in a reduced incidence of BKD lesions compared to controls. In more recent studies, Evelyn et al., (1987) employed formalin killed bacteria and used various immunization procedures to vaccinate two species of Pacific salmon (i.e., coho, O. kisutch and sockeye, O. nerka). They found that the vaccinated fish were not protected against challenge and concluded that Oncorhynchus species may not benefit from currently vaccination to the degree seen with Salmo species.

These studies were conducted with Oncorhynchus kisutch and utilized heat killed R. salmoninarum, soluble antigen(s) (SA) produced by the bacteria, and a polysaccharide extracted from the cell walls of the bacteria as immunogens. These immunogens were used in combination with immunostimulatory agents such as formalin killed Vibrio angulillarum cells, and its cell extract, Freund's complete adjuvant (FCA) (killed M. tuberculosis, H37Rv suspended in mineral oil), and M. chelonii as a replacement for the M. tuberculosis. It has previously been shown that formalin killed V. anguillarum is an effective vaccine in fish culture and an extract of its cell wall is a potent polyclonal activator of salmonid lymphocytes (Yui and Kaattari, 1987). Freund's complete adjuvant has also been used for fish immunization as a non-specific immunostimulatory agent. Soluble antigen(s) were

chosen because they are produced in large quantities (Turaga et al. 1987a) during culture of the bacterium and have been found to exhibit tixigenic properties (Turaga et al. 1987b).

MATERIALS AND METHODS

Animals

Fingerling coho salmon (Oncorhynchus kisutch) (50-100 g) were kept in ambient (12°C) temperature pathogen-free well water in 220 gallon circular tanks at the Oregon State University Fish Disease Laboratory (OSU-FDL), Corvallis, Oregon. The fish were fed on Oregon Moist Pellets. Unless otherwise stated, fish of the above mentioned weight range were used and maintained as described throughout the study. The disease challenges were performed on salmon housed in 30 liter tanks at both OSU-FDL and at the Corvallis Environmental Protection Agency (EPA) facility . The effluent from these tanks was chlorinated.

Buffers

Phosphate buffered saline (PBS), pH 7.2 and 0.01 M, was prepared by dissolving 7.5 g of NaCl, 0.245 g of KH_2PO_4 and 0.809 g of Na_2HPO_4 in 1 liter of distilled water. Tris buffered saline (TBS), pH 8.2, was prepared by dissolving tris-base 6.07 g, EDTA 0.3 g and NaCl 8.7 g in one 1 of distilled water.

Bacterial Growth

Vibrio anguillarum and *Mycobacterium chelonii* were grown in tryptic soy broth (Gibco, MI) at room temperature. Formalin fixation of *Vibrio* cells was accomplished by addition of formaldehyde to achieve a 0.3% concentration in the culture. This was then incubated at room temperature for 24 hr. The bacterial cells were washed 3 times with sterile PBS (2000 x g for 20 min. at 4oC) and resuspended in PBS for use in the conjugation procedures.

Mycobacterium chelonii required constant agitation throughout the culture period to prevent the cells from clumping. Prior to conjugation, the cells were heat-killed by incubating them at 560C for one hour. The cells were subsequently washed as above, and resuspended in PBS.

Preparation of Soluble Antigen from *Renibacterium salmoninarum*

Five hundred ml of the sterile KDM-2 medium in 2.8 L Erlenmeyer flasks were inoculated with a 5% (v/v) inoculum of a bacterial culture which was in a late log phase of growth. The culture flask was then incubated on a reciprocating shaker at 17°C for 10-14 days. As these cultures grew to late log phase they were centrifuged at 4000 x g for 30 min. The supernatant was removed and concentrated by passage through a PTGC

10000 NWML filter packet in a Minitan Ultrafiltration apparatus (Millipore Corp., Bedford, MA). Typically ten-fold reductions of the original volume were achieved. This supernatant was further concentrated by ammonium sulphate (Sigma, MO) precipitation (3 13 g of ammonium sulphate/1000 ml of concentrated supernatant). The salt was added gradually over a period of one hour with constant stirring, while holding the supernatant on an ice bath. This solution was stirred for an additional 3 hours at 4oC. The precipitate was removed by centrifugation at 400 x g for 30 minutes at 4°C, and resuspended in 10 ml of PBS. Using a saturated ammonium sulfate (SAS) solution, two additional 50% precipitations were performed. The final precipitate was then resuspended in 10 ml of PBS and dialyzed extensively at 4°C against 3 liters of PBS with three changes of the buffer over a period of 16 hours. The preparation was then filter sterilized using a 0.45 µm filter (Corning, Corning, NY). The protein content of the preparation was determined by the method of Lowry et al. (195 1).

Preparation of *Vibrio anguillarum* Extract

The *Vibrio anguillarum* extract (VE) was prepared (Yui and Kaattari, 1987) from *Vibrio anguillarum* strain SL-174 which had been formalin killed and stored frozen. Fifty ml thawed packed cells were suspended in 10 volumes of 2% saline and placed in a boiling water bath for two hours. Cells were washed three times

in 2% saline, centrifuged at 3,000 x g for ten min at 4oC , resuspended in 95% ethanol, and incubated for 48 hr at 37°C. The cells were then washed two times in acetone, centrifuging at 3000 x g for 10 min and the resulting paste was dried overnight at 370C. The dried paste was ground to a fine powder with mortar and pestle and stored at 4oC. The soluble VE used for these studies was prepared by resuspending the powder in PBS at 10 mg/ml and placing the tube in a boiling water bath for one hour with frequent agitation. This suspension was then centrifuged at 1000 x g to remove particulates and filter sterilized. Protein concentrations were determined by the method of Lowry et al. (1951).

Polysaccharide Extraction from Renibacterium salmoninarum

The methods followed *were* basically that of Fiedler and Draxl (1986) and Schleifer and Kandler (1967). Live bac terial cells were obtained from 5 liters of culture medium and stored at -200C. After thawing, the cells were heat-killed by incubation in a boiling water bath for 30 min. The cells were washed with PBS and finally resuspended in distilled water. Using a Bead-Beater[R] Cell disrupter (Biospec Products, Bartlesville, OK) and 0.1 mm diameter glass beads (one lb.), the cells were disrupted with 20, one minute bursts with a five minute interval between each burst. The preparation was examined under a light microscope to determine if any intact cells remained. This cell wall

preparation was centrifuged at 1500 x g for 20 min. The supernatant, containing the cell walls, was isolated and centrifuged at 12000 x g for 30 min and washed once with distilled water. The final pellet was resuspended to a volume of 10 ml in distilled water. Trypsin (0.5 mg/ml in distilled water) (Sigma, St. Louis, MO.) was added to the suspension and incubated at 370C for 24 hr. The cell walls were washed four times with distilled water at 12000 x g for 30 min. The preparation was stored at this stage at -200C until the next step.

Formamide extraction of the cell walls was performed according to the method of Perkin (1965). The frozen cell walls were thawed and washed again with distilled water. The pellet was resuspended to 15 ml with formamide (Sigma, St. Louis, MO.) and heated for 30 min at 170°C, under reflux condensing conditions. The preparation was then cooled on ice and 2.5 volumes of an acid/alcohol solution (5 parts of 2N HCl/95 parts of ethanol) were added to the preparation and centrifuged at 100,000 x g for 30 min. The supernatant was then diluted with 10 volumes of acetone. A white precipitate was allowed to form by incubation at 4oC for 48 hr. The precipitate was pelleted by centrifugation at 1000 x g for 10 min and washed with distilled water. The final pellet was resuspended in 1 ml of distilled water and lyophilized.

Antibody Titration of Vaccinated Fish Sera

The method used was basically that of Voller et al. (1978). Briefly, 100 µl of soluble antigen (SA) was coated on the wells of an EIA plate (Coming, New York, NY) and incubated overnight at 17oc. After blocking the wells with 3% bovine serum albumin (BSA) in 0.1% tween 20 in TBS (TTBS) (100 µl/well) for an hour, the wells were washed three times with TTBS followed by another three washes with TBS. One hundred µl of different dilutions of hyperimmune anti-SA coho serum in TTBS were incubated for one hour in the wells along with the dilutions of the sera from vaccinated fish diluted in TTBS . The plate was washed as described above, and an appropriate dilution of biotinylated (Kendall et al. 1983) anti-salmonid immunoglobulin monoclonal antibody, 1-14 (DeLuca et al., 1983) in TTBS (100 µ l/well) was added and incubated for an hour at room temperature. After washing the plate, 100 µl of a 1/100 dilution of streptavidin coupled to horseradish peroxidase (Sigma, St. Louis, MO) was added to each well and incubated for 45 min. The wells were washed again and 100 µl of the substrate solution was added. The color development was measured spectrophotometrically at 405 nm. The substrate solution consisted of: 10 ml of citrate buffer (pH 4.0), 10 µl of hydrogen peroxide and 75 µl of a 10 mg/ml solution of 2,2'-Azinobis, 3-ethyl benzthiazoline sulfonic acid (ABTS) in distilled water.

Bacterial agglutination assay

The method used is basically that of Campbell et al. (1970). On a 96 well plate 50 µl of PBS was placed in each well. Fifty µl of sera from immunized and normal coho salmon were added to the first two columns of the wells. Serial two fold dilutions of the sera was made in the remaining ten columns. Fifty µl heat killed *R. salmoninarum* suspension (1.0 O.D. at 520 nm) in PBS was added to each well from the second column onwards. The plate was allowed to incubate at room temperature for two hours and checked for agglutination. If no agglutination had occurred at this time, the plate was then transferred to a 170C incubator an checked every six hours for 24 hours.

Coniugation Methods of Antigens from R. salmoninarum to other Adjuvants

The following methods were used for the conjugation of adjuvant material to *R. salmoninarum*.

A. Coniugation with Tannic Acid: The method was basically that of Campbell et al. (1970). *Vibrio anguillarum* cells (0.5 ml packed cells) were washed 3 times with PBS (pH 7.2). Phosphate buffered saline (PBS) was prepared by mixing 100 ml of saline with 100 ml of phosphate buffer consisting of 24 ml of 0.15 M KH_2PO_4 and 76 ml of 0.15 M Na_2HPO_4. The cells were then diluted with 7 ml of PBS (pH 7.2). To 2 ml of this Vi bri o

suspension, 2 ml of tannic acid (0.05 mg/ml in saline) solution were added. This suspension was mixed for 10 min in a 370C water bath. The tanned cells were then washed 3 times in PBS. One ml of soluble antigen (1 mg/ml in saline), 2 ml of the above suspension, and 4 ml of PBS (pH 6.4) were mixed for 10 minutes at room temperature. PBS, pH (6.4), was prepared by mixing 100 ml of saline with 100 of phosphate buffer consisting of 32.2 ml of 0.15 M Na_2HPO_4 and 67.8 ml of 0.15 M KH_2PO_4. To block remaining protein binding sites on the Vi br i o cells, the conjugated cells were incubated for 10 min in a gelatin solution (2 mg/ml in saline). Finally, the preparation was washed 3 times with PBS and emulsified in FCA. The preparation of M. chelonii plus SA employed similar protocol was followed except that the V. anguillarum cells were replaced with M. chelonii.

B. Conjugation with Glutaraldehyde: The method followed was that of Avrameas (1969). Washed, pelleted [the pellet obtained after 5 ml of a 1.0 0. D. (500 nm) cell suspension was centrifuged] bacterial cells (heat killed R. salmoninarum) were added to Vibrio anguillarum extract (VE) (5 ml of a 0.260 mg/ml preparation), mixed well and 25% glutaraldehyde (Sigma, St. Louis, MO) was added to the mixture (10 µl/ml of the mixture). The mixture was allowed to incubate for two hours on ice with occasional stirring. The preparation was then washed three times with PBS by centrifuging at 4000 x g at 4oC for 30 min. Finally the pellet was resuspended to the original volume (5 ml) of the bacterial suspension.

Immunization Procedures and collection of sera

The primary screening for prophylactic efficiency assessed the effectiveness of SA alone, V. angullarum cells plus SA, heat killed R. salmoninarum (HKDB), HKDB plus VE and FCA only. The fish (30 animals per group) were injected intraperitoneally with 0.1 ml of each preparation. When Freund's complete adjuvant (FCA) was employed, it was emulsified 1:1 (v/v) with the vaccine preparation. The 'secondary injections, comprising the same amounts of preparations, were performed by resuspending the materials in PBS only. These preparations were administered 30 days after the primary injection. The formal immunizations incorporated triplicated tanks each containing 35 individual fish for each preparation of M. chelonii alone, M. chelonii plus SA, polysaccharide alone groups, and non-injected controls. Prior to live challenge of these groups, two fish were sacrificed from each group and were bled through caudal vein. The serum was collected and stored at -200C until titrations were performed.

Bacterial Challenge

Renibacterium salmoninarum was grown in KDM-2 medium (Evelyn, 1977) on shake flasks at 170C for 10 to 14 days. When the culture attained late log phase, the 0. D. (500 nm) of the cells was adjusted to an 0. D. of 1.0 unit on a

Spectronic 20 spectrophotometer (Baush & Laumb). Fish were challenged, 30 days after their secondary injection, with an intraperitoneal injection of 0.1 ml of this culture suspension. Mortalities were recorded daily.

RESULTS

Antibody Titration of Vaccinated Fish Sera

The anti-SA antibody titers of the vaccinated fish sera varied considerably between groups. Fish sera were collected from two fish and the average values obtained for the two fish are depicted in Fig. 7.1. Soluble antigen alone or coupled with formalin killed *Vibrio* cells demonstrated higher antibody titers compared to the other test immunogens. Uninjected controls and those individuals injected with Freund's complete adjuvant (FCA) alone did not exhibit appreciable antibody titers.

Agglutination Titers of Vaccinated Fish Sera

The sera used for specific antibody estimation were also screened to estimate bacterial agglutination titers (Table 7.1). No appreciable difference between the vaccinated groups and controls were apparent.

Survival Studies of Vaccinated Fish after Challenge

Fish were challenged 60 days post secondary immunization in the preliminary screening studies, with live R. salmoninarum (suspension at 0.5 0. D. at 500 nm; 0.1 ml/fish). The mortalities began to appear by day 53 after challenge and mortalities were recorded on each subsequent day. The percent survival for each group during the time alotted for the challenge are depicted in Figs. 7.2-7.9. Relative percent survival was calculated (Table 7.2) for these groups as suggested by Amend (1981). A single injection (1°) or two injections (2°) of heat-killed kidney disease bacterium (HKDB) or heat-killed R. salmoninarum alone [RPS of 0 (1°) and 29 (2°) on day 104] and Vibrio anguillarum extract (VE)-coupled HKDB [RPS of 4 (1°)and 5 (2°) on day 104] demonstrated no appreciable affect on survival compared to controls. Soluble antigen (SA) alone [RPS of 29 (1°) and 20 (2°) on day 104], SA plus Vibrio aneuillarum cells [RPS of 57 (1°) and 34 (2°) on day 104], and FCA alone [RPS of 67 (1° and 2°) on day 104] demonstrated better protection, compared to controls. One intriguing observation is that FCA alone appears to offer the best protection against the challenge. The formal immunization studies demonstrated equivalent and statistically significant protection given by M. chelonii alone (RPS of 67 at day 35 but of 7 at day 55) and soluble antigen coupled to M. chelonii (RPS of 67 at day 35 but of 6 at day 55) but poor or no protection was

confered by the polysaccharide alone group (RPS of 22 on day 35 and 2 on day55).

DISCUSSION

This study was designed to assess the effectiveness of various forms of antigen and adjuvants for the prophylactic treatment of BKD. The preliminary screening utilized different immunogens incorporated in Freund's complete adjuvant (FCA) as the vehicle. It was of particular interest to note that FCA alone conferred the best protection (RPS of 67 on day 104 after challenge) (Table 7.2) followed by *V. anguillarum* cells plus SA and SA alone. Heat killed *R. salmoninarum* resulted in the least protection. This finding suggests that non-specific cellular immunity may be the most protective form of immunity. Such non-specific prophylactic effects by FCA have previously been observed in salmonid fish (Olivier et al. 1985). Furthermore, the incorporation of SA together with FCA resulted in an obvious decrease in the RPS [67 (FCA 2°) vs 20 (SA + FCA, 2°) on day 104] (Table 7.2). This difference suggests that SA may have toxic properties and thus if injected prior to challenge may exacerbate the artificial challenge. Indeed this exacerbation appear to diminish the protective value of FCA alone as evidenced by the decrease in RPS. Such toxicity does seem likely in view of studies revealing its immunosuppressive effects on salmonid lymphocytes in *vitro* (Turaga et al. 1987b) its

association with decreasing hematocrit values during infection (Turaga et al. 1987), hemagglutination activity (Daly and Stevenson, 1987) and leukoagglutination properties (Wiens and Kaattari, manuscript in preparation). Alternatively, soluble antigen may also be responsible for such exacerbation by inducing the formation of immune complexes, and thus responsible for the pathology as seen in BKD (Turaga et al., manuscript in preparation). If this is indeed the case, the generation of antibody response to SA may in itself be detrimental to prophylaxis. This may suggest why no correlation of antibody titer with protection was observed. In fact FCA demonstrated the lowest antibody titer on ELISA and yet demonstrated the greatest degree of protection.

Based on the preliminary screening studies *M. chelonii* (a salmonid mycobacterium) was used in place of the killed *M. tuberculosis*, H37Rv, more commonly used in FCA. Incorporation of the *M. tuberculosis* in the mineral oil adjuvant was cost prohibitive and thus *M. chelonii*, was substituted for *M. tuberculosis* and was used without the mineral oil vehicle. Use of this preparation in an aqueous suspension would facilitate future large scale immunization procedures such as by bath or hyperosmotic infiltration techniques (Smith,1988). Furthermore, such modifications of FCA preparation protocols have previously been demonstrated to still be prophylactic for other fish diseases (Olivier et al. 1985). When these researchers used *M. bu tyricum* in mineral oil alone, it was found to confer protection against

lethal a challenge of Aeromonas salmonicida. In our studies, M. chelonii in aqueous form demonstrated significant protection on day 35, but no difference in RPS after 55 days (Table 7.3). The statistically significant delay in the death rate is felt to be an important indication of protection since intraperitoneal injection of the live pathogen, although considered by many to be an excessive and unnatural challenge procedure, it is currently the only reliable means by which to secure an artificial infection. It is reasonable to assume that because protection is afforded by M. chelonii alone, non-specific cellular immunity may play an important role in controlling BKD.

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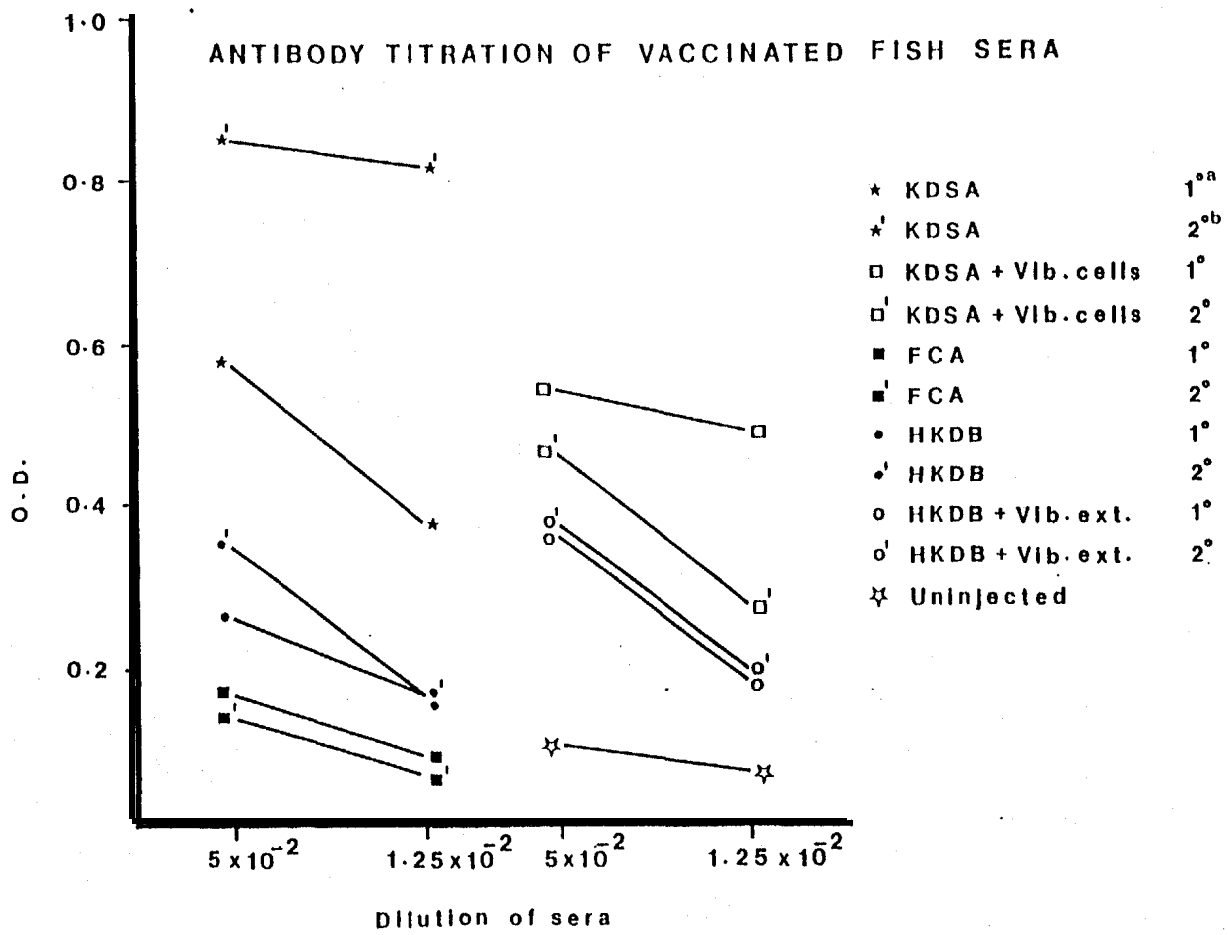
Fig. 7.1. Antibody titration of vaccinated fish sera. ELISA titers are depicted as average values of sera from two fish. The sera were diluted 1/20 and 1/80 in TTBS (M&M). FCA, Freund's complete adjuvant; KDSA, soluble antigen; Vib. cells, formalin killed *Vibrio anguillarum* cells; Vib. ext., *V. anguillarum* extract; HKDB, heat killed kidney disease bacterium (*R. salmoninarum*) were the immunogens. [a]Primary injection and [b]secondary injection.


ANTIBODY TITRATION OF VACCINATED FISH SERA
1·0
0·8
0·6
0·4
0·2
O.D.
5 x 10⁻²
1·25 x 10⁻²
5 x 10⁻²
1·25 x 10⁻²
Dilution of sera
KDSA 1°a
KDSA 2°b
KDSA + Vib. cells 1°
KDSA + Vib. cells 2°
FCA 1°
FCA 2°
HKDB 1°
HKDB 2°
HKDB + Vib. ext. 1°
HKDB + Vib. ext. 2°
Uninjected

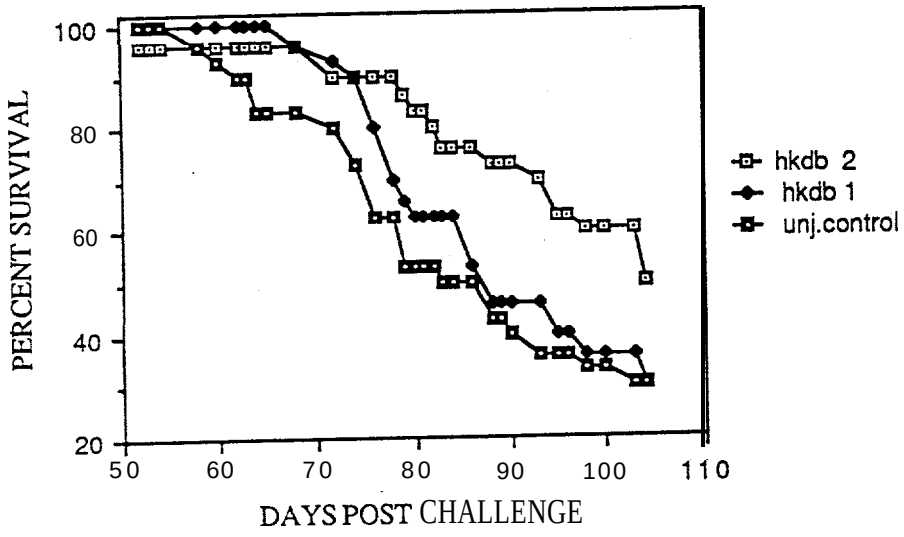


Fig. 7.2. Vaccine trial with heat killed kidney disease bacterium (HKDB) (R. salmoninarum). Percent survival vs no. of days post challenge was depicted. Legends 1 and 2 indicate primary and booster injectioned groups and unj. control is uninjected control group. Mortalities began to appear on day 52, post challenge.

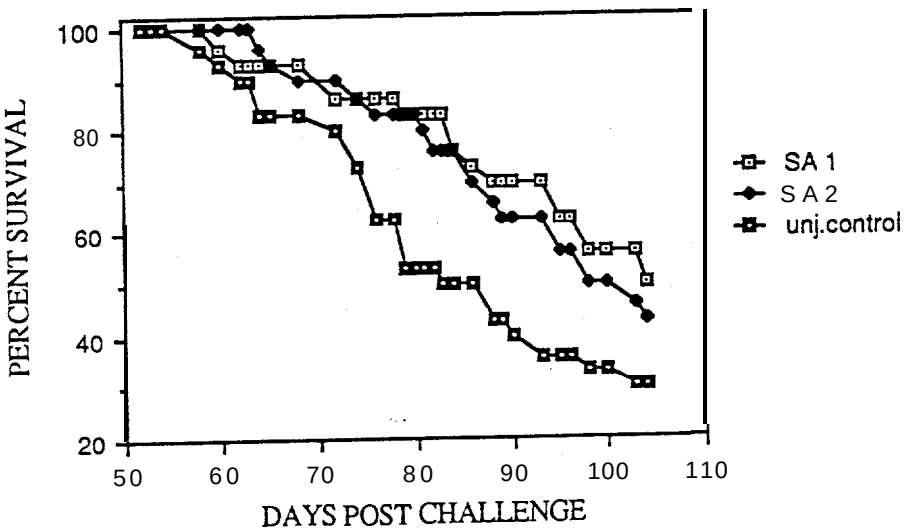


Fig. 7.3. Vaccine trial with soluble antigen (SA). Percent survival vs no. of days post challenge was depicted. Legend 1 and 2 indicate primary and booster injectioned groups and unj. control is uninjected control group. Mortalities began to appear on day 52, post challenge.

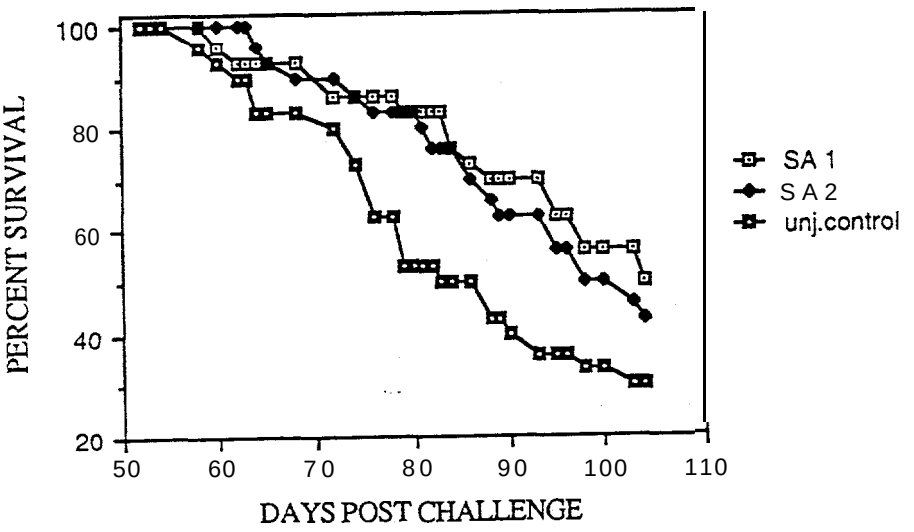


Fig. 7.4. Vaccine trial with Vibrio cells conjugated with SA via tannic acid. Percent survival vs no. of days post challenge was depicted. Legends 1 and 2 indicate primary and booster injectioned groups and unj. control is uninjected control group. Mortalities began to appear on day 52, post challenge.

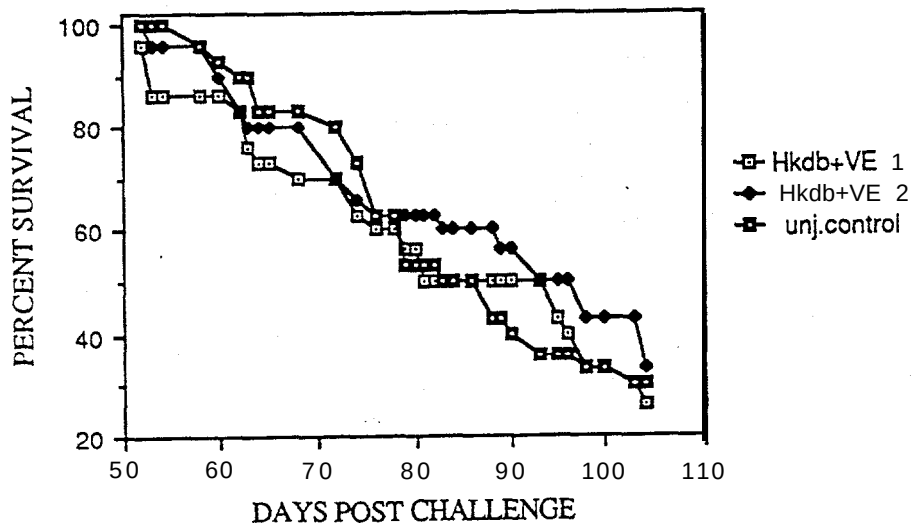


Fig. 7.5. Vaccine trial with heat killed kidney disease bacterium (HKDB) (R. salmoninarum) conjugated to Vibrio extract (VE) via glutaraldehyde. Percent survival vs no. of days post challenge was depicted. Legends 1 and 2 indicate primary and booster injectioned groups and unj. control is uninjected control group. Mortalities began to appear on day 52, post challenge.

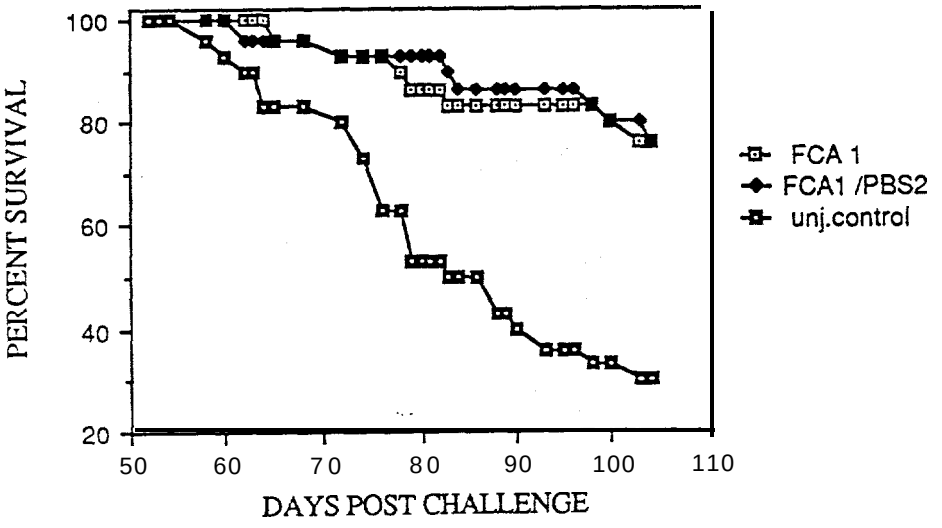


Fig. 7.6. Vaccine trial with Freund's complete adjuvant (FCA) alone. Percent survival vs no. of days post challenge was depicted. Legends 1 and 2 indicate primary and booster injectioned groups and unj. control is uninjected control group. The booster injection consisted of the buffer, PBS alone. Mortalities began to appear on day 52, post challenge.

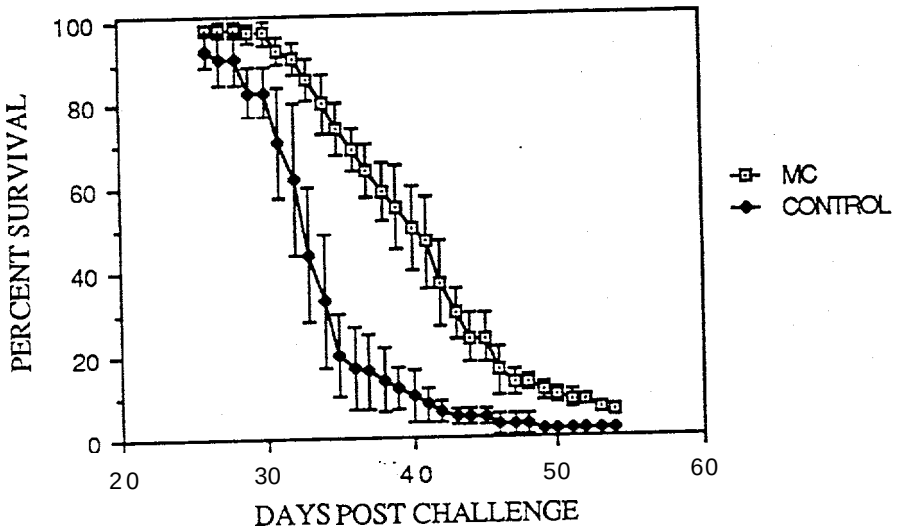


Fig. 7.7. Vaccine trial with heat killed M. ~~chelonii~~ (MC). Percent survival vs no. of days post challenge was depicted. Each point represens an average of triplicate tanks and one S.E. about the mean. Mortalities began to appear on day 26, post challenge.

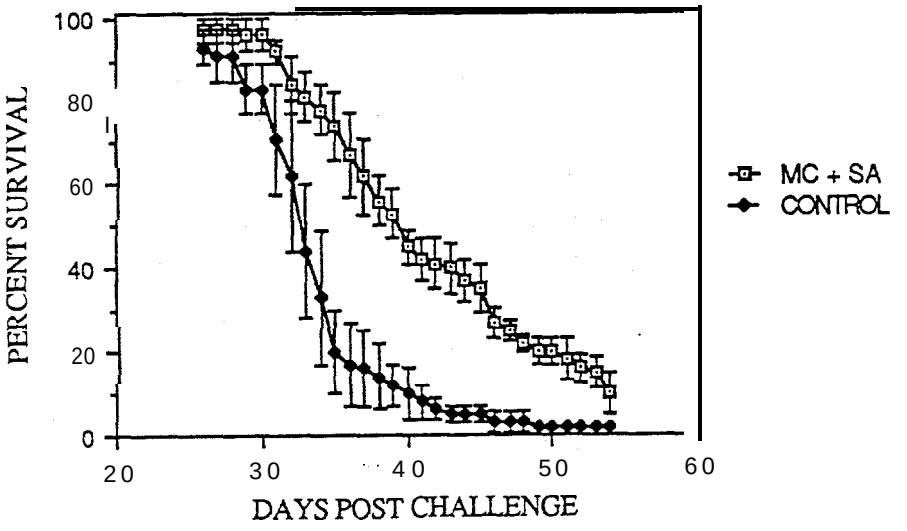


Fig. 7.8. Vaccine trial with heat killed M. ~~chelonii~~ (MC) coupled with soluble antigen (SA). Percent survival vs no. of days post challenge was depicted. Each point represens an average of triplicate tanks and one S.E. about the mean. Mortalities began to appear on day 26, post challenge.

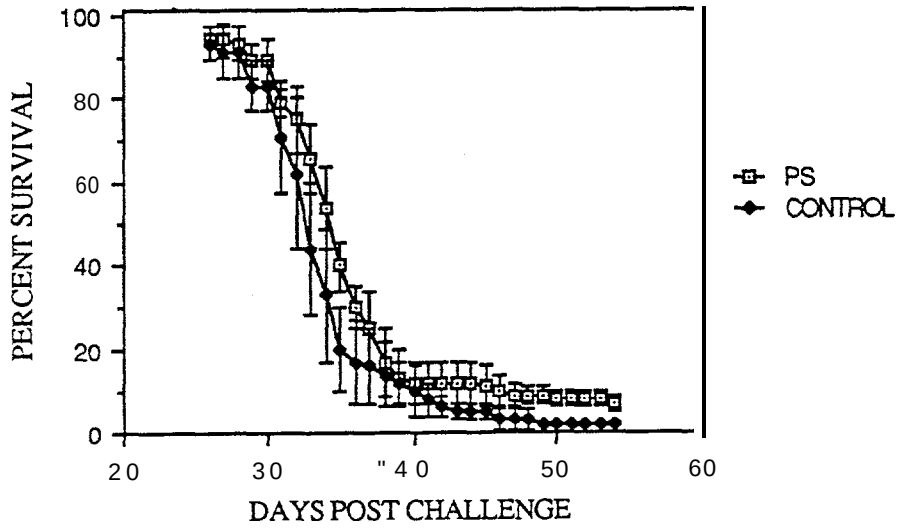


Fig. 7.9. Vaccine trial with polysaccharide (PS) extracted from R. salmoninarum. Percent survival vs no. of days post challenge was depicted. Each point represens an average of triplicate tanks and one S.E. about the mean. Mortalities began to appear on day 26, post challenge. .

TABLE 7.1. AGGLUTINATION TITERS OF VACCINATED FISH SERA

Serum source	Titer
FCA (1°)[a]	1:64
FCA (1° FCA/ 2° PBS)[b]	1:64
SA (1°)	1:64
SA (2°)	1:128
Vib. cells + SA (1°)	1:128
Vib. cells + SA (2°)	1:128
VE + HKDB (1°)	1:128
VE + HKDB (2°)	1:128
HKDB (1°)	1:128
HKDB (2°)	1:128
Uninjected control	1:64

a = titer, 60 days post primary injection
b = titer, 30 days post secondary injection

FCA = Freund's complete adjuvant
SA = soluble antigen produced by R. ~~salmoninarum~~
Vib. cells = formalin killed ~~Vibrio aneuillarum~~ cells
VE = V. anguillarum extract
HKDB = heat killed kidney disease bacterium (R. ~~salmoninarum~~)

Table 7.2. RPS[1] values of preliminary screening.

Vaccine group	Day 80[2]	Day1043
FCA 1°	85.7	67
FCA 1°/PBS 2°	71.0	67
Vib. cells + SA 1°	78	57
Vib. cells + SA 2°	64	34
SA 1°	64	29
SA 2°	64	20
HKDB 1°	21	0
HKDB 2°	64	29
VE + HKDB 1°	6	4
VE + HKDB 2°	21	5

1. RPS (relative percent survival) = $1 - \frac{\text{\% mortality in vaccine gr.}}{\text{\% mortality in control gr.}} \times 100$

2. day 80 post challenge
3. day 104 post challenge

FCA = Freund's complete adjuvant
Vib. cells = formalin killed *V. anguillarum* cells
SA = soluble antigen produced by *R. salmoninarum*
VE = *V. anguillarum* extract
HKDB = heat killed *R. salmoninarum*

Table 7.3. RPS[1] values for formal immunizations.

Vaccine group	Day 35[2]	Day 55[3]
M. chelonii alone	67	7
M. chelonii + SA	67	6
Polysaccharide	22	2

1. RPS (relative percent survival) = $1 - \frac{\text{\% mortality in vaccine gr.}}{\text{\% mortality in control gr.}} \times 100$

2. day 35 post challenge

3. day 55 post challenge

MANUSCRIPT #14

BACTERIAL KIDNEY DISEASE AND FURUNCULOSIS VACCINES

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BACTERIAL KIDNEY DISEASE AND FURUNCULOSIS VACCINES

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Prevention and treatment of bacterial kidney disease (BKD) and furunculosis are major goals of fish disease specialists around the world. This paper discusses pitfalls and possibilities in the quest to develop efficacious vaccines against BKD and to improve on available vaccines against furunculosis.

Furunculosis and bacterial kidney disease (BKD) are two salmonid diseases which have presented particularly difficult problems in terms of vaccine design. Development of useful vaccines for these diseases, as opposed to those used to combat vibriosis and enteric redmouth disease, will require extensive knowledge of the pathogenic processes as well as the basic immunologic mechanisms involved in inducing prophylaxis. Although causative agents of furunculosis and BKD are distinctly different organisms, common routes may be available for the development efficacious vaccines for both diseases. We describe here some of the possible reasons as to why Pacific salmon do not develop a strong prophylactic state in response to these organisms, and suggest approaches that may be productive.

Although only few accounts of attempts at immunization for BKD have been published (1,2,3,4), there are data suggesting that protection may be most readily achieved in Salmo species (*Salmo salar* or *Salmo gairdneri*). To date no studies have been reported that demonstrate substantial immunity in *Oncorhynchus* species. Some studies suggest that the use of an adjuvant may be required (2). The reported attempts to

produce a furunculosis vaccine, however, are much more extensive although the consistency of inducing immunity still has been elusive. The history of these reported vaccine attempts to prevent furunculosis began with the work of Duff (5) who demonstrated that cutthroat trout (Salmo clarki) vaccinated orally with a chloroform-killed preparation of A. salmonicida cells were protected against .a subsequent bath challenge. Several attempts followed this effort (Table I), and yielded generally inconsistent results. The most promising oral vaccine was the furunculosis soluble antigen (FSA) vaccine described by Klontz and Anderson (6) which was efficacious in the laboratory and early field trials. This vaccine was, however, inconsistent in continued trials and experiments by other investigators (7).

The advent of immersion vaccines and their immediate successes with Vibrio anguillarum and Yersinia ruckeri infections changed the focus of vaccinations against furunculosis away from oral delivery. However, this change did not improve on the consistency of A. salmonicida vaccine efficacy, nor did immersion result in high relative per cent survival values in the experiments which were reported as successful. This was confusing because of the apparent general similarity of the three organisms and the diseases they cause. Thus, more detailed examinations of the antigens of A. salmonicida that may have the potential to be used as vaccine material were required.

Initial investigations devoted to, the identification of protective cellular antigens were initiated by Udey and Fryer (7) who reported an additional outer membrane layer (A layer) that was associated with virulence in furunculosis. The A layer was subsequently shown to facilitate autoaggregation and tissue adherence, increased survival in

serum (11) and possibly aided in iron sequestering (12). Comparisons between the protective capabilities of the A protein, the major component of the A layer, and the outer membrane lipopolysaccharide (LPS) were conducted (13). In general, antibodies against LPS were not protective. This is in contrast to the vaccines used for Y. ruckeri and V. anguillarum where antibodies against LPS are thought to be sufficient for protection (14). Examinations of passive and active immunity have demonstrated that A layer is a potential protective antigen (13,15).

Analysis of the secreted virulence factors produced by A. salmonicida began with reports by Dahle (16) who investigated proteases produced by A. salmonicida and Aeromonas hydrophila. Ellis et al. (17) demonstrated that injections of supernatant from A. salmonicida cultures produced pathology similar to that produced during infection by the organism. The two major factors associated with this pathology were the serine protease and trout cell specific hemolysin (18,19) but other factors have been identified which may contribute more subtly to the pathogenic process. These include other proteases (20,21,22,23) and cytolysins (24,25,26,27). Extracellular factors have been used as immunogens with successful results. Cipriano (28) reported that a component of the extracellular products (ECP) isolated by diethylaminoethyl cellulose chromatography was protective upon intraperitoneal immunization. The antigenic fraction was cytolytic in vitro, and the immunizing component was reported to be a 65,000 molecular weight glycoprotein. Various authors have used protease as an immunogen with reported success (29,30). . Recently, Ellis et al. (31) demonstrated that rabbit antibody against ECP from a protease-producing strain was protective while antisera

deficient in antibodies against the protease were less protective. Relative protection was the compared by LD_{50} values.

A possible complication in using such materials for vaccines is that they may be aggressins, or toxins which act to interfere with or destroy the function of the immune system. Thus, simple injection of these components may actually exacerbate disease symptoms rather than protect the fish. This could be overcome by the generation of toxoids; however, caution must be taken because neutralization procedures may destroy essential, antigenic as well as toxigenic determinants. Information on possible toxins involved in BKD pathology, and thus potential candidates for vaccines, have only recently been published. In recent years Bruno and Munro have published data demonstrating that components of the R. salmoninarum cell wall are capable of causing decreased hematocrits and splenomegaly. Their data suggest that these components may act to affect red blood cells (32). More recent studies of Daly and Stevenson (33) have demonstrated a hemagglutinin activity of components released from the R. salmoninarum cell wall. This component has been identified as the 57 kD "F" antigen orginally described by Getchell et al. (34). Such studies have also been conducted in our laboratory (Kaattari), in which we have found a direct correlation between the amount of soluble antigens (of which the 57 kD species is a major component) elaborated and significant decreases in the hematocrit of infected fish (35). Although the direct demonstration of hemolytic activity on fish red blood cells has yet to be demonstrated, factors released from R. salmoninarum are capable of binding red blood cells and exerting pathologic effects. Of particular relevance to the possible induction of immune dysfunction are the studies conducted by our laboratory (Kaattari - 35) which demonstrate that soluble factors can at

concentrations normally found in infected Pacific salmon can suppress the in vitro antibody response to trinitrophenylated - lipopolysaccharide (an antigen which is unrelated to Renibacterium salmoninarum). It was also. observed that this treatment caused a decrease in the numbers of adherent cells in vitro. This observation is of importance because adherent cells have been found to be an essential cell in the generation of antibody responses in fish (36, Tripp and Kaattari in prep) while also serving as a target for R. salmoninarum cells (37). Also, infected fish demonstrate a reduced ability to generate antibody producing cells as compared to normal fish. If components do express these aggressin-like activities in vivo it would be essential to inactivate their toxigenic determinants prior to their use as immunogens. Failure to inactivate these determinants would result in prototype vaccines that may actually contribute to the pathology, rather than enhance immunity. This may be the case in certain vaccine studies where shortened mean times to death are found upon immunization (38, T. P. T. Evelyn - personal communication).

The development of vaccines for these diseases could profit from a search for the crucial components of those proven bacterial vaccines which are essential for conferring immunity. All of these vaccines have been developed for gram negative infections and, furthermore, the associated endotoxin or lipopolysaccharide (LPS) constituent of the outer membrane of the cell wall has been identified as a sufficient component to induce protection (14,39). This is logical since LPS is a cellular antigen, thus any immune response that is induced would be directed toward the organism. Further, LPS is known in a variety of animals included Pacific salmon to be a potent immunostiumlant (40,41,42,43,44). However, R. salmoninarum is a gram positive bacterium and does not have LPS in its cell wall. But, A.

salmonicida, being a gram negative bacterium, does possess LPS and this LPS does stimulate antibody production (45). Although A. salmonicida does induce anti-LPS antibodies, these antibodies may not be capable of elaborating the appropriate effector function that would result in destruction of the bacteria (46).

Direct conjugation of the LPS molecule to cellular antigens from R. salmoninarm or A. salmonicida could be employed in the development of a vaccine. To relate how this might be accomplished we will describe the functional anatomy of the LPS molecule. Lipopolysaccaride is a molecule which is basically comprised of three major components : A) O-antigen (polysaccharide), B) Core polysaccharide and C) Lipid A. (Fig la)
The specificity of the the immune response induced to this molecule is mediated through the recognition of the O-antigen polysaccharide while the lipid A portion is responsible for the immunostimulatory function (40,47). A potent antibody response can be easily induced in mammals simply by the conjugation of other antigens or haptens to the polysaccharide end of the molecule (48,49). This procedure has been found to induce excellent antibody responses in fish to haptens (36,50,51). Therefore, since it has been previously demonstrated that haptenated LPS can induce a good anti-hapten antibody response, one route to successful immunoprophylaxis may be to conjugate cellular or secreted antigens from A. salmonicida or R. salmoninarum to the LPS of V. annuillarum or E. coli (Fig. lb). The ability of LPS-modified antigens to generate strong antibody responses may be useful in overcoming the low agglutinin titers reported for R. salmoninarum in Oncorhvnchus species (4). One choice for such an antigen from R. salmoninarum might be the 57 kD protein. Possible components from A. salmonicida may include the A protein and the major

protease. Both have been identified as protective antigens (13,20,31). Although this has not been a consistent result it is suggestive that under particular conditions these antigens may be sufficient to induce immunity.

Another aspect of vaccine design, as it relates to humoral responses for fish, is that fish may not have the capacity to produce antibodies to the same wide variety of antigens as can mammals. This has been demonstrated immunochemically with defined antigens for trout (52,53) as well as other species (54). More importantly, the recent work of Ellis and Hastings (55) indicates that this apparent restriction may also apply to the salmonid antibody response produced to A. salmonicida. In one of our laboratories (Kaattari) we have observed that the number of antigens recognized by antibody produced to R. salmoninarum in coho salmon (Oncorhynchus kisutch) is less than the number recognized by rabbit antiserum produced to the same antigens (Fig. 2). The reasons for this restriction may reside in the structure of the immunoglobulin gene family as has been postulated by DuPasquier (56). This is not a restriction in the quantitative amount of antibodies produced, but rather in the number of specificities generated. Although the above experiment was conducted on a very limited basis, when considered along with the other previously described data, a need for populational studies using a variety of salmonid species becomes apparent. These studies should be designed to determine if certain populations or species are genetically incapable of producing an antibody response consisting of the specificities needed to protect a fish. It is important, however, to realize that there is no reason to believe that the antibody responses to V. anguillarum or Y. ruckeri are any less restricted than those to A. salmonicida or R. salmoninarum and yet simple vaccines , against these organisms are successful. This may indicate that this

limitation of the antibody repertoire is of little importance in generating a prophylactic response, or alternatively, that if these responses are comparably limited in their repertoires, those specificities induced that are precisely those needed for neutralization of the offending agent.

One observation introduced by Evelyn (4) that may be of particular interest with respect to this theory is the idea that there may be an inability to induce prophylaxis to BKD in Oncorhynchus species as opposed to Salmo one distinct possibility that should be explored, therefore, is whether Salmo species can produce a protective antibody which Oncorhynchus cannot. This would confirm the importance of the genetic capacity of a Pacific salmon to respond to R. salmoninarum

Before much work is done with respect to these genetic considerations it is essential that the salmonid humoral response be more thoroughly examined. Questions as to which method of antibody assessment is the best measure of a protective antibody response need study. There is no a priori reason to assume that agglutinin titers, immunodiffusion bands, or ELISA titers correlate with the total antibody response to any disease agent or with neutralization of the pathogen.

One area of immunological research which needs more development is the assessment of cell-mediated immunity to these pathogens. This is of particularly important if future work demonstrates that certain fish do not have the genetic capacity to produce a protective humoral response. Most important among the cell-mediated responses are those which give rise to enhanced phagocytic activity. The function of the macrophage is not only essential in the phagocytosis of bacterial pathogens but also in the processing and presentation of the antigen to other cells of the immune system (57,58,59). This itself may be crucial to the development of a

complete antibody response. For example, this may occur if an antigen cannot be presented properly due to toxin-induced macrophage dysfunction, or simply due to an inability to interact with the appropriate histocompatibility antigens that may preclude the triggering of the B lymphocyte which is capable of producing a protective antibody. Evaluation of the ability of various vaccines to enhance such cellular functions as chemiluminescent activity (60), phagocytic indices, and migration inhibition (61) are of particular importance since macrophage function is probably crucial to the control of BKD.

In summary, what is primarily needed for the development of reliable and consistently effective vaccines against R. ~~salmoninarum~~ and A. salmonicida are rigorous and thorough studies of the immunologic capacity of the Pacific salmon, and a more in depth understanding of the antigenic components of these bacteria. We are still at the formative stages in our resolution of the BKD problem, and although a commercial vaccine is available for furunculosis (62), the general concensus is that significant improvements are possible and that a truly efficacious vaccine that can be used in a variety of situations has not been produced. Furthermore, although research on both pathogens has identified several cellular or secreted factors which may have a function in virulence, attempts to use these as immunogens producing protective active immuntiy have been generally unsuccessful. This disappointing “take home lesson” stresses that an important virulence component of the these organisms is their immune avoidance mechanisms. Research on virulence factors or potential antigens must address immune avoidance before any real progress is to be achieved. Investigations into antigen proarations and novel delivery systems are also necesssary for further progress in this difficult area.

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Table 1. Development of a Oral Furunculosis Vaccine

Investigator	Treatment	Species	Protection
Duff (5)	$CHCl_3$ killed	CT[a]	Yes
Sniezko et al. (9)	Heat-killed	BkT[b]	No
Spence et al. (8)	Formalin	CS[c]	Not Sig.
Klontz, Anderson (6)	FSA	cs	±
Udey, Fryer (7)	FSA, Formalin	CS	No
Smith et al. (10)	Formalin	BnT[d]	Yes

[a]Cutthroat trout (Salmo clarki)
[b]Brook trout (Salvelinus fontinalis)
[c]Coho salmon (Oncorhynchus kisutch)
[d]Brown trout (Salmo trutta)

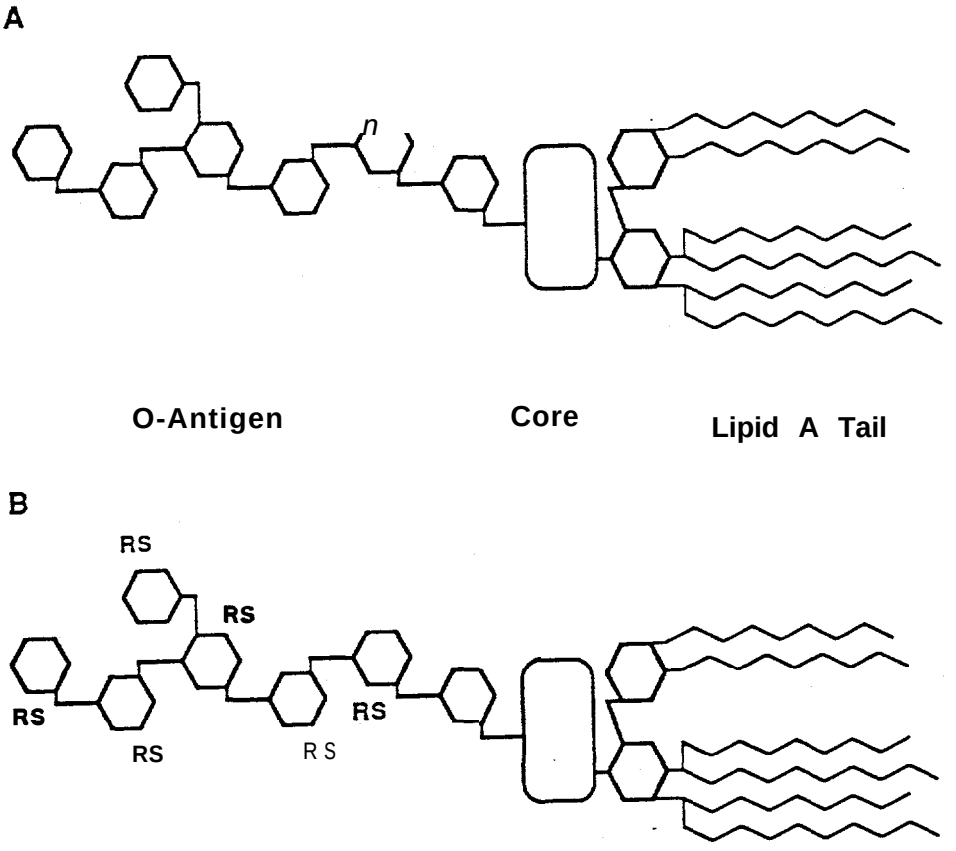

Figure 1. a) Schematic representation of a lipopolysaccharide molecule. b) Schematic representation of a lipopolysaccharide molecule conjugated to Renibacterium salmoninarum antigens.

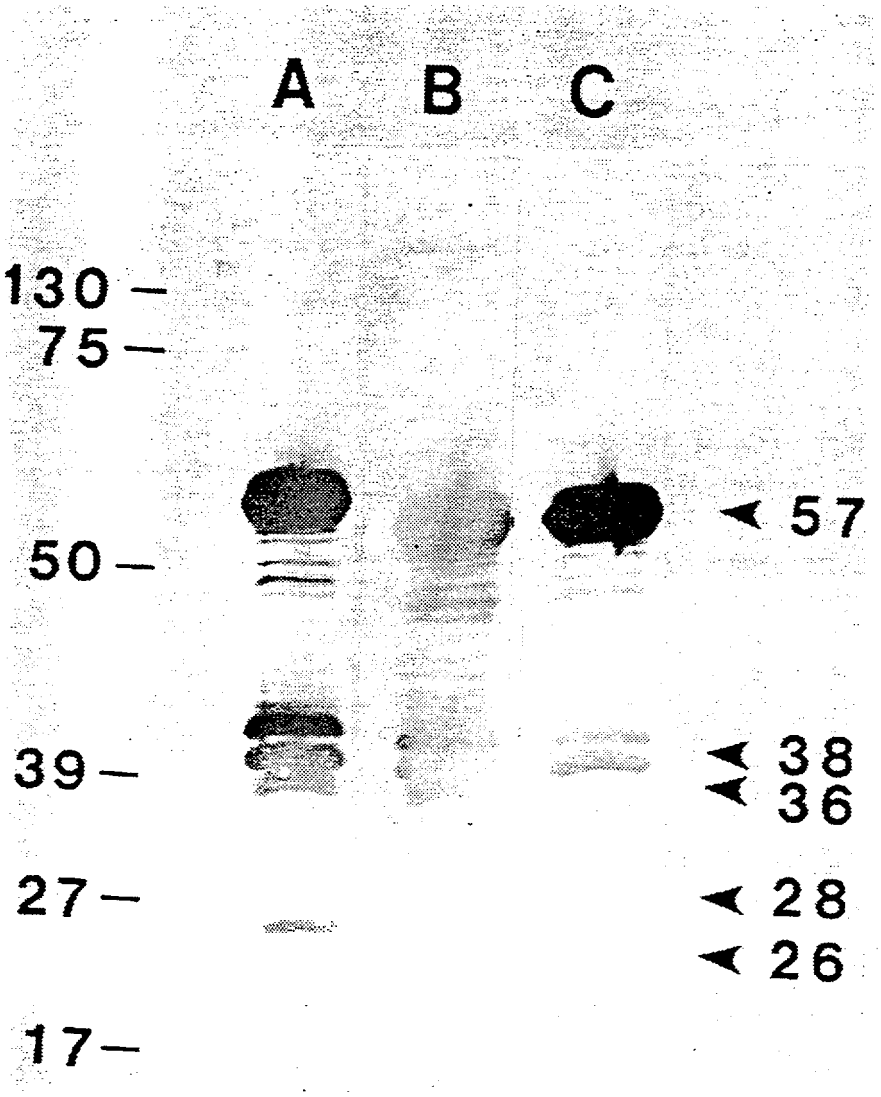

Figure 2. Western blot of Soluble Antigens (SA) from ~~Renibacterium~~ salmoninarum . Ten µg of SA was electrophoresed on a denaturing polyarylamide gel and blotted onto nitrocellulose. Blots were then incubated and developed with: Lane A - biotinylated rabbit anti-SA and streptavidin-horseradise peroxidase (SHRPO) and substrate, Lane B - rabbit and anti-SA, goat anti-rabbit HRPO, and substrate, Lane C - coho salmon anti-SA, mouse monoclonal anti-salmonid immunoglobulin, goat anti-mouse HRPO, and substrate. Values on the left indicate molecular weights of the standards in kilodaltons. Values on the right indicate molecular weight of the major SA proteins.

SUMMARY

Bacterial kidney disease of salmonids is a very complex disease which appears to exploit a variety of pathogenic mechanisms. An understanding of these mechanisms is essential to the development of efficacious vaccines. It has become well established from the studies published in this report and those of others that soluble antigens which are secreted by *Renibacterium salmoninarum* have toxigenic potential. If they are found to be responsible for mortality, the development of toxoid(s) could be paramount to the production of a vaccine.

One must, however, be circumspect in producing a vaccine. A thorough knowledge, not only of the pathogen, but also of the immune system of the host is an absolute requirement. This becomes of particular importance when dealing with fish diseases, since the field of fish immunology is still within its infancy. This lack of knowledge is particularly felt when the induction of a prophylactic immune response concommitantly leads to pathological side effects which may be as destructive as the original infection. Indeed, it appears that some aspects of BKD may be due to the induction of hypersensitivity reactions. If such immunopathologies are expressed, it is prudent to thoroughly evaluate the nature of the immunoprophylaxis to insure that these harmful sequelae do not occur.

Evaluation of a variety of antigens, adjuvants, immune responses, and survival data leads us to recommend that attempts at prophylaxis against BKD should center upon the elicitation of cellular immunity utilizing preparations of *Mycobacterium chelonii*. The choice of this species of mycobacteria was made because of its effectiveness, ease of maintenance and production, and the lack of need for its propagation within containment facilities. These assets are important to consider if large scale vaccine production is to be profitable. As can be seen from the data provided, *M. chelonii* alone is capable of producing prophylaxis to BKD, however, this is likely due to the induction of non-specific immunity and not to the existence of crossreactive antigens. Therefore, future studies should be devoted to further work on the induction of specific immunoprophylaxis incorporating this agent. This should include:

I. Neutralization of the toxigenic activity of the 57 kD protein and its conjugation or admixture to *M. chelonii*.

II. Conjugation of whole *R. salmoninarum* cells to *M. chloneii*.

Alternatively the development of vaccines for these diseases could profit from a search for the crucial components of those proven bacterial vaccines which are essential for conferring immunity. All of these vaccines have been developed for gram negative infections and, furthermore, the associated endotoxin or lipopolysaccharide (LPS) constituent of the outer membrane of the cell wall has been identified as a sufficient component to induce protection. This is logical since LPS is a cellular antigen, thus any immune response that is induced would be directed toward the organism. Further, LPS is known in a variety of animals, including Pacific salmon, to be a potent immunostimulant. However, *R. salmoninarum* is a gram positive bacterium and does not have LPS in its cell wall. Therefore direct conjugation of the LPS moleccule to the cellular antigens of *R. salmoninarum* could be employed in the development of a vaccine.

Another area which requires extensive development is that of the assesssment of cell-mediated immunity to pathogens such as *R. salmoninarum*. Most important among the cell-mediated responses are those which give rise to enhanced phagocytic activity. The function of the macrophage is not only essential in the phagocytosis of bacterial pathogens but also in the processing and presentation of the antigen to other cells of the immune system.

It is essential that any future goals can only be efficiently achieved with a thorough groundwork of knowledge on the humoral aspects of protective immunity in salmonids as well as the development of methodologies to assess specific cellular immunity. This would facilitate not only vaccine development for BKD, but also for a variety of viral, fungal, and parasitic diseases.

ASSEMBLY LIST

Page 1 of 2

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REMARKS

DEVELOPMENT OF A VACCINE FOR BACTERIAL KIDNEY DISEASE IN SALMON

Final Report

By

Dr. Stephen Kaattari, Project Leader
Mr. Prasad Turaga, Graduate Research Assistant
Mr. Gregory Wiens, Graduate Research Assistant

Department of Microbiology
Oregon State University 97331-3804

Prepared for

Alan Ruger, Project Officer
Bonneville Power Administration
Division of Fish and Wildlife
PO Box 3621
Portland OR 97208

Project No. DE-A179-84BP16480

August 31, 1989

DEVELOPMENT OF A VACCINE FOR BACTERIAL KIDNEY DISEASE IN SALMON

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Department of Microbiology
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Prepared for

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U.S. Department of Energy
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P.O. Box 3621
Portland, OR 97208-3621

Project No. 84-46
Contract Number DE-AI79-84BP16480

August, 1989

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(Previously BPA 100)

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Remarks

Development of a Vaccine for Bacterial Kidney Disease in Salmon

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REMARKS

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Oregon State University

Final Report on a Vaccine for Bacterial Kidney Disease in Salmon

Final Report

Illustration

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Final Report

U.S. Department of Energy
Bonneville Power Administration

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14. Justification: BPA is required by the Northwest Power Act (PL 96-501) to "protect, mitigate and enhance fish & wildlife" impacted by hydroelectric development and operation within the Columbia River basin. BPA projects fulfill those requirements. They are required by contract to produce annual progress & final reports to keep the scientific community abreast of activities to keep XXXXXXXXXXXXXX Congress and the NW Power Planning Council apprised of project progress; to prevent duplication of effort and to provide a basis for further efforts throughout the region.

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