TRACEY BETH SCHOCK
DNA-mediated Vaccination of Aquatic Species
(Under the direction of BRANSON WILLIAM RITCHIE)

DNA-mediated vaccines consist of plasmid DNA that contains an inserted sequence encoding an immunogenic protein of a target pathogen. In this study, the humoral immune response to DNA-mediated vaccines was evaluated by vaccination with a β-galactosidase reporter DNA vaccine in both tilapia (*Oreochromis niloticus*) and Atlantic bottlenose dolphins (*Tursiops truncatus*). Neither aquatic species elicited a measurable humoral immune response to the experimental vaccine using test procedures. Studies for alternative reporter genes and methods of optimized vaccination are currently underway. Finally, a specific DNA-mediated vaccine was engineered and tested for expression of dolphin morbillivirus (DMV) genes. The hemagglutinin and fusion proteins of DMV were amplified and cloned into a eukaryotic expression vector that was shown to transcribe mRNA specific to each gene. Documenting that the transfected cells are secreting proteins will require further research

INDEX WORDS: DNA-mediated vaccines, tilapia, dolphins, dolphin morbillivirus

DNA-MEDIATED VACCINATION OF AQUATIC SPECIES

by

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DEDICATION

I would like to dedicate my Master's Thesis to my parents, Jeff and Margaret.

"I am your child.

Wherever you go, you take me too.

Whatever I know, I learned from you.

Whatever I do, you taught me to do.

I am your child.

And I am your chance.

Whatever will come, will come from me.

Tomorrow is won, by winning me.

Whatever I am, you taught me to be.

I am your hope.

I am your chance.

I am your child."

- Barry Manilow and Marty Panzer

All That Matters

"The only treasure in the life we live is in the measure of the love we give."

- Cliff Richard

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DNA-mediated Vaccines

Introduction

DNA-mediated vaccination is an area of rapidly advancing research. This novel technology has been dubbed the "third revolution" in vaccine development. DNA-mediated vaccines represent an innovative means of expressing antigens for the generation of an immune response. They consist of bacterial plasmids with inserted genes encoding proteins of a pathogen. Observations in 1990 that plasmid DNA could directly transfect animal cells *in vivo* sparked exploration of the use of plasmids to induce immune responses by direct injection into animals of DNA encoding antigenic proteins[1]. The development of this immunization strategy was spurred by poor immunogenicity of standard vaccines and the threat of unacceptable risks with use of traditional vaccines against deadly or debilitating disorders, namely malaria, human immunodeficiency virus, herpes virus and hepatitis C virus, just to name a few. Since vaccines, in general, are effective ways to prevent disease, there is a call for safer, less expensive and easier to produce vaccines for emerging and re-emerging diseases both in human and veterinary medicine.

Construction

DNA-mediated vaccines are made of small circular plasmids that can replicate in bacteria producing high yields. Vectors for vaccine use should contain control elements, such as a strong viral promoter and a polyadenylation/ termination sequence, that are necessary to facilitate expression of the vaccinating protein in eukaryotic cells[2]. An antibiotic resistant gene is needed to confer antibiotic-selected growth in *Esherichia coli*. The plasmid is altered to carry genes specifying one or more immunogenic proteins of a targeted pathogen. By choosing only the most antigenic proteins, genes that would

enable the pathogen to reconstitute itself and cause disease are disabled. Once constructed, the vaccine vector is introduced into bacteria where many copies of the vector are produced. The plasmid DNA is then purified from the bacteria by separating the small circular plasmid DNA from the larger bacterial DNA and other impurities. The purified DNA, which is a stable molecule, dissolved in a saline solution is the vaccine.

For the production of an optimal vector, the most frequently used regulatory elements are those known to mediate high levels of gene expression under mammalian cell culture conditions or in transgenic mice[2]. These include the human cytomegalovirus immediate/early promoter (CMV)[3], the Rous sarcoma virus (RSV) long terminal repeat (LTR)[4], and the SV40 early promoter[5] used in conjunction with the SV40 or bovine growth hormone 3'-untranslated region (BGH 3'-UTR) transcript termination/ polyadenylation sequences[6]. Some vaccine vectors also contain an intron because expression of many mammalian genes may be dependent on, or may be increased by, the insertion of this intron[7]. In several different experiments, the plasmid utilizing a CMV promoter and intron A elicited the best immune response[2]. However, it is probable that no single construct will be optimal for all genes.

Delivery

DNA-mediated vaccines can be administered by a variety of techniques. For example, syringe administration of DNA by intramuscular injection (IM), intradermal injection (ID), intravenous injection (IV), and intrasplenic injection (IS); gene gun inoculation into the skin; and mucosal delivery via oral and intranasal routes have all induced immune responses and/or protection in animal models[8]. Of these inoculation routes, the more widely used are IM injection and the gene gun. The gene gun is an efficient means of intradermal gene delivery. Gold particles of 0.9-2.6 μm are coated with vector DNA and shot into the skin by a particle accelerator or compressed helium[9]. This method directly delivers the plasmid-coated gold particles into the cytoplasm of the cells, whereas IM injection releases the DNA into the extracellular space[10]. Needle injection into muscle tissue is a faster, easier and less expensive mode of immunization.

However, injection conditions, such as needle gauge, injection speed, injection angle, and injection volume as well as composition and temperature of the injected fluid, can strongly influence the efficiency of gene delivery and cause variability in the observed immune responses[11-13]. For example, it has been demonstrated that injection along the longitudinal axis of the mouse quadriceps muscle and parallel to the myofibers resulted in up to 200-fold higher reporter gene expression when compared to perpendicular injection[14]. In regard to dose of DNA, the gene gun delivery method requires much less DNA, nanogram quantities, compared to IM injections that require up to 5,000-fold more plasmid to induce a similar immune response[15]. As reported by Feltquate et al.[16], the method, gene gun versus needle injection, rather than the route of the immunization, IM or ID, was a major determinant of the induced immune response. Gene gun delivery, IM or ID, led primarily to a Th2 response with IgG1 antibodies against influenza HA while a Th1 response with IgG2a antibodies was raised by DNA injection to the same sites. In primates, however, Th1 responses appear to inhibit the vaccine[17]. Gene gun inoculation has its advantages over IM injection of DNA, but it is a more expensive and tedious method for immunization.

Antigen Presentation

Because all of the DNA inoculation techniques mentioned above resulted in immune responses and/or protection, it is theorized that a DNA-mediated vaccine may not require specific cells to express antigen. If this is true, the expression of an antigen by any cell could provide a platform for the transfer of antigen to professional antigen-presenting cells (APCs)[8]. DNA-mediated vaccine studies have mainly utilized skin or muscle as an immunization target. Studies in rodents on the transfection efficiency of injected DNA have demonstrated that muscle is 100-1000 times more permissive that other tissues for the uptake and expression of DNA[1,11]. Tissues also differ in the efficiency with which they present antigens to the immune system. Tissues, such as the skin and the mucosal linings of the respiratory tract and the gut, serve as barriers against the entry of pathogens and have associated lymphoid tissues that provide high levels of

local immune surveillance[18,19]. The skin is important in immune surveillance since approximately 5% of the epidermal cells represent Langerhans cells that can take up antigen in the skin with subsequent transport to local lymph nodes[20].

There are now at least three mechanisms by which the antigen encoded by plasmid DNA is processed and presented to elicit an immune response: (a) direct priming by myocytes and keratinocytes; (b) direct transfection of professional APCs (i.e. dendritic cells (DCs)); and (c) cross-priming in which plasmid DNA transfects a somatic cell and/or professional APC and the secreted protein is taken up by other professional APCs and presented to T cells. Several studies with bone marrow-chimeric mice have demonstrated that bone marrow-derived APCs play a key role in the induction of the immune response after DNA-mediated vaccination[21,22]. Subsequent studies were designed to define the specific type of APCs regulating these immune responses. The results provided evidence that isolated DCs, but not B cells or keratinocytes from DNA-mediated vaccinated mice, were able to efficiently present antigen to T cells *in vitro*[23]. These data suggest that the contribution to priming the immune responses after DNA-mediated vaccination involves a small number of directly transfected DCs[24].

Immune Responses

It is well established that DNA-mediated vaccination can induce antibodies, helper T cell responses, cytotoxic T cell (CTL) responses, and protective immunity in various animal models[2]. However, the processes involved in the uptake of DNA by cells in the inoculated animal have yet to be elucidated. The macrophage scavenger receptor is thought to play a likely role, because these receptors bind a range of polyanions including sequences of bacterial DNA[25]. Once inside the cells, DNA must be transported out of the endosome/lysosome into the cytoplasm to avoid digestion[8]. It then must be transported into the nucleus before transcription can occur. Here, the viral protein encoded in the plasmid is made and is presented to the immune system in two ways to induce a cellular immune response and a humoral immune response.

The humoral immune response generates primarily antibodies, which attack pathogens outside of cells. Copies of antigenic protein exit the cell where B-lymphocytes bind to the free proteins and then multiply. Upon antigen binding, the activated B cell releases antibodies that during an infection would attach to the pathogen and mark it for destruction. Other B cells become memory cells that fight the pathogen if it circulates outside cells. Prior to the activation of B cells, several preliminary steps must occur. Professional antigen presenting cells (APCs) must ingest the antigenic protein excreted from cells. Here the protein is chopped up by proteases and the peptides are displayed on major histocompatibility complex (MHC) class II molecules. Helper T cells (CD4+) must recognize both the peptide complexes and co-stimulatory molecules found only on APCs. Once activated, the CD4+ cells secrete cytokines, which finally activates the B cells bound to the antigenic protein.

On the other hand, the cellular arm of the immune response eliminates cells that are colonized by a foreign invader. In this case, peptide fragments are displayed with MHC class I molecules. Cytotoxic T cells (CD8+) (CTLs) bind to this MHC-peptide complex, which stimulates multiplication and destruction of bound cells and others displaying the same MHC-peptide complex. Some activated CTLs become memory cells to protect against future infection. As in the humoral response, priming of cellular responses involves APCs, although there is now evidence that both cross-priming and direct transfection of APCs induce CTL responses[26]. Before CTLs can respond to antigens on infected cells, APCs must present the antigenic peptide on MHC class I molecules along with co-stimulatory molecules. Then the CTLs bind this complex and cytokines are released from helper T cells prime the CTLs for cell killing.

The type of cytokines secreted depends on the type of helper T cell that is bound to the MHC-peptide complex[27]. In mice, cytokines such as IL-2 and γ-interferon (IFN-γ) produced by type 1-like helper T cells (Th1) support development of cellular immune responses, including CTL and IgG2a immunoglobulin isotype. Cytokines like IL-4, IL-5, IL-6 and IL-10 produced by type 2-like helper T cells (Th2) promote B cell activation

and immunoglobulin class switching, which is predominantly IgG1 isotype. Similar types of helper T cells have been characterized in humans as well as subsets that may produce both Th1- and Th2-like cytokines[28]. T cell help elicited during infection by a pathogen can have a critical effect on the outcome. For example, infection by *Leishmania* is lethal in mouse strains that develop Th2 responses to the infection, whereas strains that develop Th1 responses become immune[29]. As previously mentioned, most DNA-mediated vaccines induce a Th1 response although, vaccination by gene gun produces a predominantly Th2 response. It is important to know that the type of T cell help a particular vaccine may elicit is critical for the efficacy of the vaccine.

The immune responses elicited from DNA-mediated vaccination have led to long-term immunity. One year after vaccination, mice remained fully protected against a lethal dose of homologous influenza A virus[30]. As well, the immunizing protein has been shown to express in cells for the lifetime of a mouse[31]. It is assumed that the antigenic load achieved by a virulent agent induces a sufficiently large pool of memory T and B cells to provide long-lasting protection[32]. There is some evidence that B cell memory requires persistent antigen. This may be possible by antigen-antibody complexes deposited in the form of immunocomplexes on follicular dendritic cells of lymph nodes. Once antibody titers decline, these complexes are thought to be released, which, in turn, stimulates an additional response[32]. Booster immunizations have had little influence on the magnitude of the immune response, although, follow-up immunization with a conventional vaccine has had a clear boost effect[17,32].

Safety

The primary safety concern for DNA-mediated vaccines is their potential to integrate into host cellular DNA[2,33,34]. Integration is insertional mutagenesis and has the potential to activate oncogenes or inactivate tumor suppressor genes. Plasmids are known to integrate into cellular DNA when transfected in actively dividing cells *in vitro*, but *in vivo* the integration rate is lower due to barriers to cellular uptake including fibrous tissue architecture and extracellular nucleases[35]. Evidence to date suggests that

integration is an extremely rare event, if it occurs at all, with the currently used plasmids[31,36,37].

Another safety concern of DNA-mediated vaccines is the potential for inducing immunological tolerance and autoimmunity. The effect of presenting the immune system with relatively small amounts of antigen over a prolonged period, especially in young children with immature immune systems, has been investigated. Evidence suggests that immunological tolerance does not occur[2,38]. Autoimmune responses might occur as a result of immune-mediated destruction of cells expressing the antigen. DNA-mediated immunizations by IM injection indicate that only a small number of cells in the muscle are transfected[1]. Immune destruction in this part of the muscle would be unlikely to have a significant effect on the performance of the injected muscle. These cells are replaced by normal cellular turnover and are quickly repaired. When the cells expressing foreign genes are destroyed, there may be a release of cellular constituents capable of inducing autoimmune responses[2]. However, this destruction occurs in the natural course of viral and bacterial infections as well as in normal processes of tissue remodeling. It is unlikely that DNA vaccines will pose any greater risk in this regard than conventional viral or bacterial vaccines.

Another potential problem is the induction of anti-DNA antibodies. Immune responses to DNA itself are known to occur in systemic lupus erythematosus (SLE) and it seems possible that the injection of a bacterially grown and modified DNA could result in an immune response with undesirable cross-reactions with host DNA[34]. It has been reported that such antibodies are not detectable[39]. As well, several facts suggest this scenario is not likely to occur. First, purified double stranded DNA does not readily induce anti-DNA antibodies. Only denatured DNA complexed with methylated bovine serum albumin and administered with complete Freund's adjuvant will induce anti-DNA antibodies[40]. Second, anti-DNA antibodies do circulate in normal mice and humans[41]. These antibodies do not cross-react with mammalian DNA. Therefore, nonpathogenic

anti-DNA antibodies are found in most humans[2]. Preliminary data suggests that DNA-mediated vaccines are safe for veterinary and human use.

Advantages

DNA-mediated vaccines have a number of advantages compared to traditional vaccines. They are similar to live attenuated vaccines and recombinant vectors because they produce the immunizing proteins in the host. However, DNA-mediated vaccines are unlike live vaccines in that they do not cause infection[41]. DNA-mediated vaccines can induce the expression of antigens that resemble native viral epitopes more closely than do traditional vaccines. Many viral proteins have folded structures that can be disrupted during purification. Most antibodies recognize folded structures. If a vaccine contains a misshapened protein, the antibody response will recognize the disrupted structure, rather than the normal protein present on the pathogen[30]. Thus, the vaccine may fail to protect against the invading pathogen.

DNA-mediated vaccines can raise both cytolytic T cell (CTL) and antibody responses. By producing the immunogen in host cells, processes and presentation by both class I and class II major histocompatibility complex molecules of the immunogen can induce both arms of the immune system. Most subunit vaccines raise antibody responses only. It is important to raise CTLs to directly kill pathogen-containing cells and DNA-mediated vaccines can elicit both responses. Similar to live vaccines, DNA-mediated vaccines can raise long-term immune responses from only one inoculation[41]. DNA-mediated vaccines can also influence an immune response towards one of the two different types of T cell help mentioned earlier. This can help control autoimmune disease (an inappropriate Th1 response) and allergies (an inappropriate Th2 response).

One of the main advantages of DNA-mediated vaccines is the ease with which they can be constructed[32]. DNA-mediated vaccines can be produced using similar fermentation, purification and validation techniques. This simplifies vaccine development and production. These vaccines can also be constructed to contain genes

from several different pathogens on the same plasmid[30]. This procedure may reduce the number of different vaccines required.

In contrast to many traditional vaccines, DNA-mediated vaccines remain stable at both high and low temperatures[32]. This facilitates storage, distribution and administration of the vaccine without the need for constant refrigeration. Specific DNA sequences for target pathogens can be recovered from tissues of infected animals saving months of trying to culture a microbe from a diseased tissue. The ability to make a vaccine directly from the tissue of an infected animal allows the construction of vaccines for microorganisms that fail to grow in cultures, for example hepatitis B virus and papillomaviruses[41].

Protection by DNA-mediated Vaccination in Animal Models

DNA vaccines have been effective in generating immune responses and protection against a wide variety of viral, bacterial, parasitic infections and cancer. The first disease for which protective immunity was demonstrated in mice was influenza[42]. Since then, protection against viral challenge has been demonstrated in chickens, ferrets and pigs immunized with DNA encoding the hemagglutinin (HA) protein of influenza A virus[43,44,45], rabbits immunized with papillomavirus DNA-mediated vaccines[46,47], and guinea pigs immunized with herpes simplex virus (HSV) DNA-mediated vaccines[48,49]. Mice immunized with various DNA-mediated vaccines protect against several viruses including bovine herpes virus[50], lymphocytic choriomeningitis virus (LCMV)[51], respiratory syncytial virus (RSV)[52], influenza B virus[53], and rotavirus[54]. Cows are protected following immunization with DNA encoding bovine herpes virus-1[50]. Fish species, such as Atlantic salmon and rainbow trout, develop protection against infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia (VHS)[55-60] when immunized with a DNA-mediated vaccine. Cats and dogs immunized with DNA encoding the rabies virus glycoprotein G have been protected against the rabies virus[61], and dogs immunized with a DNA-mediated vaccine were protected against canine distemper virus[62]. DNA-mediated vaccines have proven effective in protecting horses

against vesicular stomatitis[63] and rhesus macaques against the measles virus[64]. These observations suggest the breadth of species in which this technology has proven to be effective.

Injection of DNA plasmids is an effective means of expressing bacterial proteins *in situ* and of providing immunity in animal models. DNA-mediated vaccines encoding proteins of *Mycoplasma pulmonis* conferred protection[65]. Mice are protected from *Mycobacterium tuberculosis* and *M. bovis* challenge following immunization with a DNA-mediated vaccine[66-68]. A DNA-mediated vaccine against the spirochetal agent of Lyme disease, *Borrelia burgdorferi*, has protected mice against challenge with this bacterium[69]. The protective portion of tetanus toxin, fragment C, was used in a DNA-mediated vaccine to protect against *Clostridium tetani*[70]. DNA-mediated vaccines hae also been tested for *Chlamydia trachomatis*[71], *Chlamydophila psittaci* in turkeys[72], and the chronic lung infection caused by *Pseudomonas aeruginosa*[73]. DNA-mediated vaccines for bacterial diseases are feasible as shown in animal models. Unfortunately, multiple injections have been necessary to maintain the immune responses against some bacterial pathogens.

DNA-mediated vaccines also have been used to stimulate immunity against parasites. Antibodies have been raised successfully against proteins from a metazoan parasite, *Schistosoma japonicum*[74], and three protozoans, *Leishmania major*[75,76], *Plasmodium yoelii*[77-80], and *Trypanosoma cruzi*[81], by intramuscular injection of plasmid DNA.

In treating cancer, DNA-mediated vaccines are a promising strategy because the antigenic phenotype varies widely among different cells within the same tumor mass, thus, immunization with a vaccine that stimulates immunity to a broad array of tumor antigens expressed by the entire population of malignant cells is likely to be more efficacious than immunization with a vaccine for a single antigen[82]. Vaccination with DNA encoding the human carcinoembryonic antigen (CEA) elicited protective responses to evade subsequent challenge with CEA-expressing tumor cell lines[83]. Similar results

occurred with DNA containing the MUC-1 gene, which encodes the polymorphic epithelial mucin (PEM), associated with breast, pancreatic and colon cancers[84]. In a recent study, DNA vaccines containing proteins of vaccinia virus provided 100% protection against subcutaneous growth of tumors while a recombinant vaccinia vaccine protected only 40% of the mice[85]. These results have promising implications in the future design of antigen-specific cancer immunotherapy.

Optimizing and Enhancing DNA-mediated Vaccinations

Despite the generation of many impressive responses to injected DNA in various animal models, the underlying mechanisms are still only partially understood, exemplified by the less efficient DNA-mediated vaccines in larger animal models and humans[86]. In the past 5 years, the advancement of DNA-mediated vaccine technology has focused on optimizing and enhancing this technique to better understand the mechanisms involved to produce more efficacious vaccines.

Vector Optimization

One of the most important considerations in optimizing a DNA-mediated vaccine is the appropriate choice of a vector. Increasing gene expression depends on the regulatory elements (promoter-enhancer complex, and transcription termination signals). Several other modifications have been examined. To express multiple genes in the same cell, dicistronic or multicistronic vectors with internal ribosome entry sites were studied. These vectors could be useful in constructing multivalent vaccines coding for two or more different antigens from the same or different pathogens[87].

Optimizing codon usage for eukaryotic cells can also enhance expression of antigens. The use of selective codons in a particular gene correlates with efficiency of gene expression[88]. For example, a plasmid expressing listeriolysin O, in which codons frequently used in murine genes were substituted for the native codons for the encoded antigen led to enhanced CTL and protective immunity[89].

A plasmid may also be engineered so that the encoded protein is either secreted or localized to the interior of the cell. Several studies show that the type and magnitude of

the immune response depends on whether an antigen is secreted, bound on the surface of the cell, or retained within the cell. For example, secreted proteins induced higher IgG titers than the same antigen localized either on the cell membrane or within the cell[90-92]. These studies demonstrated a bias in theTh2 response with comparable levels of antigen-specific IFN-γ when DNA-mediated vaccines expressed either secreted or intracellular antigen. This suggests that cellular localization of the antigen after DNA immunization may play a role in modulating immune responses, but this role may also depend on the nature of the antigen and the model system used[92].

Optimizing Cytotoxic T Lymphocyte Responses

Engineering the antigen to target specific cellular compartments can enhance CTL responses. An example is the use of N-terminal ubiquitination signals, which target the protein to proteasomes, leading to rapid cytoplasmic degradation and presentation via the MHC class I pathway. A DNA-mediated vaccine encoding β-galactosidase that was fused with ubiquitin was more effective at inducing CTL responses than was a plasmid encoding β-galactosidase alone[93]. This has also been shown in studies with HIV Nef[94] and LCMV nucleoprotein[95].

Another approach to optimized delivery is the design of vectors that use the E3 leader sequence from adenovirus, which facilitates transport of antigens directly into the endoplasmic reticulum for binding to MHC class I molecules. The addition of this leader sequence appeared to improve CTL responses for certain antigens, but did not improve CTL in all model systems[96]. Endoplasmic reticulum targeting of T cell epitope DNA-mediated vaccines may not enhance the immune response for all antigens.

By engineering vaccines to be minimal-epitope DNA-mediated vaccines, epitope-specific CTL responses can be elicited. Several groups have successfully used this technique to produce effective viral vaccines[96-100]. The minimal-epitope vaccines could function alone or when linked to other epitopes. This approach can generate a broader immune response than a DNA-mediated vaccine encoding for a single antigen and may also lead to a single vaccine against multiple pathogens.

Cytosine-Phosphate-Guanosine Motifs

Immunostimulatory CpG motifs are present in bacterial DNA. These motifs activate B cells and, in turn, result in antibody production, stimulation of cytokine-producing cells, and activation of the innate immune system. CpG motifs in plasmid vectors contribute to the immunogenicity of DNA-mediated vaccines[101-103]. However, CpG motifs appear to be limited in there ability to augment antibody and cytokine production *in vivo* such that excess CpG motifs may actually reduce immunogenicity[103]. For example, introducing 16 additional CpG motifs into the plasmid backbone improved the humoral immune response by the DNA-mediated vaccine, whereas introducing 50 motifs was detrimental. These findings were observed in mice. Unfortunately, these same motifs are less effective when tested on cells of human, monkey, or chimpanzee. Recent evidence suggests that two different human cell types respond to oligodeoxynucleotides (ODN) and different CpG motifs are required to stimulate these distinct cell populations[104,105].

An important feature of CpG motifs is their ability to stimulate multiple types of immune cells. They improve antigen-presenting function by monocytes, macrophages, and DCs, induce proliferation of B cells and boost antibody production by antigen-activated lymphocytes[106]. Studies are needed to identify sequence motifs that are optimally active, to determine whether different motifs can be used to regulate elements of the immune system, and to establish where in the plasmid these immunostimulatory sequences can be introduced for maximum response.

Adjuvants

Genetic adjuvants are vectors coding for a cytokine, costimulatory molecule or a ligand. Adjuvants are used to modulate immune responses to DNA-mediated vaccines. In the last few years, a large number of publications have resulted from the use of cytokines, chemokines, and co-stimulatory genes[107]. The granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulates growth and differentiation of various cells and can have consequences on both the humoral and cellular immune response[8].

The GM-CSF gene has been the most widely used in DNA-mediated vaccines encoding cytokines with reported enhancements of antibody, helper T cell, and CTL responses[91,108-110]. Other cytokine gene constructs, just to name a few, that have been demonstrated to increase the potency of immune responses to DNA-mediated vaccines include IL-2, which is a Th1-type cytokine that stimulates the growth and differentiation of T cells and immunoglobulin production from B cells[109-112]; IL-12, which promotes cellular immune responses through the differentiation of Th1 cells[108,110,111,113]; and IL-1, which has a wide variety of effects on immune and inflammatory responses[111,114].

A different approach has been to use DNA constructs encoding co-stimulatory molecules, B7-1/B7-2 or CD80/CD86, that are known to be important for providing a signal to T cells during contact of T cells with APCs. DNA-mediated vaccines encoding B7-1 augmented the immune responses of coexpressed antigens[115-117]. From this evidence, it is clear that cytokines, chemokines and co-stimulatory molecules can be used to modulate DNA-mediated vaccines.

Prime/Boost Strategies

Prime-boost vaccination involves priming with DNA-mediated vaccines and boosting with either recombinant protein or with attenuated recombinant viral vectors including fowl poxvirus (FPV) and modified vaccinia virus Ankara strain (MVA)[10,17]. Prime-boost strategies aim at augmenting immune responses to pathogens, which are not completely inhibited by DNA-mediated vaccination alone, i.e. HIV envelope proteins and malarial proteins[106]. In two separate studies that used rhesus macaques, it was shown that antibody production could be substantially increased in monkeys vaccinated with DNA encoding an HIV-1 envelope protein followed by a protein boost[118,119]. DNA-mediated priming followed by boosting with recombinant MVA led to high frequency CTL responses and protection of mice against a *Plasmodium berghei* sporozoite challenge[120,121]. This prime-boost strategy is not necessary for all antigens to achieve high-titer antibodies.

Administration

A variety of routes of DNA-mediated vaccines have been studied. Pathogens enter hosts via mucosal surfaces and the mucosal administration of vaccines is less invasive compared to injection with a syringe. Mucosal immunizations should result in both mucosal and systemic immune responses, including serum antibodies and CTLs[8]. Thus, the potential for mucosal DNA-mediated vaccination has been investigated. The first suggestion that mucosal immunization with DNA could result in the induction of immunity was reported by Fynan et al. after intranasal inoculation of mice with influenza hemagglutinin DNA[43]. They found that, despite no detectable antibody responses, the mice were protected from virus challenge. Since then, mucosal delivery of DNA-mediated vaccines has been accomplished intranasally[122,123], intratracheally[124], by aerosol[125], by genital tract immunization[126], and by oral administration[127,128]. Mucosal DNA-mediated vaccines are advantageous in generating mucosal IgG and IgA responses[122,126]. While further research in this area is needed, induction of broad based immunity appears promising.

Carrier-Mediated Approaches

It has been said that a majority of the DNA injected intramuscularly is degraded by extracellular deoxyribonucleases[129]. Protecting plasmid DNA from extracellular degradation by introducing it directly into target cells should optimize DNA uptake. Several methods of carrier-mediated DNA transfection have been successful. Liposomes, bilayered membranes of polar and nonpolar molecules, entrap DNA-mediated vaccines. They can then be directly delivered to antigen presenting cells (APCs)[130]. Antibody augmentation has been observed via intramuscular injection and intranasal administration[131,132]. Other carrier molecules are cochleates. These are rigid calcium-induced spiral bilayers of anionic phospholipids[106]. These stable carriers contact the target cell membrane, where fusion takes place allowing the delivery of the plasmid DNA into the cytosol. It has been reported that the induction of strong CTL and antibody responses occurs after parenteral or oral administration[133].

Another potentially exciting means of DNA delivery is the use of biodegradable polymer microparticles. Because mucosal environments are so harsh, plasmid DNA trapped in polylactide-co-glycolide (PLG) can evade the digestive enzymes and induce both mucosal and systemic immune responses[128,134].

Delivery of DNA can also be accomplished by attenuated intracellular bacteria. Intracellular bacteria, carrying the DNA, undergo phagocytosis by APCs, delivering plasmid DNA into the host cell cytosol. Three strains of bacteria have been used for delivery of DNA-mediated vaccines: *Shigella flexneri*[135], *Salmonella typhimurium*[136], and *Listeria monocytogenes*[137]. These bacteria carriers have led to induction of strong antigen-specific humoral and cellular responses.

More recently, development of a plasmid DNA-based expression system has enabled direct DNA administration rather than infection with recombinant alphavirus particles containing vector RNA[138]. Alphaviruses are arthropod-borne togaviruses with a positive-polarity and single stranded RNA genome that can replicate in a large number of animal hosts[106]. Using these plasmids for DNA-mediated vaccinations of mice against influenza A, HSV-1, and hepatitis B virus, induced a significant increase in cellular and humoral immune responses compared to conventional DNA-mediated vaccination[139-141]. This mode of gene delivery allows expression of heterologous proteins at higher levels than occurs with DNA-mediated vaccines[141].

Morbillivirus

Introduction

For centuries, morbillivirus infections have had a substantial impact on both humans and animals. Measles virus (MV), introduced by the Europeans, killed Native Americans, and it still remains a significant cause of childhood mortality, particularly in developing countries[142]. The cattle plagues of the 18th and 19th centuries in Europe were introduced by traders from the East. Rinderpest virus (RPV) was introduced into Africa from India in the 1890s, with devastating effects on domestic and wildlife species[143]. International efforts are under way to eradicate both MV and RPV. Another

morbillivirus disease of small ruminants, peste des petits ruminants virus (PPRV), is endemic in west Africa. In recent years, the virus has spread across the Middle East and southern Asia[144]. In carnivores, canine distemper virus (CDV) causes serious disease in many species, both free-ranging and domesticated. It is controlled by vaccination in domestic dogs and farmed mink, but it may be impossible to eradicate the virus because of its global distribution and wide variety of host species. In the past 13 years, new morbilliviruses with significant ecological consequences for marine mammals have been discovered including phocine distemper virus (PDV) in seals and the cetacean morbillivirus (CMV) in dolphins, whales and porpoises[145]. In 1994, a morbillivirus infecting horses and man was identified in Hendra, Australia and named equine morbillivirus (EMV)[146,147]; but upon further research, Wang and colleagues suggest that EMV is not as similar to morbilliviruses as first reported[148].

Genus

The genus *Morbillivirus* belongs to the family *Paramyxoviridae*. This virus family has two subfamilies: the *Paramyxovirinae* and the *Pneumovirinae.* The *Paramyxovirinae* contains three genera including *Morbillivirus.* When viewed through the electron microscope, morbilliviruses display the typical structures seen in other members of this family.

Virus Structure

Morbilliviruses contain a lipid bilayer envelope that is derived from the plasma membrane of the host cell by budding. The viruses are spherical in shape with a diameter of 150-350 nm. Glycoprotein spikes of approximately 8-12 nm are embedded in the surface membrane of the envelope. These can be visualized by electron microscopy (EM). Within the viral membrane is the nucleocapsid core. This core has a characteristic herringbone appearance and contains the single-stranded RNA genome of approximately 15,000 to 20,000 nucleotides.

Virus Genome

Morbilliviruses have a non-segmented helical genome (Figure 1). Six genes are encoded within the genome. The gene order for morbilliviruses is 3' the nucleoprotein (N), the phosphoprotein (P), the matrix protein (M), the fusion protein (F), the hemagglutinin protein (H) and the polymerase protein (L) 5'. Each gene contains 5' and 3' conserved transcriptional control regions. Between the end of the gene and the boundary with the subsequent gene, there is an intergenic region three nucleotides in length. The intergenic triplet, CUU (in the positive antigenome sense), is highly conserved for all morbilliviruses[149-152]. The intergenic sequences are believed to play a role in the transcription process.

Viral Proteins

Morbilliviruses contain four structural proteins (N, P, L, M), two non-structural proteins (C, V) and two surface glycoproteins (H, F). The N protein encapsidates the RNA genome, creating an RNAse-resistant environment for protection. The P and L proteins associate with the N protein and function in transcription and replication. The P protein was named for its highly phosphorylated nature and plays a central role in all RNA synthesis. This gene contains overlapping reading frames, which are used to encode two additional, but non-structural, proteins, the C and V proteins[153]. This configuration organizes a large amount of genetic information into a small genome. The C protein is a small basic protein that is expressed from the P gene from an open reading frame (ORF) that overlaps the N-terminal portion of the P gene. P gene mRNAs are cotranscriptionally edited, occurring downstream of the C protein ORF, where the V protein expresses a highly conserved cysteine-rich domain that fuses to the N-terminal domain of the P gene[154]. The function of this structure is still unclear.

Together with the L protein, the P protein forms the viral polymerase (P-L). The L protein is the largest protein of the virus. Due to its size and its 5' distal location of a transcriptional map, meaning it is last to be transcribed; the L protein is the least abundant of the structural proteins. Structure-function studies have not been reported for

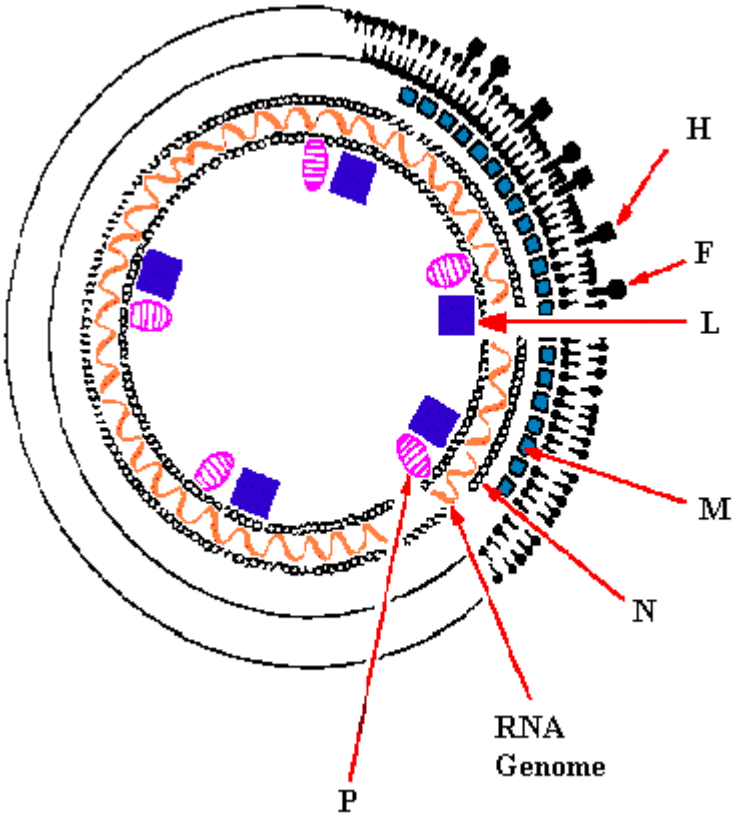

Figure 1. A representation of the morphology of the virus family *Paramyxoviridae*, which includes the *Morbillivirus* genus.

this protein. On the other hand, the M protein is the most abundant protein in the virion. It forms the inner coat to the viral envelope and serves as a bridge between the surface viral glycoproteins and the nucleoprotein core. M plays a central role in the formation of new virions[155].

Two integral membrane proteins are found in all paramyxoviruses, one involved in cell attachment (H) and the other involved in fusion of the viral envelope with cellular membranes (F)[154]. The H protein is a type II integral membrane protein that attaches to host cell receptors. Unlike other paramyxoviruses, the *Morbillivirus* H glycoprotein does not have neuraminidase activity. This activity may not be required for virus release from the cell surface. The fusion protein is initially made as an inactive precursor (F_o) that is

cleaved by a host-cell proteolytic enzyme to release the new N terminus of F_1. This forms a biologically active protein consisting of the disulfide-linked chains F_1 and F_2[154]. F proteins are type I integral membrane proteins that span the membrane once and contain a cleavable signal sequence at the N terminus. The function of F proteins is to mediate virus entry into the cell as well as cell-to-cell spread of the virus.

Antigenic Variation

The morbilliviruses are closely related antigenically. Cross-reactivity between MV, CDV, RPV, and PPRV has been found in tests using polyclonal sera[156]. Using monoclonal antibodies against MV and CDV, cross-reacting epitopes between RPV, MV and CDV have been found on the H, F, N, and P structural components[157]. Cross reactivity has also been shown for dolphin morbillivirus (DMV), porpoise morbillivirus (PMV), CDV and PDV[158,159]. Throughout the virus family, the H protein shows the greatest variability among the morbilliviruses, whereas, the F gene is highly conserved[160].

Virus Replication

Replication occurs in the cytoplasm. On adsorption of the virus to the cellular receptor, CD46, the viral membrane fuses with the cellular plasma membrane and the virus is released into the cytoplasm. Fusion occurs by binding of H to its receptor. This triggers a conformational change in F to release the fusion peptide[161]. The mechanisms of uncoating are unknown. After fusion of the viral envelope with the host cell plasma membrane has occurred, the infecting nucleoplasmids enter the cytoplasm carrying multiple copies of the P-L polymerase. RNA synthesis begins as soon as the genome encounters the ribonucleotide triphosphates in the cytoplasm, beginning with the antigenome leader RNA. The transcripts are 5' capped and polyadenylated at the 3' end. After translation of the primary transcripts and accumulation of the viral proteins, antigenome synthesis begins in order for genome replication to pursue. Genome replication and encapsidation take place at the same time preventing termination for the full-length (-) genome to be encapsidated. The nucleocapsids are thought to be assembled in two steps: first, association of free N subunits with the genome to form

helical RNP structures and second, the association of the P-L complex[162]. The envelope is assembled at the cell surface. The virus buds from the apical surface of polarized epithelial cells. The integral membrane proteins are synthesized in the endoplasmic reticulum and undergo conformational maturation before transport through the secretory pathway to the plasma membrane. The M proteins bring the assembled ribonucleoprotein core to the appropriate patch on the plasma membrane to form a budding virion.

Cetacean Morbillivirus

Since 1987, morbillivirus infections have been described in epizootics among marine mammals belonging in the *Cetacea* order. The three morbilliviruses that are known to infect cetaceans are dolphin morbillivirus (DMV), porpoise morbillivirus (PMV), and pilot whale morbillivirus (PWMV). PMV and DMV are antigenically and genetically similar and probably represent different strains of the same virus species[163]. Whereas, the novel morbillivirus, PWMV, is phylogenetically related to, but distinct from, PMV and DMV[164]. Collectively, cetacean morbilliviruses are more closely related to the ruminant morbilliviruses and measles virus than to the distemper viruses[150,151,163-165].

Cetacean morbillivirus has infected several species of marine mammals in waters all over the world. These species include harbor porpoise (*Phocoena phocoena*), pygmy sperm whale (*Kogia breviceps*), striped dolphins (*Stenella coeruleoalba*), Atlantic spotted dolphin (*S. frontalis*), dusky dolphin (*Lagenorhynchus obscurus*), Atlantic white-sided dolphin *(L. acutus*), white-beaked dolphin (*L. albirostris*), Pacific striped dolphin (*L.* obliquidens), Risso's dolphin (*Grampus griseus)*, long-beaked common dolphin (*Delphinus capensis*), short-beaked common dolphin (*D. delphis*), Fraser's dolphin (*Lagenodelhis hosei*), Atlantic bottlenose dolphin (*Tursiops truncatus*), long-finned pilot whale (*Globicephala melas*), short-finned pilot whale (*G. macrorhynchus*), false killer whale (*Pseudorca crassidens*), pygmy killer whale (*Feresa attenuata*), fin whale (*Balaenoptera physalus*), and minke whale (*B. acutorostrata)*[163-178]. The bodies of water

impacted by these infected species include the North Atlantic, the Mediterranean, the South Pacific, the Black Sea, the Gulf of Mexico, and the Pacific Ocean around Japan.

Dolphin Morbillivirus

Molecular and antigenic data suggest that DMV is closest to the reputed morbillivirus ancestor[150,166], implying that it may be the 'archevirus' of the genus and may have infected cetaceans for hundreds of thousands or even millions of years. The wide geographic and species distribution of CMV, the occurrence of potentially large numbers of hosts among cetacean species from polar waters to tropical seas for approximately 5 million years[179] and the migratory habits of these marine mammals, also argue in favor of this hypothesis.

DMV Pathogenesis

Excretion and Transmission

Studies of the distribution of morbillivirus antigen in tissues of cetaceans, such as the lacrimal gland, lung, epithelia of pharynx, stomach, bile ducts, penis, prepuce, urinary bladder, renal pelvis, skin, and mammary gland, suggests there is high potential for viral excretion from body orifices and skin[180-184]. Virus shedding may occur in all body secretions one week after infection and frequently before clinical signs, although because the virus is secreted into seawater, it will be diluted and partly or completely inactivated[168]. Most likely, transmission through inhalation of aerosolized virus shed by infected individuals will lead to lateral spread of the virus. Detection of viral antigen in the male reproductive tract of a harbor porpoise[181], and occasionally in the mammary glands of striped dolphins and bottlenose dolphins[182,183], suggests the possibility of venereal and vertical transmissions, respectively.

Clinical Signs

Most marine mammals with morbilliviral disease wash ashore dead or strand in a moribund state and die shortly thereafter. When found alive, the cetaceans usually are in poor body condition, which results in reduced flotation[168]. Skin lesions and erosions of the buccal mucosa are common[182], as well as respiratory distress, lethargy, and decreased

mobility. Some infected dolphins have been found repeatedly striking their bodies against rocks, possibly as a result of brain damage[168].

Pathology

Bronchopneumonia is usually the most prominent post-mortem finding[182]. Subpleural emphysema and dilatation of subpleural lymphatics have been seen, as well as enlarged and edematous lung-associated lymph nodes[182,184]. Some dolphins have been found with large hemorrhagic necrotic lesions in the cerebral cortex and ulcerative stomatitis. Other pathologies identified in dolphins are septicemia, including edema of internal organs and accumulation of large quantities of serosanguinous fluid in the pleural and peritoneal cavities, and also pulmonary, myocardial, hepatic and pancreatic fibrosis[182]. Non-purulent meningoencephalomyelitis is also apparent in DMV infections[167].

Histologically, the most prominent lesions of DMV are interstitial pneumonia, nonsuppurative meningoencephalitis and lymphoid depletion[183,185]. Eosinophilic intranuclear and intracytoplasmic inclusion bodies and syncytial cells can be found in affected tissues, mainly lung, neurons, glial cells, lymphatic cells, but have also been found in the epithelium of the pharynx, esophagus, stomach, intestines, biliary ductules, urinary bladder, renal pelvis, ureter, penile urethra, prepuce, oviduct, vagina, mammary and lacrimal glands, oviduct, skin and pancreatic cells[180-183]. Fibroplasia of alveolar septa was commonly seen in cetaceans, suggesting a chronic course of infection.

Diagnosis

Diagnosis of morbillivirus infection in dolphins can be made by histopathological and immunocytochemical methods[171,181-183], antigen capture enzyme linked immunosorbent assay (ELISA)[158,186], virus neutralization assay[158], virus isolation[158,163], and RT-PCR[187].

Threat

Dolphin morbillivirus has caused mass mortality in free-ranging populations of dolphins. For example, more than 50 % of the inshore bottlenose dolphin population off

the U.S. east coast may have died during the 1987-90 epizootic[171]. The geographic spread of this virus is causing concern for cetacean populations. At least two distinct morbilliviruses are present in feral populations of harbor porpoise, and striped and bottlenose dolphins in the Northern Hemisphere. It appears likely that these, or other undiscovered morbilliviruses, will continue to pose a threat to naïve cetacean populations in many regions of the world[188]. Van Bressem and colleagues state that the high death rate, the persistence of cetacean morbillivirus in several populations and the natural history of other members of this genus indicate that CMV may have long term effects of the dynamics of cetacean populations either as enzootic infections or recurrent epizootics[165]. Although there is evidence suggesting morbilliviruses may be transmitted between aquatic mammals belonging to different orders[189], it is impossible to predict the occurrence of future epizootics until more is known about the epidemiology of morbillivirus infections in marine mammal populations.

Conclusion

Morbillivirus infections pose a substantial risk to many species of aquatic mammals. To date, there are no cetacean specific vaccines available for morbillivirus. There is, therefore, a need for development of safe and efficacious vaccines for these important marine mammals.

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CHAPTER 1

EVALUATION OF THE HUMORAL IMMUNE RESPONSE TO DNA-MEDIATED VACCINATION IN TILAPIA (*OREOCHROMIS NILOTICUS*)

Introduction

Tilapia (*Oreochromis niloticus)* are an economically valuable and widely cultured fish, both as a food source and as a biological model for scientific research[1]. Culture of tilapia is a lucrative business worldwide, with farms producing more than one hundred million pounds of fish annually. Bacteria can cause high mortality on tilapia farms. The same bacteria affecting tilapia can cause infections in humans. Because of the growing concern about antibiotic residues in food for human consumption, as well as the increase in antibiotic resistant microorganisms, vaccination has gained notoriety as a safe, inexpensive and effective means to protect animals from infectious diseases. Current vaccine strategies have only been successful against some bacterial pathogens. No vaccines are currently available for viral or parasitic pathogens affecting tilapia. The ideal vaccine for aquaculture must be effective in reducing morbidity and mortality, be inexpensive to produce and license, provide immunity of long duration, and be easily administered.

DNA vaccines are most likely to meet these requirements. These vaccines are composed of naked DNA molecules that code for an antigen under the control of a eukaryotic promoter. DNA-mediated vaccination has proven to induce long-term foreign protein expression while inducing humoral and cell-mediated immunity without autoimmunity or integration in fish[2]. These studies have used plasmids containing a reporter gene, the most widely used being *lacZ* which expresses β-galactosidase[2-5]. The potential for DNA vaccines has been successfully evaluated for several species of fish,

including Japanese flounder (*Paralicthys olivaceus)*[6], Atlantic salmon *(Salmo salar)*[7] and rainbow trout (*Oncorhyncus mykiss*)[8]. Where *S. salar* and *O. mykiss* nucleic acid vaccine studies have worked well as intramuscular (IM) vaccines protecting against infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia (VHS)[7-12].

DNA vaccines have several practical and immunological advantages that may make them very useful in aquaculture. From a practical point of view, they are relatively inexpensive and easy to produce, and require a standardized process. Also, DNA is a stable molecule, which can accommodate varying culture temperatures. Immunologically, DNA inoculations can induce strong and long-lasting humoral and cellular immune responses, in some cases without additional administration, making this technique appealing to fish farmers. The study reported here evaluated the humoral immune response to DNA vaccination in tilapia (*O. niloticus)* using a β-galactosidase reporter gene plasmid.

Materials and Methods

Fish

Thirty purebred tilapia (*Oreochromis niloticus)* were housed in a 20-gallon tank at a temperature of 23^0C at the Department of Biomedical Sciences and Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University (Blacksburg, VA). Tilapia were first grouped according to weight, so a similar number of fish at the same weight range were in each group. Then the fish were randomly allocated into 3 groups of ten fish each.

Plasmid Preparation

Two expression plasmids were used in this study. The test plasmid, pCMVβ, is a eukaryotic/mammalian reporter plasmid that contains the gene for β-galactosidase and the human cytomegalovirus (CMV) immediate-early enhancer/promoter (Figure 2, Clonetech, Inc., Palo Alto, California, USA; 7.2 kilobases, Genebank accession no. U02451). The control plasmid, pCI, is an "empty" mammalian expression plasmid that has the CMV promoter and cloning site but lacks a gene for expression (Figure 2,

Promega, Inc., Madison, Wisconsin, USA; 4.0 kilobases, Genebank accession no. U47119). Plasmids were prepared at the University of Georgia according to manufacturer's instructions, using large-scale plasmid preparation kits to produce endotoxin- and pyrogen-free products (Qiagen, Inc., Valencia, California, USA). Purified plasmids were stored at – 20^0C in sterile phosphate-buffered saline (PBS), pH 7.4, at a concentration of 1 μg/μl. The day prior to use, thawed plasmid stocks were diluted 1:1 with sterile PBS to a working concentration of 0.5 μg/μl.

DNA Inoculations and Collection of Sera

Group 1 fish each were inoculated with 50 μg of the test plasmid, pCMVβ. Group 2 fish each were inoculated with 50 μg of the control plasmid, pCI, and group 3 fish each were injected with 100 μl sterile PBS as a diluent control. All inoculations were administered intramuscularly (IM) on the left side of the body, caudo-lateral to the dorsal fin. Pre-immune sera were collected from the caudal vein prior to the initial vaccination (Day 0) and then every other week for a total of five blood samples for each fish. Sera were stored at – 20^0C prior to immunoassay.

ELISA Immunoassay

An indirect enzyme-linked immunosorbent assay (ELISA), using β-galactosidase (Sigma, St. Louis, Missouri, USA) as antigen, was designed to detect tilapia antibodies generated against the plasmid gene expression product. Each well of an eight-well microtiter strips (EIA/RIA, high binding, Costar, Cambridge, Massachusetts, USA) was coated of β-galactosidase at 100 μg/ml in carbonate-bicarbonate buffer, pH 9.6. The coating process was performed at 4^0C for a minimum of 18 hours. After coating, wells were rinsed once using wash buffer (PBS, 0.05% Tween 20). Tilapia pre-immune sera and immune sera were diluted in 50 μl of conjugate buffer (PBS, 0.1% Tween 20, 5% Carnation™ non-fat dry milk). Serial two-fold dilutions, starting at 1:20 and ending at 1:1280, were performed in duplicate for each serum sample. Tilapia sera and antigen were incubated with rocking for 30 minutes at 37^0C followed by three rinses in wash

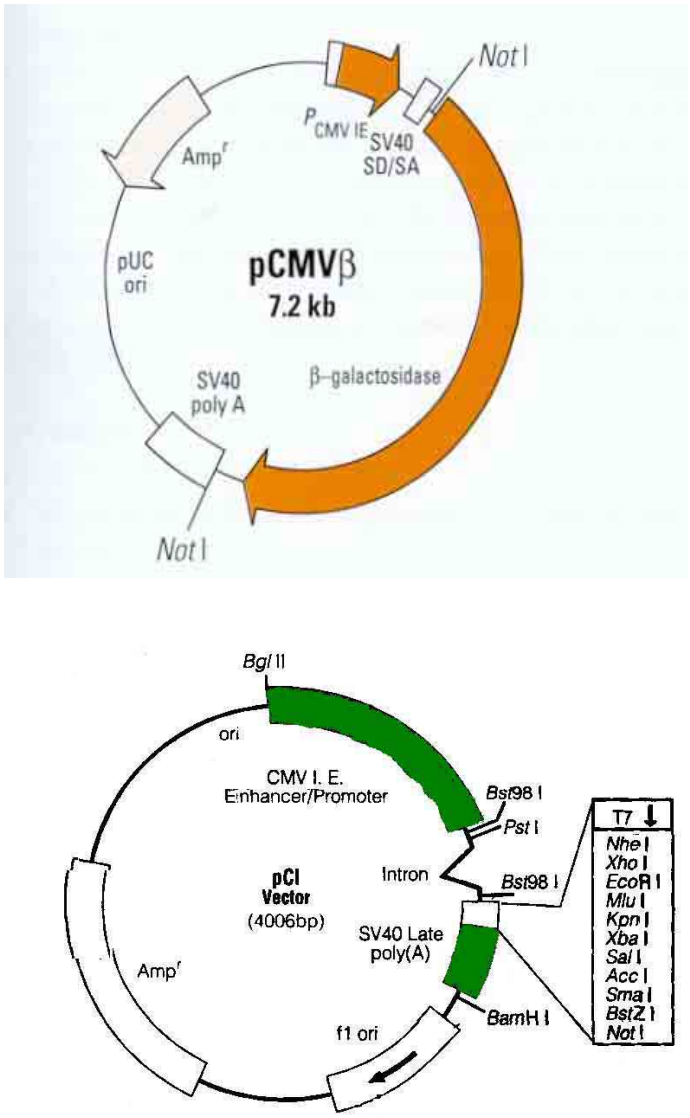

Figure 2. Test plasmids for assessment of the humoral immune response in tilapia to DNA-mediated vaccination. Top: pCMVβ, containing β-galactosidase reporter gene; Bottom: pCI, an empty plasmid. Both vectors contain the CMV immediate/early enhancer/promoter.

buffer. The secondary rabbit anti-tilapia IgG antibody was diluted 1:10,000 in conjugate buffer and added to test wells at 50 µl per well. The wells were then incubated for 30 minutes at 37^0C with rocking, followed by three rinses with wash buffer. Commercial goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) was used as the tertiary antibody conjugate (Gibco BRL, Rockville, Maryland, USA). Tertiary antibody was

diluted 1: 40,000 in conjugate buffer and added to test wells at 50 μl per well. Wells were incubated at 37^0C for 30 minutes with rocking, followed by three final washes. enzyme activity was detected using 50 μl per well of an o-phenylenediamine (OPD) (Sigma) solution (1 mg/ml in 100mM citric phosphate buffer, pH 5.0, 30% H_2O_2). Substrate color development was stopped after 20 minutes at 25^0C by the addition of 30 μl of 2 M H_2SO_4. Wells were read for absorbance at 490 nm using an automated plate reader (V-max Kinetic Plate Reader; Molecular Devices Corp., Sunnyvale, California, USA).

Eleven different tilapia sera were assayed in one ELISA experiment. A series of negative and positive controls were performed for each ELISA. The positive control utilized commercial anti-β-galactosidase sera generated in rabbits (Sigma, St. Louis, Missouri, USA) and an appropriate corresponding secondary antibody-HRP conjugate. A substrate-only negative control was included to detect any endogenous peroxidase activity. Twenty-two conjugate-only negative control wells were generated for each ELISA experiment to monitor for non-specific secondary antibody binding.

Statistical Analysis

Tilapia anti-β-galactosidase antibody titers were calculated as the reciprocal of the highest dilution that was two standard deviations above the average optical density of the 22 conjugate control wells. Geometric mean anti-β-galactosidase antibody titers were calculated for control plasmid and test plasmid groups at 0, 14, 28, 42, and 54 days post inoculation. Each anti-β-galactosidase antibody titer was log-transformed and single factor analysis of variance (ANOVA) was employed to evaluate the differences in antibody titers among the three experimental groups, with significant differences recognized at $p < 0.03$. Geometric mean titers were generated from the raw data to account for biological data being skewed to the right. Standard deviations of all data were calculated.

Table 1. β-galactosidase antibody titers determined by ELISA from inoculation of three tilapia groups 1) pCMVβ, 2) pCI, and 3) saline control.

pCMVβ					
Fish #	**10/24/98**	**11/7/98**	**11/21/98**	**12/5/98**	**12/19/98**
3	800	800	400	1	3200
4	1	400	1	1	200
9	1	200	100	100	400
11	1	1	1	1	100
17	1600	3200	100	800	1600
18	1600	1600	800	800	6400
21	3200	200	200	200	800
23	100	1	400	1	800
28	100	100	200	1	200
30	200	200	200	200	800
Geometric mean	81.611431	129.3454	79.621434	17.41101	696.4405

pCI					
Fish #	**10/24/98**	**11/7/98**	**11/21/98**	**12/5/98**	**12/19/98**
2	200	100	1600	1600	6400
5	1600	1600	3200	800	6400
8	1	1	200	1	200
15		400	1600	3200	3200
16	400	1600	6400	1600	1600
19	1600	100	1600	100	1
24	200	100	800	1	400
26	400	6400	3200	3200	6400
27	400	800	1	200	1600
29	800	800	6400	3200	1600
Geometric mean	258.99152	289.9119	878.84843	258.6908	878.8484

Saline control					
Fish #	**10/24/98**	**11/7/98**	**11/21/98**	**12/5/98**	**12/19/98**
1	1	200	100	200	400
6	400	200	1	1	100
7	400	1600	1	200	6400
10	100	400	800	200	3200
12	400	200	400	200	800
13	1600	6400	6400	400	3200
14	6400	1600	1600	100	800
20	1	100	1	1	200
22	400	100	1	1	1
25	100	100	100	100	200
Geometric mean	138.62897	373.2132	44.827549	38.07308	382.541

Results

Circulating anti-β-galactosidase antibodies in the test plasmid-injected tilapia showed no significant difference from the empty plasmid and the diluent control β-galactosidase titers (p=0.6945) (Figure 3). As shown in table 1, the highest ELISA antibody titer detected was 1: 6,400. This occurred in all study groups, even in pre-

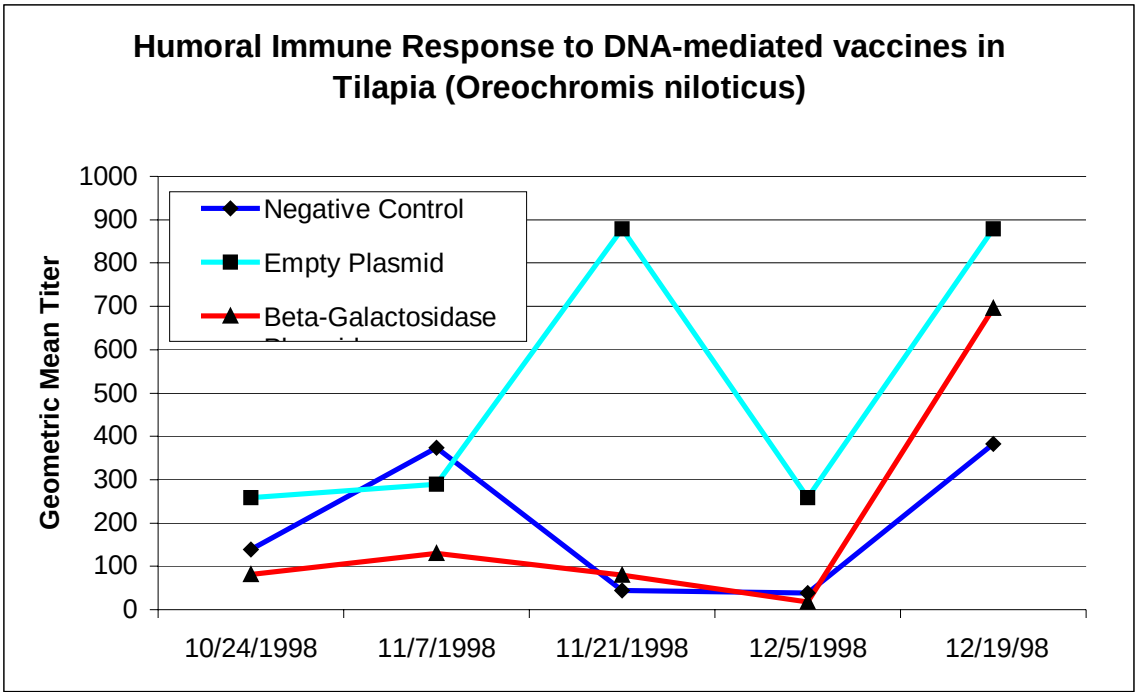

Figure 3. Geometric mean anti-β-galactosidase antibody titers of saline control (100 µl, IM), empty plasmid control, pCI, (50 µg, IM), and test plasmid, pCMVβ, (50 µg, IM) tilapia treatments. Tilapia were inoculated with treatments on 24 October 1998.

bleeds of saline control fish. Both the saline control and empty plasmid groups produced varying β-galactosidase antibody titers from week to week.

Discussion

This study assessed the humoral immune response to DNA-mediated vaccination with a β-galactosidase reporter gene in *Oreochromis niloticus.* We chose the β-galactosidase gene, driven by an efficient and commonly used human cytomegalovirus immediate-early enhancer/promoter, because of its inherent degree of safety and proven utility as a reporter system[2-5]. With this test system, we were able to examine the humoral immune responses following DNA-mediated immunization of commonly farmed fish.

We used an indirect ELISA to screen tilapia sera for antibodies to β-galactosidase. Each group of tilapia had varying anti-β-galactosidase titers prior to

inoculation (Table 1). Unfortunately, the sera were not analyzed until the entire experiment was completed. At the onset of an experiment, background titers for the immunogenic reporter protein should be of primary concern. Using test animals with low background titers could prove more effective in analysis of the humoral immune response in tilapia as has been shown in other species of fish[2,6-8].

Throughout the study, anti-β-galactosidase antibody titers fluctuated from week to week among the tilapia in each group (Table 1). ANOVA results ($p = 0.6945$) showed no significant difference among the groups. The geometric mean titers of the empty plasmid group, pCI, had the highest anti-β-galactosidase titers detected in the study. The pre-vaccination variation in the β-galactosidase titers may be due to endogenous β-galactosidase activity in *Vibrio* spp.[13,14]. Because *Vibrio* spp. is ubiquitous in aquatic systems and is identified as a fish pathogen, it is likely that the tilapia used to run this experiment had been exposed to this bacteria.

There are other possible explanations to the oscillating anti-β-galactosidase antibody titers. First, a wide variety of bacterial species comprise the normal flora of fish. Because β-galactosidase is a bacterial enzyme, it is possible that the immune system of fish may encounter this protein in its consistent interaction with normal flora. Therefore, tilapia may possess low levels of circulating anti-β-galactosidase antibodies. Second, portions of bacterial DNA, called unmethylated CpG dinucleotides, have been shown to cause potent immune activation and are being investigated as nonspecific immune enhancers for vaccine applications in higher and lower vertebrates, including humans, mice and fish[5, 15]. Because plasmids are of bacterial origin, tilapia receiving the control plasmid may have been affected by CpG-like immune stimulants, which could have resulted in an increase in their anti-β-galactosidase antibody titers.

Our data suggests that a β-galactosidase reporter gene may not be the optimal system for evaluating the humoral immune response of aquatic species. Possibly, immunization with a different reporter gene, that is novel to tilapia, may provide a more

suitable model, such as green fluorescent protein (GPF)[6,16] or the luciferase gene[17]. Perhaps the use of fish promoters, such as carp β-actin[17], would stimulate the immune system in tilapia better than viral promoters. Such a promoter should contain efficient regulatory signals of fish origin to drive transcription of the immunogenic protein.

Although it is important to induce optimal immune responses to DNA-mediated vaccines, other important issues, such as production cost for large numbers of animals will be determinants for the potential application of this technology in commercial fish farms. Lower doses, of nanogram quantities, which have proven effective in protection of IHNV in *O. mykiss*[10] will reduce cost. Alternative methods of administration may also help make production more cost efficient. Intramuscular injection is not practical for vaccine inoculation in the aquaculture industry. Therefore, oral delivery could be a potential option. Preliminary studies have identified intracellular bacteria as carrier systems for DNA-mediated vaccines[16]. Since several species of the genus *Listeria* can infect fish, a reporter plasmid carried by *L. monocytogenes* was used to infect various fish cell lines. Strong expression of the reporter protein suggests that these gram-positive bacteria could be carriers for vaccine administration to fish via oral route.

The aquaculture industry needs to increase its production and efficiency to meet the increasing consumer needs for fish products. Thus, there is a critical need for effective vaccines for the prevention of important infectious diseases of these aquatic animals. DNA-mediated vaccine technology is well on its way to becoming the most useful treatment for commercial fish farms.

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CHAPTER 2

ASSESSMENT OF THE HUMORAL IMMUNE RESPONSE OF ATLANTIC BOTTLENOSE DOLPHINS (*TURSIOPS TRUNCATUS)* TO DNA-MEDIATED VACCINATION

Introduction

The Space and Naval Warfare Systems Center San Diego (SPAWARSYSCEN) trains and cares for approximately one hundred marine mammals, including California sea lions (*Zalophus californianus)* and Atlantic bottlenose dolphins (*Tursiops truncatus)*. Due to their worldwide travels and their life in captivity, these animals are susceptible, and may be exposed, to a variety of potential pathogens. Because of the value of these animals, the Navy is dedicated to providing them the highest level of care, including a comprehensive preventive medicine program.

Vaccination of individuals within a population is perhaps the single most effective preventive medicine tool. Currently, there are no efficacious vaccines available to protect these animals from commonly encountered pathogens. Traditional vaccination protocols have had limited success, at best, in preventing infectious diseases in marine mammals. This presents a challenge to the Navy's veterinary staff, particularly since infectious diseases are among the most prevalent causes of morbidity and mortality in marine mammals[1,2]. Thus, there is a critical need to develop effective, safe vaccines for use in marine mammals.

Immunization using nucleic acid in the form of a DNA-mediated vaccine represents a potentially efficacious and low-risk method for vaccination of valuable aquatic species against common pathogens causing high mortality or morbidity. The ideal plasmid would encode for a protein product that induces long-lasting, protective

immunity against the target pathogen. To date, several plasmid vaccines have been engineered to contain specific gene sequences that encode for immunogenic, nonpathogenic proteins of infectious organisms, including canine distemper virus, influenza A virus and the virus causing measles[3-5]. The uptake and expression of protective proteins from nucleic acid vaccines have been observed in several species, including fish, penguins, pigs, cows, and horses[6-9]. Thus, DNA vaccine technology may be a valuable tool in a marine mammal preventive medicine program.

The goal of this research is to demonstrate that a selected aquatic species, *T. truncatus*, is capable of immunologically responding to foreign proteins that are transferred in the form of plasmid DNA. Knowledge gained from this work will directly apply to future development of plasmid vaccines against specific organisms causing diseases of cetaceans, such as *Erysipelothrix* sp., *Brucella* sp., caliciviruses, and morbilliviruses.

Materials and Methods

Pilot Study

Animals

Three Atlantic bottlenose dolphins (*Tursiops truncatus)*, Buster, Bugs and Nihoa, were housed with the Navy Marine Mammal Program at SPAWARSYSCEN in San Diego, CA. The veterinary and husbandry staff coordinated the vaccination and blood collection schedules as part of their normal, daily routines. The dolphins were randomly assigned to the treatment groups to prevent biased sampling.

Plasmid Preparation

Two expression plasmids were used in this study. The test plasmid, pCMVβ, is a eukaryotic/mammalian reporter plasmid that contains the gene for β-galactosidase and the human cytomegalovirus (CMV) immediate-early enhancer/promoter (Figure 4, Clonetech, Inc., Palo Alto, California, USA; 7.2 kilobases, Genebank accession no. U02451). The control plasmid, pCI, is an “empty” mammalian expression plasmid that has the CMV promoter and cloning site but lacks a gene for expression (Figure 4,

Promega, Inc., Madison, Wisconsin, USA; 4.0 kilobases, Genebank accession no. U47119). Plasmids were prepared at the University of Georgia according to manufacturer's instructions, using large-scale plasmid preparation kits to produce endotoxin- and pyrogen-free products (Qiagen, Inc., Valencia, California, USA). Purified plasmids were stored at – 20^0C in sterile phosphate-buffered saline (PBS), pH 7.4, at a concentration of 1 μg/μl.

DNA Inoculations and Collection of Sera

Buster received 50 μg of the test plasmid, pCMVβ. Nihoa was immunized with 50 μg of the control plasmid, pCI. All inoculations were administered in phosphate buffered saline via intramuscular (IM) injection on the left side of the body, caudo-lateral to the dorsal fin. Pre-immune sera were collected prior to the initial vaccination (Day 0) and then post-injection sera were collected every other week for one year. Three repeat vaccinations were given every 4 weeks. Sera were stored at – 20^0C prior to immunoassay.

ELISA Immunoassay

An indirect enzyme-linked immunosorbent assay (ELISA), using β-galactosidase (Sigma, St. Louis, Missouri, USA) as antigen, was designed to detect bottlenose dolphin antibodies generated against the plasmid gene expression product. Each well of an eight-well microtiter strips (EIA/RIA, high binding, Costar, Cambridge, Massachusetts, USA) was coated of β-galactosidase at 100 μg/ml in carbonate-bicarbonate buffer, pH 9.6. The coating process was performed at 4^0C for a minimum of 18 hours. After coating, wells were rinsed once using wash buffer (PBS, 0.05% Tween 20). Pre-immune sera and post-innoculation sera were diluted in 50 μl of conjugate buffer (PBS, 0.1% Tween 20, 5% Carnation™ non-fat dry milk). Serial two-fold dilutions, starting at 1:10 and ending at 1:640, were performed in triplicate for each serum sample. Dolphin sera and antigen were incubated with rocking for 30 minutes at 37^0C followed by three rinses in wash buffer. The secondary rabbit anti-*T. truncatus* IgG antibody was diluted 1:4,000 in

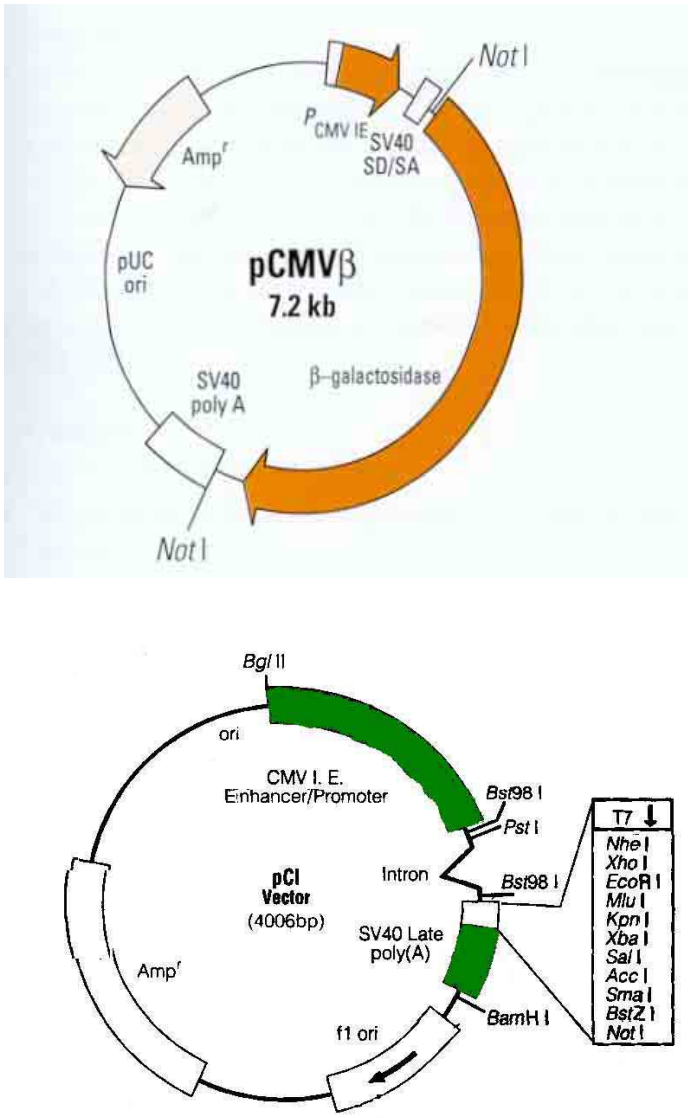

Figure 4. Test plasmids for assessment of the humoral immune response in bottlenose dolphins (*Tursiops truncatus*) to DNA-mediated vaccination. Top: pCMVβ, containing β-galactosidase reporter gene; Bottom: pCI, an empty plasmid. Both vectors contain the CMV immediate/early enhancer/promoter.

conjugate buffer and added to test wells at 50 μl per well. The wells were then incubated for 30 minutes at 37^0C with rocking, followed by three rinses with wash buffer. Commercial goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) was employed as the tertiary antibody conjugate (Gibco BRL, Rockville, Maryland, USA).

Tertiary antibody was diluted 1: 10,000 in conjugate buffer and added to test wells at 50 μl per well. Wells were again incubated at 37^0C for 30 minutes with rocking, followed by three final washes. Enzyme activity was detected using 50 μl per well of an o-phenylenediamine (OPD) (Sigma) solution (1 mg/ml in 100mM citric phosphate buffer, pH 5.0, 30% H_2O_2). Substrate color development was stopped after 20 minutes at 25^0C by the addition of 30 μl of 2 M H_2SO_4. Wells were read for absorbance at 490 nm using an automated plate reader (V-max Kinetic Plate Reader; Molecular Devices Corp., Sunnyvale, California, USA).

Twelve different sera samples from either Buster or Nihoa were assayed in one ELISA experiment. Negative and positive controls were performed for each ELISA. The positive control used commercial anti-β-galactosidase sera generated in rabbits (Sigma, St. Louis, Missouri, USA) and an appropriate corresponding secondary antibody-HRP conjugate. Pre-immune sera, from the test dolphins, were used as a negative control for each dolphin, as well as sera from a dolphin not participating in this study. A substrate-only negative control was included to detect any endogenous peroxidase activity. Thirty-six conjugate-only negative control wells were generated for each ELISA experiment to monitor non-specific secondary antibody binding.

Statistical Analysis

Anti-β-galactosidase antibody in dolphin sera was calculated as the reciprocal of the highest dilution that was two standard deviations above the average optical density of the pre-immune sera.

Repeat Study

Two Atlantic bottlenose dolphins were used in a subsequent vaccination study in which dose and route of injection were changed from the original protocol. Bugs received 500 μg of the test plasmid, pCMVβ, while Nihoa was again immunized with the control plasmid, pCI, but this time with a dose of 500 μg. Inoculations were administered by an ultrasound guided intramuscular injection in the longissimus muscle

near the cervical lymph node. The vaccination schedule was identical to the pilot study. The sera samples were analyzed by ELISA for β-galactosidase antibody production as detailed previously.

Results

Pilot Study

Results of the pilot study suggest that β-galactosidase antibodies were not produced by the test cetacean, as there was not a significant difference in the β-galactosidase antibodies produced by the control cetacean (Table 2, Figure 5). Buster, the dolphin vaccinated with the test plasmid, had a peak titer of 1:40, at 6 weeks post-vaccination. This titer returned to baseline levels by 8 weeks post-vaccination. Based on this data, another study was conducted in which the dose and route of inoculation were changed.

Table 2. β-galactosidase antibody titers from inoculations of two *Tursiops truncatus* with pCMVβ, containing the β-galactosidase gene or pCI, as the empty plasmid control.

Buster (pCMVβ)			**Nihoa (pCI)**		
Week	**βgal titer**	**Vax dose**	**Week**	**βgal titer**	**Vax dose**
0	20	50 μg	0	20	50 μg
2	20		2	10	
4	20	50 μg	4	1	50 μg
6	40		6	1	
8	10	50 μg	8	10	50 μg
10	10		10	10	
12	--	50 μg	12	--	50 μg
14	20		14	1	
16	20		16	1	

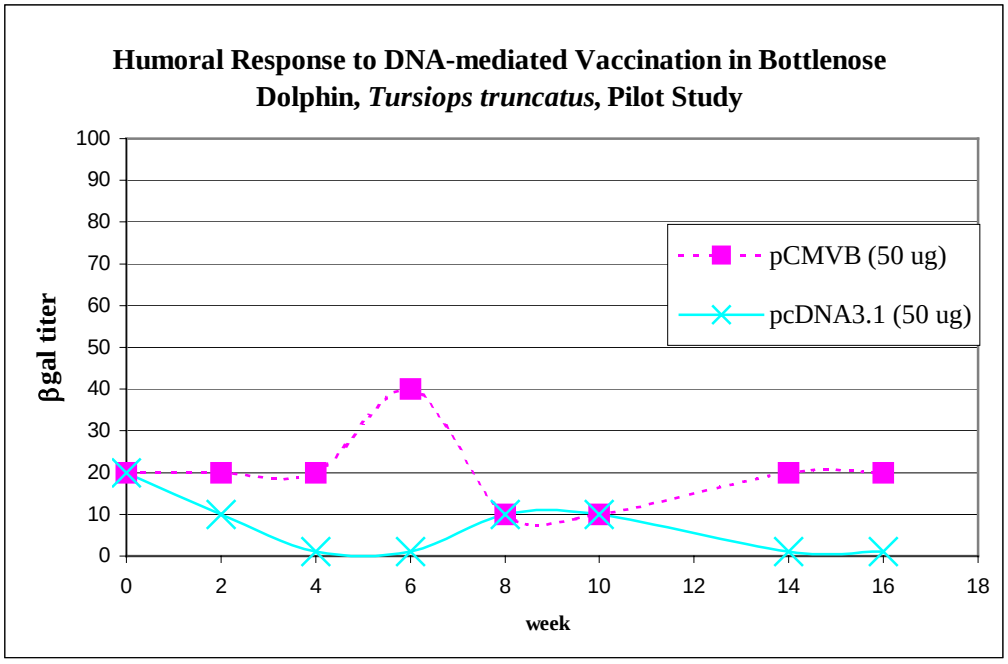

Figure 5. Anti-β-galactosidase antibody titers of empty plasmid control, pCI, (50 μg, IM.), and test plasmid, pCMVβ, (50 μg, IM) *Tursiops truncatus* treatments. Dolphins were inoculated week 0, 4, 8, and 12.

Repeat Study

β-galactosidase antibody titers were detected in Bugs (Table 3, Figure 6); however, the post-vaccination titers were not significantly different (4 fold or greater increase) than the pre-vaccination titer. The negative control dolphin, Nihoa, displayed low background anti-β-galactosidase titers, as previously documented. Because Bugs had pre-vaccination antibodies to β-galactosidase, we were unable to assess the humoral immune response to the test vaccine.

Table 3. β-galactosidase antibody titers from inoculations of two *Tursiops truncatus* with pCMVβ, containing the β-galactosidase gene or pCI, as the empty plasmid control.

Bugs (pCMVβ)			Nihoa (pCI)		
Week	**βgal titer**	**Vax dose**	**Week**	**βgal titer**	**Vax dose**
0	1000	500 μg	0	20	500 μg
1	1000		1	1	
2	1000		2	40	
4	1000	500 μg	4	1	500 μg
6	1000		6	10	
8	1000	500 μg	8	1	500 μg
10	1000		10	1	
12	1000	500 μg	12	1	500 μg
14	1000		14	1	
16	1000		16	1	

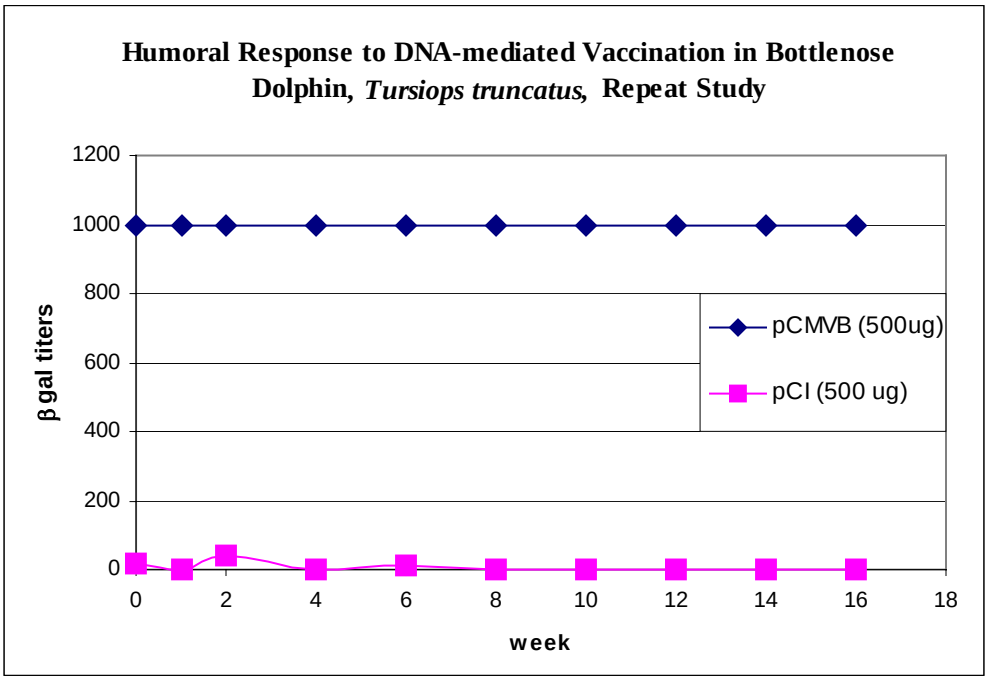

Figure 6. Anti-β-galactosidase antibody titers of empty plasmid control, pCI, (500 μg, ultrasound IM), and test plasmid, pCMVβ, (500 μg, ultrasound IM) *Tursiops truncatus* treatments. Dolphins were inoculated week 0, 4, 8, and 12.

Discussion

A successful, preventive medicine program is needed to protect captive marine mammals, many of which represent critically endangered or threatened species. Infectious diseases, such as *Erysipelothrix* sp., *Brucella* sp., caliciviruses, and morbilliviruses, are the most prevalent causes of morbidity and mortality in these marine mammals[1,2]. Plasmid vaccine technology is an effective, new way to safely and economically protect humans and animals from diseases caused by viruses, bacteria and parasites. Prior to this study, no research has been reported to detemine how marine mammals would respond to plasmid vaccines.

In this pilot study, the primary objective was to investigate specific humoral immune responses induced by immunization with a plasmid in Atlantic bottlenose dolphins (*Tursiops truncatus*). The test plasmid contained the β-galactosidase reporter gene under the control of a mammalian virus transcription promoter (CMV). This plasmid has proven useful in assessing humoral immune responses stimulated by DNA-mediated vaccination in other species[10]. We expected to observe a measurable antibody response to the expressed immunizing protein.

In the pilot study, the dolphin receiving the test plasmid did not produce a significant amount of β-galactosidase antibodies compared to the antibodies produced by the control dolphin (Table 2, Figure 5). However, we were able to document that plasmid vaccines could be administered to dolphins without causing obvious adverse side effects.

Based on the pilot study, we theorized that we might be able to induce a humoral immune response from by altering the dose of the plasmid and route of the injection. In other large mammals and humans, milligram amounts of plasmid injected intramuscularly are needed to produce an immune response[12]. An adult bottlenose dolphin average weight is approximately 300-650 kg. Because of the size of these cetaceans, in the repeat study we used a plasmid concentration of 500 μg/ml. It is also important for the injected plasmids to be taken up by antigen presenting cells that will transport the produced antigen to lymph nodes, where immune responses are initiated.

Unfortunately, reports in the literature about the lymphoid system of cetaceans are sparse, and inadequate. Dr. Cowan and his colleagues at the University of Texas Medical Branch, in Galveston, Texas have provided a recent and in-depth study of the morphology of the lymphoid organs of *T. truncatus*[13]. Yet, no immunologic studies have been performed and published on this system in any cetacean species of which we are aware. So, based on this data, we injected the plasmids into the longissimus muscle in the cervical region. This injection site is closely associated with direct drainage to the pre-scapular lymph nodes.

Evaluation of anti-β-galactosidase antibody titers for the repeat study revealed that the dolphin, receiving the β-galactosidase reporter plasmid, demonstrated high β-galactosidase antibody titers; however, testing archived serum samples from this dolphin prior to vaccination also showed similar titers to β-galactosidase. Unfortunately, with a baseline β-galactosidase antibody titer of 1:1000, we were unable to assess the humoral immune response to DNA vaccination in this study.

Subsequent to these studies, high β-galactosidase titers have been seen in other aquatic species, possibly due to environmental exposure to *Vibrio* spp (unpublished data). *Vibrio* spp. genome contains the β-galactosidase gene[14,15]. Since *Vibrio* spp. are ubiquitous in aquatic systems, it is highly likely that *T. truncatus* and other aquatic species frequently encounter these pathogens. Thus, β-galactosidase may not be the best reporter gene for studying plasmid vaccination in aquatic species.

Alternative strategies for optimization of nucleic acid vaccines are an industry priority. Although, intramuscular injection of DNA vaccines elicits both humoral and cellular immune responses in mice, these vaccines are less efficient in larger animal models and humans[16]. It is imperative to investigate cytotoxic lymphocyte (CTL) responses in conjunction with the humoral immune response, because in larger animals, DNA vaccines appear able to prime strong, broad CTL but only modest antibody responses[17]. These variances in response may be the result of lower transfection

efficiencies caused by differences in muscle structure. Dolphin muscle and skin has been immunologically characterized in comparison to human skin to determine if Langerhans type cells for antigen capture and presentation are present in dolphins[18]. Direct immunization into lymphoid tissues may be preferable to IM injection. This novel technique has enhanced immunogenicity of DNA vaccines by 100- to 1,000-fold in mice[19].

Until it is demonstrated that dolphins will mount an immune response to a foreign antigen that is presented in the form of a plasmid vaccine, further development of this class of vaccines specifically targeted against marine mammal infectious agents is not possible.

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CHAPTER 3

CONSTRUCTION OF A DOLPHIN MORBILLIVIRUS DNA-MEDIATED VACCINE[1]

[1] Schock, T.B., Stanton, J., and Ritchie, B.W. To be submitted to Virology.

Introduction

In the past 13 years, morbillivirus infections have been recovered during epizootics among marine mammals belonging in the *Cetacea* and *Pinnipedia* orders. Four morbilliviruses are now known to infect various marine mammals: canine distemper virus (CDV) in seals[1,2], phocine distemper virus (PDV) in seals[3], dolphin morbillivirus (DMV) in dolphins and whales, and porpoise morbillivirus (PMV) in porpoises[4].

From 1987 to 1988, more than half of the population of inshore bottlenose dolphins (*Tursiops truncatus)* along the Atlantic coast of the United States was estimated to have died during the first recognized marine morbilliviral epizootic[5]. In 1987, CDV killed thousands of Siberian seals (*Phoca sibirica*) in Lake Baikal, Russia[1]. A devastating epizootic in 1988, killed approximately 17,000 harbor seals (*Phoca vitulina*) in the North Sea[3,6]. About the same time, an outbreak of PMV killed small numbers of harbor porpoises (*Phocoena phocoena*) in northwestern Europe[4,7]. From 1990 to 1991, a DMV epizootic killed thousands of striped dolphins (*Stenella coeruleoalba)* in the western Mediterranean[7-9]. From 1993 to 1994, another DMV epizootic occurred in *T. truncatus* in the Gulf of Mexico[10]. More recently, morbilliviral infection has been reported in cetaceans in the Pacific, namely common dolphins *(Dolphinus delphis)* and Pacific striped dolphins (*Lagenorhynchus obliquidens)*[11,12]. The most recent addition to the morbillivirus-infected group is a long-finned pilot whale (*Globicephalus melas)* of the U.S. Atlantic coast[13].

The Space and Naval Warfare Systems Center San Diego (SPAWARSYSCEN) trains and cares for approximately one hundred marine mammals, including California sea lions (*Zalophus californianus)* and Atlantic bottlenose dolphins (*Tursiops truncatus)*. These animals perform tasks in oceans all over the world. Due to their worldwide travels and their life in captivity, these animals are susceptible to infectious agents such as morbillivirus. With evidence that morbilliviruses of aquatic animals may cross barriers between species of different orders[14], the Navy is in need of a method of protecting these valuable animals against morbillivirus.

Vaccination of individuals within a population is perhaps the single most effective preventive medicine tool. Currently, there are no efficacious vaccines available to protect these animals from harm. Traditional vaccination protocols have had limited success, at best, in preventing infectious diseases in marine mammals. Thus, there is a critical need to develop effective, safe vaccines for use in marine mammals.

Immunization using nucleic acid in the form of a DNA plasmid represents a potentially powerful, efficacious, and low-risk method for vaccination of aquatic species against common diseases associated with high mortality or morbidity. The ideal plasmid would encode for a protein product that induces long-lasting, protective immunity against the parent pathogen. To date, several plasmid vaccines have been engineered to contain specific gene sequences that encode for immunogenic, nonpathogenic proteins of infectious organisms, including canine distemper, influenza and measles[15-17]. The uptake and expression of protective proteins from nucleic acid vaccines have been observed in several domestic and exotic animal species, including fish, penguins, pigs, cows, and horses[18-21]. Thus, DNA vaccine technology may be an effective tool in a marine mammal preventive medicine program.

The goal of this research is to construct a DNA vaccine to protect specifically against DMV.

Materials and Methods

Virus

Dolphin morbillivirus (DMV) +RNA was obtained from Dr. Jerry Saliki at Oklahoma State University (Stillwater, Oklahoma). The viral RNA was extracted from infected Vero cells supplied by Dr. Juan Lubroth at Plum Island, USDA, APHIS (Orient Point, New York).

Amplification of Hemagglutinin (H) and Fusion (F) Genes of DMV

Reverse transcription and polymerase chain reaction (RT-PCR) were preformed on total RNA of DMV infected Vero cells using Titan™ One Tube RT-PCR system (Roche, Indianapolis, Indiana). Primers used to amplify the gene products were

identified by the computer program Oligo (Molecular Biology Insights, Cascade, Colorado), using published DMV sequence data (Accession no. for H gene: Z36978; accession no. for F gene: Z30086; accession no. for L gene: NC_001921 (Canine distemper virus), NC_001498 (Measles virus), Z30697 (Rinderpest virus)) . For the hemagglutinin (H) gene, the forward primer sequence is 5'AAGCTTCTTTAGTGATTCTGTGCGTAT 3' and the reverse primer sequence is 5'CTCGAGATAGCAGTMTCAGTCATTARCC 3'. To facilitate cloning, a *Hin*dIII restriction site was added to the forward primer and a *Xho* I site was added to the reverse primer. The fusion gene was amplified using 5'ACTTTTCATCTGGTCGTCAACA 3' as the forward primer and 5'CCTGGTATGCCCTTGTAGAATG 3' as the reverse primer. Amplification of both target nucleic acid sequences was carried out by 1 cycle at 50^{o}C for 60 minutes, followed by a cycle at 94^{o}C for 2 minutes, then 30 cycles of 94^{o}C for 1 minute, 55^{o}C for 1 minute and 30 seconds, then a cycle at 68^{o}C for 3 minutes, and finally 1 cycle for a 7 minute extension at 68^{o}C. Gene products were identified on a 1% agarose/ethidium bromide gel run at 100 volts for 45 minutes. The products were purified by QuickStep™ PCR Purification Kit (Edge Biosystems, Gaithersburg, Maryland) and confirmed by sequencing.

Cloning the Gene Products into a Eukaryotic Expression Vector

The F and H genes were initially cloned into a TOPO TA Cloning® vector (pCR® 2.1-TOPO®) (Invitrogen, Carlsbad, California) (TA-F and TA-H, respectively) and transformed into One Shot® chemically competent *Escherichia coli*. Positive clones were analyzed by alkaline lysis miniprep, followed by a restriction digest by *Eco*RI to identify vector and insert. A 1% agarose/ethidium bromide gel allowed visualization of cloned products as well as the digested vector and insert.

The fusion and hemagglutinin inserts of the TA clones were subcloned into an expression vector produced by the DNA vaccine company, Vical, Inc. (San Diego, California). Primers were designed to amplify the gene insert of the TA clones and to contain restriction sites for ease of ligation into the eukaryotic expression. The forward

primers include a consensus Kozak translation initiation sequence (GCCGCCACC)[22]. Primer sequences for the F gene were as follows: forward 5' GGCGTCGACGCCGCC ACCATGGCCGCAGTAACGGCG 3' and reverse 5' GAGGCGGCCGCTCACAAGG ATCTTACATATGATT 3'; and for the H gene: forward 5' GCGGTCGACGCCGCCA CCATGTCTTCTCCGCGTGACAAGGTCGAGGCA 3' and reverse 5' CCAGCGGC CGCCTAACGGCTGCAGCTCATAT 3'. The PCR amplified products were digested with *Sal*1 and *Not*1, which allowed gel purification of the inserted gene products with Genelute Agarose Spin Columns (Sigma, Saint Louis, Missouri). The purified products were then ligated into the linearized (*Sal*1 and *Not*1) eukaryotic expression vector using T4 DNA ligase (Promega, Madison, Wisconsin). This expression vector contains the human cytomegalovirus (CMV) immediate-early enhancer/promoter, an intron A, and a rabbit β-globin/proudfoot terminator sequence. The plasmids were prepared according to manufacturer's instructions, using large-scale plasmid preparation kits to produce endotoxin- and pyrogen-free products (Qiagen, Santa Clarita, California). Purified plasmids were stored at -20^0C in sterile phosphate-buffered saline (PBS), pH 7.4. Once constructed and confirmed by sequencing, the F and H expression vectors, vrDMVF and vrDMVH respectively, were analyzed for protein expression.

Antibodies

A fluorescent antibody assay was validated to determine the antibodies to be used to test for protein expression in transfected cells. DMV infected Vero cells were fixed on Supercell™ culture slides (Fisher Scientific, Pittsburgh, Pennsylvania) with cold 80% acetone for 15 minutes. The primary antibodies used were a monoclonal (MAb) mouse anti-DMV Ig and a polyclonal mouse anti-CDV Ig, both obtained from Dr. Saliki. These antibodies were doubly diluted from 1:10 to 1:320 in 1X PBS and incubated on the slides for 30 minutes at 37^0C in a CO_2 incubator. The slides were then washed 3 times with 1X PBS-0.05% Tween 20. The secondary antibody, a goat anti-mouse IgG conjugated with Fluorescein Isothiocyanate (FITC) (Sigma), was incubated at a 1:50 dilution in 1X PBS for 30 minutes at 37^0C in a CO_2 incubator. Finally, the slides were washed as above and

viewed on a fluorescent microscope. A slide fixed with CDV infected Vero cells was used as the positive control and a slide with only Vero cells as the negative control.

Protein Expression Assays

Transfection

African green monkey kidney cells (COS-7) (ATCC, Manassas, Virginia) were plated on 6 well cell culture plates (Costar, Corning, New York) with Dulbecco's Modified Eagle Medium (DMEM) (BioWhittaker, Walkersville, Maryland) and 10% fetal bovine serum (FBS). When the cells were 70-90% confluent, they were transfected with the plasmids using lipofectAMINE PLUS™ reagent according to the GIBCO BRL protocol (Life Technologies, Rockville, Maryland). Briefly, 1 μg of plasmid DNA vrDMVH, vrDMVF, pCMVβ (Clonetech, Inc., Palo Alto, California 94303) were each diluted in 100 μl OPTI-MEM© I (Life Technologies) with 6 μl PLUS reagent. The solutions were mixed and incubated at 25^0C for 15 minutes. The negative control contained OPTI-MEM© I and PLUS reagent but no plasmid DNA. A second solution was made diluting 4 μl lipofectAMINE™ reagent in 100 μl OPTI-MEM© I. The solutions were mixed and incubated at 25^0C for 15 minutes. During incubation, the COS-7 cells were washed 2 times with OPTI-MEM© I. The solutions were mixed with OPTI-MEM© I to a volume of 1 ml and added to the appropriate wells for 3 hours at 37^0C in CO_2. After 3 hours, additional medium and 20% FBS were added to the wells. The cells were assayed for protein expression after 48 hours incubation.

Western Blot

Initially, pelleted DMV was resuspended in PBS. DMV infected Vero cells were subjected to 2 freeze-thaw cycles, sonicated on ice for 10 seconds and the cells were pelleted by low-speed centrifugation. The virus was concentrated from the supernatant by centrifuging it at 125,000 X g for 1 hour at 4^0C. The pellet was resuspended in 300 μl PBS. Laemmli sample buffer (Bio-Rad, Hercules, California) containing 2% 2-mercaptoethanol was added to the virus sample and boiled for 2 minutes. The sample was loaded into 10% Tris-HCl gels (Bio-Rad) for sodium dodecyl sulfate-

polyacrylamide gel electrophoresis (SDS-PAGE). Following SDS-PAGE, proteins were transferred onto nitrocellulose (Bio-Rad) and blocked with 2% skim milk in Tris buffered saline/ 0.1% Tween 20 (TBS-T) for 30 minutes. The nitrocellulose was probed with the mouse anti-DMV Mab or the polyclonal mouse anti-CDV antibody at a 1:100 dilution in TBS-T for 16 hours at 20^0C. Bound antibody was detected using a goat anti-mouse IgG antibody conjugated to alkaline phosphatase (Sigma) and visualized with BCIP/NBT phosphatase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, Maryland).

For analysis of protein expression by vrDMVF and vrDMVH, transfected COS-7 cells were washed and then frozen in PBS. Protein was extracted from the cells by 2 freeze-thaw cycles. The supernatants and DMV were diluted 1:1 in Laemmli sample buffer containing 2% 2-mercaptoethanol and loaded into 12% Tris-HCl gels for SDS-PAGE. Proteins were then transferred onto nitrocellulose and blocked with 2% skim milk in TBS-T for 1 hour. The membrane was probed with convalescent sera that has a DMV serum neutralization (SN) titer of 1:192 or with negative dolphin sera with a DMV SN titer of less than 1:8 (both supplied by Dr. Cynthia Smith and Dr. Saliki). The serum was diluted 1:400 in TBS-T and incubated for 1 hour at 20^0C. Bound antibody was detected using Protein A conjugated to alkaline phosphatase (Calbiochem, La Jolla, California) and visualized with BCIP/NBT phosphatase substrate.

mRNA Detection

RNA was extracted from the supernatants of the COS-7 cells transfected with vrDMVF, vrDMVH, and the no DNA control according to manufacture's instructions of the High Pure RNA Isolation Kit (Roche), which includes a DNase treatment. A small portion of the extract samples was also treated with RNase. RT-PCR was performed on the extracts and the RNase treated samples using the Titan™ One Tube RT-PCR system with specific primers that amplify approximately 500 base pairs of the F or H genes of DMV. The primers used for the F gene began at the 1590^{th} nucleotide 5' CTAACTGC GCATCGGTACTC 3' and ended at the 2126^{th} nucleotide 5' GCCTAGGTTTTGGTGT TACGG 3'. The H gene was amplified with primers beginning at the 986^{th} nucleotide

5’ CATAGGGGGTGGTTTGAGTAATC 3’ and ending at the 1569th nucleotide 5’ CCTTTGTAGGCAATACA 3’. RT-PCR parameters were the same as above. DMV RNA was the positive control while the no DNA tranfected cells RNA extract was the negative control. PCR was also performed on the RNA extracts. Samples were run on 1% agarose gels and stained with either ethidium bromide or SYBR green for visualization. RT-PCR was also performed on the DNase treated, extracted RNA of the vrDMVF transfected cells using a LightCycler© to verify mRNA production by analysis of melting peaks.

Results

Plasmid Construction

The H and F genes of DMV were successfully amplified from infected Vero cells and cloned into the pCR® 2.1-TOPO® vector. The H gene product is 2929 base pairs (bps) long and the F gene product is 1927 bps long. When cloned into the TA vector,

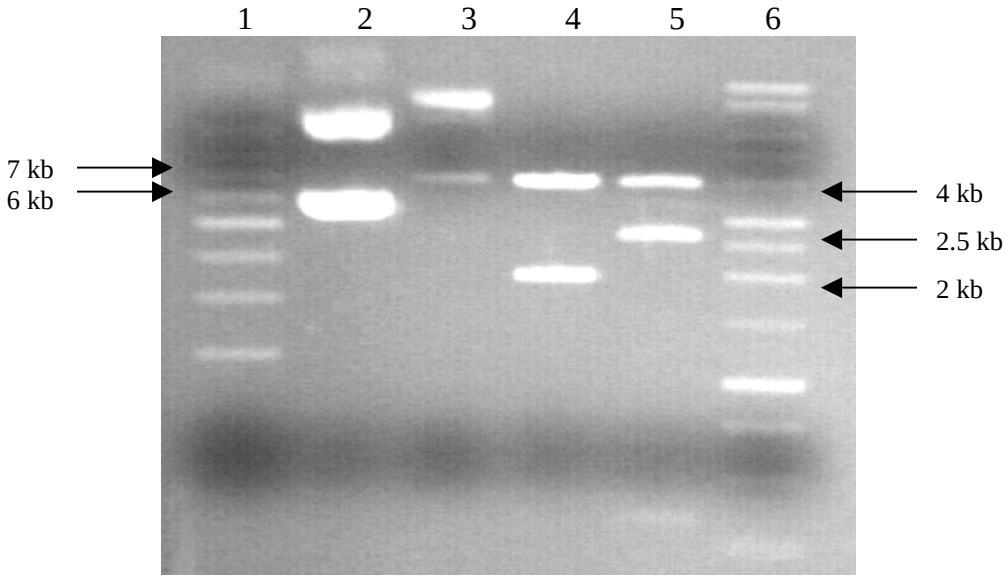

Figure 7. A 1 % agarose/ EtBr gel of TA-F and TA-H clones. Lane 1: Supercoiled ladder (arrows denote sizes of ladder), Lane 2: TA-F clone, Lane 3: TA-H clone, Lane 4: TA-F clone digested with *Eco*RI, Lane 5: TA-H clone digested with *Eco*RI, Lane 6: 1 kilobase ladder (arrows denote sizes of ladder).

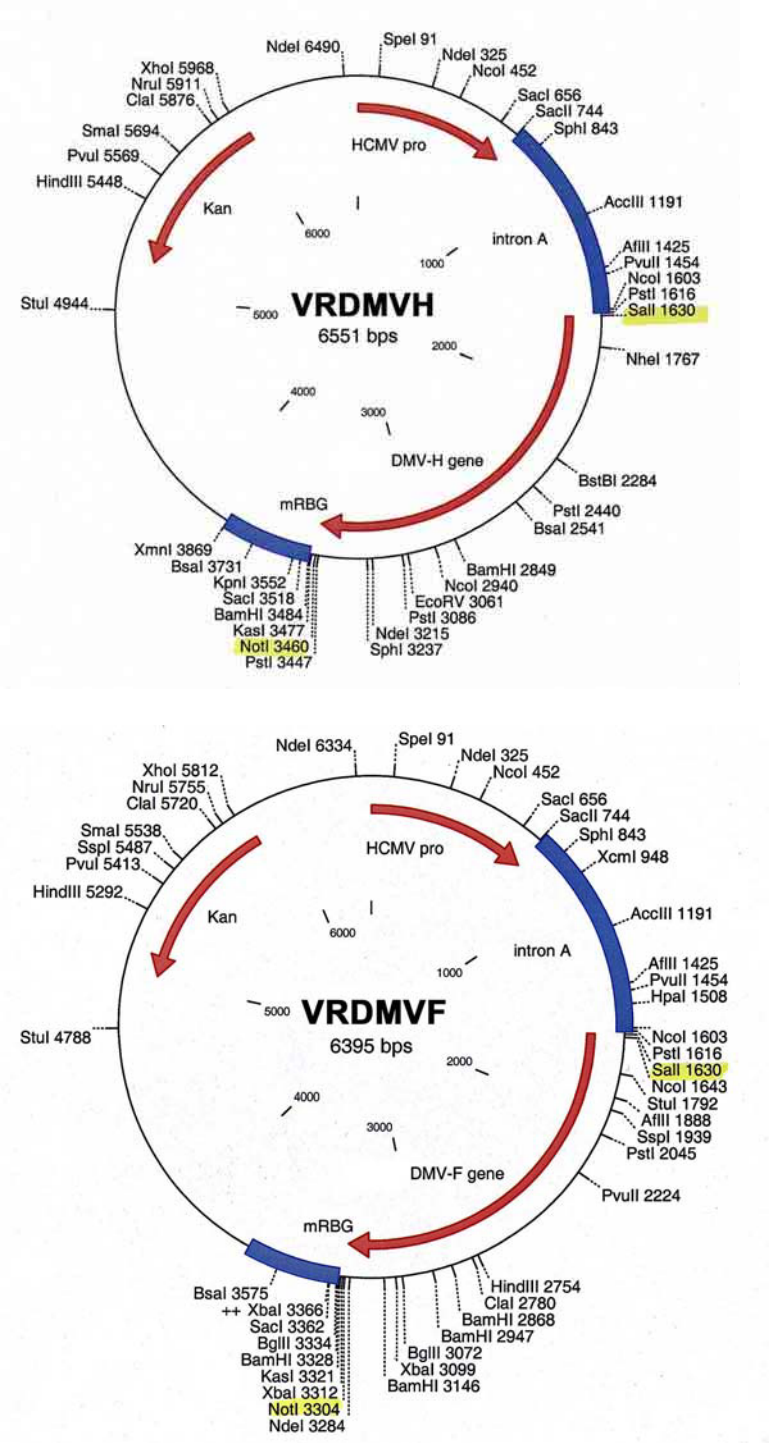

Figure 8. Maps of DNA-mediated vaccines for dolphin morbillivirus, vrDMVH and vrDMVF. The highlighted restriction sites are the sites at which the gene products were ligated into the vectors.

TA-F is 5827 bps and TA-H is approximately 6829 bps in size (Figure 7). Once the sequences of the genes were confirmed, they were cut out of the TA vector, gel purified and ligated into the eukaryotic expression vector from Vical (Figures 8). The vrDMVH vector has a size of 6551 bps and the vrDMVF vector is 6395 bps (Figure 9). Completed ligation was determined by restriction digestion with *Bam*HI for vrDMVH and *Hin*dIII for vrDMVF. Correct size products for vrDMVH are 635 and 5916 bps. The F gene inserted into the expression vector resulted in 2424 and 3971 bps products after digestion.

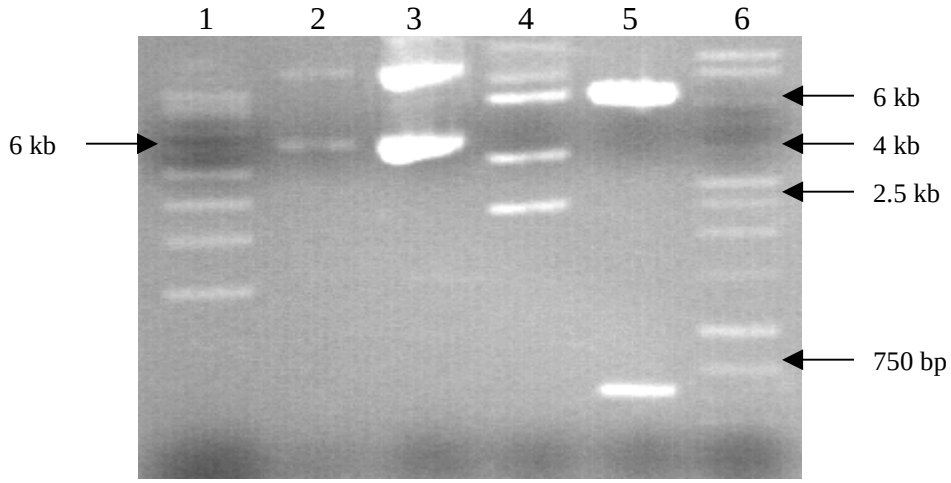

Figure 9. A 1% agarose/ EtBr gel of vrDMVF and vrDMVH. Lane 1: Supercoiled ladder (arrows denote ladder sizes), Lane 2: vrDMVF, Lane 3: vrDMVH, Lane 4: vrDMVF digested with *Hin*dIII, Lane 5: vrDMVH digested with *Bam*HI, Lane 6: 1 kilobase ladder (arrows denote ladder sizes).

Antibodies

A fluorescent antibody assay detected DMV in infected Vero cells only with the specific mouse anti-DMV Mab (Figure 10). The polyclonal mouse anti-CDV, that has been used to detect DMV in competitive enzyme linked immunosorbent assays (ELISAs)[23], did not identify the virus in the infected cells. The mouse anti-DMV Mab was then used in the protein expression assays.

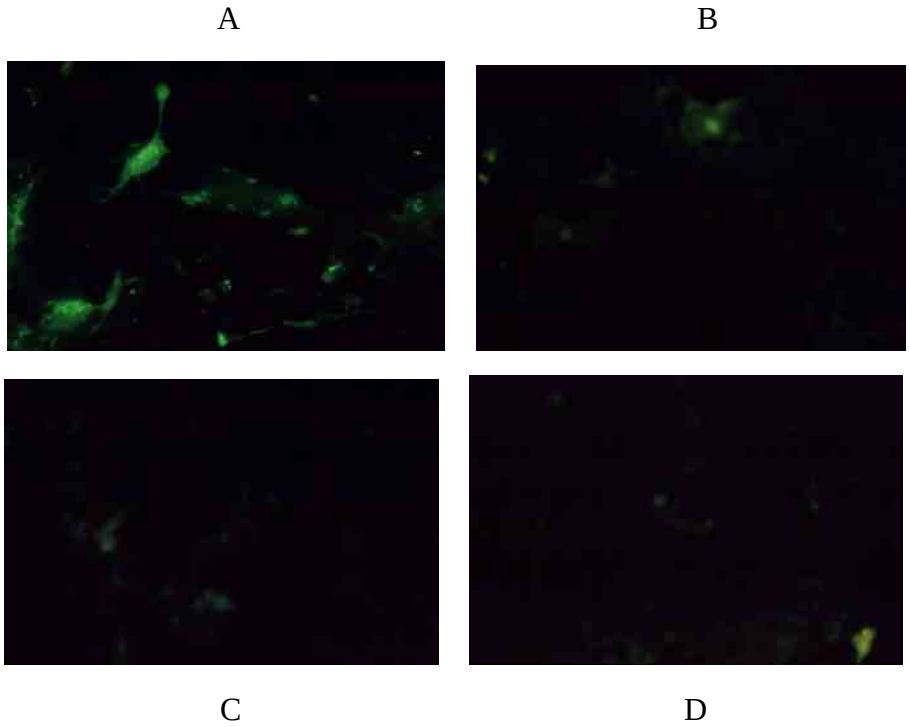

Figure 10. Fluorescent antibody assays with DMV infected Vero cells using a mouse anti- DMV Mab (A) and a polyclonal mouse anti-CDV antibody (C). B and D are uninfected Vero cells assayed with the mouse anti-DMV and mouse anti-CDV antibodies, respectively. Photographs were taken with a fluorescent microscope at 40X.

Protein Expression Assays

The ability of the DMV DNA-mediated vaccines to express antigen *in vitro* was investigated in COS-7 cells. The DNA-mediated vaccines were transiently transfected into COS-7 cell monolayers using Lipofectamine Plus and the expression of F and H proteins of DMV were analyzed by western blot.

Western Blot

A western blot was performed on pelleted DMV and probed with both the mouse anti-DMV Mab and the polyclonal mouse anti-CDV antibody. The DMV specific monoclonal antibody identified only the phosphoprotein (P) of DMV with a band at 72

kilodaltons (kDa) (Figure 11). The polyclonal antibody did not cross-react with any of the DMV proteins.

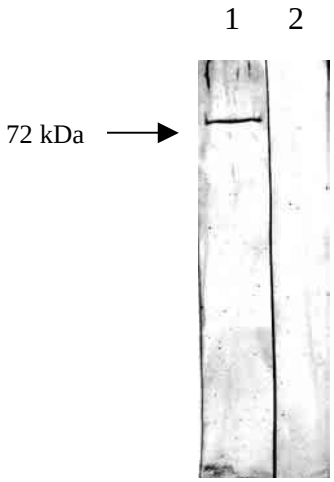

Figure 11. Western blot of DMV with a DMV specific monoclonal antibody. Lane one: DMV proteins, Lane 2: no virus control.

In order to determine if protein was being expressed in COS-7 cells using the constructed plasmids, convalescent dolphin sera with a DMV SN titer of 1:192 was used. Immunoglobulins were detected with alkaline phosphatase conjugated Protein A, a cell wall constituent of *Staphylococcus aureus* that binds the immunoglobulins of several species of vetebratess including cetaceans, nonspecifically[24]. Unfortunately, the dolphin serum nonspecifically bound to some of the cellular proteins from the transfected COS-7 cells. Bands of the correct sizes for the F and H proteins could not be distinguished from those of the no DNA transfected cells (data not shown). However, purified DMV proteins were detected banding at 72 kDa (P protein), 68 kDa (H protein), 60 kDa (F protein), and 58 kDa (N protein) (Figure 12).

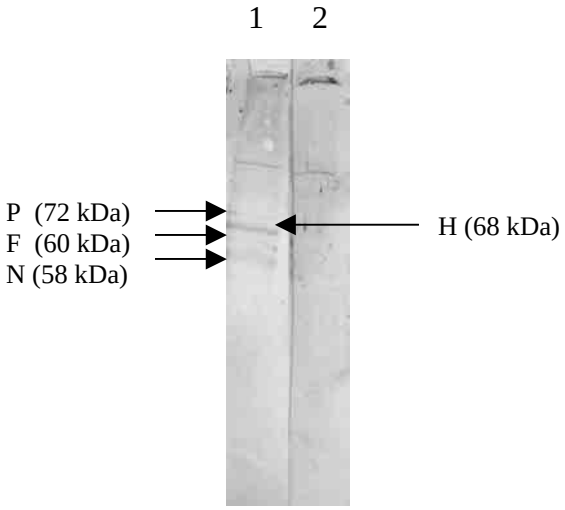

Figure 12. Western blot of DMV proteins detected with convalescent dolphin sera. Lane 1: DMV proteins incubated with dolphin sera with a DMV SN titer of 1:192. Lane 2: DMV proteins incubated with dolphin sera with a DMV SN titer of < 1:8.

mRNA Detection

Because proteins could not be detected with a western blot, RT-PCR was performed to determine if mRNA was being transcribed by the vrDMVH and vrDMVF vectors. Specific primers of 536 base pairs identified the F gene and primers of 583 base pairs amplified the H gene from the RNA extracts treated with DNase but did not amplify the genes using PCR (Figure 13) or RNase treated extracts (Figure 14). Products for the F mRNA could only be seen when the agarose gel was stained with SYBR green, which is the most sensitive DNA detection method (Figures 13 and 14). Because of the small amounts of the F mRNA detected from the extracts, melting peaks produced from RT-PCR using a LightCycler© was used to verify that the products from the DNase treated RNA extracts of vrDMVF transfected cells were correct (Figure 15). DMV RNA and extracted RNA from transfected cells had the same melting peaks occurring at 83.73^0C and 83.83^0C, respectively.

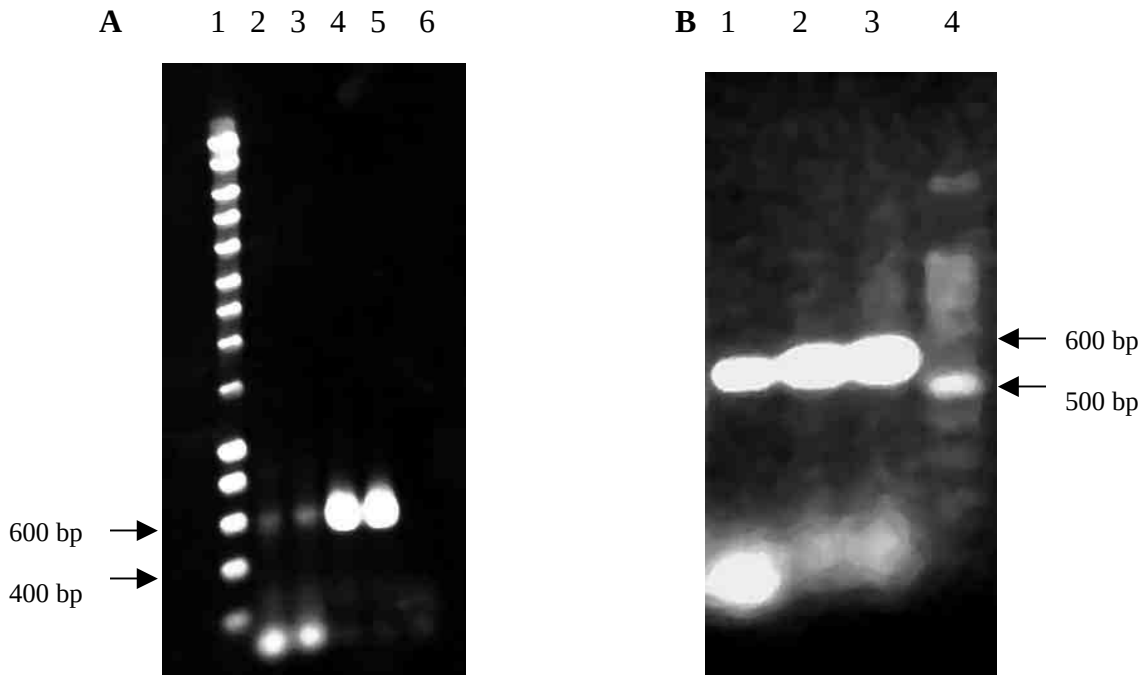

Figure 13. mRNA detection by RT-PCR and PCR on RNA extracts from vrDMVH and vrDMVF transfected cells. A: F mRNA by agarose gel stained with SYBR green. Lane1: 1 kb ladder, Lanes 2 + 3: extracted RNA from vrDMVF transfected cells, Lanes 4 + 5: DMV RNA positive controls, Lane 6: extracted RNA PCR control. B: H mRNA by agarose gel stained with ethidium bromide. Lane 1: DMV RNA positive control, Lanes 2 + 3: extracted RNA from vrDMVH transfected cells, Lane 4: 1 kb ladder.

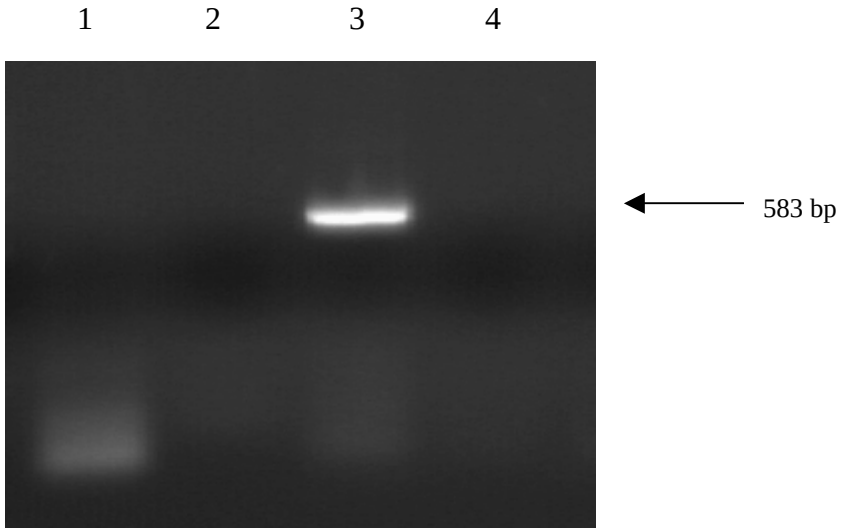

Figure 14. 1% agarose/ ethidium bromide gel of RT-PCR products from DNase and RNase treated RNA extractions of vrDMVF and vrDMVH transfected COS-7 cells. Lane 1: vrDMVF RNA extraction with DNase, Lane2: vrDMVF RNA extraction with RNase, Lane 3: vrDMVH RNA extraction with DNase, Lane 4: vrDMVH RNA extraction with RNase.

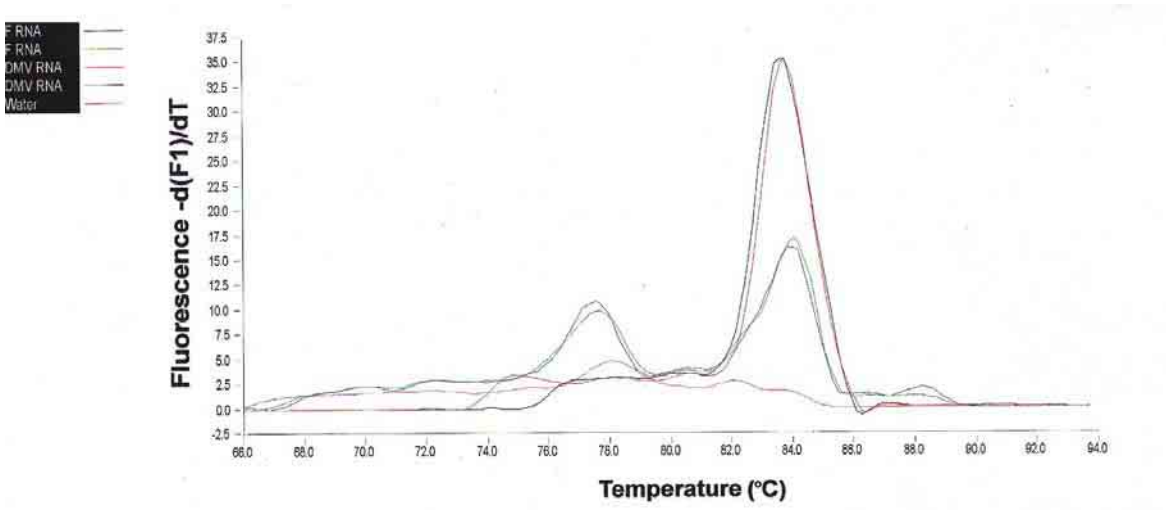

Figure 15. Melting peaks of DMV RNA and extracted RNA from vrDMVF transfected COS-7 cells by RT-PCR using a LightCycler©.

Discussion

DNA-mediated immunization has attracted interest as a potentially powerful, efficacious, and low-risk method for vaccination against common diseases associated with high mortality or morbidity. Morbillivirus has affected numerous marine mammal species in oceans world-wide. In order to protect the U.S Navy's Atlantic bottlenose dolphin (*Tursiops truncatus*) population against morbillivirus, we have constructed a DNA-mediated vaccine specific for this common cetacean disease.

Previous studies investigating DNA-mediated vaccines for other morbilliviruses have proven that they are protective when containing sequences encoding the viral glycoproteins, hemagluttinin (H) or fusion (F) proteins[17,25-26]. In this study, the H and F genes were amplified from the pCR® 2.1-TOPO® cloning vector using primers that encoded the initiation consensus sequence (GCCGCCACC) described by Kozak et al; this sequence enhances the translation process[22]. The amplified gene products were ligated into a eukaryotic expression vector that contained the CMV promoter, which has now been well accepted as the optimal promoter for use in DNA-mediated vaccines.

Other contruct modifications in this vector include an intron A, known to increase the expression of the protein in eukaryotes[27], and a rabbit β-globin/proudfoot terminator sequence.

A polyclonal mouse anti-CDV antibody that has been shown to cross-react with marine mammal morbilliviruses in a competitive enzyme linked immunosorbent assay (ELISA)[23], was used to evaluate gene expression. When used in a fluorescent antibody assay and a western blot, the polyclonal antibody did not detect DMV proteins of infected Vero cells (Figure 10). When a mouse anti-DMV monoclonal antibody was used, virus proteins were detected by the fluorescent antibody assay. This suggests that a cellular based assay requires specific antispecies conjugates. A western blot with this monoclonal antibody detected the phosphoprotein (P) of purified DMV banding at 72 kDa (Figure 11). Thus, this antibody would not be capable of detecting expression of the F and H proteins. A DMV-specific polyclonal is required in order to determine if the DNA-mediated vaccines are expressing protein.

The only DMV-specific polyclonal available is a very limited supply of convalescent dolphin sera. A western blot using this serum made identification of expressed proteins difficult because of nonspecific binding with COS-7 cellular proteins. Using Protein A as a secondary antibody compared to a dolphin anti-rabbit IgG followed by a conjugated rabbit antibody greatly reduced background and nonspecific binding. DMV proteins were identified (Figure 12), suggesting that this western blot protocol will detect the expressed proteins. A 1:400 dilution of the primary antibody resolved faint bands of the DMV proteins, while reducing the cellular nonspecific binding. With a lower dilution, DMV proteins were more easily visible, however, nonspecific binding became a problem. RNA was extracted from the transfected cell supernatants for each vector and the samples were treated with DNase and RNase. With specific primers within the F and H genes, RT-PCR identified the production of mRNA for both genes (Figure 13). Thus, the amplified products are not the result of the DNA introduced during the transfection process because the DNase treated extracts amplified the product of within

the H gene, whereas, the RNase treated samples did not amplify the product within the gene (Figure14). This suggests that the vrDMVH and vrDMVF vectors are able to replicate and transcribe mRNA from the inserted genes. PCR controls did not amplify the intended products due to DNase treatment during the RNA extractions verifying that specific mRNA was detected. The F product could only be visualized when stained with SYBR green, signifying that only small quantities of mRNA are being transcribed. RT-PCR was also performed on the same DNase treated RNA extracts from vrDMVF transfected cells using a LightCycler© to verify mRNA production (Figure 15). The melting peaks produced were of the same temperature suggesting that F mRNA is being made by the vrDMVF plasmid. The H product from the RNA extract was easily visible by staining with ethidium bromide, suggesting larger amounts of mRNA are being produced. Sequencing of the specific products will ultimately determine the success of the vrDMVH and vrDMVF plasmids to transcribe mRNA. Since mRNA is produced by both plasmids, it is expected that the mRNA is being translated into protein and should express in mammalian cells.

Some alternatives to the methods completed in this study that may enhance the display of protein expression by the vrDMVH and vrDMVF plasmids in mammalian cells include making an acetone powder from the COS-7 cells and incubating it with the dolphin sera in order to reduce the cellular nonspecific binding. A more sensitive detection system, such as with luminescent substrates, may also identify the proteins being expressed. A transcription/translation kit would demonstrate if protein is being made. Unfortunately with this system, a T7 or SP6 promoter is necessary and the Vical vector does not contain either of these promoters. Possibly, a different vector would be more compatible with the DMV F and H genes for protein expression.

There is a possibility that protein is not being expressed due to several factors. The mRNA may easily be degraded within the cell prior to translation. Protein may be made, but cellular proteases may break down these proteins as a regulatory mechanism before protein is extracted from the cells. Post-transcriptional regulation such as protein

folding and glycosylation may not have occurred preventing their detection using available antibodies.

It is imperitive that protein expression be determined before these DNA-mediated vaccines can be administered in an animal model and before it can be used to protect *Tursiops truncatus* from morbillivirus associated disease.

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