IV. PROJECT PROGRESS REPORTS

A. Immunization of Channel Catfish

Termination Report
For the Period
May 2, 1989 to April 30, 1991

COOPERATING INSTITUTIONS:

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ADMINISTRATIVE ADVISOR:

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REASON FOR TERMINATION:

Project completed.

PRINCIPAL ACCOMPLISHMENTS:

Auburn University

Objective 1A: Isolation and purification of immunocompetent antigen.

Cell extract and crude membrane protein from Edwardsiella ictaluri were used to immunize channel catfish. The antibody titer of immunized fish to cell extract and crude membrane gradually increased the first week and then more rapidly thereafter until reaching a maximum titer at four weeks post intraperitoneal injection. An anamnestic response immediately followed a booster vaccination in which the antibody titer increased in a short period of time. The antibody titers gradually declined after this point, but were still detectable 11 weeks post immunization. A whole cell formalin-killed preparation injected at 2.0×10^4 cells/fish produced lower antibody titers than 2.0×10^6 or 2.0×10^8 cells/fish, but there was no difference in antibody titers from 2.0×10^6 and 2.0×10^8 cells/injected fish. A crude membrane preparation produced similar results; antibody production resulting from 0.20 and 1.50 mg protein/fish were not different, but were higher than that stimulated by 0.02 mg protein/fish. In general, the higher the antigen concentration the higher the antibody production, but an antigen saturation point was attained. The antibody response at 25 and 30°C were similar in rate of increase and did not differ. However, antibody titer of fish at 20°C was lower than those produced at 25 and 30°C. Channel catfish exposed to E. ictaluri by immersion for two and five minutes had similar antibody response, but fish exposed for 30 minutes and eight hours had higher antibody response.

Objective 1B: Protection of channel catfish to E. ictaluri from immunization.

Cells transferred from fish immunized with E. ictaluri were used to determine the cell-mediated immune response of channel catfish, Ictalurus punctatus, and compare this response to the humoral immune response. Channel catfish (average size of 28.7 g) were immunized intraperitoneally with cell extract of E. ictaluri, and 21 days later head kidney cells from immunized and non-immunized fish were removed and transferred to other immunized and non-immunized

fish. Total white blood cell count from control fish was 2.5 x 10^6 cell/ml and 3.1 x 10^6 cells/ml in immunized fish at 21 days post immunization. The mortality was 20% in non-immunized-non-cell transferred fish, 32% in immunized-non-cell transferred fish, and 8% in both immunized-cell transferred and non-immunized-cell transferred. Head kidney cells transferred from immunized and non-immunized fish to other immunized and non-immunized fish resulted in only 8% mortality, which indicated that cell transfer played a more important role in protection than immunization alone.

Channel catfish were vaccinated intraperitoneally with cell extract, crude membrane, and a 36 kDa purified outer membrane protein from E. ictaluri. Fish were boosted 14 days later, and then 28 days after initial vaccination they were challenged with E. ictaluri. When fish were vaccinated with the 36 kDa outer membrane protein and boosted, mortality was reduced from 54.5% in the control group to 24.0% in vaccinated fish. Fish vaccinated with cell extract, crude membrane (both boosted and non-boosted), and fish not boosted with the 36 kDa protein demonstrated no degree of protection compared to control. Survivors from a natural infection of E. ictaluri demonstrated a strong relationship between degree of protection. Intraperitoneal injection of 2.0 x 10^7 cells killed 100% of fish with antibody titers of 0 to 128; 77.8% of fish with titers from 256 to 512 (medium); and 57.7% of the fish with titers of >1024 (high). The second trial using 5.1 x 10^5 cells/fish gave 72.2% mortality in fish with no detectable antibody titers, 51.3% in low antibody titer fish, 25.0% in medium antibody titer fish, and 6.5% in high antibody titer fish. These results demonstrate that channel catfish had protective antibody after they were exposed to the pathogen, but if fish are challenged with large numbers of pathogens, this protective immunity can be overwhelmed.

Application of cell extract of E. ictaluri impregnated feed showed that vaccinated fish receiving the antigen-impregnated feed every 10 days maintained their antibody titer.

USEFULNESS OF FINDINGS:

Results of the research at Auburn provide some basic knowledge to the immunity of channel catfish to E. ictaluri. It is important to know that catfish respond immunogenically to bacterial cell protein extract, and crude membrane material and that they do have an anamnestic response. Also >0.2 mg of protein was required to produce a significant immune response. Water temperature and length of exposure time to antigen are critical points to be determined. The role of cell-mediated immunity to the immune response was hinted but not proven. Protection of immunized catfish from infection of E. ictaluri was also an important key to antigen preparation (vaccine) and application. Fish exposed for two and five minutes had similar antibody response, but fish exposed for 30 minutes and eight hours had higher antibody response.

Louisiana State University

Objective 2A: Cloning of the channel catfish herpesvirus (CCV) thymidine kinase gene.

The gene encoding the previously identified unique channel catfish herpesvirus (CCV) thymidine kinase (Tk) was preliminarily located on the CCV genome. CCV genomic DNA libraries were constructed into plasmid pUC 19 and cosmid pHC 79. Analysis of $CaCl_2$, DEAE and cationic liposome mediated transfection techniques using beta-galactoside expressing plasmid pON 105 on the channel catfish ovary cell line (CCO), a Tk deficient mutant of CCO (CCOBr), and the brown bullhead cell line (BB) revealed cationic liposome transfection of CCO cells to be the most effective combination. More importantly, cationic liposome mediated transfection of whole CCV-DNA onto CCO or

CCOBr cells was the only method that effectively produced infectious viral progeny. This is the first account showing purified CCV-DNA to be infectious. Subsequently, cationic liposome mediated co-transfection of cloned wild type CCV-DNA with the Tk deficient mutant of CCV (CCVAr) in marker rescue assays mapped the mutation within the 18 Kb direct repeat ends of the genome.

In addition, the polymerase chain reaction (PCR) was used (Jack Numberg, Cetus Corporation) to amplify regions flanked by sequences with homology to degenerate primers corresponding to conserved amino acid sequences among herpesvirus Tk's. Three PCR generated fragments were isolated, cloned into pUC 19 and sequenced. The corresponding amino acid sequence of the presumptive coding strand of one sequence (405) showed limited homology to the mammalian cytoplasmic and poxvirus Tk. A weak specific hybridization signal was located on the Eco RI L-fragment, which is located on the direct repeat ends of the CCV genome, when DNA-DNA hybridization analysis was performed using the purified ^{32}P nick-translation labeled 405 fragment. The combined marker rescue and PCR data mapped the Tk gene within the direct repeat region of the CCV genome. With subsequent subcloning of restriction digested cosmid clones, the Tk gene was mapped to within a 3.1 Kb fragment of the 18 Kb direct repeat ends of the genome. This location is unique among herpesviruses, indicating significant divergence from previously identified herpesvirus gene arrangements.

Objective 2B: Cloning and expression of the S-layer protein gene of Aeromonas Hydrophila.

The S-layer protein gene of A. hydrophila was cloned in the phagemid expression vector Lambda ZAP II (Stratagene, La Jolla, California). A. hydrophila genomic DNA was partially digested with Eco RI to yield fragments ranging in size from 2 to 10 kilobase pairs (Kb) and shotgun cloned into the phagemid vector Lambda ZAP II. Under isopropylthio-B-D-galactosidase (IPTG) induction of the Lac Z promoter, approximately 25,000 plaque forming units (PFU) were screened on nitrocellulose membranes using an enzyme-linked immunosorbant assay (ELISA). Thirty positive clones were identified and purified. The p Bluescript SK-plasmid was excised from the phagemid by co-infection with VCSM13 helper phage (Stratagene) and transformed into fresh host cells to produce double-stranded plasmid DNA. The transformed cells were then used for analysis of the inserts by agarose gel electrophoresis and determination of protein expression by Western blot analysis.

Two of the original 30 clones, which contained approximately a 9.4 Kb insert, expressed two proteins with molecular weights of 85 and 81 Kb. Both proteins were expressed with or without IPTG induction, indicating that the S-layer protein gene is contained within the Eco RI fragment and is under the control of its own promoter rather than the Lac Z promoter of the vector. Restriction enzyme digests of the 9.4 Kb Eco RI fragment were subsequently subcloned into p Bluescript.

USEFULNESS OF FINDINGS:

The first steps of engineering CCV as a vaccine vector were accomplished. The thymidine kinase was cloned and mapped to a 3.1 kh DNA fragment in the CCV genome. Also the immunodominant S-layer protein of A. hydrophila was cloned for insertion into the CCV-TK gene. This non-reverting mutant of CCV that expresses the bacterial antigen can provide protection against the bacterium and CCV. The research also establishes a foundation for developing a CCV based vaccine vector system that could be used with other catfish pathogens.

University of Georgia

Objective 3A: Determine if specific immune resistance to E. ictaluri can be enhanced through dietary manipulation in non-immunized, bath and orally immunized, and oral only immunized fish from each experimental diet.

Four laboratory prepared feeds were compared. These feeds were identical except for the lipid source which was beef tallow, soybean oil, menhaden oil or an equal combination of all three lipid sources. Within each diet group, macrophage function and antibody production were compared in non-immunized, bath-immunized, orally-immunized and bath followed by an oral boost.

There were significant differences among the groups in macrophage function. In general, macrophages from fish fed menhaden oil and the combination feed had an enhanced ability to kill engulfed, live E. ictaluri. Bath immunization further enhanced this killing, however, oral immunization, with our oral preparation, did not. Although the menhaden group had the highest macrophage killing activity, it had the lowest growth.

Objective 3B: Compare the above groups for survival after challenge with virulent E. ictaluri.

An attempt was made to challenge fish from each group with a live, virulent strain of bacteria. Unfortunately, it was unsuccessful in that there were very few mortalities even in the non-immunized group.

Objective 3C: Evaluate fatty acid profiles and vitamin E content of the diets and tissues of fish from each group.

Liver and muscle were removed from representative fish from each group. Fatty acid profiles of the tissues reflected the dietary fatty acid profiles. Fish fed menhaden oil had the highest percentages of the long-chained polyunsaturated n-3 fatty acids. Tissue samples to be used for vitamin E analysis were unfortunately lost during a power outage.

Objective 3D: If dietary enhancement is seen, determine least amount of time enhancing diets would have to be fed in order to balance optimal protection and cost-effectiveness.

Because of the above results, three diets (commercial, beef tallow and combination) were tested instead of the two diets proposed. Swim-up fry were received from LSU and divided into 24 groups, which included non-immunized and bath immunized (which were later orally boosted). All groups were fed commercial feed for varying times before switching to the lab diets in order to determine if time on the lab diets had any effect on protection. Groups were maintained on lab feeds four, two or one week prior to the oral boost and on these feeds until challenged three weeks later.

Using a sub-sample of fish from each group, fish were bath-challenged with a strain of E. ictaluri which killed fish within three days of injection. Using 10^{12} bacteria and increasing the time of exposure to the bacteria to two hours, there were still very few deaths. A second challenge after a rapid temperature change was attempted. Again this was not successful in killing fish. It is believed water quality parameters, fish strain and/or stress must play an important role in the bacteria's ability to enter the fish and overcome the defense mechanism. Some of these are currently being investigated.

So as to obtain some useful information from this experiment, it was decided to examine some of the actual macrophage killing mechanisms and the effects of both diet and vaccination on them. Phagocytes produce various oxygen radicals as a bactericidal mechanism against engulfed

organisms. The production of intracellular superoxide anion as well as the extracellular secretion of superoxide anion and hydrogen peroxide can be measured.

It was found that vaccination enhanced the ability of macrophages to produce intracellular superoxide anion after phagocytosis of live E. ictaluri. The production was increased 8.5x in the fish fed combo feed, 6.5x in fish fed the commercial feed and only 2.3x in fish fed the beef tallow feed. The extracellular secretion of superoxide anion and hydrogen peroxide was increased in vaccinated fish from the combo and commercial groups but not in the fish fed beef tallow.

The conclusion is that nutritional manipulation can be used to potentiate the immune response. Certain lipids appear to be very useful in the immune response and macrophage function. A combination of menhaden oil, soybean oil and beef tallow is probably the best regarding both disease resistance and growth. If lipid is used as a dietary enhancement prior to vaccination, these feeds would have to be fed for 3-4 weeks (at 24°C or above) prior to vaccination.

USEFULNESS OF FINDINGS:

This work indicates that a combination of dietary immunopotentiators and vaccination programs could significantly reduce losses due to E. ictaluri.

PUBLICATIONS:

Vinitnantharat, S. 1991. Humoral and cell-mediated immune response of channel catfish, Ictalurus punctatus, to Edwardsiella ictaluri. Ph.D. Dissertation, Auburn University, AL. 155 pp.

Plumb, J. A. and S. Vinitnantharat. 1991. Kinetics of the immune response in channel catfish to Edwardsiella ictaluri. 16th Annual Eastern Fish Health Workshop. Martinsburg, WV. June, 1991.

Hanson, L. A. 1990. Biochemical characterization and gene mapping of the channel catfish herpesvirus (CCV) encoded thymidine kinase, a selectable site for homologous recombination. Ph.D. dissertation. Louisiana State University, Baton Rouge, LA. 70803.

Awad, M. and R. L. Thune. 1991. Cloning and expression of the S-layer protein gene of Aeromonas hydrophila. Proceedings of Annual Meeting of Fish Health Section of American Fisheries Society, Portland, OR. p. 29.

Lingenfelser, J. T., V. S. Blazer and R. E. Klinger. 1991. Metabolic activation of channel catfish macrophages. 16th Annual Eastern Fish Health Workshop. Martinsburg, WV. June, 1991.

B. Enhancement of the Immune Response to Edwardsiella ictaluri in Channel Catfish

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For the Period
May 2, 1989 - September 30, 1991

COOPERATING INSTITUTIONS:

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