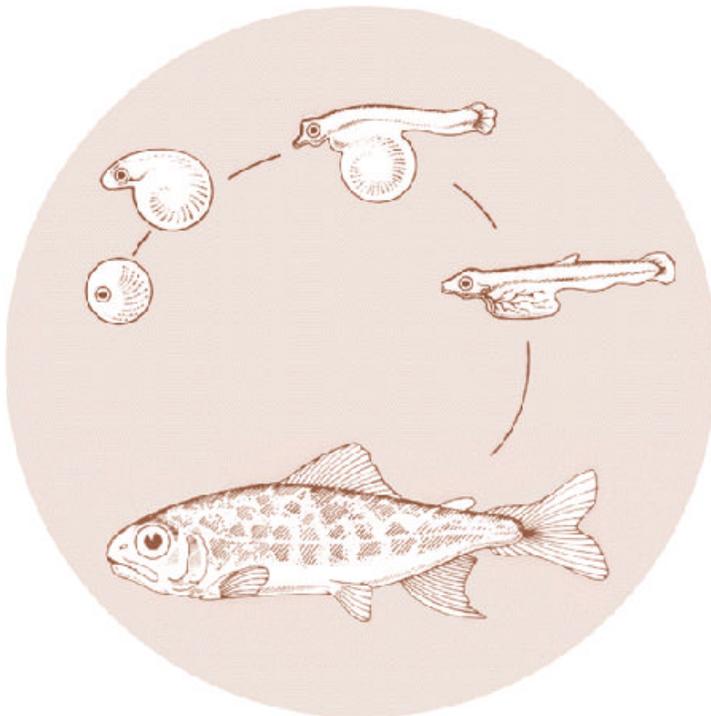


June 1987

# DEVELOPMENT OF A VACCINE FOR BACTERIAL KIDNEY DISEASE IN SALMON

Annual Report 1986



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Development of a Vaccine for  
Bacterial Kidney Disease in Salmon

Annual Report FY 1986

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## EXECUTIVE SUMMARY

Bacterial kidney disease (BRD) has been and remains a chronic contributory problem limiting the productivity of salmon of the Columbia River Basin. Control of this disease will not come easily, but it would lead to a tremendous increase in the health and numbers of our salmon populations. Vaccination of salmon of Renibacterium salmoninarum (KDB) is a potentially successful method of controlling this disease. To date, however, no successful vaccine has been developed for general use. A possible solution to this problem, and thus the goal of this research, is to isolate the antigenic components of KDB and enhance their ability to activate the host defenses. This will be accomplished by the chemical modification of these antigens with potent immunomodulatory substances. These modified antigens will then be tested for their effectiveness in inducing immunity to BKD and thereby preventing the disease.

The goal of the project's third year was to test the immunogenicity and prophylactic value in coho salmon (Oncorhynchus kisutch) of various chemical conjugates of Renibacterium salmoninarum cells and major antigens. This was accomplished by assessing the serum antibody response, the cellular-immune response (cellular proliferation), and the kinetics of mortality after lethal injections of the bacterium. An important facet of this research is the identification and isolation of virulence factors. These studies are not only important to the dissection of the mechanism of pathogenesis of bacterial kidney disease, but the purification of such a factor(s) will insure the production of a more potent vaccine.

The studies completed this year have: 1) identified antigenic material which protect; 2) identified antigenic material which can exacerbate the disease; 3) identified a possibly major mechanism of pathogenesis via the interference with antibody; 4) the general ability to produce delineated a western blot technique for identification of infected fish; 5) described the use of monoclonal antibodies for antigenic analysis; and 6) identified an unusual and dramatic effect of R. salmoninarum cells on phagocytic function.

## INTRODUCTION

### Geographical Distribution

Bacterial kidney disease (BRD) is one of the most widespread diseases in the Columbia River Basin and occurs in many parts of the United States, Canada, Europe (Fryer and Sanders, 1981) and Japan (Kimura and Awakura, 1977). The first reported identification of what was most likely *R. salmoninarum* or kidney disease bacterium (RDB), was in 1930. Smith (1964) communicates that gram positive diplococci were isolated from Atlantic salmon (*Salmo salar*) found in Aberdeenshire in the River Spey in Scotland. The first recorded cases in the United States occurred in hatcheries in the state of Massachusetts as described by Belding and Merrill (1935). Not only is this disease a very grave problem for hatchery reared salmonids, but it has also been demonstrated to occur in wild populations (Evelyn et al., 1973).

### The Pathogen

The kidney disease bacterium (KDB) is described as a non-motile, non-spore forming diplobacillus. Original attempts to isolate the organism failed due to its rather fastidious requirements for growth in culture media. The first successful attempt at cultivation utilized media with such rich supplements as beef serum and fish extract (Earp et al., 1953). Later work of Ordal and Earp (1956) made use of cysteine blood agar, which contained 20% human blood as well as 0.1% cysteine-HCl. The most recent modifications

obviates the need for blood or serum, thus reducing the cost. This medium employs 0.1% L-cysteine-HCl in Mueller Hinton medium (Wolf and Dunbar, 1959).

Superficially the organisms, especially when seen within the tissues, resemble corynebacteria, thus they have been referred to as corynebacteria and the disease as corynebacterial kidney disease (Ordal and Earp, 1956; Hunn, 1964; Wedemeyer and Rose, 1973). Certain aspects of the pathology caused by this organism resemble not only corynebacterial disease, but to some degree diseases caused by mycobacteria and listeria. However, these rather circumstantial methods of characterization were not satisfactory for rigorous taxonomic classification. Sanders and Fryer (1980) explored this taxonomic problem on the molecular level, and determined that in regards to the guanosine/cytosine (GC) content, peptidoglycan and cell wall composition, these organisms were quite unique. Due to these singular molecular characteristics, these organisms have been placed in their own genus and species, Renibacterium salmoninarum. Isolates from various regions of the world also seem to share only a single serotype (Getchell, 1983).

#### Disease Pathology

The disease caused by RDB is considered to be a chronic systemic disease, with lesions occurring through much of the viscera and musculature in advanced cases. A common route of entry for the organism has not been demonstrated, and it seems plausible that infection may occur by various routes. Wood and Wallis (1955) have demonstrated that the ingestion of infected salmon flesh by chinook salmon leads to a lethal infection.

Alternatively, eye trauma has also been suggested as a possible route of infection. Hendricks and Leek (1975) found that chinook salmon possessing exophthalmia, demonstrated granulomatous lesions behind the affected eye which contained large numbers of leukocytes and KDB. Many of the fish which possessed this exophthalmia, had no other lesions internally. However, if all fish which exhibited this exophthalmia were held in aquaria, it was found that they all succumbed to BKD within two to three months. Upon necropsy, the animals were found to have disseminated BRD lesions. It was felt that the eye may serve a primary route of infection in hatchery-reared salmonids, due to the frequency of eye trauma when the animals are maintained in raceways. A similar, but perhaps a more common route of infection, may be through general abrasion of the body surface (Wolf and Dunbar, 1959).

Although there are many organs which become infiltrated with KDB, most investigators feel that the primary target is the kidney. The hematopoietic portion of the anterior kidney appears to be especially susceptible. It is a diagnostic feature of the disease to see white granulomatous areas of infection within the kidney. When examined microscopically these areas are seen to possess KD organisms. These foci of infection are not limited to the kidney, but they are found to appear also in the spleen and liver. As the disease progresses, the reproductive organs, musculature, and brain often become infected. Externally, besides the exophthalmia, pustules or blebs may be seen above the lateral line and petechial hemorrhaging around the muscles of the peritoneum.

One of the most striking features of the internal pathology of BKD is the development of a white pseudomembrane composed of dead host tissue,

bacteria, and leukocytes. This false membrane has been seen to cover the liver, reproductive organs, spleen, and occasionally the swim bladder (Snieszko and Griffin, 1955). This phenomenon is not unlike the pseudomembrane produced in diphtheria infections in man. The formation of this pseudomembrane, however, is quite temperature dependent. It is reported that it forms at temperatures below  $8.3^{\circ}\text{C}$ , whereas at higher temperatures only necrosis is found (Smith, 1964). Attempts to isolate an exotoxin as is expressed by Corynebacteria diphtheriae have not met with success.

On the cellular level, bacterial kidney disease appears to be more similar to listerial or mycobacterial infections. A common feature of these diseases are the granulomatous reactions that occur. Like listeria and mycobacteria, KDB organisms are phagocytized by macrophages, but are not always digested by the phagocytic cells (Young and Chapman, 1978). In fact, the KDB as well as listeria and mycobacteria have been observed to multiply within the macrophage itself. In the case of mycobacteria, the cellular arm of the immune response is eventually activated to destroy the bacteria. The usual delay in this response, coupled with physiological mechanisms the pathogen uses to subvert the phagocytic response leads to a widespread infection. With regards to a specific antibody response, intracellular organisms such as KDB are thought to be relatively resistant, while within their host cell. In this situation, although the pathogen may be protected, the immune system of the host is still exposed to a continuous supply of antigen from the pathogens. As a result there is a continuous severe immune reaction which eventually destroys the surrounding host tissue in attempting to destroy the pathogen. This immune reaction leads to much necrosis and

characteristically severe granulomatous lesions. Since KDB is harbored by macrophages of the fish and these severe granulomatous reactions occur in response to the antigen of the pathogen, it is not surprising that two of the organs that are most severely affected are the spleen and kidney. Both of these organs are immune organs and contain a great number of macrophages.

#### Effect on the Kidney

It is felt that terminal cases of BKD may be fatal due to the destruction of the kidney which, in turn, may lead to an inability of the salmon to osmoregulate (Frantsi et al., 1975). This feature of BKD is extremely important in light of the evidence that salmon infected with BKD demonstrate marked increase in mortality when held in salt water as compared being held in fresh water (Banner et al., 1983). The possibility arises that even if fish seem relatively healthy or have recovered from BKD after antibiotic treatment, they may be at high risk once they enter the ocean, due to extensive kidney damage.

Kidney pathology, in salmon with BKD, looks quite similar to the pathology seen in glomerulonephritis in mammals. Glomerulonephritis could be mediated by either of two mechanisms: 1) immune complex formation between KDB antigens and anti-KDB antibodies or, 2) by a reaction of anti-KDB with crossreactive kidney antigen on the basement membrane of the glomerulus. The precise mechanism by which this condition is elicited has yet to be

discovered, however, due to the degree of bacterial infiltration that occurs throughout the body of the fish, it would seem most likely that an immune complex reaction could be occurring.

### Tolerance

It seems obvious that in most cases of BKD the salmon are responding to the pathogen, but their response seems inappropriate and incapable of overcoming the pathogenic insult. Immunological tolerance may play a role in BKD if it serves to delay or produce an inappropriate immune response.

If a foreign antigen is present within the body early in life, the animal may experience a state of immunological tolerance (Billingham et al., 1953). In this state the animal does not recognize that particular foreign antigen as being different from its own body and; therefore, it will not respond to it immunologically. This phenomenon may apply in some cases to BKD. It has been demonstrated by Evelyn et al, (1984) that eggs from females with BKD possess the pathogen within the yolk. This was demonstrated microscopically and by iodine treatment of the egg surface. The iodine treatment should have killed any pathogen on the surface of the egg or residing within the perivitelline space, but the organism persisted. If this evidence is correct, it would support the hypothesis of vertically transmitted of BKD and would indicate a route by which fry might experience conditions similar to that giving rise to neonatal tolerance in mammals.

## Induction of Protective Immunity

The appearance of circulating antibody does not correlate with protection from the disease, Although this intracellular location of the pathogen may seem inaccessible, it is not. Mycobacteria and listeria also reside within phagocytes and these pathogens can be controlled if the host is properly sensitized or immunized. Listeria, which possesses physical characteristics similar to those of KDB (Bullock et al., 1975), appears to be insensitive to specific antibody. Priming of the cell mediated immune response, however, results in the elimination of these pathogens. Induction of the cell mediated (T cell) response results in the activation of the phagocytic cell and digestion of the bacteria residing there (Mackness, 1969).

It is possible to enhance this cell mediated immune response, as well as the immune response in general through the use of adjuvants. Adjuvants utilizing bacteria such as mycobacteria or corynebacteria lead to an enhancement of the immune response to the admixed antigen, Such augmentation may aid in the control of BKD. It has been reported that intraperitoneal injections of, KDB emulsified in oil and mycobacteria (Freund's complete adjuvant) can lead to a reduction of BRD lesions and organisms (Paterson et al., 1981). Although such immunization procedures would be impractical for large scale vaccine programs, they do demonstrate that proper presentation of KDB antigen to the fish can lead to a protective state of immunity,

## MATERIALS AND METHODS

Animals. Six-12 month coho salmon (150-300 g) were kept in ambient (12<sup>o</sup>C) pathogen free well water, in 220 gallon circular tanks at the Oregon State University Fish Disease Laboratory (OSU-FDL). The fish were maintained on Oregon Moist Pellets.

Adult female New Zealand White (NZW) rabbits and BALB/c mice were maintained by the Laboratory Animal Resource Center at Oregon State University.

Bacterial strain. R. salmoninarum isolate Lea-1-74 (ATCC 33209), obtained from J. R. Rohovec, Oregon State University, Corvallis, Oregon, was used throughout the study.

Growth conditions. Bacteria were grown in either unfiltered Kidney Disease Medium-2 (KDM-2 or ultrafiltered KDM-2 (UF-KDM-2). Roth media were modified by the elimination of the bovine serum supplementation that was originally specified by Evelyn (1977). The UF-KDM-2 was prepared by passage of KDM-2 through a PTGC-10,000 NMWL filter packet in a Minitan ultrafiltration apparatus (Millipore Corp., Bedford, MA). This filtration produced media free of molecules with molecular weights greater than 10,000. Cultures were incubated for one to three weeks in low form culture flasks with constant agitation. At the end of the incubation period, the bacterial cells were centrifuged at 6000 x g for 30 minutes (4<sup>o</sup>C) and the supernatant fluid was saved for soluble antigen extraction..

Soluble antigen extraction. Culture supernatants were filtered, as described above. The retentates, or high molecular weight fractions, were concentrated by 50% saturated ammonium sulfate (SAS) precipitation. After addition of the ammonium sulfate, the solutions were stirred for 3-4 hours at 4<sup>0</sup>C. The precipitate was removed by centrifugation at 6000 x g for 15 minutes (4<sup>0</sup>C) and suspended in 10-20 ml of 0.01 M phosphate buffered saline, pH 7.2 (PBS). The solution was reprecipitated twice and the resuspended precipitate dialysed extensively against PBS. The dialysed protein extract was assayed for protein by the method of Lowry et al. (1951).

Sonication. Sonicates of bacterial cells were prepared as described by Getchell (1983). Briefly, a suspension of 10% washed cells in PBS were exposed to four bursts at 50 watts from a Sonifier Cell Disruptor (Heat Systems Ultrasonics, Plainview, NY). Particulates were removed by centrifugation at 5000 x g and the supernate filter sterilized and stored at 4<sup>0</sup>C until used.

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 immunogen and Freund's complete adjuvant (FCA). 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 -70<sup>0</sup>C.

Immunogens used were formalin-killed R. salmoninarum, washed three times in PBS and resuspended to 1.0 OD<sub>520</sub>; dialysed protein

extract from UF-KDM-2 (1.5 mg/ml); and dialysed protein extract from KDM-2 (2.2 mg/ml).

Antisera from coho salmon were produced by monthly injections of 0.1 ml of a 1:1 mixture of formalin-killed R. salmoninarum in FCA. Serum was aliquoted and stored at  $-70^{\circ}\text{C}$  until used.

Gel filtration. Standard solutions of all antigen preparations were examined with respect to their molecular weight by the use of gel filtration. Briefly, each antigen preparation was eluted through a P-150 column (Biorad, Richmond, CA). Each sample was adjusted to a concentration of 1.0 mg in 1.0 ml. Elution was conducted using PBS as the elution buffer. The fractions were monitored for protein using the Lowry method. Molecular weight standards used for the calibration of the column consisted of human IgG (150 kd), bovine serum albumin (68 kd), and cytochrome C (13 kd).

Polyacrylamide gel electrophoresis (PAGE). The method followed is basically that of Laemmli (1970). The samples were mixed with the sample buffer at a 1:1 ratio and subjected to the electrophoretic field. The samples are usually loaded in a duplicate fashion so that one half is subjected to silver staining and the other half is used for Western blotting. During the electrophoretic run, a constant 20 mA current is applied in the stacking gel and a constant 40mA during the run when the samples are in the separating gel. After completion of the experiment the gel is cut into two halves and one half is silver stained by the method of Wray et al. (81).

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Serum samples from infected and normal coho salmon were stored at  $-20^{\circ}\text{C}$ . 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% v/v glycerol, and 3 mM bromophenol blue). Then placed in a boiling water bath for 2-3 minutes. Samples were then immediately run on 83 x 103 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) (BioRad, Richmond, CA, USA).

Conjugation of horseradish peroxidase (HRPO) to antibody. Four mg of horseradish peroxidase (Sigma, St. Louis, MO) were dissolved in one ml of distilled water. To this solution, 200 ul of a 0.1 M solution of sodium meta-periodate (Sigma) in water was added and stirred at room temperature for 20 minutes. This mixture was then dialysed against 1 mM acetate huffer (pH 4.4) overnight at 4°C.

Simultaneously, a 50% saturated ammonium sulfate (SAS) globulin precipitate of 1 ml rabbit antiserum was reconstituted in 1 ml of distilled water and dialysed overnight at 12°C in 0.01 M Na<sub>2</sub>CO<sub>3</sub> pH 9.47. Twenty ul of this bicarbonate buffer was then added to the HRPO to raise it to a pH of 9.0-9.5, The rabbit globulins were then immediately added to the solution and stirred at room temperature for two hours. This conjugate was then dialysed against 0.01 M PBS with a dialysis membrane having a pore size that included molecular weight species of greater than 50000. The conjugate was stored in the dark in 0.001% merthiolate.

ELISA for the detection of soluble antigens. A method for the detection of soluble KDB antigen was developed utilizing a capture antibody technique. Briefly, individual wells of Costar EIA 1/2 well

plate (Cambridge, MA) were coated with rabbit anti-soluble antigen (from either UF-RDM-2 or KDM-2). Antigen coating was accomplished by incubating the rabbit antibody diluted in carbonate buffer (0.05 M, pH 9.5) overnight at 4°C. The precise concentration of antibody varied depending upon the experimental protocol. Prior to use, the plates were washed three times in a Tris/Tween diluent buffer (see appendix), followed by three washes in Tris buffer. Solutions containing soluble antigens or unknowns were diluted in Tris/Tween and added to the wells of the plate in 100 µl aliquots and incubated for two hours at room temperature. Following this incubation, the wash sequence was repeated. At this point 100 µl of rabbit anti-soluble antigen-HRPO was added to each well and incubated for two hours at room temperature. This incubation was followed by another standard wash and the addition of 100 µl of the substrate solution (see appendix). Elaboration of a colored product was read spectrophotometrically at 405 nm by a Biotek Automatic ELISA Reader (Burlington, VT). Absorbance readings were proportional to the amount of soluble antigen bound to the plate since the conjugate was incubated in excess.

Monoclonal antibody production. Adult female BALB/c mice (Simonsen Laboratories, Gilroy, CA) were immunized with either 100 µg soluble antigen or 1 O.D. unit of formalin-treated RDB emulsified in Freund's complete adjuvant. Sera from immunized animals were tested at one month. Animals with positive titers, as determined by the previously described ELISA, were challenged intravenously (i.v.) with 10 µg of the

same antigen in physiological saline. Animals with negative titers were boosted with the initial antigen preparation until a positive titer appeared.

Three days after i.v. challenge, single cell suspensions were prepared from the spleen and fused with an equivalent number of SP/2 myeloma cells by the aid of polyethylene glycol (Fisher, Fair Lawn, Nj). Hybridomas were selected by addition of hypoxanthine-thymidine-aminopterin (HAT) medium (Oi and Herzenberg, 1980). Productive fusions were ascertained by screening the hybridoma supernatants with the previously described EIISA. Cells from these fusions were cloned and stored in liquid nitrogen until used.

Culture medium. All in vitro cultures employed the one medium described here. Media components were purchased from Whittaker M. A. Bioproducts, Walkersville, MD, unless otherwise noted. Mishell-Dutton holding medium (HM) consisted of 100 ug/ml gentamicin and 10% fetal calf serum in RPMI 1640 (Gibco). Mishell-Dutton modified RPMI (RPMI MDM) was used for tissue culture and consisted of RPMI supplemented with: non-essential amino acids, sodium pyruvate, L-glutamine, 10% fetal calf serum (hybridoma screened), 100 ug/ml gentamicin, 50 uM 2-mercaptoethanol (MCB, Cincinnati, OH), and the nucleosides, adenosine, uracil, cytosine, and guanine (10 ug/ml, Sigma, St. Louis, MO). The nutritional cocktail was also prepared as previously described (Tittle and Rittenberg, 1978) and fed daily to the cultures as described below.

Cell Cultures. Fish were sacrificed and their spleens and/or anterior kidneys were aseptically removed and placed in holding medium. A single cell

suspension of each organ was obtained by aspiration through a one ml syringe. Organs from multiple fish were pooled to obtain the required number of cells for culture. The resulting cell suspension was incubated on ice to allow organ fragments to settle. The supernatant medium, containing a single cell suspension, was then washed two times in holding medium by centrifugation at 500 x g for 10 minutes at 4°C. The final washed cell pellet was resuspended in RPMI MDM. Lymphocytes were enumerated by the use of a hemocytometer or Coulter counter (Coulter Electronics, Hialeah, FL) adjusted for counting salmonid leukocytes. The cell suspension was then adjusted with RPMI MEM to a concentration of  $2 \times 10^7$  cells/ml and held on ice until culture. 0.2 ml aliquots of the final cell suspension were added to the wells of a 24-well, flat-bottomed, tissue culture plate (Corning, Corning, NY) containing antigen or mitogen. Tissue culture plates were then incubated in plastic culture boxes (C.B.S. Scientific, Del Mar, CA) in an atmosphere of 7% CO<sub>2</sub> at 16°C. The cultures used for PFC assays were maintained by adding 25 ul of cocktail on alternate days.

Plaque-forming Cell Assay. Single cell suspensions of  $2 \times 10^7$  cells/ml were prepared in RPMI MDM, as described above. Aliquots of 0.2 ml were added to 0.2 ml of the appropriate dilution of antigen in RPMI MDM or in medium alone. Cultures were fed 50 ul of feeding cocktail on alternate days until harvest. Cells secreting anti-trinitrophenyl (TNP) or anti-soluble antigen antibodies were detected by a modification of the Cunningham plaque assay (Cunningham and Szenberg, 1968). 100 ul (40 ul for final assays) of the lymphocyte suspension, 25 ul (10 ul ) of a 10% suspension of TNP-sheep red blood cells (TNP-SRBC; Rittenberg and Pratt, 1969) in modified barbital buffer

(MBB), and 25 ul (10 ul of steelhead serum, diluted in MBB, were mixed in individual wells of 96-well microtiter plate (Linbro, McLean, VA). The contents of each well was pipetted into a slide chamber, sealed and incubated for 1-2 hours at **16°C**. Plaques were then enumerated under low power with the aid of a dissecting microscope. KD SRBC were produced by chromic chloride conjugation (Vyas et al., 1968).

Mitogen Assays. For the mitogen assays, 50 ul of the cell suspension ( $1 \times 10^6$  cells/ml) were placed in individual wells of a 96-well flat bottom tissue culture plate with 50 ul of mitogen (Vibrio extract or phytohemagglutinin) or culture medium. The plates were then incubated in gas boxes under 7%  $\text{CO}_2$  at **17°C**. Twenty-four hours before harvest each well was pulsed with 1 **uCi** of tritiated thymidine (methyl- $^3\text{H}$ , ICN Biomedicals, Irvine, CA) in 50 ul of RPMI MDM. 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 liter toluene, after Etlinger et al., 1976), and counted on a Beckman liquid scintillation counter (EL 3800). Data are reported as mean counts per minute (cpm) +/- standard error, of triplicate cultures, or as stimulation indices (SI) defined as experimental cpm/control cpm.

Glutaraldehyde conjugation. This form of conjugation basically follows the procedure of Avrameas (1969). Briefly, the bacterial suspension is diluted with phosphate buffered saline (PBS - pH 7.2) to a concentration of  $3 \times 10^8$  cells/ml. To two-five ml of this suspension, one ml of KD soluble antigen (2 mg/ml) is added and mixed in the suspension. Twenty ml of 25%

glutaraldehyde (Sigma, St. Louis, MO) is added and the suspension is allowed to react for 2 hrs. on ice. The preparation is dialysed overnight in PBS at 4°C. The suspension is then centrifuged at 6130 x g for 20 minutes and the supernatant removed. The cellular pellet resuspended to the original cellular concentration of  $3 \times 10^8$  cell/ml.

Conjugation with cyanuric chloride. This method of conjugation is taken from that of Scibienski et al. (1977). Briefly, 0.5 ml of Vibrio extract is added to 1 ml of 0.15 M  $\text{NaHCO}_3$  and kept constantly stirring while held on ice. Cyanuric chloride (0.2 ml of 10 mg/ml) in acetone is added to the reaction mixture. The mixture is then stirred for 30 minutes on ice. KDB cells (2 ml of a 1.0 O.D. solution in  $\text{NaHCO}_3$ ) are added to the solution. This mixture is then incubated for four hours at room temperature, while stirring. The cells are centrifuged at 6130 x g for 30 minutes at 4°C. The cells are resuspended to the original volume with PBS and dialysed in PBS overnight at 4°C.

Conjugation with ethyl-carbodiimide (ECDI). Soluble antigen (1.5 mg) was added to 9.5 mg of ECDI. This solution was mixed well then added to 3 ml of formalin-killed vitro cells (1.0 O.D. at 500 nm). This suspension was stirred for 1 hr. at room temperature. Six mg of glycine was then added to the suspension to terminate the reaction. The suspension was stirred for an additional 30 min. The suspension was then dialyzed extensively against PBS to remove excess ECDI-glycine.

Conjugation with tannic acid. Vibrio cells (0.5 ml packed cells) were washed 3 times with PBS (pH 7.2). PBS (pH 7.2) was prepared by mixing 100 ml of saline with 100 ml of phosphate buffer consisting of 24 ml of 0.15 M  $\text{KH}_2\text{PO}_4$

and 76 ml of 0.15M  $\text{Na}_2\text{HPO}_4$ . The cells were then diluted with 7 ml of PBS (pH 7.2). To 2 ml of this Vibrio suspension 2 ml of tannic acid (0.05 mg/ml saline) solution were added. This suspension was mixed for 10 minutes in a  $37^\circ\text{C}$  water bath. The tannic cells were then washed 3 times in PBS (pH 7.2). One ml of soluble antigen (1 mg/ml), 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 ml of phosphate buffer consisting of 32.2 ml of 0.15 M  $\text{Na}_2\text{HPO}_4$  and 67.8 ml of 0.15 M  $\text{KH}_2\text{PO}_4$ . To block remaining protein binding sites on the Vibrio cells, the conjugated cells were incubated for 10 minutes in a gelatin solution (2 mg/ml saline).

Chemiluminescence. Single cells suspension of anterior kidney lymphocytes were prepared described above. In a 5 ml polystyrene test tube, 500 ul of luminol (LKB) was added to 300 ul of stimulant (1 O.D. R. salmoninarum, 1 mg/ml soluble antigen, or 1 O.D. Vibrio cells). Prior to light monitoring on a LKB luminometer, 200 ul of the anterior kidney cell suspension was added.

## RESULTS AND DISCUSSION

Electrophoretic analysis of soluble and sonicated antigens. In a previous annual report (Kaattari et al., 1984) we demonstrated the existence of at least three major soluble antigens; 60000 (60KD), 34000(34RD), and 26000(26KD) molecular weight proteins. The possibility that the 34KD and 26KD could add up to a 60KD protein, and since the monoclonal antibody studies (see below) demonstrate antigenic identity among these proteins, it is of importance to purify these proteins for further comparison. Furthermore, as will be demonstrated later, since biological activity is associated with these proteins, it is important to determine which of these proteins possess such activity.

Figure 1 demonstrates that the soluble antigens are consistently produced from batch to batch. Although batch 2 (Fig. 1 - C, D) lanes are weaker than batch 3 (E, F), major common proteins are seen to occur. Furthermore, the more intense banding patterns (E, F) reveal a 48-50 KD and a 30-34 KD protein.

Electrophoresis under native, or non-denaturing conditions (Fig. 2) revealed additional information about the soluble antigens as well as the effect of sonication of whole *R. salmoninarum* cells. Since native PAGE separates proteins on the basis of molecular weight and charge, the dispersing effect that different charges among different proteins could demonstrate an additional dimension of heterogeneity of the soluble antigens. Since this gel is run under non--denaturing conditions, it must be realized that the molecular weight markers (Fig, 2 - A, B, E, I, L, P, Q) do not represent true molecular

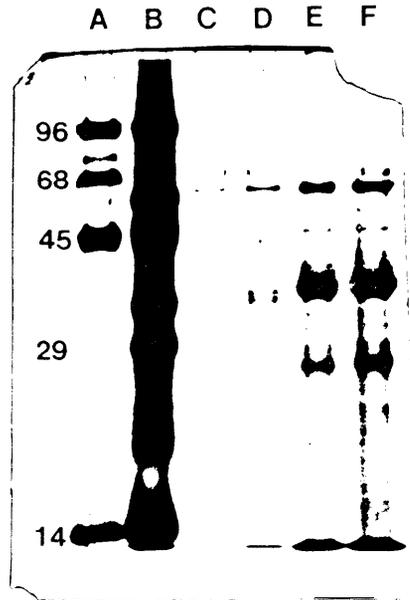


Figure 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of *R. salmoninarum* soluble antigen preparations. A = molecular weight markers; phosphorylase (96 KD), bovine serum albumin (68 KD), ovalbumin(45 KD), carbonic anhydrase (29 KD), lysozyme (14 KD). B = pre-stained markers (not defined). C = 4 ul of soluble antigen (batch 2, 1 mg/ml); D = 8 ul (batch 2, 1 mg/ml); E = 4 ul (batch 3, 1.2 mg/ml); F = 8 ul (batch 3, 1.2 jg/ml)