GRANT #: N00014-05-1-0696
PRINCIPLE INVESTIGATOR: Dr. Bobby L. Middlebrooks
INSTITUTION: The University of Southern Mississippi
GRANT TITLE: Evaluation of a DNA Vaccine Specific for the 54 kDa Protective Antigen of *Erysipelothrix rhusiopathiae*
AWARD PERIOD: December 1, 2004 through December 31, 2006

OBJECTIVES: The work described in this report is based on discussion with Dr. Linda Chrisey following the catastrophic loss of reagents and samples as a consequence of the effects of Hurricane Katrina. The work is a full departure from the previously approved and funded research, which for both practical reasons (related to loss of reagents as a result of the hurricane), and for programmatic reasons (reflecting changes in personnel and reduced interest in the previously approved research aimed at development and evaluation of a DNA vaccine for *E. rhusiopathiae*). The research represents the most feasible, as well as the most useful, expenditure of funds and research effort, since it capitalizes on research techniques and products developed in previous years of funding.

Objective 1: Use of hybridoma tissue culture batch reactors to produce monoclonal antibodies from existing hybridomas specific for *Tursiops truncatus* immunoglobulin isotypes IgG (combined reactivity for IgG1 and IgG2) and IgA in quantities adequate to conduct quantitative assays of serum samples of interest.
Objective 2: Purification of *T. truncatus* IgG1 and IgG2 using Protein G and thiophilic affinity columns
Objective 3: Purification of *T. truncatus* IgM using Sephacryl S-300HR
Objective 4: Production of monoclonal antibodies specific for *T. truncatus* gamma1, gamma2, and mu heavy chains
Objective 5: Production of polyclonal antiserum against *T. truncatus* IgA, IgM, and IgG (combined)
Objective 6: Statistical analysis of samples assayed by this laboratory

APPROACH: The purpose of this project was to purify known immunoglobulin isotypes/subclasses from *Tursiops truncatus* serum. The resulting purified *T. truncatus* immunoglobulins were used for the production of monoclonal and polyclonal antibodies specific for these immunoglobulin isotypes. The monoclonal and polyclonal antibodies produced can be used in two ways. First, they can be used as diagnostic reagents to quantify immunoglobulin isotype levels in serum or milk. Secondly, they can be used for detection of immunoglobulin isotypes in tissues via immunohistochemistry.

ACCOMPLISHMENTS:
Objective 1: Two hybridomas have previously been produced in this laboratory, one against *T. truncatus* gamma heavy chain (specific for both gamma1 and gamma2) and one against the alpha heavy chain. Aliquots of these hybridomas stored in liquid nitrogen tanks were exposed to uncontrolled/unknown conditions for an extended period following Hurricane Katrina as a result of region-wide power failure. It was therefore necessary to re-screen these aliquots for continued viability and production of antibody of desired specificity (i.e. against gamma heavy chain or alpha heavy chain). Representative aliquots of each hybridoma to be tested were thawed and cultured so the media could be harvested for re-screening. The harvested media was tested for antibody production via Isostrip (mouse monoclonal antibody) isotyping kits (Roche). Isostrip analysis showed that both hybridomas were producing antibody. The hybridoma specific for *T. truncatus* IgG gamma heavy chain showed a mouse isotype IgA with kappa light chain while the hybridoma specific for *T. truncatus* IgA alpha heavy chain showed a mouse isotype IgG1 with kappa light chain (Figure 1). Initially, an ELISA was used to determine continued specificity to the gamma

heavy chain or the alpha heavy chain. A 96-well microtitre ELISA plate was coated with a 1:100 dilution of either purified *T. truncatus* IgG or IgA. The plate was incubated overnight at room temperature. The plate was washed and the media from both hybridomas was added to their respective wells. After 30 minute incubation at 37 degrees Celsius, the plate was washed again and a 1:10,000 dilution of biotinylated goat anti-mouse IgG, IgA, and IgM (polyvalent, Sigma M6149; biotinylated using Pierce EZ-Link Biotinylation Kit Catalog Number 21435). After another 30 minute incubation at 37 degrees Celsius, the plate was washed again, and a 1:10,000 dilution of ExtrAvidin Alkaline Phosphatase conjugate was added (Sigma E2636). Following another 30 minute incubation at 37 degrees Celsius, the plate was washed a final time, and a para-nitrophenolphosphate was added as a chromogenic substrate. The plate was incubated for another 30 minutes at room temperature and read by a microplate reader at 405 nm. The results of the ELISA showed marginal reactivity between *T. truncatus* IgA and the monoclonal antibody against the alpha heavy chain. There was no detectable reactivity between *T. truncatus* IgG and the monoclonal antibody against the gamma heavy chain. The antibodies from the hybridoma media may need to be purified in order to have sufficient quantities for reactivity in ELISA's.

Following the testing of the hybridoma media in an ELISA, a western blot was performed using media from each of the hybridomas. *T. truncatus* IgG and IgA were purified from serum via Protein G and Jacalin affinity chromatography. Purified IgG and IgA were electrophoresed in SDS on two separate gels with replicate wells for 35 minutes at 200 V. Following SDS-PAGE, the gels were blotted to two separate nitrocellulose membranes for one hour at 100 V. The membranes were immunostained using a 1:10 dilution of hybridoma media (one membrane was used for each hybridoma being tested) followed by a 1:1000 dilution of alkaline phosphatase-labeled anti-mouse polyvalent immunoglobulins (i.e. anti-mouse IgG, IgM, and IgA). The hybridoma secreting mouse IgG against dolphin IgA showed reactivity with the heavy chain of purified dolphin IgA. However, the hybridoma secreting mouse IgA against dolphin IgG showed marginal activity with the heavy chains of both dolphin IgG and dolphin IgA (Figure 2).

Despite the limited reactivity of these hybridomas, they were continuously cultured in order to stockpile a large quantity of media, addressing the specifics of objective 1. **To date, approximately 500 ml of media from the hybridoma against the dolphin gamma heavy chain have been collected. Also, approximately 100 ml of media has been collected from the hybridoma secreting antibody against the alpha heavy chain of the dolphin.**

Objective 2: Prior to any purification, *T. truncatus* serum had to be pooled and clarified due to the extended period during which they were exposed to thawing and elevated temperatures resulting from power losses in the aftermath of Hurricane Katrina. The sera were pooled, and then centrifuged two times at 10,000 x *g*, each time retaining the supernatant. Following centrifugation, the samples were then filtered using a 0.45 µm syringe filters. The pooled serum was then filter sterilized using a 0.2 µm filter apparatus (Fisher 09-740-28E). The filtered serum was applied to a Sepharose Protein G 4 Fast Flow affinity column (GE Healthcare). The unbound fraction of proteins was eluted with phosphate buffered saline (PBS, 0.1 M sodium phosphate, 0.15 M sodium chloride, pH 7.3). After 3-4 column volumes were passed through the column, the bound proteins (containing IgG) were eluted with 1-3

column volumes of a 0.1 M glycine (pH 2-3) elution buffer and neutralized with 100 µl/ml of 1 M Tris base (Figure 3). The presence of protein was detected by measuring the absorbance at 280 nm. The IgG fraction was verified by SDS-PAGE (Figure 6). We have putatively identified this IgG as subclass IgG1. The unbound proteins from the initial procedure were concentrated by centrifugation and passed a second time through the Protein G column in order to remove any remaining IgG1. Once, the unbound proteins, thought to be "exhausted" of IgG by the Protein G column, were concentrated a second time by centrifugation. They were then applied to a T-Gel Adsorbent (Pierce Kit 44916) thiophilic adsorption chromatography column per the instruction included with the kit. The bound protein fraction from the T-gel was detected by measuring absorbance at 280 nm (Figure 4). The bound fraction was verified by SDS-PAGE (Figure 6). **We have putatively identified this IgG as subclass IgG2.**

Objective 3: Serum was clarified by the methods described in Objective 2. A molecular weight/gel filtration column was prepared using GE Healthcare Sephacryl S-300HR. The column was packed to a gel height of 65 cm (344.5 ml gel volume) using PBS as a buffer. Whole *T. truncatus* serum was passed through the Sephacryl S-300HR gel filtration matrix. Samples were collected using 1 ml fractions. The fractions were assayed for protein content at 280 nm. Two major peaks were detected. The initial peak was suspected to contain purified IgM (Figure 5). Verification was performed by SDS-PAGE showed that IgM was present in the first peak (Figure 6). **Sephacryl-300HR has thus proven to be among the most efficient methods of obtaining purified *T. truncatus* IgM**

Objective 4: T. truncatus IgG1, IgG2, and IgM were purified by the various chromatography methods described above. There was only enough material to attempt a single trial for each isotype. Balb/c mice were used to produce the monoclonal antibodies. Two mice were immunized per immunogen and two immunizations trials per immunogen for each fusion attempt. An emulsion of 0.05 ml of TiterMax Gold adjuvant and 0.05 ml of immunogen (0.3 to 2.5 mg/ml) was prepared. The balb/c mice were injected on days zero and 14 with 25 µl of the emulsion in two sites at the base of the tail using a 27 gauge needle. On day 21 (0.1 ml) was drawn (27 gauge needle) from the tail and the serum was tested for antibody titers. If the titers were low (<1/100) then the mice were boosted again (25 µl of the emulsion injected in two sites at the base of the tail) immediately ant the titers were rechecked seven days later. Once titers reached adequate levels, the use of adjuvant for subsequent injections was not required. Twenty-one to 28 days after acceptable titers are achieved; the mice were injected intraperitoneally with 20-100 µg of the soluble immunogen and injected intravenously with the same amount of immunogen the following day. Three days later the mice were sacrificed by exposure to carbon dioxide and the spleens removed for hybridoma fusion.

The extracted spleen was passed through a sterile metal sieve into a sterile PBS solution with 1% antibiotic antimycotic solution to obtain the splenocytes. It is important to note that this procedure was done entirely under a laminar flow hood. The red blood cells in the solution were lysed by using an ammonium chloride solution (0.15 M in PBS) and incubating the cells for 5 minutes. The cells were washed in serum free/HEPES free media. The spleen cells and myeloma cells were counted so that a 10:1 myeloma:spleen cell ratio wax prepared for use in the fusion process.

Fusion was initiated by adding a 50% polyethylene glycol solution slowly (800 µl over one minute) to the mixture of spleen and myeloma cells in a round bottomed, glass tube which was being swirled in a 37°C water bath. Serum free/HEPES free media was added slowly (5 ml over three minutes). The cells were then be resuspended in complete media containing hypoxanthine, aminopterin, and thymidine (HAT) and then plated out in a 96-well plate at a 125 µl per well. The plates were incubated in a CO_2 environment at 37°C. After three days, the cells were fed with HAT media. After day eight, the media was switched to media containing only hypoxanthine and thymidine (HT) which was used for two weeks. Screening for antibody production by ELISA began on day 14. If antibody was detected, the hybridomas were allowed to grow in regular media (without supplements).

Three separate hybridoma fusion procedures were performed, one for each of the isotypes. **Unfortunately, none of the hybridomas produced from these procedures survived the clonal expansion process.** In one instance, a contamination event destroyed all hybridomas being produced in the laboratory at that time including those being produced against *T. truncatus* IgG1. In another instance, the CO_2 environmental incubator failed to pump CO_2 into the unit resulting in the death of the cells lines being produced against *T. truncatus* IgM. Lastly, the hybridomas being produce against *T. truncatus* IgG2 did not survive the media switch from HAT to HT media for unknown reasons.

Objective 5: Pooled *T. truncatus* immunoglobulin was prepared using half ammonium sulfate precipitation. Polyclonal rabbit antiserum against *T. truncatus* IgA, IgM, and IgG (combined) was produced by injection of the precipitated pooled immunoglobulin faction. A blood sample (5 ml) was taken using a 22 gauge needle prior to immunization. The serum was collected and kept to determine whether the animal produced antibodies to the immunogen. The animal was initially immunized with a mixture of 0.5 ml Complete Freund's adjuvant (CFA) and 0.5 ml of immunogen. This emulsion was injected (21 gauge needle) in 0.25 ml amounts intramuscularly over 4 different areas using major muscle groups of the rabbit. Fourteen days later the animal was injected with a mixture of 0.5 ml Incomplete Freund's Adjuvant (IFA) and 0.5 ml of immunogen. This emulsion was also injected in 0.25 ml amounts intramuscularly over four different areas. Ten days later the animal was bled by venipuncture (~10 ml) using a 22 gauge needle. After this initial series of immunizations with adjuvant, no further adjuvant was used in monthly booster injections of immunogen (0.5 ml) alone which were given intramuscularly on alternating hind legs of the rabbit. Blood was drawn by venipuncture ten days after each monthly booster injection. The serum was collected and store at -4 degrees Celsius until further use.

The resulting antiserum was applied to a Protein G affinity column (Pierce) to purify the IgG fraction. The eluted rabbit IgG with specificity against *T. truncatus* immunoglobulin was concentrated using a Millipore centrifuge filter to a concentration of 310 mg/mL. The IgG purity was verified using SDS-page.

T. truncatus immunoglobulin isotypes IgG and IgM were purified from serum using Protein G and gel filtration using the methods described in the previous objectives. *T. truncatus* IgA was purified by applying whole dolphin serum to a Jacalin affinity column. The eluate from

the column containing IgA was then concentrated using a Millipore centrifuge filter at 3000 x *g* to a concentration of 16.89 mg/mL. The absorbance of each fraction was read at 280 nm. The immunoglobulin isotypes were verified by SDS-PAGE.

To analyze the reactivity of the rabbit antiserum, the purified *T. truncatus* immunoglobulins were first electrophoresed at 200 V for 35 minutes. Each immunoglobulin isotype, including a control of lane of previously collected rabbit IgG, was duplicated on the gel. The duplicate isotype sets were then electroblotted to a nitrocellulose membrane at 100 V for one hour. One set of isotypes was incubated with the purified IgG from the rabbit anti-*T. truncatus* as the primary antibody. The second set was incubated with serum obtained from the rabbit prior to immunization with *T. truncatus* pooled immunoglobulin. Both sets were treated with alkaline phosphatase-labeled anti-rabbit IgG as the secondary antibody (Sigma). The isotype sets were then stained with BCIP-NBT tabs. Each membrane showed mild reactivity on the control lane at the IgG heavy chain site. The pre-immune rabbit serum membrane showed no further reactivity with dolphin immunoglobulin isotypes. **The membrane immunostained with anti-*T. truncatus* immunoglobulin (of which a significant stock is now available) showed heavy reactivity for each purified isotype at both the heavy and light chains.**

Objective 6: There is nothing to report on this objective due to the parties involved failing to collaborate on the research, i.e. no samples were submitted for our analysis.

CONCLUSIONS:

Hybridomas previously produced in this laboratory with specificity for *T. truncatus* IgA and IgG were successfully produced in batches and were shown to have retain at least some of their reactivity post-Hurricane Katrina. Purification of immunoglobulins was successful. IgG1 and IgG2 were separated using Protein G affinity chromatography and thiophilic adsorption chromatography respectively. Also, *T. truncatus* IgM was purified using the Sephacryl S-300HR gel filtration column. The laboratory was also successful in producing polyclonal antibodies in rabbits with specificity for *T. truncatus* immunoglobulins.

Unfortunately, monoclonal antibodies for the newly purified immunoglobulin isotypes IgG1, IgG2, and IgM were unable to survive the clonal expansion phase of the hybridoma production trials. There was only enough time to produce enough material for one trial of each isotype. Also, there failed to be collaboration between parties involved in the statistical analysis of data between samples in this lab and samples assayed elsewhere. Thus, there is nothing to report on this objective

SIGNIFICANCE:

These studies have provided information on how various isotypes of *Tursiops truncatus* immunoglobulins, specifically IgG1, IgG2 and IgM can reliably and consistently be purified and used as immunogens for the production of isotype specific monoclonal and polyclonal antibodies. The antibodies so produced are necessary tools for use in diagnostic testing in veterinary assays for various applications.