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Biochemical and Immunological Characterization of the Immobilization Antigens of *Ichthyophthirius multifiliis*
(Under the direction of Dr. HARRY W. DICKERSON)

The pathogenic ciliate *Ichthyophthirius multifiliis* (Fouquet) infects a wide range of freshwater fish and causes the disease Ichthyophthiriasis (Ich) or "white spot". Fish that survive infection acquire immunity to subsequent challenge and produce specific serum antibodies that immobilize the parasite *in vitro*. Cell surface protein antigens targeted by these antibodies are referred to as immobilization antigens (i-antigens). Using immobilizing mouse monoclonal antibodies as ligands, the i-antigens of *I. multifiliis* were purified by immunoaffinity chromatography. The biochemical characteristics of purified i-antigens were studied with SDS-PAGE, MALDI-TOF, Western blotting, and Edman degradation, and the immunogenicity of purified i-antigens were confirmed by injecting rabbit and channel catfish (*Ictalurus punctatus***)** to produce immobilizing antisera. Subunit vaccines comprised of the purified i-antigens induced protective immunity in juvenile channel catfish when administered with Freund's adjuvant or a CpG oligodeoxynucleotide. Moreover, i-antigen subunit vaccines elicited immobilizing antibodies and conferred protection only against homologous parasites, which supports a model for immunity mediated by immobilizing antibodies. The i-antigen genes were introduced into a novel expression system, the free-living non-pathogenic ciliate *Tetrahymena thermophila.* The recombinant i-antigens were either targeted to the host cell surface in full-length form or secreted into the culture medium in a truncated form lacking the GPI-anchor addition site. The recombinant i-antigens had the same antigenicity and immunogenicity as i-antigens produced by the parasite. Live cells of *Tetrahymena* over-expressing *Ichthyophthirius* i-antigens were effective vaccines against

I. multifiliis. DNA vaccines containing the i-antigen genes elicited serum antibody response and conferred protective immunity against *I. multifiliis.* These results clearly indicate that the i-antigens of *I. multifiliis* are protective antigens and good candidates for the development of vaccines against *I. multifiliis*.

INDEX WORDS: *Ichthyophthirius multifiliis*, channel catfish (*Ictalurus punctatus*), immobilization antigen, immunization, subunit vaccine, recombinant vaccine, DNA vaccine, Freund's adjuvant, CpG oligodeoxynucleotide, *Tetrahymena thermophila*.

BIOCHEMICAL AND IMMUNOLOGICAL CHARACTERIZATION OF THE IMMOBILIZATION ANTIGENS OF *ICHTHYOPHTHIRIUS MULTIFILIIS*

by

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DEDICATION

This dissertation is dedicated to my beloved wife, parents, parents-in-law, whose love, support, and encouragement made this possible.

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CHAPTER 1

LITERATURE REVIEW

1. Biology of *Ichthyophthirius multifiliis*

The ciliate, *Ichthyophthirius multifiliis* (Fouquet), is one of the most pathogenic protozoan parasites of fish. It is well known as the etiological agent of Ichthyophthiriasis (Ich) or "white spot disease" and infects a wide range of freshwater fish. The disease is highly contagious and particularly severe when fish are crowded. Thus, it causes deaths not only in wild fish communities, but also results in significant economic losses in the commercial aquaculture of channel catfish, rainbow trout, carp, eels, tilapia and ornamental species (62). Morbidity in enclosed warm water systems with species such as channel catfish usually reaches 100%.

Epizootics of *I. multifiliis* in carp were described as early as the 10th century in China's Sung Dynasty (Cited by (54)). In 1876 Fouquet described this ciliate in detail and proposed the name *Ichthyophthirius multifiliis*. *I. multifiliis* is placed in the subkingdom *Protozoa*, phylum *Ciliophora*, class *Oligohymenophorea*, subclass *Hymenostomata*, order *Hymenostomatida*, suborder *Ophryoglenina*, family *Ichthyophthiriidae*, genus *Ichthyophthirius*.

I. multifiliis has been isolated in tropical, subtropical and temperate regions extending north to the Arctic circle (96), and appears to parasitize all freshwater fish and in rare cases, even amphibia, such as striped marshfrog (*Limnodynastes peronii*) (42).

Like other ciliates, *I. multifiliis* has distinguishing characteristics that include: nuclear dimorphism (a characteristic transcriptionally active horseshoe-shaped macronucleus and at least one transcriptional silent germline micronucleus), cilia, microtubular infraciliature located below the cell membrane, and a cytostome (31).

I. multifiliis is an obligate parasite with a life cycle that consists of a fish-associated feeding stage (trophont) and a free-living infectious stage (theront). Theronts, ~30 x 50 μm in size and pyriform to fusiform in shape with a tapered posterior end, swim actively in water up to 4 days until they find fish. Theronts respond chemotactically to components in fish mucus (10). After approaching the surface epithelium of the gills and skin, theronts penetrate within five minutes to the basal layer (23). During invasion, theronts secrete a layer of mucoid material, and enter the host by moving through the epidermal layers (40). After penetration, theronts transform into host-associated feeding trophonts. In response to infection, the epidermis of the host fish proliferates to produce the typical grey-white vesicular lesions, hence the common name of white spot disease. Trophonts feed until they have matured (up to 800 μm in diameter) or the host dies. The duration of feeding depends on water temperature, health of the host and the body region infected (38). At 22 ^{o}C, trophonts feed on channel catfish for about 4~7 days (21). The mature tomonts leave the skin, attach to a suitable substrate, secrete a cyst, and divide five to nine times to produce 200 to 1000 daughter cells, referred to as tomites. Tomites differentiate into theronts. Development takes approximately 18 to 24 hours at 22 ^{o}C. The theronts bore through the cyst wall and exit to complete the life cycle.

Infection by *I. multifiliis* is limited to the epidermal layers of the skin, gills, and buccal cavity. The parasites do not penetrate beyond the epithelial basal lamina (39). Low

or moderate infections cause mild histopathological changes. Heavy infections, however, induce host tissue necrosis and extensive epithelial hyperplasia. Fish die from loss of osmoregulatory balance and impaired gaseous exchange (54).

2. Prevention and control of *I. multifiliis*

I. multifiliis is difficult to control, especially under intensive aquaculture conditions. Although infective theronts are vulnerable to a number of chemicals, trophonts within the epithelium are protected from most agents added to water. A simple physical treatment is dilution or removal of the theronts from the water. The repeated transfer of fish to different aquaria is effective but is impractical for large-scale fish culture.

Chemotherapy is often used for control of the parasite. Current treatments include the use of formalin, malachite green, chloramine-T, and toltrazuril (45). These compounds are not always effective, and their use is facing more restrictive regulations in food fish species. These compounds kill only the free-swimming infective (theront) and replicative (tomont) stages, but are ineffective against the fish-associated (trophont) stage (31). Chemical treatments impose additional stress on infected fish, which increases mortality. Some of the most effective chemicals against the parasite are prohibited from use on food fish for human consumption. For example, malachite green is a confirmed teratogen that causes the development of abnormal structures in the embryo (93) and is not approved for fish consumed by humans.

Prophylactic immunization against *Ichthyophthrius* would be a practical alternative to chemical treatment, based on the fact that fish surviving infection develop immunity to subsequent parasite challenge (14, 24, 31, 52, 72).

3. Host immunity against *I. multifiliis*

3.1. Innate immunity

Surface mucus is the first line of the fish defense system. The mucus layer is not simply a physical barrier against invasion by the parasite, but a primary barrier containing humoral factors, such as protease, lectin, agglutin, and complement that inhibit parasite infection. Infection by *I. multifiliis* elicits epithelial cell proliferation and the skin becomes infiltrated with granulocytes, macrophages, and lymphocytes (25, 97). Studies on non-specific cellular responses indicated that, in channel catfish infected with *I. multifiliis*, nonspecific cytotoxic cells (NCC, the fish equivalents of mammalian natural killer cells) increase in the peripheral blood of moribund fish. NCC activity also increases as measured by lysis of mammalian tumor cells (37, 46, 47). NCC can lyse *Tetrahymena pyriformis in vitro* that have been either chemically deciliated or immobilized with specific anti-sera (46). There is no direct evidence, however, of an effect by NCC on live *I. multifiliis* theronts. Overall, as in other vertebrates, innate mechanisms in fishes play an important but poorly understood role in resistance to parasites.

3.2. Acquired immunity

While disease caused by *Ichthyophthirius* is usually lethal, fish that survive infection become resistant to subsequent challenge (75). The ability to develop acquired immunity against *I. multifiliis* has been demonstrated in various fish species, including: carp (4, 52, 53, 58), white catfish (5), rainbow trout (100), channel catfish (32), tilapia (92), and tropical ornamentals (72). Generally, fish that survive sub-lethal or controlled infection become resistant to subsequent challenge (11, 14, 15, 75). Complete protection is also induced by intraperitoneal injection of live theronts. Moreover, the surface

immobilization antigen of *I. multifiliis* elicits protective immunity in channel catfish when injected intraperitoneally with complete Freund adjuvant or CpG oligodeoxynucleotide (101, see chapter 2 and 3). Resistance is seen within 3-4 weeks and lasts for a period of at least 12 months (11).

3.2.1. Humoral antibody responses

Evidence for a humoral antibody response against *I. multifiliis* exists in several fish species, including carp (53), rainbow trout (100); and channel catfish (15). Sera from immune fish immobilize free-swimming tomonts of *I. multifiliis in vitro,* and strong correlation exists between antibody levels against *I. multifiliis* membrane antigens and the ability of sera to immobilize live parasites *in vitro* (14). The antigens responsible for immobilization have been identified (29) and are referred to as immobilization antigens, or i-antigens. Immunoflurescent microscopy reveals that these proteins are present on plasma and ciliary membranes. Serum and mucus antibodies from immune channel catfish recognize these proteins on Western blots. Furthermore, immuno-histological studies have determined that membrane antigens on cilia, plasma and cortical mucocysts are the major targets for antibodies from immune fish (24, 105).

Mucus from *I. multifiliis* immune fish contains antibodies that react with i-antigens on dot blot assays or ELISA (105). The origin of these antibodies, whether they are produced locally or transported from serum, is still unknown. A secretory form of antibody, structurally distinct from systemic antibody, was reported in a marine fish species (71). In channel catfish, serum and mucosal antibodies are structurely similar, although production of mucosal antibody appears to be elicited without a concurrent rise

in serum antibodies when fish are surface exposed to antigen suggesting a distinct mucosal antibody response (70).

3.2.2. Cellular responses

All vertebrate immune responses are mediated by leukocytes. Fish leukocytes are classified as lymphocytes, monocytes/macrophages, and granulocytes based on morphology, ultrastructure, and cytochemical staining (83). There is currently a paucity of cell surface markers available to identify functional sub-populations of fish leukocytes.

Fish lymphocytes equivalent to mammalian B cells are responsible for the antibody response to *Ichthyophthirius* and other parasitic ciliates. The existence of B cells has been directly demonstrated in many teleosts using mAb specific for fish Ig and by the identification of Ig heavy and light chain genes (76). Some studies showed fishes have the functional equivalents of T cell sub-populations (reviewed by (80)). A cell-mediated response against *I. multifiliis* was proposed based on immuno-suppression by corticosteroid by (58). Following administration of corticosteroids, immunized carp became fully susceptible to infection while serum antibody levels against the parasite remained high. In addition to suppressing cell-mediated immunity, corticosteroids also inhibit Ig production from B cells (73). A delayed-type hypersensitivity reaction against *Ichthyophthirius* was reported in goldfish (*Carassius auratus*) immunized by intraperitoneal injection of live theronts (88). Recently, two types of catfish alloantigen-dependent cytotoxic T cells were cloned from peripheral blood leukocyte (PBL) taken from a fish immunized *in vivo* and stimulated *in vitro* with the allogeneic B cell line. Studies to characterize their recognition and cytotoxic mechanisms found that catfish

cytotoxic T cells show heterogeneity with respect to target recognition and cytotoxic mechanisms (107).

3.3. Passive immunity

Hines and Spira (52) and Ling *et al*. (68) published reports stating that naïve fish held in the same aquaria with immune individuals are protected against *Ichthyophthirius* infection. They hypothesized that passive immunity is mediated through mucus from immune fish released into the water. Subasinghe (91, 92) found that tilapia immunized against *Ichthyophthirius* conferred a small degree of protection to fry.

Lin *et al*. (66) showed that following intraperitoneal injection of mouse mAb specific to i-antigens into naive fish, IgG class mAbs provide strong passive protection against lethal challenge. IgM class mAbs failed to protect. ELISA and *in vitro* immobilization assays showed that IgG class mAbs exist in both the serum and mucus of test fish, whereas, no passively transferred IgM class mAbs or fish Ig specific to i-antigens was detected in mucus. Passive immunization experiments using tetrameric (IgM-like) antibodies from the sera of *I. multifiliis* immune channel catfish did not protect against lethal challenge by *I. multifiliis*, although the transferred fish antibody remained at relatively high levels in the blood for up to one week (105). Also, no passive protection occurred with the transfer of leukocytes from immune channel catfish. Because passively transferred fish antibodies did not get to skin, the immobilizing antibodies in mucus must be produced locally.

4. Mechanisms of acquired immunity

A model of acquired immunity against *I. multifiliis* based on antibody was first proposed by Hines and Spira (52), who suggested that immobilization of theronts by

mucus antibodies in mucus blocks penetration of the parasite into the host. Other possible protective mechanisms based on antibodies include complement activation, and/or antibody-dependent cell-mediated events (review, (28)).

When Cross and Matthews (23) directly examined *I. multifiliis* on the tailfins of immune carp, they found that the parasite invaded into the epithelium, but within 2 hours ~80% of the parasites had disappeared. Clark *et al*. (19) found similar results in passive immunity studies using immobilizing mouse mAbs. The exit of *I. multifiliis* began within 3 hours of passive transfer of i-antigen-specific mouse mAbs and was completed by 12 hours. The response to mouse mAbs requires i-antigen cross-linking on the parasite surface. Fab_2 fragments have the same effect as intact antibody, while monovalent Fab fail to protect (the activity of Fab could be restored by subsequent i.p. injection of bivalent goat anti-mouse Ig) (19). Parasites that left the host had mAb on their surface and could divide to form new theronts. Based on results from these experiments, Clark and Dickerson have proposed a mechanism of humoral immunity that involves a novel effect of antibody on parasite behavior (13). They suggest that after penetrating the superficial mucus barrier of the skin where the antibody concentration is too low to cause immobilization, theronts are forced to exit fish prematurely, rather than being immobilized or killed in response to immobilizing antibody binding. The precise mechanism that could account for this premature exit is unclear, but a number of possibilities exist, including: (1) i-antigens cross-linking at the cell surface triggering a signal cascade that leads to a change in development state (e.g. trophont to tomite); (2) antibodies short-circuiting normal metabolic pathways leading to exit; or (3) antibody binding eliciting a chemotactic aviodance response. Where the cutaneous antibodies come

from is still unclear. Their source could be either lymphocytes present locally in the skin, or lymphoid tissues located outside the skin from which antibody is transported to the epithelium.

5. Immobilization antigens

As shown by Hines and Spira in 1974, sera from immune fish immobilize *Ichthyophthirius in vitro* (52). With the idea that the antigens responsible for immobilization play a role in acquired immunity, considerable effort has been spent in attempts to define the target antigens responsible for this phenomenon (14, 15, 29, 67). Using mouse monoclonal antibodies, these antigens have been identified as a class of highly abundant surface membrane proteins on *I. multifiliis* cells and cilia. These antigens are structurally related to the well-studied immobilization antigens of the free-living ciliates, *Paramecium* and *Tetrahymena* (9, 12, 18, 20, 21, 33, 36, 78, 79, 82, 89, 90). Binding of antibodies to these proteins cause cilia to clump and inhibits cell motility. When injected into rabbits, affinity-purified antigens elicit the production of immobilizing antisera (67).

Based on immobilization with specific antisera, five i-antigen serotypes, designated A-E, are presently known (28). The majority of these (10 of 12) fall into 3 groups (serotypes A, C and D). The epitopes responsible for immobilization are distinct for each serotype.

The antigenic determinants (or epitopes) responsible for immobilization are conformational based on the fact that most immobilizing monoclonal antibodies fail to react with the i-antigens on Western blots (67). Although the epitopes responsible for immobilization are unique to a given strain, the i-antigens of different serotypes react in

Western blots with heterologous antisera (30). Using a cDNA probe encoding the 48 kDa i-antigen of G1, Southern hybridization analysis of genomic DNA from G1, G1.1 and G2 isolates suggested that these i-antigens are products of a related gene family with approximately 85% homology at the nucleotide sequence level (30).

Xu (1995) found that *I. multifiliis* release soluble 48 and 60 kDa i-antigens (106). Following proteolytic cleavage with trypsin, *S. aureus* protease V8, and endoprotease Lys-C, peptide maps of the membrane-associated and soluble i-antigens are indistinguishable. Furthermore, migration patterns of the soluble and membrane-associated antigens are altered to the same extent in non-reducing versus reducing conditions on SDS-PAGE, and both forms are minimally glycosylated. Comparisons of peptide maps of the 48 and 60 kDa antigens suggest that the two proteins are themselves related. These results indicate that the membrane and soluble proteins represent the same antigens. The existence of membrane and soluble forms of surface antigens has been observed in other parasitic protoza, including *Trypanosoma* (7, 98).

To characterize the i-antigens in more detail, the gene encoding the 48 kDa antigen of *I. multifiliis* isolate G1 has been cloned (17, 20) and designated *IAG48[G1]*. This gene encodes a single, uninterrupted reading frame that predicts a protein precursor of 442 amino acids having a theoretical MW of 45,025 Da. The first 20 amino acids at the N- terminus of the predicted protein are mostly hydrophobic amino acids, which would suggest that this region specifies a signal peptide. Immediately downstream of the signal peptide, the deduced protein contains a sequence identical to the N- terminal amino acid sequence of the 48 kDa i-antigen protein as determined by Edman degradation. At the C-terminus of the predicted protein, there is a stretch of 14 mostly hydrophobic amino acids

separated by a short spacer from 3 small amino acids (CAS). This type of sequence is characteristic of a GPI-anchor addition site. A P-loop domain exists at position 316-323 with a sequence of G-[X_4]-GKS, which is found in a wide variety of proteins that bind ATP or GTP (85). The most striking feature of the predicted protein, however, is five homologous segments with an average of 80 amino acids. This type of repeated structure is characteristic of i-antigens of the related free-living ciliates, such as *Tetrahymena* and *Paramecium* (77, 94). The repeats are characterized by 6 invariant Cys residues that fall into register when the homologous segments are aligned. There are 14 motifs having the order, C-$X_{2,3}$-C, as well as 4 larger elements having the order, C-X_2-C-X_{20}-C-X_3-C-X_{20}-C-X_2-C. The C-$X_{2,3}$-C motif is common to a large and diverse family of proteins that bind zinc and other metal ions (6). Anomalous codon usage exists in *Ichthyophthirius*, where the conventional stop codons, UAG and UAA, encode glutamine instead. There are 23 UAA and 1 UAG interspersed throughout the 48kDa cDNA open reading frame. *Tetrahymena* and *Paramecium* have the same codon usage (12, 56). Interestingly, a search of the SWISSPROT database shows similarities between the 48-kDa i-antigen and several other protozoan membrane proteins, most notably the i-antigens of *Tetrahymena thermophila*, and the variant specific proteins (VSPs) of *Giardia lamblia*. A striking feature of all these proteins is the presence of numerous C-$X_{2,3}$-C motifs embedded within higher order repeats. A comparison of the predicted sequence of the 48-kDa i-antigen with the *Giardia* VSP, A6-S1, reveals a high degree of similarity in the overall spacing of these motifs, with 29 of a possible 30 cysteine residues in the *I. multifiliis* protein overlapping with the identical amino acid in the VSP gene product (74). The association of C-$X_{2,3}$-C motifs with zinc-finger proteins (6, 86), as well as the ability of

the *Giardia* VSPs to bind zinc *in vitro* suggests that the 48-kDa i-antigen may have metal binding properties as well. The role of metal ions in the majority of zinc-binding proteins is structural. Tetrahedral coordination of zinc by cysteine, or some combination of cysteine and histidine residues, results in stabilization of short protein folds ("fingers", or "knuckles") that act as functional domains in protein-protein or protein-DNA interactions (6, 86). Although *Ichthyophthirius* and *Giardia* are widely diverged from an evolutionary standpoint, a possible structure/function relationship between the i-antigens and VSPs is intriguing, given the fact that they are abundant surface membrane proteins on highly motile protozoan cells.

In addition to the sequence for the 48-kDa protein, genes that encode two ~52-kDa i-antigen variants of the G5 have been recently cloned and designated *IAG52A[G5]* and *IAG52B[G5]*. These two genes encode proteins of 468 and 460 amino acids respectively, and have the same overall structure as the 48-kDa i-antigen. Like the 48-kDa protein, the ~52-kDa variants have hydrophobic signal peptides at their ends, and a series of tandem repeats with marked cysteine periodicity. The presence of hydrophobic domains at the N- and C-termini are consistent with the i-antigens being GPI-anchored proteins. The tandem repeats are a characteristic feature of all i-antigens including those of *Paramecium* and *Tetrahymena* (33, 79). Along with the signal peptides, there are several regions of primary sequence that appear to be highly conserved between the 48- and ~52-kDa gene products, most notably, a stretch of 7 amino acids (KKLTSGA) that is present near the C-termini of all three.

The N-terminal sequence predicted by *IAG52A[G5]* differs markedly from the actual sequence obtained by Edman degradation from i-antigens purified from the G5

isolate by affinity chromatography with immobilizing mAbs. The *IAG52A[G5]* gene specifies a protein with the same N-terminal amino acid sequence obtained by Edman degradation from affinity purified G5 i-antigens. These results suggest that more than one i-antigen is present in the G5 strain despite the fact that only one protein of ~52-kDa is seen in Western blots of total protein fractionated by one-dimensional SDS-PAGE. Interestingly, the proteins predicted by the two G5 i-antigen genes are quite different in terms of their primary sequence, although both appear to be recognized by the same immobilizing monoclonal antibody (namely, G3-61) and rabbit antibody against purified G5 i-antigens. The fact that the N-terminal sequence predicted by *IAG52A[G5]* gene did not show up following Edman degradation would suggest that this gene is expressed at low levels (or not at all).

6. Development of vaccines against *I. multifiliis*

Aquaculture has grown into an important industry, and infectious diseases pose a constant and costly threat to fish farms. Antibiotics provide a means to control bacterial diseases but for parasitic diseases, chemotherapy is not as effective, because of their more complex life cycles. Chemicals also induce severe stress in infected fish, and some of the most effective chemicals against parasites are prohibited from use on food fish.

The first commercial fish vaccine was licensed in 1976 for preventing enteric redmouth (ERM) disease, caused by the gram-negative bacterium *Yersinia ruckeri* (99). Since then, vaccination has become an important method for prevention of infectious diseases in farmed fish. Most commercial vaccines are inactivated vaccines administered by injection or immersion. Bacterial infections caused by Gram-negative bacteria, such as *Vibrio* sp., *Aeromonas* sp., and *Yersinia* sp., have been effectively controlled by

vaccination. With furunculosis, the success is attributed to the use of injectable vaccines containing adjuvants. Vaccines against virus infections, including infectious pancreatic necrosis, have also been used in commercial fish farming. Vaccines against several other bacterial and viral infections have been studied and found to be technically feasible (48).

Prophylactic immunization against *I. multifiliis* is possible because fish surviving infection become resistant to subsequent parasite challenge. Attempts to immunize fish with various preparations of whole parasites have been made. With advances in recombinant DNA technology and the understanding of molecules involved in protective immunity, a new generation of *I. multifiliis* vaccines built on immunogenic molecules are being developed, along with the exploration on new adjuvants and vaccine delivery methods. A summary of the development of *I. multifiliis* vaccines is given below:

6.1. Whole cell vaccines (killed versus live)

Parker (1965) reported that goldfish injected with killed (freeze-thawed) tomites were protected when challenged with lethal dose of live parasites (75). Similarly, Areerat (1974) found protection following injection of formalin-fixed trophonts (3). However, Burkart *et al.* (1990) were unsuccessful in their attempts to vaccinate channel catfish by i.p. injection with either killed cells or cilia, and their study demonstrated that live vaccines were much more effective than killed preparations when compared under the same experimental conditions. In that study immune protection lasted for 13 months (11).

Other studies also found that live parasite conferred protection. Fish vaccinated by infection with sublethal doses of parasites (52, 69, 75) or with potentially lethal doses followed by treatment (5, 14, 57, 65, 72), were protected against lethal challenge. Furthermore, Dickerson *et al.* (1985) found that: following i.p. inoculation, theronts

establish infection in the peritoneum where they grow for a period of about 21 days until they become surrounded by granulomatous tissue and die. An immune response is elicited that is capable of completely blocking surface challenge (32).

Using live parasites as vaccines, cross-protective immunity against heterologous challenge has been demonstrated. Channel catfish immunized by sublethal infection with *I. multifiliis* isolate G1.1 (serotype B) or G2 (serotype C) had cross-protective immunity against heterologous challenge (65). Similarly, catfish immunized with either G3 (serotype D) or G4 (serotype C) were completely protected from lethal challenge with a heterologous isolate (60). Sera from immune fish had high immobilization antibody titers (>1:2000) against the homologous isolate, but did not immobilize heterologous isolates. Furthermore, sera tested by *in vitro* immobilization assay did not show seroconversion to heterologous isolates at 30-60 days post-challenge. These results suggest that: although immobilization elicited by i-antigens is serotype specific, the protection against other serotypes is not. This cross protection might be attributed to either conserved, non-immobilizing epitopes on i-antigen proteins of different serotypes, or other protective antigens.

Although vaccination trials from different laboratories using different fish species indicated that live cells of *I. multifiliis* elicit protective immunity, it is impractical to use live vaccines because *I. multifiliis* is an obligate parasite and difficult to collect it in large quantities from infected fish. There is also the danger of outbreaks in populations exposed to the live pathogen itself.

Goven *et al.* (1980, 1981) published findings on protection against *I. multifiliis* following immunization of channel catfish with cilia from the free-living protist

Tetrahymena pyriformis (43, 44). Wolf and Markiw (1982) were able to demonstrate similar protection against *I. multifiliis* (as well as *Ichthyobodo necatrix*) in rainbow trout by vaccination with live *Tetrahymena thermophila* (104). Ling *et al*. (1993) found that goldfish immunized with *Tetrahymena pyriformis* were protected against *Ichthyophthirius* as well as a number of other ciliated parasites, including *Chilodonella cyprini* and *Triehodina* spp. (69). However, other studies conducted by Dickerson *et al.* (1984) (27), Burkart *et al.* (1990) (11), and Houghton *et al.* (1992) (57) using the same strategies were not able to achieve the same positive results. Dickerson *et al.* (1984) found that while immunization with *Tetrahymena* cilia conferred at most a decreased "days to death", protection against lethal challenge was not afforded (27). Furthermore, no evidence was found for cross-reacting surface antigens between *T. pyriformis* and *I. multifiliis* using immobilization assays, ELISA, and Western blotting (14, 67).

6.2. Subunit vaccine

Experimental evidence supports the hypothesis that i-antigens play an important role in eliciting protective immunity. First, fish produce serum antibodies against i-antigens (14, 15, 18, 23, 24, 67). Second, both Western blotting data, and *in vitro* immobilization assays with fish mucus indicate that antibodies against these proteins are present at the site of infection (that is, at the surface of fish) (105). Finally and most importantly, i-antigen specific mouse mAbs provide virtually complete passive protection against infection (18, 19, 66).

The immobilization antigens from *I. multifiliis* isolates G1 and G3 have been purified by affinity chromatography using immobilizing mAbs against *I. multifiliis* as

ligands (66). In this study, i-antigens themselves are shown to elicit protective immunity when injected into channel catfish (see chapter 2 and 3).

6.3. Recombinant vaccine

As mentioned above, *I. multifiliis* is an obligate parasite, which precludes producing sufficient antigens for large-scale vaccine production directly from the parasite. Thus, it is necessary to produce recombinant antigens in other organisms.

One of the main obstacles in producing *I. multifiliis* i-antigen in heterologous organisms, however, is its anomalous codon usage. In *Ichthyophthirius*, the conventional stop codons, UAG and UAA, encode glutamine. Thus, the base-triplets UAA and UAG encoding this amino acid in *Ichthyophthirius* terminate translation in other organisms commonly used for expressing foreign genes. Nevertheless, after modifying these codons, the gene encoding the 48-kDa i-antigen of *I. multifiliis* isolate G1, *IAG48[G1]*, was expressed in *E. coli* (87). Using PCR-based mutagenesis, the first 8 TAA and TAG stop codons within the first 414 bp of the 1.2 kb cDNA for 48-kDa i-antigen were changed to glutamine codons. The modified cDNA fragment was subcloned into an expression plasmid and transformed into *E. coli*. A 23kDa protein was detected on Western blot using immobilizing rabbit anti-serum against G1 isolate. The immunogenicity of the recombinant protein was not tested for the ability to elicit protection.

He *et al* (1997) expressed a synthesized gene fragment of *I. multifiliis* i-antigen in *E. coli* (50). Based on the gene sequence of *IAG48[G1]*, a 316 bp gene fragment containing a potential antigenic epitope of the 48 kDa immobilization antigen of *I. multifiliis* was synthesized. The gene construct was introduced into *E. coli* and expressed as a fusion protein, glutathione S-transferase-iAgI (GST-iAgI). Antisera from catfish

immunized with this fusion protein reacted with an *I. multifiliis* protein of about 48kDa on Western blot. A group of 22 goldfish were immunized by i.p. injection of 15 μg of this fusion protein with CFA and boosted by same dosage without adjuvant. One week after boosting, fish were challenged with 5000 theronts/fish. The immunized group had 95% survival, but the control group (injected with PBS and CFA) had 55% survival. This result is questionable, however, due to the small sample size and low mortality in the control group. For a vaccination trial with the mortality in control group less than 40%, the group size should be over 40 in order to see a statistical significant difference ($p<0.05$) (59).

Tetrahymena, a free-living ciliated protozoan, has great potential for developing a vaccine against *I. multifiliis*. *T. thermophila* is non-pathogenic and is generally regarded as an environmentally safe organism (103). *Tetrahymena* cell lines can be grown in large volume cultures, reach a remarkably high density of $> 5 \times 10^6$ cells per milliliter in a relatively short time (generation time 1.4 hours), and tolerate wide ranges of tonicity and temperatures (41). The *Tetrahymena* expression system offers distinct advantage over *E. coli* or other conventional expression systems for expressing *Ichthyophthirius* genes. First, *Tetrahymena* has the same anomalous codon usage (UAA and UAG encoding glutamine) as *Ichthyophthirius*. Second, faithful protein targeting and post-translational modification are more likely to occur since *Tetrahymena* is a ciliate in the same order, *Hymenostomatida*, with *Ichthyophthirius.* Finally, *Tetrahymena* confers a degree of non-specific immunity against *I. multifiliis* infection (27). Thus, expression of an *I. multifiliis* gene in *T. thermophila* should not only allow the production of large amounts of antigen at relatively low cost, but also allow the use of *T. thermophila* as a live vaccine.

Although using *Tetrahymena* as an expression system has advantages mentioned above, methods that allow reliable expression of foreign genes have been lacking until recently. Using a mutant strain of *T. thermophila* carrying a negative selectable allele of a β-tubulin gene, foreign genes can be directed to this locus by homologous recombination. The gene encoding the 48-kDa i-antigen of *I. multifiliis* serotype A has been introduced into the *BTU1* locus of *T. thermophila* (41). The coding sequence *IAG48[G1]* of the 48 kDa G1 i-antigen was subcloned between the flanking sequences of *BTU1*, and the result *btu1-4::IAG48[G1]* fragment was also introduced into *Tetrahymena* by biolistic bombardment. The stable transformants containing *IAG48[G1]* at the *BTU1* locus were selected by growth in taxol. When the transformed cell was analyzed by Western blot using rabbit polyclonal antiserum against affinity purified 48 kDa G1 i-antigen, a ~50 kDa protein was detected. The expression level of i-antigen is 260 pg/ug of total cell protein. Indirect immunoflurescent microscopy showed the i-antigen was expressed on the surface of *Tetrahymena*.

In addition to the expression of the full-length i-antigen on the cell surface of *T. thermophila*, a secretory form of recombinant i-antigen was produced by *T. thermophila* transformed with the gene *C19/IAG48[G1]* (16). The *C19/IAG48[G1]* (derived from the *IAG48[G1]*) lacked the coding region for the putative GPI-anchor addition site at the C-terminus. Elimination of the coding region for the C-terminal GPI-anchor addition site results in constitutive secretion of the recombinant protein rather than membrane retention (35). Obviously, to target the recombinant 48-kDa i-antigen to the exterior of cells would facilitate purification of recombinant protein. Furthermore, transformants

that constitutively secrete i-antigens could potentially be used as live vaccine for bath immunization of fish.

The expression level of the recombinant i-antigen has been increased by using an inducible promoter from the metallothionein gene of *T. thermophila* (MTT) (Yan Gao and Jacek Gaertig, unpublished). The promoter of MTT contains multiple metal regulatory elements, which can be induced by heavy metals, such as cadmium. Protein expression under the control of MTT promoter can be significantly increased when the cadmium concentration in the medium is elevated. In *T. pigmentosa*, MT-mRNA level increased more than 40-fold within 30 minutes of cadmium treatment (84).

The evaluation of a recombinant vaccine against *I. multifiliis* has achieved very encouraging results (Chapter 4).

6.4. DNA vaccines

Since their inception in 1992, DNA vaccines have gained wide attention for the control of infectious diseases (34, 95). They have been shown to be effective against a wide range of pathogens, particularly when immunogenic proteins have been identified for the pathogen in question. In this approach, the gene(s) coding for the protective protein antigens are cloned in appropriate plasmid vectors under strong promoters and injected into the host. The injected plasmid DNA is then taken up and expressed by antigen-presenting cells; the result is the elicitation of an immune response against the expressed foreign antigen.

All DNA vaccine vectors have 5 features in common: a bacterial origin of replication that allows amplification of plasmid; a prokaryotic selectable marker gene; the sequence encoding an antigenic protein or polypeptide; eukaryotic transcription

regulatory elements (strong viral promoter/enhancer) to direct high levels of gene expression in a wide host range; and a poly(A) sequence to ensure appropriate termination of the expressed mRNA (26).

DNA vaccination has three advantages over conventional technologies. First, the manufacturing technology (plasmid DNA preparation) is very simple and fit for all vaccines. Second, DNA vaccines contain CpG motifs which act as an adjuvant to enhance immune response. Finally, DNA vaccines induce a mixed Th_1 and Th_2 response, although a predominately Th_1 response is reported more often (26).

DNA vaccines have great potential in the field of aquaculture. The first DNA vaccine in fish was reported by Anderson *et al*. (1), who showed that rainbow trout fry injected with the glycoprotein gene of infectious haematopoietic necrosis virus (IHNV) were more resistence to subsequent challenge with the virus. Heppell *et al*. (51) demonstrated that rainbow trout fry injected with plasmids containing the glycoprotein gene of viral haematopoietic septicaemia virus (VHSV) alone, or together with plasmids containing the nucleocapsid protein gene of this virus, were strongly protected against challenge with the virus. Boudinot *et al*. (8) showed that combined DNA immunization with the glycoprotein gene of viral hemorrhagic septicemia virus and infectious hematopoietic necrosis virus induces specific protective immunity and nonspecific response in rainbow trout. Studies also showed strong expression of reporter genes in muscle cells of fish injected with plasmid DNA (2, 49, 81). These results suggest that DNA vaccine should be as successful for fish as they are for other animals.

The i-antigens of *I. multifiliis* are highly immunogenic and there is substantial evidence for their role in protection. Genes encoding i-antigen variants from two

serotypes of *I. multifiliis* have been cloned and characterized. All of these previous efforts made possible the development of an i-antigen-based DNA vaccine against *I. multifiliis*. The i-antigen gene from *I. multifiliis* G5 isolate, *IAG52A[G5]*, was modified to replace its ciliate-specific codons (UAA and UAG encoding glutamine instead of stop codons) with channel catfish preferred codons, CAA and CAG, respectively. Additionally, two truncated i-antigen genes were obtained by deleting the coding sequence for either the N-terminal signal peptide or C-terminal GPI addition site. These three version of i-antigen genes were cloned into the eukaryotic expression vector pcDNA3.1 and evaluated in channel catfish (Chapter 6).

6.5. Methods of vaccine delivery

The development of vaccination methods is based on an understanding of basic immunological principles and their application. The currently available methods for administrating fish vaccines include injection, immersion, and oral administration (99).

Intraperitoneal injection is the most effective way to induce a high degree of protective immunity, and is widely used throughout the salmon industry (55). However, injection causes unaviodable stress to fish, and mass vaccination by injection is time and labor intensive.

Immersion vaccination is widely practiced with very small fish because it is easy and cost effective. In this method and its variations (including spray, hyperosmotic dip, and flush exposure), fish are dipped directly into a solution that contains immunogens. A disadvantage of this method is the requirement for considerable amount of a vaccine to obtain the appropriate response.

Oral administration is the ideal method for immunization of farmed fishes. Oral vaccines are mixed with food either by top dressing or by incorporating into food. This method does not stress fish, and does not require excessive time and labor. Furthermore, oral delivery induces mucosal immunity, which protects against microorganisms that infect through mucosal surfaces. However, this delivery method may not exclude the possibility of oral tolerance, which has been indicated in carp, trout and salmon when fish were fed repeatedly with protein antigens (80). Also, new techniques to protect protein antigens from proteolysis in fish gut and to increase antigen uptake in circulation are required for oral administration. Lavelle (64) orally immunized rainbow trout with antigen encapsulated in poly(DL-lactide-co-glycolide) microparticles. The result showed the encapsulated antigen was partially protected against proteolysis and the serum antibody level increased.

In this study, the recombinant vaccine against *I. multifiliis* was administrated to channel catfish by injection and immersion, and the serum antibody response and protection was studied (see chapter 4).

6.6. New adjuvants

Vaccines have become an important tool in the prevention of fish diseases in aquaculture. While many immunogens are effective alone, particularly those (such as LPS) that elicit innate immunity by themselves, most protein antigens require the use of adjuvants. There is no universal adjuvant, and the action of a given adjuvant relies on different mechanisms. Some adjuvants are thought to act by creating depots at the site of inoculation from where the antigen is slowly released and presented to the immune system over a prolonged period of time. Other adjuvants (such as CpG ODN) are non-

specific immune activators that can be used to augment immune responses in an antigen-specific fashion when included in vaccines. Many adjuvants have both these properties, such as Freund's complete adjuvant, in which the mineral oil acts as an antigen depot and the mycobacterial cell wall components act as non-specific immune activators. In addition to augmentation of qualitative aspects of the immune response (i.e. strength and kinetics), adjuvants also play a role in determining the type of immune response generated. Development of the appropriate type of immune response is essential for successful immunization. Strong cell-mediated immunity associated with a Th_1-type immune response is thought to be essential for the control of intracellular pathogens whereas strong humoral immunity, which can be found with both Th_1 and Th_2 type immune responses, appears to be essential for the control of extracellular pathogens (102).

Freund's complete adjuvant (FCA) has been an important model adjuvant commonly used in animal research as it can augment both humoral and cellular immune responses to a wide range of antigens. However, its side effects in fish include fecundity problems, growth reduction, and melanisation with adhesions in the peritoneal cavity causing downgrading at the time of harvest. Thus, the discovery of alternative adjuvants is currently an important area of vaccine research.

Immunostimulatory oligodeoxynucleotides containing the dinucleotide CpG motif (CpG ODNs) have promise as adjuvants for both DNA and protein vaccines. In mammalian systems, CpG ODN stimulate cytokine production by cells of the innate immune system, and act as potent B-cell mitogens when used at high concentration *in vitro* (63). *In vivo*, CpG ODNs at low concentration induce strong antibody responses against co-administered antigens. In goldfish, CpG ODNs have recently been shown to

stimulate antibody production against a model antigen (β-galactosidase) suggesting that the receptors for bacterial DNA are evolutionarily conserved (61). The low toxicity of CpG ODNs relative to Freund's (102) makes them an attractive choice as potential adjuvants in fish vaccines.

CpG oligodeoxynucleotides were compared with complete Freund's adjuvant for the ability to stimulate humoral responses to i-antigen, and to elicit protective immunity. The results showed that a subunit i-antigen vaccine administered with CpG ODN conferred comparable immunity to vaccine administered with Freund's adjuvant (see Chapter 5).

7. Summary

The parasitic ciliate *Ichthyophthirius multifiliis* offers a useful system for the study of cutaneous immunity against an infectious microorganism. Naive fish usually die following infection, but animals surviving sublethal parasite exposure become resistant to subsequent challenge. This resistance correlates with the presence of humoral antibodies in the sera and cutaneous mucus of immune fish. A mechanism of immunity has been elucidated in which antibody- mediated cross-linking of surface proteins (referred to as immobilization antigens or i- antigens) triggers a response by the parasite resulting in its exit from the host. Several i-antigens have been purified and characterized, and their genes cloned. This has made it possible to prove directly the important role that i-antigens play in protective immunity, and to construct and produce vaccine-candidates based on these antigenic molecules. The successful expression of *I. multifiliis* i-antigen genes in the non-pathogenic, free-living ciliate *T. thermophila* provides a promising method for future prophylactic immunization against *I. multifiliis*.

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CHAPTER 2

SURFACE IMMOBILIZATION ANTIGEN OF THE PARASITIC CILIATE *ICHTHYOPHTHIRIUS MULTIFILIIS* ELICITS PROTECTIVE IMMUNITY IN CHANNEL CATFISH (*ICTALURUS PUNCTATUS*) [1]

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ABSTRACT

Channel catfish (*Ictalurus punctatus*) that survive infection with the parasitic ciliate *Ichthyophthirius multifiliis* acquire immunity to subsequent challenge and produce specific serum antibodies that immobilize the parasite *in vitro*. Cellular surface protein antigens targeted by these antibodies are referred to as immobilization antigens (i-antigens). Using immobilizing mouse monoclonal antibody as ligand, the i-antigen of *I. multifiliis* isolate G5 was purified to homogeneity by immunoaffinity chromatography, and its immunogenicity confirmed by inoculating rabbit and channel catfish to produce immobilizing antisera. To test the purified i-antigen as a subunit vaccine, channel catfish fingerlings were injected intraperitoneally (ip) with purified i-antigen at a dose of 10 μg/fish in complete Freund's adjuvant on day 1, followed by a second ip injection of the same amount of i-antigen in incomplete Freund's adjuvant on day 15. Negative control fish were immunized similarly with either BSA, or an immobilization-irrelevant *I. multifiliis* protein. On day 84, fish were challenged with *I. multifiliis* G5 live theronts at a dose of 15,000 cells per fish. Seventy-two percent of fish immunized with i-antigen survived challenge. All negative control fish died within 16 days of exposure. There was a significant difference in the median days to death between the negative control fish injected with BSA and the fish that died following vaccination with i-antigen. Fish injected with i-antigen developed high immobilizing antibody titers in serum. This is the first demonstration of a direct role for i-antigens in eliciting protective immunity

suggesting that these proteins by themselves serve as effective subunit vaccines against *I. multifiliis.*

Keywords: *Ichthyophthirius multifiliis,* channel catfish (*Ictalurus punctatus*), immobilization antigen, immunization, subunit vaccine.

INTRODUCTION

The obligate parasitic ciliate *Ichthyophthirius multifiliis* is one of the most common and destructive protozoan pathogens of freshwater fish. The free-swimming, highly motile infective theront penetrates into the epithelia of the skin and gills where it transforms into a large (500 μm) feeding trophont. After a period of growth it leaves the host and replicates within a protective cyst in the aqueous environment. Although the disease (commonly referred to as "Ich" or "white spot disease") is usually fatal, fish that survive infection develop immunity to subsequent parasite challenge (3, 10, 13, 17, 22). Our laboratory is focused on elucidating the mechanisms of this protective immune response.

The first observation that sera from immune fish immobilize the parasite *in vitro* was reported in 1974 (17), where it was postulated that this effect corresponds to protection *in vivo.* It was subsequently found that antibody binding to parasite cell and ciliary surface antigens causes immobilization (3, 4). The target antigens of immobilization have been purified by immunoaffinity chromatography (20) and characterized as a class of highly abundant, glycosyl-phosphytidyl-inositol (GPI)-anchored, surface membrane proteins (5). These proteins (referred to as immobilization antigens, or i-antigens) are analogous in structure to the surface antigens found on the free-living ciliates *Paramecium* and *Tetrahymena* (2, 24). To date, ten different *I. multifiliis* isolates have been classified into five immobilization serotypes (A-E) based on *in vitro* immobilization (11).

Experimental evidence supports the hypothesis that immobilizing antibodies play a role in protective immunity. Channel catfish passively immunized by intraperitoneal

(ip) injection of immobilizing mouse monoclonal antibodies (mAbs) are protected against subsequent lethal challenge (19). Furthermore, parasites colonized in the epithelia of naive fish are induced to leave following the injection of i-antigen-specific mAbs or Fab_2 fragments. This response requires cross-linking of surface i-antigen by bivalent antibody at sub-immobilizing concentrations (7). Mouse IgG antibodies reach surface epithelia of fish within 12 hours of intravenous (iv) or ip injection. Immobilizing mouse IgM antibodies or fish serum antibodies (tetrameric 750-kDa IgM-like molecules), however, are not found in surface mucus of fish following passive transfer. Presumably this is due to their large molecular mass, which precludes transport to the skin. Nevertheless, specific immobilizing antibodies have been detected in the skin of actively immunized fish and these are postulated to protect by the same mechanisms as those of passively administered mouse antibodies (26).

An important goal of *I. multifiliis* research is the development of an effective and practical vaccine to protect fish from infection. Fish have been successfully immunized in the laboratory by intraperitoneal injection of live theronts (1), or by surface exposure followed with treatment (4). Parasites introduced into the peritoneal cavity establish infection and grow for about 21 days before they become surrounded by granulomatous tissue and die (15). Interestingly, intraperitoneal infection elicits an immune response that effectively blocks surface challenge. While live parasites elicit protection under controlled circumstances, such vaccines are not practical for large-scale field use because *I. multifiliis* is an obligate parasite and difficult to grow in large quantities. Also, the danger of inadvertent outbreaks exists if live parasites are used for vaccination. For these reasons we have investigated the use of purified i-antigen as a subunit vaccine. While

passive immunization experiments have suggested that the i-antigens of *I. multifiliis* play an important role in immunity, direct evidence that these proteins by themselves stimulate protective immunity had not been established until now. Here we demonstrate that the vaccination of channel catfish with affinity-purified i-antigens elicits protective immunity against a potentially lethal parasite challenge.

MATERIALS AND METHODS

Propagation and collection of parasites. The *Ichthyophthirius multifiliis* G5 isolate used in this study has been characterized previously (5, 19), and is currently maintained by serial passage on juvenile channel catfish (*Ictalurus punctatus*) (23). Live theronts were obtained using previously described methods (12). Five heavily infected fish with visible parasites over the entire body surface were placed in a beaker containing 3 L carbon-filtered water, and trophonts were dislodged from fish skin. Parasites were collected by a 200-mesh sieve, transferred into 100 mL carbon-filtered water, and allowed to develop into theronts (18–20 h at 22 °C). Theronts were passed through a 400-mesh sieve, harvested by centrifugation at 1000 *g* for 2 minutes, and washed once in 50 ml carbon-filtered water. Cells were used immediately or pellets were frozen in liquid nitrogen and stored at –70 °C.

Purification of protein antigens from *I. multifiliis*. Two mouse monoclonal antibodies, mAbs G3-61 and G3-74, were used for the preparation of immunoaffinity columns and the subsequent purification of *I. multifiliis* protein antigens. These mAbs (IgG1) were generated against the *I. multifiliis* G3 isolate (7, 19). MAb G3-61 specifically immobilizes the *I. multifiliis* G3 isolate as well as the G5 isolate, and was used to purify i-

antigen from *I. multifiliis* G5 in this study. G3-74 is a non-immobilizing antibody that was used to purify a 14-kDa immobilization-irrelevant protein from *I. multifiliis G5*.

Immunoaffinity chromatography was performed as described previously (20) with modifications. Briefly, 10 mg of mAb purified on a protein A agarose column (Boehringer Mannheim, Mannheim, Germany) was coupled to a 2 mL AminoLink^{TM} column (Pierce, Rockford, IL, USA) following the manufacturers' protocols. Parasite membrane protein was solubilized in 1% (v/v) Triton X-114 (Sigma, MO, USA) in 10 mM pH 7.5 Tris-HCl buffer and isolated by phase separation at 30^0 C as described previously (12). Five mL of parasite membrane protein (2-5 mg/mL in 10 mM, pH 7.5 Tris-HCl buffer) was applied to the affinity columns. Unbound proteins were washed off the columns with 20 mL 10 mM pH 7.5 Tris-HCl buffer. Antigens were eluted from the affinity columns using 10 mM pH3.0 glycine buffer, neutralized with 1 M pH 7.5 Tris-HCl buffer, and their protein concentration determined by either absorbance at 280 nm or by the BCA method (Pierce, Rockford, IL, USA).

SDS-PAGE and Western blotting. One-dimensional SDS-PAGE and Western blotting were done using standard protocols (20). A mouse monoclonal antibody against channel catfish Ig heavy chain (MAF13) was provided by Dr. Norman W. Miller, Department of Microbiology, University of Mississippi Medical Center, Jackson, MS. The conjugate of this mAb and alkaline phosphatase was prepared and used as a secondary antibody in Western blotting and ELISA.

MALDI-TOF. To determine the molecular mass of the purified G5 i–antigen, matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry was performed on a Hewlett-Packard G23051 MALDI-TOF mass

spectrometer located at the Complex Carbohydrate Research Center, University of Georgia. The analytic parameters used were: laser energy 7.00 (0.07) µJ, vacuum 1.26e-006 torr, polarity positive, and matrix Sinapanic acid.

Experimental fish. Channel catfish (*Ictalurus punctatus*) fingerlings (10-15 g) with no previous history of exposure to *I. multifiliis* were obtained from a local hatchery. Prior to immunization, fish were treated with formalin (25 ppm) to remove any possible external parasites and maintained in a flow-through system. Fish were fed daily with commercial trout starter feed (Purina, USA). Water temperature was maintained at 20-23 ^{0}C, and water quality (NH_3, NO_2, and pH) monitored daily with standard test kits.

Production of immobilizing antisera. Antisera were prepared in rabbits and channel catfish. Animals were bled before immunization to test for background immobilizing activity. Two adult female New Zealand White SPF rabbits were each injected intradermally with 100 µg of purified i-antigen in complete Freund's adjuvant (CFA), and boosted 3 weeks later with intradermal injection of 100 µg i-antigen in incomplete Freund's adjuvant (IFA). Blood was collected by cardiocentesis 3 weeks after the second injection. Ten channel catfish weighing 15-20 g each were injected twice intraperitoneally with 10 µg of purified i-antigen in CFA and IFA respectively at a 2-week interval, and bled from the caudal vein/arteries 4 weeks after the second injection. Sera from rabbit and channel catfish blood were heat inactivated at 56 ^{0}C for 30 minutes and stored at –80 ^{0}C.

Vaccination procedure. Channel catfish were distributed into eight 38-liter aerated and conditioned aquaria at a density of 35 fish per aquarium. The vaccine trial consisted of four groups, each consisting of 70 randomly assorted fish (two aquaria per

group). Fish were immunized by intraperitoneal injection of protein or live theronts using a 1 mL syringe fitted with a 26 gauge needle. Fish in group 1 were immunized on day 1 with 10 µg of i-antigen in CFA, and on day 14 with 10 µg of i-antigen in IFA. Fish in group 2 were immunized on day 1 with 10 µg bovine serum albumin (BSA) in CFA, and on day 14 with 10 µg of BSA in IFA. Fish in group 3 were immunized on day 1 with 10 µg of 14-kDa protein, and on day 14 with 10 µg of 14-kDa protein in IFA. Fish in group 4 were immunized on day 1 with 8000 live theronts without adjuvant and on day 35 with 10,000 live theronts without adjuvant.

Challenge. A small-scale experiment was carried out to determine the proper number of theronts for challenge. Small subgroups (ranging from 2 to 7 fish each) from each treatment group were challenged with three different doses of theronts (5000, 10,000, or 15,000) on day 28 following a standard protocol (14). In the large-scale trial the remaining fish in the four treatment groups were exposed to 15,000 theronts/fish on day 84. Aquaria were equipped with biological filtration, and water quality and temperature were monitored daily. Mortalities were recorded daily in each group until all fish died or recovered from infection.

Collection of catfish cutaneous mucus and serum samples. Channel catfish were anesthetized with 100-200 ppm MS-222 (tricaine methane sulfonate, Argent Chemical Laboratories, Redmond, WA) and gently wiped over both lateral surfaces with cotton swabs. Each mucus-saturated swab was soaked in 0.1 mL of ice-cold phosphate buffered saline (PBS; 135 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.5) and then squeezed out the solution. The PBS-mucus solution was centrifuged at 14,000 x g for 5 min at 4^0 C and the supernatant was collected as the mucus sample.

Mucus samples were normalized to a protein concentration of 100 µg/mL, supplemented with 1% BSA, and stored at -80^0 C until use. Blood sample was taken from anesthetized fish immediately after mucus sample collection. Bleeding, preparation, and heat inactivation of sera were carried out as described previously (4).

***In vitro* Immobilization assays.** Assays were carried out to determine serum immobilizing antibody level as previously described (4) with minor modification. Briefly, serum samples were prepared in 96-well microtitre plates as a series of doubling dilutions in 50 % PBS. Each well contained 50 µL diluted serum. Approximately 100~200 live theronts in 50 µL of carbon-filtered water were added to each well and incubated at room temperature (RT, 20-22 ^{0}C). Immobilization was determined by observation under a dissection microscope, and titers were expressed as the inverse of the highest dilution in which all of theronts were immobilized after incubation for 30 minutes at RT. Pre-immune serum samples were used as negative controls.

Detection of mucus antibodies by ELISA. To absorb antigen to plates, individual wells of ELISA plates (Falcon 3911Microtest III, Becton Dickinson, USA) were filled with 50 µL of purified i-antigen (20 µg/mL) in 25 mM sodium acetate buffer (pH 7.5) and incubated overnight at 4 ^{0}C. Control wells were coated with 2% (w/v) BSA. Non-specific protein binding was blocked by overnight incubation at 4 ^{0}C with 100 µL of 5% (w/v) BSA in Tris buffered saline with Tween 20 (TBST; 20 mM Tris-HCl, 50 mM NaCl, 0.05% v/v Tween 20, pH 7.5). Mucus samples (50 µl per well) were added to the plates and incubated for 1 hour at RT. Following three washes with TBST, mouse mAb anti-catfish Ig (MAF13) coupled to alkaline phosphatase (1:1000 dilution in 2% w/v BSA in TBST) was added (50 µL/well) and incubated for 1 hour at RT. After three washes

with TBST, substrate p-nitrophenylphosphate was added, and plates were incubated for 1 hour in the dark at RT. The reaction was stopped with 4 N NaOH and plates were read at 405 nm with ELISA reader.

Statistical Analyses. Differences in the median days to death (MDD) among groups were calculated by the one way analysis of variance (ANOVA). Differences in the proportion of survivors among groups were calculated by the z test. All calculations were done using the SigmaStat statistical software (Jandel Scientific Software, CA, USA).

RESULTS

Immunization with purified immobilization antigen. The i-antigen from *Ichthyophthirius multifiliis* isolate G5 was purified to homogeneity by immunoaffinity chromatography using mouse monoclonal antibody (mAb) G3-61. This mAb was originally produced against *I. multifiliis* isolate G3, but was previously shown to immobilize both G3 and G5 isolates and confer passive protection against the G5 isolate (19). The i-antigen purified from *I. multifiliis* G5 comprises a single peptide chain. Its approximate Mr is 55 kDa under reducing condition and 46 kDa under non-reducing condition as determined by SDS-PAGE (FIG.2.1). Its more exact size by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry analysis is 44.3 kDa (FIG.2.2). To confirm its functional identity, the affinity-purified protein was used to immunize two rabbits whose sera were then incubated with live parasites. The rabbit antisera immobilized the free-swimming theronts at a titer of 1280, and recognized both reduced and non-reduced i-antigen on Western blots at a dilution of 1: 10,000. When G5 i-antigen was used to immunize channel catfish, a similar result was obtained with a

notable exception. Fish antisera immobilized live theronts at a titer of 640, and recognized only non-reduced i-antigen protein on Western blots at a dilution of 1: 2,000. These results indicate that affinity-purified G5 i-antigen is highly immunogenic to both rabbits and channel catfish. Catfish, however, produce antibodies that react only with conformational antigenic epitopes.

The 55-kDa i-antigen of *I. multifiliis* G5 was administered to channel catfish as a subunit vaccine with Freund's adjuvant to test if the purified protein could elicit protective immunity against homologous *Ichthyophthirius* infection. Preliminary experiments have shown that the purified protein without adjuvant is not an effective immunogen (Wang, unpublished results). Forty to fifty fish per group were tested in a large-scale vaccination trial by exposure to 15,000 theronts/fish on day 84 after the initial injection. As shown in FIG.2.3, seventy-two percent of fish (n=50) immunized with 10 µg of affinity purified 55-kDa i-antigen in Freund's complete and incomplete adjuvant survived lethal challenge with the homologous parasite. Negative control fish immunized with an immobilization-irrelevant 14-kDa *I. multifiliis* protein or BSA died by day 16 following exposure. *I. multifiliis* infection was visible on all fish in negative control groups at 6 days after the challenge. Statistical analysis using the z-test showed that there was no significant difference between the survival of the i-antigen injected group and the group vaccinated with live theronts ($p=0.250$). There was a significant difference, however, between the survival of groups vaccinated with i-antigen or live theronts and negative control groups injected with BSA or the 14kDa protein ($p<0.001$). The median-days-to-death (MDD) of fish that died in groups immunized with i-antigen or live

parasites was significantly greater ($p < 0.001$, Kruskal-Wallis test ANOVA) than the MDD of the control fish injected with BSA (Table 2.1).

Detection of i-antigen-specific serum and mucus antibody. The amount of serum and mucus antibody detected by the immobilization assay and ELISA, respectively, correlated positively with the level of protection. Channel catfish immunized with i-antigen or live parasites had high serum immobilizing serum antibody titers ranging from 160 to 640 beginning 2 weeks after the first injection, while fish injected with BSA or the 14-kDa protein had no detectable immobilizing antibody response (Table 2.2). Furthermore, at 9 months after challenge, the titer of immobilizing serum antibody in fish that survived challenge was 640. Fish were all resistant to re-challenge with 15,000 theronts per fish at that time (results not shown).

Mucus antibodies against the i-antigen were not at a sufficiently high concentration to immobilize parasites but were detectable by ELISA. As shown in Table 2.2, fish immunized with either purified G5 i-antigen or live theronts developed specific mucus antibodies against G5 i-antigen at week 11.

Antibodies from *I. multifiliis*-immune fish and immobilizing mouse mAb recognize conformation-dependent epitopes. When analyzed by SDS-PAGE (see FIG.1), the i-antigen protein migrates at different mobilities under reducing or non-reducing conditions (with or without β-mercaptoethanol, respectively). Under reducing conditions the protein has a Mr of 55 kDa. Under non-reducing conditions its Mr is 46 kDa. This is likely due to intramolecular sulfhydryl bonds existing between repeated cysteine residues (8). As shown by Western blot analyses (FIG.2.4), immobilizing mAb G3-61 and polyclonal antisera from immune channel catfish only recognize the non-

reduced form of the i-antigen. In contrast, polyclonal antisera from rabbits immunized with purified G5 i-antigen recognize the polypeptide under both reducing and non-reducing conditions. Reactivity of antisera or antibodies against i-antigens on blot membranes could be eliminated by pre-incubation of antisera or antibodies with high concentrations of *I. multifiliis* cilia or live theronts (data not shown). These results indicate that immobilizing antibodies recognize conformation-dependent epitopes on the i-antigen molecule and that these epitopes elicit the predominant antibody response in fish.

DISCUSSION

The results presented here demonstrate that vaccination of naïve channel catfish with the purified i-antigen of *Ichthyophthirius multifiliis* elicits immunity, thus confirming its role as a protective immunogen. Previous studies have strongly implicated an involvement for i-antigens in the immune response against this highly pathogenic protozoan parasite. *I. multifiliis*-immune fish produce serum antibodies against i-antigens and mucus collected from skin (a primary site of parasite infection) contain antibodies that react with the proteins on Western blots (3, 4, 6, 9, 10, 22). There is considerable evidence supporting the existence of a separate mucosal immune system in fishes (26) and we postulate that specific antibodies targeting i-antigens in the skin and gills are responsible for protection against *I. multifiliis* (11). Passive transfer studies with mouse immobilizing mAbs support a model of surface immunity mediated by antibodies (7, 20). It is not yet clear whether fish antibodies generated in response to infection are transported to surface sites from central or regional lymphoid tissue or produced locally.

Two aspects of the fish immune response to i-antigens are apparent from this and other studies in our laboratory. First, i-antigens elicit protective immunity only when presented in context with live parasites or associated with adjuvants (1). It appears that inflammation and its associated “danger signals” are necessary elements for initiation of the acquired immune response. Similar results were found in other fish species immunized with individual protein antigens (21). This is of practical significance for the further development of vaccines where an adjuvant system will be needed that is easy to administer (preferably orally or by immersion), causes minimal tissue damage, and elicits an appropriately modulated inflammatory response. Second, fish serum antibodies (like mouse immobilizing mAbs) do not recognize i-antigens on Western blots run under reducing conditions (20). Intramolecular sulfydryl bonds are most likely responsible for maintaining the conformation required for binding of immobilizing antibodies. Antibodies from rabbits immunized with purified i-antigen and Freund's adjuvant recognize both reduced and non-reduced forms of the protein on Western blots. Different sites or alternate mechanisms of antigen processing and presentation might account for this difference from the fishes’ response. The fact that channel catfish were injected intraperitoneally while rabbits were inoculated intradermally could be a contributing factor. In any case, it appears that fish generate a homogeneous antibody response with regard to the recognition of conformational epitopes.

It is clear that immobilizing antibodies prevent infection, but it is also known that immunity generated in response to natural infection with *I. multifiliis* protects fish against challenge by parasites with heterologous i-antigen serotypes (18). This raises an interesting question with regard to: whether non-immobilizing epitopes on i-antigen

molecules elicit protection against parasites of heterologous serotype, or other protective antigens exist. To explore further the role of i-antigens in cross-protection, we are currently testing groups of channel catfish injected with affinity purified i-antigens from two distinct serotypes (A and D) against challenge with parasites of homologous or heterologous serotype.

The experimental vaccine used in this study consisted of i-antigen that was affinity purified directly from the parasite. While this native protein clearly served as an effective vaccine, it would be extremely difficult to collect enough for the production of a vaccine for large-scale field use. A heterologous system for the production of recombinant i-antigen proteins is clearly required. Toward this end, the gene encoding the 48-kDa i-antigen of *I. multifiliis* strain G1 has been cloned and expressed in the free-living ciliate *Tetrahymena thermophila* (16). *Tetrahymena* has great potential for use in vaccine development. *Tetrahymena* cell lines can be grown in large volume cultures, reach a remarkably high density of $> 5 \times 10^6$ cells per milliliter in a relatively short time (generation time 1.4 hours), and tolerate wide ranges of tonicity and temperatures. Importantly, *T. thermophila* is non-pathogenic and is generally regarded as an environmentally safe organism (25). Thus, successful expression of an *I. multifiliis* gene in *T. thermophila* should allow the production of large amounts of antigen in a purified form, at relatively low cost. In addition to its use in the production of purified antigens, transformed *T. thermophila* can also be used as a live vaccine applied either as a bath or by injection into the animal host. We have found that up to 1×10^6 *T. thermophila* cells can be injected into the peritoneal cavity of channel catfish fingerlings weighing 10-15 grams, where they survive for several days without causing adverse effects (X. Wang and

H. W. Dickerson, unpublished results). Expression of native *Ichthyophthirius* proteins on the surface of *Tetrahymena* may provide the most efficient means of exposing fish to antigens, short of infection with the live parasite itself. The use of *T. thermophila* as an expression system could have widespread implications in the development of fish vaccines.

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FIG.2.1. Purified i-antigen of *Ichthyophthirius multifiliis* G5 isolate. G5 i-antigen was purified by immunoaffinity chromatography with immobilizing mAb G3-61 coupled to AminoLink™ matrix. The purified i-antigen was resolved by 10 % SDS-PAGE and stained with silver nitrate. Lane 1: total membrane proteins of *I. multifiliis* G5 isolate, 10 μg; lanes 2 and 3: reduced purified G5 i-antigen, 0.5 μg; lane 4: non-reduced purified G5 i-antigen, 0.5 μg. Protein standards (molecular mass in kilodaltons) are indicated to the left. The Mr of the reduced G5 i-antigen is ~55 kDa, and that of the non-reduced G5 i-antigen is ~46 kDa.

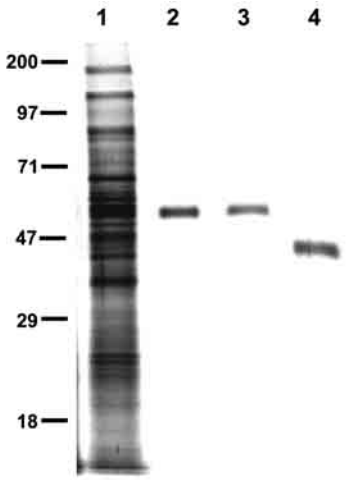

1
2
3
4
200
97
71
47
29
18

FIG.2.2. Analysis of the molecular mass of the G5 i-antigen by MALDI (Matrix-assisted laser desorption/inonization). MALDI-TOF mass spectrometry was performed on a Hewlett-Packard G23051 MALDI-TOF mass spectrometer to determine the molecular mass of the purified G5 i–antigen. The analytic parameters were: laser energy 7.00 (0.07) μJ, vacuum 1.26e-006 torr, polarity positive, and matrix Sinapanic acid. Under these conditions the molecular mass of the G5 i-antigen was determined to be 44387.4 Da indicated by the major peak (the small peak is half the value of the molecular weight of the G5 i-antigen).

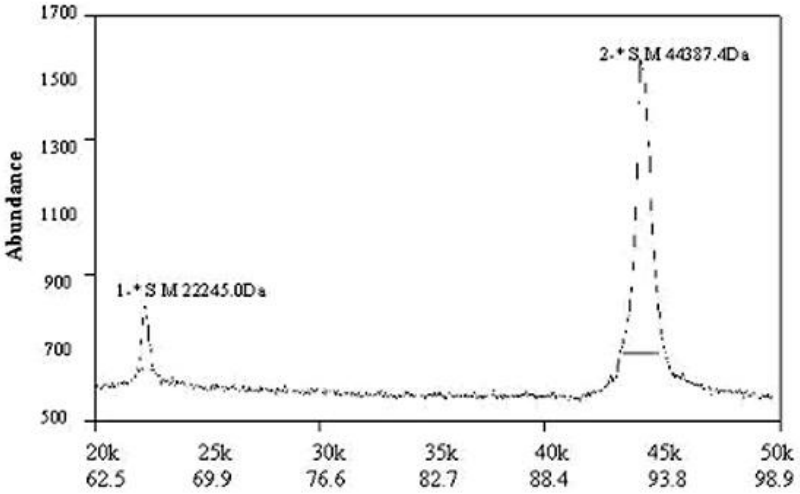

1700
1500
1300
1100
900
700
500
Abundance
2-* S M 44387.4Da
1-* S M 22245.0Da
20k
62.5
25k
69.9
30k
76.6
35k
82.7
40k
88.4
45k
93.8
50k
98.9

FIG.2.3. Vaccine challenge experiment. All groups were challenged with 15000 live *I. multifiliis* theronts per fish at day 84 after the first injection. Seventy-two percent of fish (n=50) immunized with 10 µg of affinity purified 55-kDa i-antigen in Freund's adjuvant, and 59.2 % of fish (n=49) immunized with live *I. multifiliis* theronts survived challenge. Negative control fish injected with an immobilization-irrelevant 14-kDa *I. multifiliis* protein or BSA in Freund's adjuvant died by day 16 following challenge.

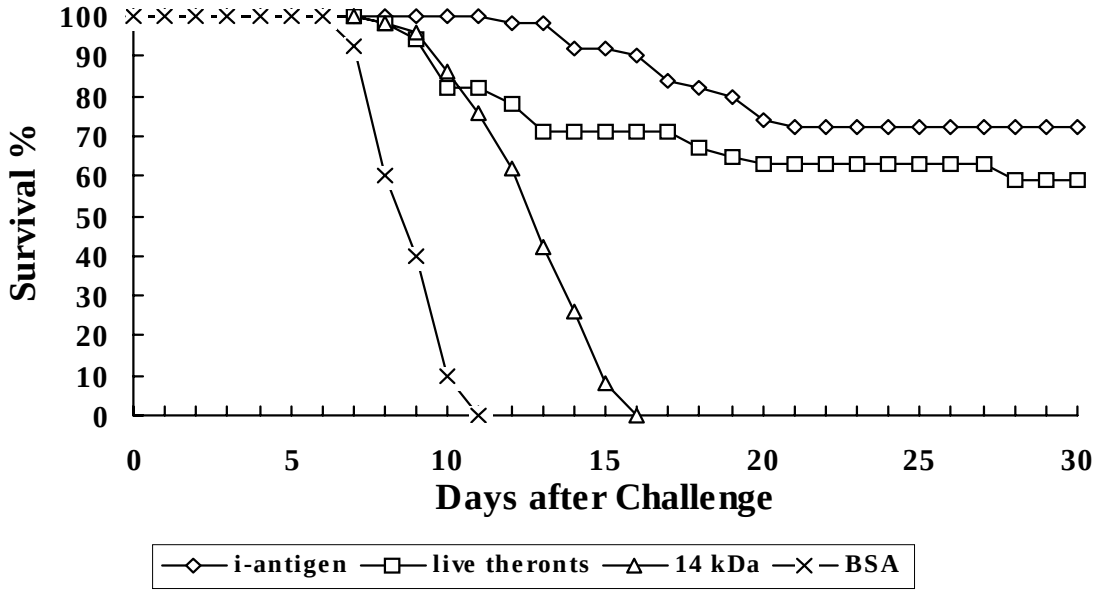

Survival %
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
Days after Challenge
i-antigen
live theronts
14 kDa
BSA

Table 2.1. Survival of immunized channel catfish following challenge.

Vaccine[a]	No. of challenged[b] / No. of survived	Survival %	MDD[c]	25%[d]	75%[e]
i-antigen	50 / 36	72.0	17	14	20
live theronts	49 / 29	59.2	12	10	18
14kDa	50 / 0	0	13	12	15
BSA	40 / 0	0	9	8	10

[a] Channel catfish were injected with protein antigens or live theronts as described in Materials and Methods.

[b] 15000 theronts / fish by surface exposure.

[c] Median days to death, which was calculated by Kruskal-Wallis One Way Analysis of Variance on Ranks. There is a significant difference in MDD among the treatment groups ($p < 0.01$).

[d] lower quartile.

[e] upper quartile.

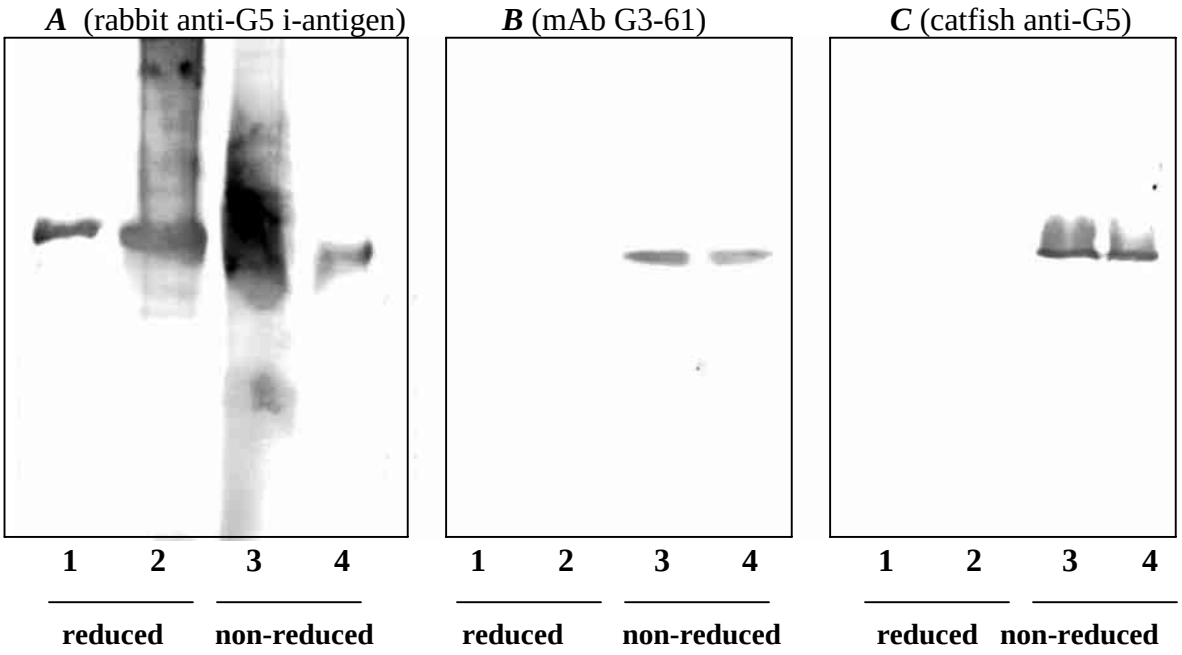
Table 2.2. Serum and mucus antibody responses over time.

Immuogen	Serum antibody[a] (titer)				Mucus antibody[b] (OD_{405nm})			
	Week 2	7	9	11	2	7	9	11
Live theonts	160	240	480	480	0.011	0.008	0.039	0.085
i-antigen	120	640	640	480	0.018	0.004	0.027	0.049
14 kDa	0	20	0	0	0.032	0.007	0.017	0.004
BSA	0	0	0	0	0.011	0.007	0.012	0.011

[a] Determined by *in vitro* immobilization assay. The value is the median titer (n=6).

[b] Determined by ELISA. The value is the median value of optical density (OD) at 405nm (n=6).

FIG.2.4. Western blot analyses of the G5 i-antigen. In the three blots, lane 1 is the reduced purified G5 i-antigen (0.7 µg); lane 2 is the reduced G5 membrane protein sample (5 µg); lane 3 is the non-reduced G5 membrane protein sample (5 µg); lane 4 is the non-reduced purified G5 i-antigen (0.7 µg). Blot ***A*** was probed with rabbit anti-G5 i-antigen serum (1:5000), blot ***B*** was probed with monoclonal antibody G3-61 (1:1000), and blot ***C*** was probed with fish anti-G5 serum (1:500). The secondary antibodies are goat anti-rabbit IgG, rabbit anti-mouse IgG, mouse mAb anti-catfish Ig (MAF13), conjugated with alkaline phosphatase. Rabbit antiserum against purified G5 i-antigen recognizes both reduced and non-reduced G5 i-antigens. Immobilizing mAb G3-61 and fish anti-G5 serum only recognize the non-reduced forms of the protein.


A (rabbit anti-G5 i-antigen)
B (mAb G3-61)
C (catfish anti-G5)
1 2 3 4
1 2 3 4
1 2 3 4
reduced non-reduced
reduced non-reduced
reduced non-reduced

CHAPTER 3

IMMUNIZATION OF CHANNEL CATFISH (*ICTALURUS PUNCTATUS*) WITH *ICHTHYOPHTHIRIUS MULTIFILIIS* IMMOBILIZATION ANTIGENS ELICITS SEROTYPE-SPECIFIC PROTECTION[1]

[1]Wang, Xuting, Clark, Theodore G., Noe, Jane, and Harry W. Dickerson. To be submitted to *Journal of Fish and Shellfish Immunology*.

ABSTRACT

Surface immobilization antigens (i-antigens) were purified from two strains of *Ichthyophthirius multifiliis* (NY1 and G5) that represent different i-antigen serotypes, namely A and D, respectively. The efficacy of the purified antigens as subunit vaccines was then tested in challenge studies using parasites of the homologous or heterologous serotype. Three groups of juvenile channel catfish (70 animals per group) were immunized with i-antigens from either the G5 or NY1 isolates, or with bovine serum albumin (BSA) as a control. Proteins were injected intraperitoneally (ip) at a dose of 10 μg/fish with complete Freund's adjuvant on day 1, followed by a second injection in incomplete Freund's adjuvant on day 15. Fish immunized with the purified i-antigens developed high titers of serum immobilizing antibodies whereas sera from BSA-injected control fish did not. Fish antisera immobilized parasites of the homologous, but not the heterologous strain, and recognized the corresponding i-antigens on Western blots run under non-reducing conditions. On day 36, each group was divided into 2 subgroups (n=30). One subgroup was challenged with G5 parasites, and the other was challenged with NY1 parasites. When challenged with G5 parasites, 70% of fish immunized with the G5 i-antigens survived. When challenged with NY1 parasites, 33.3% of fish immunized with the NY1 i-antigens survived. All BSA-injected control fish died, as did all fish injected with the purified antigens and challenged with the non-homologous parasite strain. Statistical analyses indicated significant differences among test and control groups with regard to the mean days to death (MDD). While the results of these studies clearly support a role for i-antigens in protection, active immunity in response to natural infection is not serotype-specific. The utility of i-antigens, as well as the existence of

other potential vaccine candidates for the prevention of ‘white-spot’ disease, are discussed.

Keywords: *Ichthyophthirius multifiliis,* channel catfish (*Ictalurus punctatus***),** immobilization antigen, immunization, serotype cross protection, subunit vaccine.

INTRODUCTION

The parasitic ciliate *Ichthyophthirius multifiliis* infects all species of freshwater fish and causes significant losses in the aquaculture industry (1). Current strategies for its control depend largely on the use of chemicals such as formalin to kill water-borne stages of the parasites and are not always effective. Moreover, chemical treatment is becoming restricted due to increasing concern over environmental pollution. Vaccination against *I. multifiliis* has been proposed as an alternative to chemical treatments based on the fact that fish develop immunity following controlled infection (2). Nevertheless, attenuated strains of *Ichthyophthirius* are not yet available, and vaccination with wild-type parasites is impractical due to a risk of inadvertent outbreaks. To eliminate that risk, we are investigating the use of subunit vaccines consisting of *I. multifiliis* surface protein antigens referred to as immobilization antigens.

Immobilization antigens (i-antigens) are 40- to 60- kDa polypeptides bound to the plasma membrane through a glycolipid anchor (3). These proteins when injected into animals elicit the production of antibodies that immobilize the live parasite *in vitro* (4, 5). At least five different serotypes of *I. multifiliis* have now been identified (namely, A through E) based on antibody-specific immobilization (6, 7). Intraperitoneal injection of channel catfish (*Ictalurus punctatus*) with affinity-purified i-antigens of *I. multifiliis* serotype D elicits immobilizing antibodies as well as active immunity against parasites of the same serotype (8). Based on passive immunization studies with mouse monoclonal antibodies, i-antigen-mediated immunity is thought to involve a novel mechanism in which parasites are forced to exit fish prematurely in response to antigen clustering at the

cell surface (9). This mechanism is serotype-specific and involves antibodies directed against the conformational epitopes responsible for immobilization of parasites *in vitro*.

If this were the sole effector mechanism responsible for protection against *I. multifiliis,* one would expect that infection with the parasite itself would also elicit serotype-specific immunity. This is not the case, however, as fish exposed to theronts of a given serotype develop solid immunity against lethal challenge with heterologous strains (10, 11). In contrast with serotype-specific monoclonal antibodies, rabbit polyclonal antisera against affinity-purified i-antigens of serotypes A and D recognize antigenic determinants that are shared among i-antigens of all strains (12). It is therefore possible that these shared epitopes contribute to cross-protection seen with live parasite infections. To test this idea, we immunized fish with purified i-antigens obtained from *I. multifiliis* serotypes A or D, and challenged vaccinates with parasites of either the homologous or heterologous serotype. As reported here, the purified antigens appear to elicit serotype-specific protection.

MATERIALS AND METHODS

Experimental fish. Channel catfish (*Ictalurus punctatus*) fingerlings (10-15 g) with no history of exposure to *I. multifiliis* were obtained from a local hatchery. Prior to immunization fish were treated with formalin (25 ppm) for 24 hours to remove external parasites, and then held in a flow-through system. Fish were fed to satiation once daily with commercial trout starter feed (Purina, USA). Water temperature was maintained at 20-23 ^{0}C, and water quality (NH_3, NO_2, and pH) monitored daily with standard test kits.

Propagation and collection of parasites. Two *I. multifiliis* isolates, G5 and NY1, were used in this study. Each represents a different serotype based on *in vitro* immobilization with type-specific mono- and polyclonal antibodies. Parasites cultures were maintained separately by serial passage on juvenile channel catfish (13). The G5 isolate is immobilization serotype D and has been described previously (3, 14). NY1 was isolated from infected rainbow trout at a hatchery in upstate New York. It is immobilization serotype A.

Five heavily infected fish were placed in a beaker containing 3L carbon-filtered water, and trophonts were dislodged from the skin. Free-swimming trophonts were collected on a 200-mesh sieve, transferred to 100 mL of carbon-filtered water, and allowed to develop into theronts (18–20 h at 22 °C). Theronts were passed through a 400-mesh sieve, harvested by centrifugation at 1000x*g* for 2 minutes, and washed once in 50 mL carbon-filtered water. Cells were used immediately, or stored as frozen pellets at –70 °C.

Purification of immobilization antigens from *I. multifiliis*. Mouse monoclonal antibodies, G3-61 and 10H3, were used for the preparation of immunoaffinity columns and the subsequent purification of *I. multifiliis* antigens. MAb G3-61 was developed initially against the G3 parasite strain (14) and immobilizes isolates belonging to serotype D (including the G5 strain). MAb 10H3, on the other hand, was developed originally against the G1 parasite strain (15) and immobilizes parasites belonging to serotype A (including the NY1 strain).

Immunoaffinity chromatography was performed as described previously (15) with modifications. Monoclonal antibody was purified by binding to protein A agarose

(Boehringer Mannheim, Mannheim, Germany). Antibody (20 mg) was then coupled to a 2 mL CarbonLink^{TM} column (Pierce, Rockford, IL, USA) following the manufacturer's protocols. Parasite membrane proteins were solubilized in 1% (v/v) Triton X-114 (Sigma, MO, USA) in 10 mM pH 7.5 Tris-HCl buffer and isolated by phase separation at 30^0 C as described previously (5). Five milliliters of parasite membrane protein (2-5 mg/mL in 10 mM, pH 7.5 Tris-HCl buffer) was applied to the affinity columns. Unbound proteins were removed from the columns by washing with 20 mL of 10 mM Tris-HCl buffer (pH 7.5). Immobilization antigens were eluted with 10 mM glycine buffer (pH3.0), and immediately neutralized with 1M Tris-HCl buffer (pH7.5). Protein concentrations were determined either by absorbance at 280 nm, or using a BCA colorimetric assay (Pierce, Rockford, IL, USA) with BSA as a standard.

N-terminal amino acid sequence analysis. N-terminal amino acid sequences of the NY1H, NY1L, and G5 i-antigens were determined by Edman degradation. For the G5 antigens, protein eluted from monoclonal antibody affinity columns were dialyzed against HPLC water and directly sequenced as below. For NY1H and NY1L antigens, total membrane protein from NY1 parasites was isolated by extraction with Triton X-114 (5) and fractionated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. Protein was then electroblotted onto two sheets of PVDF membrane (0.45 micron. Pierce, Rockford, IL, USA) that were stacked together. The membrane closest to the gel was stained with Coomassie Bright Blue, while the one furthest away was probed sequentially with mAb 10H3, followed by rabbit anti-mouse IgG-alkaline phosphatase conjugate in order to localize the NY1H and NY1L antigens. Coomassie blue stained bands corresponding to those recognized by mAb 10H3 were cut

from the membrane with a razor blade and submitted to the Molecular Genetics Instrumentation Facility at the University of Georgia. Edman degradation was carried out on an ABI Procise 494 sequencer (Applied Biosystems, Foster City, CA, USA).

Antibody and antisera. Rabbit anti-serum against the affinity purified G5 i-antigens were prepared in two adult female New Zealand White SPF rabbits, each injected intradermally with 100 µg of purified i-antigen in complete Freund's adjuvant (CFA), and boosted 3 weeks later with intradermal injection of 100 µg i-antigen in incomplete Freund's adjuvant (IFA). Blood was collected by cardiocentesis 3 weeks after the second injection. Serum was heat inactivated at 56 ^{0}C for 30 minutes and stored at –80 ^{0}C.

Channel catfish antisera against the NY1 parasite strain were obtained from six channel catfish that survived natural infection with NY1. Fish serum was heat inactivated at 56 ^{0}C for 30 minutes and stored at –20 ^{0}C.

SDS-PAGE and Western blotting. One-dimensional SDS-PAGE and Western blot analyses were done using standard protocols (15). Protein samples were prepared either under reducing conditions (i.e. with β-mercaptoethanol in Laemli buffer) or under non-reducing conditions (i.e. without β-mercaptoethanol in Laemli buffer). A mouse monoclonal antibody against channel catfish Ig heavy chain (MAF13) was provided by Dr. Norman W. Miller, Department of Microbiology, University of Mississippi Medical Center, Jackson, MS, USA. This mAb was conjugated with alkaline phosphatase as previously described (14) and used as a secondary antibody in Western blotting.

Vaccination procedure. Channel catfish were distributed randomly into eight 38-liter aerated and biologically conditioned aquaria at a density of 35 fish per aquarium.

The vaccine trial consisted of three groups, each having 70 randomly assorted fish (two aquaria per group). Fish were immunized with G5 i-antigen, NY1 i-antigen, or BSA. The proteins were injected ip at a dose of 10 µg/fish with CFA, followed by a second ip injection in IFA after a 14-day interval. Fish were challenged with live parasites five weeks after the second injection. Each group of fish was subdivided into 2 smaller groups (30 fish each), one of which was challenged with the G5 isolate and the other with the NY1 isolate.

Collection of channel catfish serum samples. Channel catfish were anesthetized with 100-200 ppm MS-222 (tricaine methane sulfonate, Argent Chemical Laboratories, Redmond, WA) and blood samples were collected from the caudal vein/arteries. Blood was allowed to clot for 1 hour at room temperature (RT, 20-22 ^{0}C) or overnight at 4 ^{0}C. Serum was collected after centrifugation at 1500xg for 10 minutes, heat inactivated at 56 ^{0}C for 30 minutes, and stored at –20 ^{0}C.

Immobilization assays. Assays to determine titers of immobilizing antibodies in fish serum were carried out essentially as described by Clark et al. with minor modification (4). Briefly, serum samples were prepared in 96-well microtitre plates as a series of doubling dilutions in 50 % phosphate buffered saline (PBS; 135 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.5). Each well contained 50 µl of diluted serum, to which ~200 live theronts in 50 µl of dechlorinated water were added. Immobilization was observed with a dissection microscope, and titers were expressed as the inverse of the highest dilution in which all of theronts were immobilized after incubation for 30 minutes at RT. Pre-immune serum samples were used as negative controls.

Parasite challenge studies. Immunized fish were challenged by surface exposure to a potentially lethal dose (15000 cells per fish) of live *I. multifiliis* theronts. Groups of fish were transferred to a 3L beaker containing 100 mL of charcoal-filtered water per fish. Live theronts were added, and fish were maintained for 2 hrs with aeration. Following exposure, fish were placed into aquaria supplied with individual biological filters. Water quality and temperature were monitored daily. Mortalities were recorded daily in each group.

Statistical Analyses. Differences in the mean days to death (MDD) among groups were calculated by the one way analysis of variance (ANOVA). Differences in the proportion of survivors among groups were calculated by the z test. All calculations were done using the SigmaStat statistical software (Jandel Scientific Software, CA, USA).

RESULTS

Affinity purification of i-antigens

The i-antigens of *I. multifiliis* isolates NY1 and G5 were purified by affinity chromatography on matrices that contained mouse mAbs 10H3 and G3-61, respectively. These mAbs strongly immobilize live *I. multifiliis* organisms and are used to define i-antigen serotypes. Eluted protein fractions were subjected to SDS-polyacrylamide electrophoresis under non-reducing conditions and stained with silver nitrate (Figure 3.1). The G5 i-antigens appeared as a single protein with apparent molecular mass of ~ 46 kDa. NY1 i-antigens consisted of three prominent protein bands with M_rs of ~ 45, 39 and 34 kDa, and a less abundant band of ~ 29 kDa.

Characterization of i-antigens by Western blot analysis

As shown in Figure 3.2, rabbit antiserum generated against affinity purified G5 i-antigens reacted in Western blots with both the G5 (panel D) and NY1 (panel A) proteins. This result is consistent with previous findings (6) and indicates that the i-antigens of different serotypes have conserved antigenic determinants. Under reducing conditions, the G5 proteins ran as a single band of ~ 55 kDa, while those of NY1 migrated as three distinct bands of approximately 56-, 46- and 42 kDa (Figure 3.2). Under non-reducing conditions, the apparent M_rs of these proteins shifted to ~ 46 kDa for the G5 antigens, and ~ 45-, 39- and 34 kDa for the NY1 proteins. The 29-kDa protein detected by silver stain in the affinity purified NY1 i-antigen fraction (Figure 3.1) was not recognized by rabbit antiserum in Western blots.

While the rabbit polyclonal antiserum against G5 proteins reacted with both reduced and non-reduced i-antigens of the G5 and NY1 strains, immobilizing mAbs (G3-61 and 10H3), as well as sera from fish vaccinated with the purified proteins, recognized only non-reduced i-antigens of the homologous serotype. Figure 3.2 shows Western blotting profiles of non-reduced G5 antigens reacted with mAb G3-61 (panel E), or with immune sera from fish vaccinated with affinity purified G5 proteins (panel F). No signals were detected when proteins were fractionated under reducing conditions and incubated with the same antibodies. As in the case of the G5 proteins, mAb 10H3, as well as immune sera from fish vaccinated with the NY1 antigens recognized only non-reduced NY1 proteins on Western blots (Figure 3.2, panels B and C). These same antibody preparations failed to react with the reduced NY1 proteins. We had previously shown that immobilizing mAbs 10H3 and G3-61 recognize conformational epitopes unique to

serotypes A and D, respectively (14, 15). Interestingly, antibodies in fish are directed primarily toward these same conformational epitopes. It is also worth noting that immune fish sera (as well as mAb 10H3) only reacted with two of the three protein bands recognized by the rabbit anti-G5-i-antigen polyclonal antiserum. As shown in Figure 3.2, those were the 45- and 39- kDa proteins (designated NY1H and NY1L in panels B and C, respectively).

Comparison of the N-terminal amino acid sequences of i-antigens

To further characterize i-antigens of the different isolates, N-terminal amino acid sequences of the G5 i-antigen, and the NY1H and NY1L polypeptides were determined using the Edman degradation method. Table 3.1 lists the N-terminal sequences derived by Edman degradation from the parasite i-antigens characterized to date. The NY1H and NY1L polypeptides share the same N-terminal amino acid sequence. With the exception of a single mismatch at position 4, the N-termini of these proteins are also identical to the equivalent region of the 48- kDa i-antigen of isolate G1 (another member of serotype A) (10). We presume the (P to Q) mismatch is an artifact of Edman degradation, since the gene for the 48 kDa antigen specifies a proline at position 4 (as in the NY1 proteins) (14). Along with the serotype A proteins, Table 3.1 shows a comparison of the N-terminal sequences of serotype D i-antigens obtained from isolates G5 and G3. These sequences are also identical, and share roughly ~ 40% similarity with N-termini of the serotype A proteins. A comparison of the N-termini of the serotype A and D i-antigens shows a conserved V--P-G-----G-S/TD-GAAD motif.

Serum antibody response of immunized channel catfish is serotype-specific.

As shown in Figure 3.4, juvenile channel catfish injected with affinity purified i-antigens developed serum antibodies that strongly immobilized *I. multifiliis* theronts *in vitro*. Antibody titers became apparent within a week of injection, and reached their highest levels (1:640 - 1:1280) by week 4. Consistent with the results of Western blotting analyses (Figure 3.2), the ability of immune sera to immobilize parasites was serotype-specific. Antisera from fish immunized with G5 antigens immobilized only G5 theronts, while antisera from fish vaccinated with the NY1 proteins immobilized only NY1 theronts (Figure 3.4). Fish injected with BSA had no detectable immobilizing antibody titers.

Immobilization antigens elicit serotype-specific immunity.

To determine whether purified i-antigens elicited immune protection, groups of 70 channel catfish were vaccinated with either the G5 or NY1 proteins. Following vaccination, each group was divided into 2 subgroups (n = 30 fish), one of which was challenged with G5 theronts, and the other with NY1 theronts.

Seventy percent of fish immunized with G5 antigens survived challenge with G5 parasites, and 33.3% of fish immunized with the NY1 proteins survived challenge with NY1 parasites (Figure 3.3A and 3.3B). All fish in the BSA control groups died within 26 days after challenge. Moreover, all fish immunized with the purified antigens and challenged with the heterologous serotype died.

Although a substantial number of fish in the test groups challenged with homologous parasite strains died (particularly in the case of the NY1 vaccinates), a statistically significant difference in the relative percent survival (RPS) and the mean

days-to-death (MDD) was found for both groups in comparison with control fish injected with BSA alone (Table 3.2 column 5). The days-to-death difference is a further indication of serotype-specific protection by the purified antigens.

DISCUSSION

The results reported here clearly demonstrate that protective immunity elicited by purified i-antigens is serotype-specific. Protection was achieved when fish were challenged with parasites of the homologous serotype, but immunity was restricted to that serotype. This result is consistent with passive immunization studies in which mouse mAbs directed against conformational epitopes associated with immobilization *in vitro,* conferred strong protection on fish in a serotype-specific manner (14, 16). While these findings support a role for i-antigens in protective immunity, they stand in sharp contrast with the fact that immunity in response to parasite infection extends across serotypes (8, 9). Cross-protection has been now demonstrated in studies with the G1.1 and G2 isolates (serotypes B and C) (9), as well as with the G3 and G4 strains (serotypes D and C, respectively) (8). In the past, we could not rule out the possibility that antibodies directed solely towards the i-antigens afford protection across serotypes, primarily because the i-antigens of different serotypes have conserved linear epitopes. The findings presented here, however, argue that this is not the case. The fact that the purified i-antigens elicited only serotype-specific immunity strongly suggests that cross-protection (in response to infection) results from antibodies directed toward other parasite proteins. Alternatively, some fundamentally different (non-antibody-driven) mechanism of protection may come into play. Although a role for non-specific cytotoxic cells in immunity toward *I.*

multifiliis has been suggested (17), parasites that reside within the skin appear to resist cell-mediated killing (18), and immunity against extracellular pathogens, in general, requires a strong antibody response. Thus, it is likely that, along with the i-antigens, other parasite proteins may elicit the production of protective antibodies. This has obvious implications for vaccine development since broad-based protection using the i-antigens alone will require multivalent vaccines. On the other hand, the recently developed proteomics technology (e.g. highly reproducible two-dimensional SDS-PAGE of whole cell proteins and mass spectrometry with database search) to screen whole parasite proteome provides an opportunity to identify additional *I. multifiliis* antigens suitable for vaccine development.

Immunoblotting of proteomes with convalescent or hyperimmune sera can be used to identify the major immunodominant proteins (the "immunome") present in solubilized parasite extracts (19). This methodology provides a new tool to identify antigens that are only present at a particular stage of the parasites life cycle (e.g., the *I. multifiliis* infective theront or feeding trophont stages). Additionally, using a proteomics approach one can identify post-translational modifications of proteins such as phosphorylation, glycosylation, acylation, and methylation. Such modifications are often crucial for the correct functioning of proteins *in vivo*, and are increasingly recognized as targets for new drug discovery (20). A preliminary proteomics study on the identification of the antigens that elicit cross-protective, non-serotype specific immunity against *I. multifiliis* has shown that there are about ten proteins that react with immune fish sera on Western blots (Wang and Dickerson, unpublished results). These antigens will be further characterized by

matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, and their immunogenicity tested in channel catfish.

While a proteomics-based approach may eventually lead to the identification of other vaccine candidates, the i-antigens by themselves confer very high levels of protection and are, at present, the best hope for the development of recombinant subunit vaccines. Clearly, vaccine formulations based on these proteins must be comprised of multiple i-antigens to elicit protection against the different serotypes encountered in the field. The selection of i-antigens for inclusion in multivalent vaccines should be based on the prevalence of the various *I. multifiliis* serotypes in nature. To date, five different immobilization serotypes of *I. multifiliis* have been described (7). The majority of these isolates (eight out of eleven) belong to serotypes A and D, indicating that an i-antigen-based vaccine should contain at least the proteins of these serotypes.

While N-terminal amino acid sequence analysis (along with Western blotting data) indicate that the i-antigens from isolates that comprise a given serotype are virtually the same, some interesting differences are worth noting. In particular, the NY1 isolate appears to express at least three (M_rs = ~ 56-, 46-, and 42 kDa), and possibly four i-antigen polypeptides, while the G1 isolate (also a member of serotype A) had previously been shown to express only two i-antigen polypeptides of ~ 60- and 48 kDa (13). The differences in apparent molecular mass between the two higher molecular weight proteins of the G1 and NY1 isolates, respectively, is relatively trivial and may be attributed to experimental error. The presence of additional antigens in the NY1 strain is nevertheless interesting, as is the fact that only the 56- and 46- kDa polypeptides react with mAb 10H3 on Western blots. The failure of the two smaller NY1 polypeptides to react with mAb

10H3, despite having been isolated by affinity chromatography with bound 10H3, is difficult to understand. Obviously, the smaller polypeptides may represent contaminants of the purification procedure. Alternatively, SDS treatment, even in the absence of a reducing agent, may be sufficient to destroy the antigenicity of the smaller proteins. It is worth noting in this regard that gene fragments corresponding to at least three i-antigens of varying size have recently been isolated from the NY1 strain (C.C. Wang and T.G. Clark, unpublished).

Finally, it was evident in these studies that protection conferred by NY1 i-antigens was significantly less than that seen with the G5 antigens when measured by relative percent survival and MDD following challenge with the homologous parasite strains (see Table 3.2). Since animals were challenged with equal numbers of parasites of the two strains, the lower survival of the NY1 infected groups could be due to a difference in virulence between strains. Indeed, this idea is supported by the fact that the MDD of control fish immunized with BSA and challenged with NY1 parasites was significantly less ($p<0.05$, Student Newman Keuls method) than that of similar control fish challenged with G5 parasites. Although additional studies will be required to determine whether NY1 is in fact more virulent, the lower level of protection afforded by the purified NY1 antigens may have simply been due to an overwhelmingly high challenge dose. Should a difference in virulence actually be found, comparisons between isolates could bring to light the underlying mechanisms responsible for the pathogenesis of parasite infection.

ACKNOWLEDGMENTS

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Figure 3.1. **Silver stained SDS-PAGE**. Lane 1: non-reduced total membrane proteins of *I. multifiliis* G5, 10 μg; lane 2: purified non-reduced G5 i-antigen, 1.0 μg; lane 3: non-reduced total membrane proteins of NY1, 10 μg; lane 4: non-reduced purified NY1 i-antigen 1.0 μg.

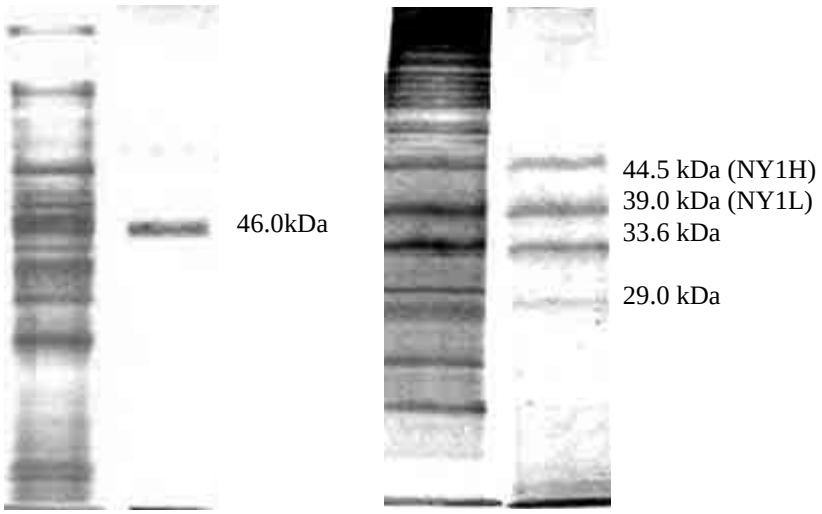
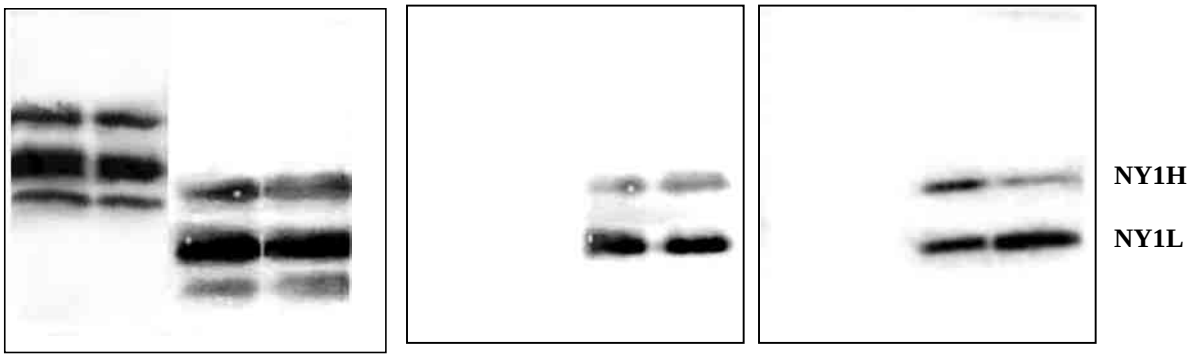

46.0kDa
44.5 kDa (NY1H)
39.0 kDa (NY1L)
33.6 kDa
29.0 kDa
1
2
3
4

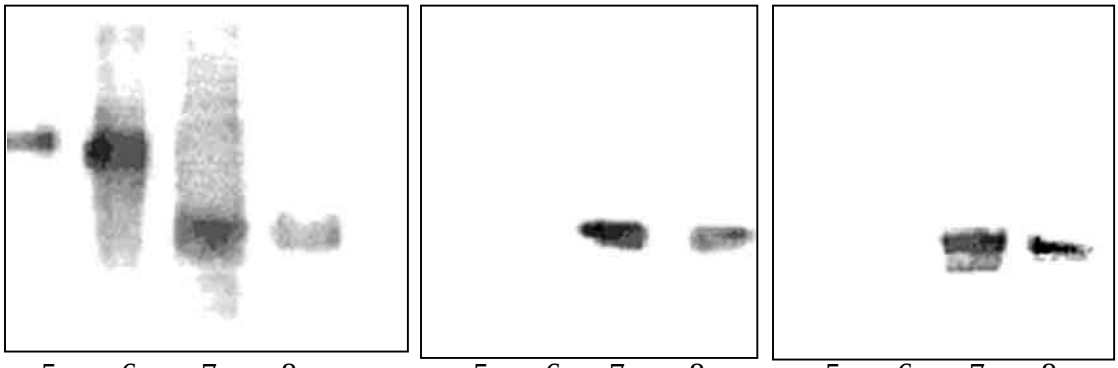
Figure 3.2. Characterization of i-antigens by Western blotting. Proteins were electrophoresed on SDS-polyacrylamide gels, transferred to nitrocellulose filters and probed with rabbit antiserum against G5 i-antigen (**panels A** and **D**), mouse mAb 10H3 (**panel B**), immune fish sera against *I. multifiliis* NY1 (**panel C**), mouse mAb G3-61 (**panel E**) and immune fish sera against *I. multifiliis* G5 (**panel F**). The following samples were probed: NY 1 i-antigen reduced (**lane 1**) and non-reduced (**lane 4**); NY1 membrane proteins reduced (**lane 2**), and non-reduced (**lane 3**); G5 i–antigen reduced (**lane 5**) and non-reduced (**lane 7**); and G5 membrane proteins reduced (**lane 6**), non-reduced (**lane 8**). Each lane was loaded with either 20μg membrane proteins or 2μg purified i-antigens. Rabbit antisera reacted with protein antigens under both reducing and non-reducing conditions (**panels A** and **D**). Mouse monoclonal and fish antibodies recognized antigens only under non-reducing conditions (**panels B, C, E,** and **F**),

A (Rabbit anti-G5 i-antigen) **B** (mAb 10H3) **C** (Channel catfish anti-NY1)



1 2 3 4 1 2 3 4 1 2 3 4

D (Rabbit anti-G5 i-antigen) **E** (mAb G3-61) **F** (Channel catfish anti-G5)



5 6 7 8 5 6 7 8 5 6 7 8

Figure 3.3A. Survival of channel catfish following challenge with the *Ichthyophthirius multifiliis* G5 isolate. Three groups (n = 30) of fish were immunized with purified G5 i-antigen, NY1 i-antigen, and bovine serum albumin (BSA), and challenged with theronts of *I. multifiliis* G5 isolate.

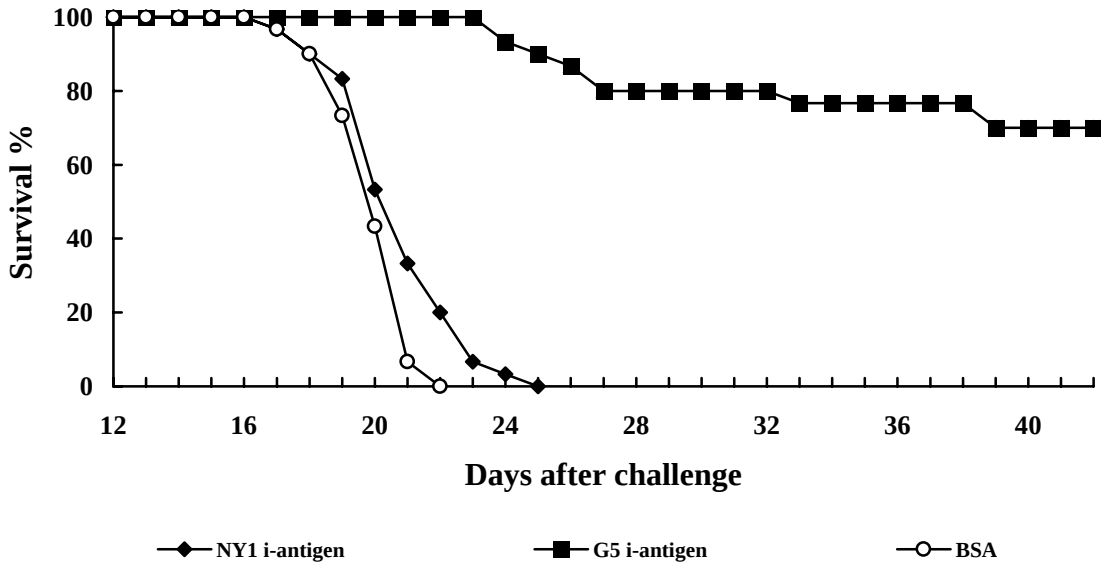

100
80
60
40
20
0
Survival %
12
16
20
24
28
32
36
40
Days after challenge
NY1 i-antigen
G5 i-antigen
BSA

Figure 3.3B. Survival of immunized channel catfish following challenge with the *Ichthyophthirius multifiliis* NY1 isolate. Three groups (n = 30) of fish were immunized with purified G5 i-antigen, NY1 i-antigen, and bovine serum albumin (BSA), and challenged with theronts of *I. multifiliis* NY1 isolate (15000 cells per fish).

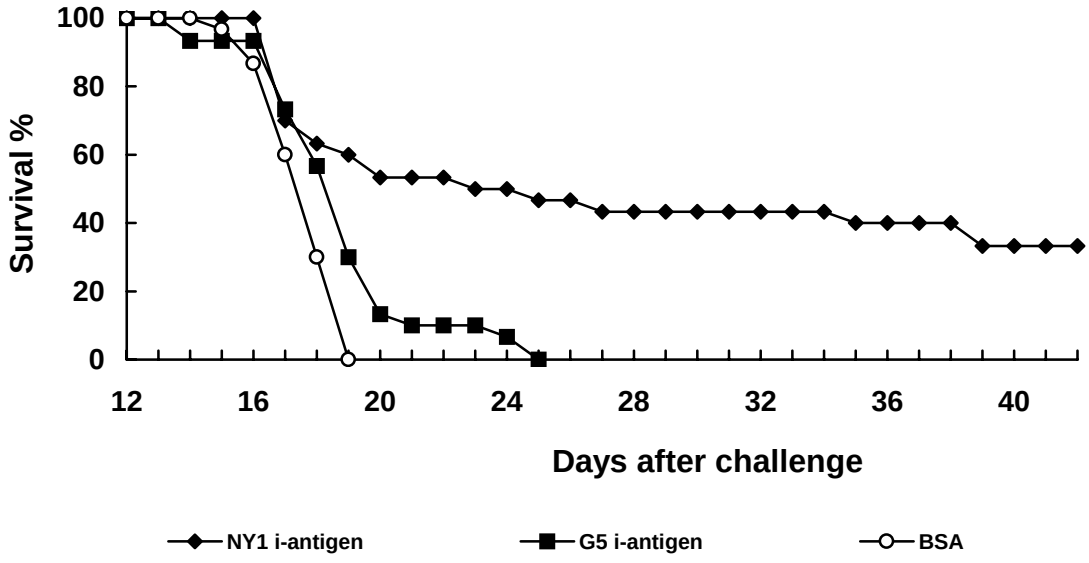

100
80
60
40
20
0
Survival %
12
16
20
24
28
32
36
40
Days after challenge
NY1 i-antigen
G5 i-antigen
BSA

Figure 3.4. **Serum antibody response of immunized channel catfish.** The serum antibody levels of immunized fish at different times after injection were determined by *in vitro* immobilization assays. Pooled heat-inactivated sera from six randomly selected fish were tested at each time point. The six assays performed at each time point are presented in the figure as "x"/ "y", in which "x" stands for the serum from the fish immunized with "x", and "y" is the isolate of *I. multifiliis* incubated with the serum in the immobilization assay. There were no detectable immobilization antibody titers in control fish. Also, sera from fish immunized with the G5 or NY1 i-antigen immobilized only parasites of the same serotype.

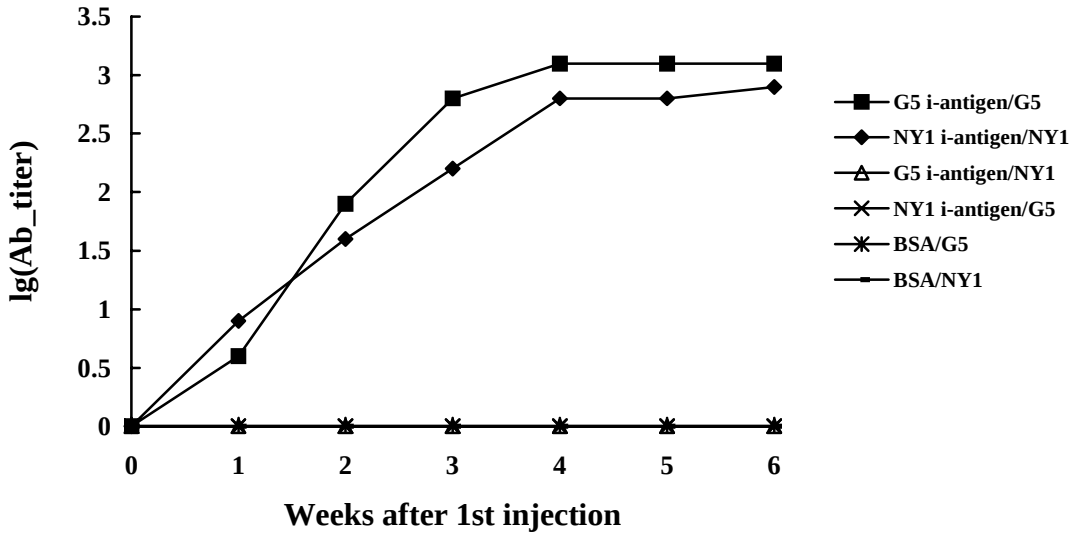

3.5
3
2.5
2
1.5
1
0.5
0
lg(Ab_titer)
0
1
2
3
4
5
6
Weeks after 1st injection
G5 i-antigen/G5
NY1 i-antigen/NY1
G5 i-antigen/NY1
NY1 i-antigen/G5
BSA/G5
BSA/NY1

Table 3.1. The N-terminal amino acid sequences of the *I. multifiliis* i-antigens.

Isolate	Serotype	N-terminal AA sequence of i-antigen
G1*	A	VPCPDGTQTQAGLTDVGAADLGTC
G1**	A	XPXQDGTQTQAGLTDVGAADLGTCVVC
NY1H	A	VPXPDGTQTQAGLTDVGAXD
NY1L	A	VPYPDGTQTQAGLTDVXAXD
G3**	D	VNXPNGAAIANGQXDTGAA
G5	D	VNXPNGAAIANGQSDTGAADXTQDGA

* The deduced N-terminal sequence of the G1 48 kDa i-antigen (GenBank, AF140273. (21));

** Lin, T., Clark, T. G., and Dickerson, H.W., unpublished;

"x" means not determined, and “bold” letters represent conserved amino acid residues for all isolates.

Table 3.2. I-antigen Vaccination and Statistical Analysis

Group	Immunogen[a]	Challenged with[b]	Fish number survived[c]	MDD[d] ± SD	RPS (%)[e]
1	NY1 i-antigen	NY1 theronts	10	21.8 ± 7.5	33.3
2	G5 i-antigen	NY1 theronts	0	18.9 ± 2.5	0
3	BSA	NY1 theronts	0	17.7 ± 1.2	0
4	NY1 i-antigen	G5 theronts	0	20.9 ± 1.8	0
5	G5 i-antigen	G5 theronts	21	29.3 ± 6.1	70
6	BSA	G5 theronts	0	20.1 ± 1.2	0

[a] Catfish were injected ip with protein antigens as described in Materials and Methods.

[b] Fish were challenged at a dose of 15000 theronts / fish by surface exposure.

[c] Thirty fish were challenged in each group.

[d] Mean days to death, which was calculated by One Way Analysis of Variance. All Pairwise Multiple Comparison (Student-Newman-Keuls Method) shows that there is a significant difference ($p < 0.05$) in MDD between the paired groups of: 1 vs 2, 1 vs 3, 1 vs 5, 2 vs 5, 3 vs 4, 3 vs 5, 3 vs 6, 4 vs 5, and 5 vs 6. No significant difference between the paired groups of: 1 vs 4, 1 vs 6, 2 vs 3, 2 vs 4, 2 vs 6, and 4 vs 6.

[e] Relative percent survival; RPS = (1 - % mortality in vaccinated group / % in control group) x 100 (22). The z-test analysis shows that there is a significant difference ($p < 0.01$) in the RPS of the paired groups of: 1 vs 2, 1 vs 3, 1 vs 4, 1 vs 5, 1 vs 6, 5 vs 2, 5 vs 2, 5 vs 4, and 5 vs 6.

CHAPTER 4

VACCINATION AGAINST *ICHTHYOPHTHIRIUS MULTIFILIIS* USING *TETRAHYMENA* EXPRESSING RECOMBINANT IMMOBILIZATION ANTIGENS[1]

[1]Wang, Xuting, Gao, Yan, Gaertig, Jacek, Clark, Theodore G., and Harry W. Dickerson. To be submitted to *Vaccine*.

ABSTRACT

Laboratory vaccine trials with affinity-purified surface immobilization antigens (i-antigens) of the pathogenic ciliate *Ichthyophthirius multifiliis* have shown that these proteins elicit protective immunity in channel catfish. In order to obtain sufficient material for vaccine studies, the i-antigen genes have been introduced into a novel heterologous expression system, the free-living non-pathogenic ciliate *Tetrahymena thermophila* in a previous study (Gaertig, J., Y. Gao, T. Tishgarten, T. G. Clark, and H. W. Dickerson. 1999. Nature Biotechnology. 17**:**462-465). By targeting i-antigen coding sequence into *BTU1* locus of *T. thermophila*, the recombinant i-antigen proteins were either displayed on the cell surface in full-length form or secreted into the culture medium in a truncated form lacking a GPI-anchor addition site. In the present study, the expression level of i-antigen was dramatically enhanced (>30-fold) using an inducible promoter from the metallothionein gene of *T. thermophila*. To test the immunogenicity of recombinant i-antigens produced in *Tetrahymena*, channel catfish were injected intraperitoneally twice at 2-week intervals with either live *T. thermophila* cells expressing i-antigen or purified recombinant i-antigen proteins. Immunized fish produced specific serum antibodies that immobilized live *I. multifiliis* parasites *in vitro* and recognized native i-antigens on Western blots. Moreover, 80-100% of fish immunized with live *Tetrahymena* over-expressing *Ichthyophthirius* i-antigens were protected from lethal change with parasites of homologous serotypes. These results indicate that recombinant i-antigens produced in *Tetrahymena* have the same antigenicity and immunogenicity, and

live cells of *Tetrahymena* over-expressing *Ichthyophthirius* i-antigens are effective vaccines against *I. multifiliis*.

Keywords: *Ichthyophthirius multifiliis, Tetrahymena thermophila,* channel catfish (*Ictalurus punctatus***),** immobilization antigen, immunization, recombinant vaccine.

INTRODUCTION

The common ciliated protozoan parasite, *Ichthyophthirius multifiliis*, infects all species of freshwater fish and causes significant economical loss in the food and ornamental fish industries (11, 20). Current strategies for its control and treatment are not always effective and depend largely on the use of chemicals, which by themselves induce severe stress. Moreover, some of the most effective chemicals against the parasite are prohibited for use with food fish for human. For example, malachite green is a confirmed teratogen that causes the development of abnormal structures in the embryo (31). Although laboratory experiments have shown that the immunization of fish with live *I. multifiliis* provides complete protection against lethal challenge (1, 7, 9, 12, 24), this would be an impractical approach in the field.

Studies in this laboratory have revealed a mechanism of protective immunity in which parasites exit the skin in response to antibody binding (5). The target antigens are a class of surface membrane proteins referred to as immobilization antigens, or i-antigens (10). Recently, we showed that immobilization antigens (i-antigens) purified from *I. multifiliis* G5 isolate confer protection against a lethal challenge with the homologous strain when injected into juvenile channel catfish in combination with Freund's adjuvant (32). Nevertheless, because *I. multifiliis* is an obligate parasite, an efficient heterologous protein expression system is clearly required to produce enough antigen for a practical vaccine.

Tetrahymena has great potential for the development of fish vaccine. *Tetrahymena* cell lines can be grown in large volume cultures, reach a remarkably high density of $> 5 \times 10^6$ cells per milliliter in a relatively short time (generation time 1.4

hours), and tolerate wide ranges of tonicity and temperatures. Importantly, *T. thermophila* is non-pathogenic and is generally regarded as an environmentally safe organism (34). Thus, expression of an *I. multifiliis* gene in *T. thermophila* would allow the production of large amounts of antigen at relatively low cost. *T. thermophila* displaying *I. multifiliis* i-antigen on its surface could possibly also be used as a live vaccine.

Toward this end, the gene encoding the 48-kDa i-antigen of *I. multifiliis* serotype A has been introduced into the *BTU1* locus of *T. thermophila*. The recombinant protein was either produced and targeted to the cell surface in a full-length form (13) or secreted into the culture medium in a truncated form lacking GPI-anchor addition site (4).

To enhance the expression level of foreign genes in *Tetrahymena*, an inducible promoter from the metallothionein gene of *T. thermophila* (*MTT*) has been used. The promoter of the *MTT* contains multiple metal regulatory elements, which can be induced by heavy metals, such as cadmium. Protein expression under the control of the *MTT* promoter is significantly increased when cadmium is added to growth medium. In *Tetrahymena pigmentosa*, MT-mRNA level increased more than 40-fold within 30 minutes of cadmium treatment (28). Recently, the genes encoding the i-antigens of *I. multifiliis* serotype A and D have been expressed in *T. thermophila* under the control of *MTT* promoter (Yan Gao and Jacek Gaertig, unpublished).

In this study, we characterized the recombinant *Ichthyophthirius* i-antigens produced by *Tetrahymena*, and tested the efficacy of the recombinant *Tetrahymena* as a vaccine in the channel catfish model.

METHOD AND MATERIALS

Expression of *Ichthyophthirius* i-antigen in *Tetrahymena.* Expression of the *I. multifiliis* i-antigen gene under the control of *BTU1* promoter in *T. thermophila* cells has been described previously (13). *T. thermophila* strain CU522 was used as the host strain for expressing *I. multifiliis* i-antigens. This strain has a substitution (Lys350Met) in the β-tubulin-1 (*BTU1*) gene and is sensitive to the microtubule-stabilizing agent, paclitaxel. The plasmid for transforming *Tetrahymena* cells was constructed by inserting the coding sequence for the *I. multifiliis* i-antigen between the flanking sequences of *BTU1*. The resulting plasmid pBICH3 was introduced biolistically into starved CU522 cells to "knock-out" the endogenous *BTU1* gene of the host cell. After replacement of the *Tetrahymena* β-tubulin gene with the *Ichthyophthirius* i-antigen gene by homologous recombination, stable transformants were selected by resistance to paclitaxel.

To over-express *I. multifiliis* i-antigens in *T. thermophila* cells, a cadmium (Cd) inducible *MTT* promoter (Gaertig and Gorovsky, unpublished) was used to construct plasmids for transformation. The 467-bp fragment located downstream of *BTU1* 5'-flanking sequence in pBICH3 was replaced by the 2-kb fragment of 5'-flanking sequence of the *MTT* gene. Thus, the *MTT* promoter was placed upstream of the coding sequence of the i-antigen. The resulting plasmid was introduced into the *BTU1* locus of *T. thermophila* strain CU522 by homologous recombination as described above.

The genes encoding the i-antigens of *I. multifiliis* serotype A and D (see Table 4.1) were used. The production of *I. multifiliis* i-antigens by the positive transformants was confirmed by indirect immunofluorescence assay, Western blot analysis, and immobilization test.

Indirect immunofluorescence assays. The expression of *I. multifiliis* i-antigens on the cell surface *T. thermophila* was detected by indirect immunofluorescence assays as described previously (13) with minor modification. *Tetrahymena* cells were fixed for 30 minutes with 2% of paraformaldehyde, and antibody against *I. multifiliis* i-antigen was added at a dilution of 1:100 in phosphate buffered saline (PBS, 135 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.5), followed by incubation 3 hours at room temperature. After 3 times washing with PBS, FITC labeled secondary antibody was added and incubated for 1 hour in dark at room temperature. Labeled cells were washed with PBS and mounted on slides (5 μL cells + 5 μL DABCO, 1,4-diazobicyclo-[2,2,2]-octane) for microscopy examination.

Western blot analysis. Western blot analysis was used to determine the antigenicity of recombinant i-antigens, and to detect specific serum antibodies in immunized fish. One-dimensional SDS-PAGE and Western blotting were done using standard protocols (22).

Purification of membrane-associated and secreted recombinant i-antigen. Immunoaffinity chromatography was used to purify recombinant i-antigens of *I. multifiliis* serotype A, as described previously (22, 33) with modifications. Polyclonal rabbit antibody against i-antigen of serotype D was used as ligand for affinity chromatography. Briefly, 16 mg of rabbit antibody purified on a protein A agarose column (Boehringer Mannheim GmbH Roche Molecular Biochemicals, Mannheim, Germany) was coupled to a 2-mL CarboLink™ column (Pierce, USA) following the manufacturers' protocols. To purify the membrane associated i-antigen, five mL of *Tetrahymena* membrane protein preparation (2-5 mg proteins /mL) solubilized in 1%

(v/v) Triton X-114 (Sigma) in 10 mM Tris-HCl buffer (pH 7.5) was applied to the affinity column. Unbound proteins were washed off the column with 10 volumes of 10 mM Tris-HCl. Captured i-antigen was eluted from the column using 10 mM pH3.0 glycine buffer. Purity of i-antigen fractions was checked on SDS-PAGE stained with silver nitrate. To purify the secretory i-antigen, cell culture medium was centrifuged at 3000g at 4^0C for 5 minutes and the supernatant filtered with a 0.8 μm filter, and passed through the affinity column as above.

Propagation and collection of parasites. Two *I. multifiliis* isolates, G5 and NY1, were used in this study. Each represents a different serotype based on *in vitro* immobilization, and is maintained separately by serial passage on juvenile channel catfish (25). The G5 isolate characterized as immobilization serotype D and has been described previously (4, 21). The NY1 isolate was found on infected rainbow trout from a hatchery in upstate New York (T. G. Clark, personal communication). The NY1 isolate is characterized as immobilization serotype A.

Live theronts were obtained using previously described methods (10). Five heavily infected fish with white spots over the entire body surface were placed in a beaker containing 3 liter carbon-filtered water, and trophonts were dislodged from fish skin. Free-swimming trophonts were collected by a 200-mesh sieve, transferred into about 100 mL carbon-filtered water, and allowed to develop into theronts (18–20 h at 22 °C). Theronts were passed through a 400-mesh sieve, harvested by centrifugation at 1000*g* for 2 minutes, and washed once in 50 mL carbon-filtered water. Cells were used immediately or pellets were frozen in liquid nitrogen and stored at –70 °C.

Experimental fish. Juvenile channel catfish (*Ictalurus punctatus*) (15-20 g) with no previous history of exposure to *I. multifiliis* were obtained from a local hatchery. Prior to immunization, fish were treated with formalin (25 ppm) to remove external parasites and held in a flow-through system. Fish were fed daily with commercial trout starter feed (Purina, USA). Water temperature was maintained at 20-23 ^{0}C, and water quality (NH_3, NO_2, and pH) monitored daily with standard test kits.

Immunization of channel catfish. For immunization with recombinant *Tetrahymena* live cells, each fish was injected twice intraperitoneally (i.p.) at a 14-day interval with a suspension of $1x10^6$ cells in 10 mM Tris-HCl. For immunization with purified i-antigen, each fish was injected initially i.p. with 10 μg of protein in complete Freund's adjuvant (CFA) followed by 10 μg of i-antigen in incomplete Freund's adjuvant (IFA) at a 14-day interval.

Determination of serum antibodies. The *in vitro* immobilization assay was used to determine the serum antibody response (3). Live parasites were incubated with varying dilutions of antibodies or heat-inactivated sera in 96-well plates at room temperature (22 ^{0} C) and observed under low-power magnification for cessation of swimming. The highest dilution at which complete immobilization occurred was recorded.

Challenge. Immunized fish were challenged by surface exposure to a potentially lethal dose (15000 G5 theronts or 5000 NY1 theronts per fish) of live *I. multifiliis* theronts. Each group of fish was transferred into a 3L baker with 100 mL coal-filtered water per fish. Live theronts were added, and fish were kept in challenge bakers for 2 hour with aeration. Fish were replaced into tanks supplied with individual biological filters for observation of infection and mortality.

Statistical analysis. Differences in the proportion of survivors among groups were calculated by the z test. Differences in the median days to death (MDD) among groups were calculated by a one way analysis of variance (Student-Newman-Keuls method). All calculations were done using the SigmaStat statistical program (Jandel Scientific Software, San Rafael, CA).

RESULTS

Detection of recombinant *I. multifiliis* i-antigen expressed by *T. thermophila*

Surface display of the *I. multifiliis* serotype A 48 kDa i-antigen on *T. thermophila* cells under the control of *BTU1* promoter has been detected by indirect immunofluorescence assays and reported previously (13). In this study, *T. thermophila* cells were transformed with plasmids which were enable to over-express *I. multifiliis* i-antigens of serotype A or D under the control of the *MTT* promoter. Using rabbit antibody against purified i-antigen of *I. multifiliis*, indirect immunofluorescence assays detected the recombinant i-antigen protein on the surface of *T. thermophila* cells that were transformed with the *I. multifiliis* i-antigen gene. Moreover, the expression of recombinant i-antigen proteins under the control of *MTT* promoter was strictly regulated by the present of Cd. The surface display of recombinant protein dramatically increased after cadmium induction for 6 hours (**Figures 4.1A)**, and with no detectable expression before induction **(Figure 4.1B)**.

The expression of *I. multifiliis* i-antigen by *T. thermophila* was further confirmed using Western blot analyses and *in vitro* immobilization tests. Western blot analyses using serotype-specific mouse monoclonal antibodies showed that: (1) serotype A mAb

10H3 reacted with a protein from induced MTT.G1 cells (*T. thermophila* cells over-expressing of *I. multifiliis* IAG48[G1]), and this protein had the same size of one of 2 native proteins from *I. multifiliis* NY1 isolate (**Figure 4.2A**); (2) serotype D mAb G3-61 reacted with a 46 kDa protein from induced MTT.G5A cells (*T. thermophila* cells over-expressing of *I. multifiliis* IAG52A[G5]), and this protein had the same size of native protein from *I. multifiliis* G5 isolate (**Figure 4.2B**); (3) no cross-reaction was observed.

Immobilization tests using serotype-specific mouse monoclonal antibodies showed that induced MTT.G1 cells were immobilized by mAb 10H3 (**Figure 4.3A**), induced MTT.G5A cells were immobilized by mAb G3-61 (**Figure 4.3B**), and no cross-reaction was observed. The end point titers of the immobilization by mAbs reached 1:160, and more than 90 % of recombinant *Tetrahymena* cells ceased swimming within 15 minutes.

Stability of recombinant i-antigens produced by *T. thermophila.*

After Cd induction, MTT.G1 cells were transferred into 10 mM Tris-HCl (pH7.5) buffer and kept at room temperature. Western blot analysis of the cells sampled at different time points showed recombinant i-antigen protein present in cells up to 17 days without significant degradation (**Figure 4.4**). The stability of the recombinant protein in the expression system is critical for this protein to be used for immunizing animals.

Affinity purification of recombinant i-antigens produced by *T. thermophila*

The membrane-associated and secreted forms of recombinant i-antigen of *I. multifiliis* serotype A were purified by affinity chromatography from recombinant *T. thermophila* T.G1 cell lysates and from T.sG1 cell culture supernatant respectively, using polyclonal rabbit antibody against *I. multifiliis* G5 i-antigen as ligand.

On SDS-PAGE stained with Coomassie Brilliant Blue, the sizes of the membrane-associated and secreted proteins were estimated to be 45 kDa, and 42-kDa respectively under reduced condition (**Figure 4.5**).

Serum antibody response to recombinant i-antigens

The immunogenicity of the recombinant i-antigens was tested in juvenile channel catfish. Fish were either injected i.p. with live *T. thermophila* cells expressing *I. multifiliis* i-antigens or purified recombinant i-antigens in Freund's adjuvant (see Table 4.1). Levels of serum immobilizing antibody was measured at various time points after immunization.

Fish immunized with live MTT.G1 or MTT.G5 cells developed very strong serum immobilizing antibody (up to a titer of 1:640). Most importantly, anti-sera from fish immunized with MTT.G1 only immobilized NY1 theronts (of *I. multifiliis* serotype A), and anti-sera from fish immunized with MTT.G5A only immobilized G5 theronts (of *I. multifiliis* serotype D). No cross-immobilization occurred (**Figures 4.6A and 4.6B**).

However, the recombinant i-antigens produced by T.G1 or T.sG1 induced low levels of serum immobilizing antibodies. Fish injected with the purified full-length i-antigen from T.G1 developed immobilizing antibody titers of 1:10, and those injected with live T.G1 or T.sG1 cells developed immobilizing antibody titers of 1:20. Fish injected with the purified secretory-form i-antigen from T.sG1 cells did not developed detectable immobilizing antibody (Table 4.1). Pre-immune sera and sera from control fish had no immobilizing activity.

Moreover, sera from fish immunized with recombinant *T. thermophila* live cells react serotype-specifically with both non-reduced native *I. multifiliis* i-antigens and non-reduced recombinant *I. multifiliis* i-antigens on Western blots (data not shown).

Vaccination trials

The first vaccination trial was designed to test the efficacy of the recombinant 48-kDa i-antigen produced in *T. thermophila* under the control of *BTU1* promoter.

Groups of 30 fish were injected intraperitoneally (i.p.) with purified i-antigen plus Freund's adjuvant, or live cells of *T. thermophila* following the procedure in Table 4.1, and then challenged with *I. multifiliis* NY1 parasites (15,000 theronts per fish). Control fish injected with BSA or live *Tetrahymena* cells expressing the *neo* gene developed visible signs of infection within 5 days after challenge. Fish vaccinated with purified i-antigen or live *Tetrahymena* expressing G1 i-antigen were less severely infected than controls until 2 weeks after challenge at which time they became heavily infected as a result of the second round of *I. multifiliis* proliferation. The time course of survival is shown in **Figure 4.7**. A one-way ANOVA indicated that there was a significant difference ($p < 0.01$, Student-Newman-Keuls Method) in the mean days to death (MDD) among fish in test and control groups (**Table 4.2**). These results indicate that recombinant *Ichthyophthirius* 48 kDa i-antigen produced in transformed *Tetrahymena* confers a degree of protective immunity against parasite challenge, although absolute levels of protection were relatively low (13.3%). Initially, we thought that the low level of protection in this case resulted from the fact that NY1 (as well as G1) produces more than a single i-antigen, and would require a multivalent vaccine that combined both the 48- and 60-kDa antigens to achieve full protection. However, in a recently completed vaccine trial

designed to test cross-protection between strains, we realized that the NY1 isolate is significantly more virulent than the G5 (Wang, Clark, and Dickerson, unpublished), and that our challenge dose might have been unrealistically high.

Our next vaccination trial was designed to evaluate MTT.G1 and MTT.G5 (i.e.: *T. thermophila* expressing *I. multifiliis* 48-kDa or 52-kDa i-antigen under the control of MTT promoter) as live vaccine.

Juvenile channel catfish were immunized with live cells of MTT.G1 or MTT.G5 by i.p. injection following the procedure in **Table 4.3**, and then groups of 30 fish were challenged with *I. multifiliis* NY1 (5000 theronts per fish) or G5 parasites (15000 theronts per fish) respectively. Negative control fish immunized with live *Tetrahymena* cells expressing the *neo* gene, and positive control fish were injected i.p. with NY1 or G5 live theronts (5000 cells per fish). The result (**Figure 4.8A and 4.8B**) showed that: when challenged with NY1, 100 % of fish injected with MTT.G1 cells survived, and 96.7 % of fish injected with NY1 theronts survived; when challenged with G5, 80 % of fish injected with MTT.G5 cells survived G5 challenge, and 90 percent of fish injected with G5 theronts survived. All negative control fish died by day 14 after challenge.

These results clearly show that recombinant *T. thermophila* expressing *I. multifiliis* i-antigens under the control of a *MTT* promoter have great potential as live vaccine against *I. multifiliis* infection.

DISCUSSION

The *T. thermophila* experimental system is now well equipped with the genetic tools for mass DNA-mediated transformation (14), gene replacement (16), gene knockout

(23, 29, 30), gene expression on an episomal, high-copy number vector (15, 27), and most recently germ-line transformation (2). Because it is a related species, *Tetrahymena* offers distinct advantages for expression of *I. multifiliis* genes over more conventional expression systems such as *E. coli* or yeast (17). For example, *Tetrahymena* and *Ichthyophthirius* both use a non-standard genetic code in which the conventional UAA and UAG stop codons encode glutamine (8, 18, 19, 26). Thus, while expression of *I. multifiliis* genes (or cDNA) is relatively straightforward in *Tetrahymena,* it requires additional genetic manipulation (e.g. *in vitro* mutagenesis, or suppression) in non-ciliate systems. Similarly, faithful protein targeting, as well as secondary protein modification (which often plays a role in antigenicity), is more likely to occur in a closely related species.

The successful expression of an *I. multifiliis* gene in *T. thermophila* should allow the production of large amounts of antigen in a purified form, at relatively low cost. In addition to its use in the production of purified antigens, transformed *T. thermophila* can also be used as a live vaccine applied either as a bath or by injection into the animal host. We have found that up to $1x10^6$ *T. thermophila* cells can be injected into the peritoneal cavity of channel catfish fingerlings weighing 10-15 grams, where they survive for several days without causing adverse effects (Wang and Dickerson, unpublished results). Expression of native *Ichthyophthirius* proteins on the surface of *Tetrahymena* may provide the most efficient means of exposing fish to antigens, short of infection with the live parasite itself. The use of *T. thermophila* as an expression system could have widespread implications in the development of fish vaccines.

The methods used in this study (namely, homologous recombination into the *BTU1* locus and negative selection by growth in taxol) allowed us to generate large numbers of stably transformed cell lines with relative ease. Nevertheless, while transformants provided more than enough material for vaccine trials, the level of i-antigen expression in these cells was less than anticipated given the locus into which the gene was cloned (namely, *BTU1*). Tubulin accounts for several percent of total cell protein in *T. thermophila* and it was assumed that the level of protein synthesis directed by a transgene cloned into the *BTU1* locus would be much higher than what was actually seen (<0.03% of total cell protein).

Channel catfish vaccinated with recombinant i-antigen (either in a purified form or on the surface of transformed *Tetrahymena*) produced serum antibodies that not only immobilized *Ichthyophthirius in vitro*, but also recognized the non-reduced *Ichthyophthirius* i-antigen on Western blots. Using the inducible promoter from *MTT*, the expression level of i-antigen was increased dramatically (over 30-fold compared with using *BTU1* promoter), and the protection efficacy of the recombinant Tetrahymena was also increased to a very high level.

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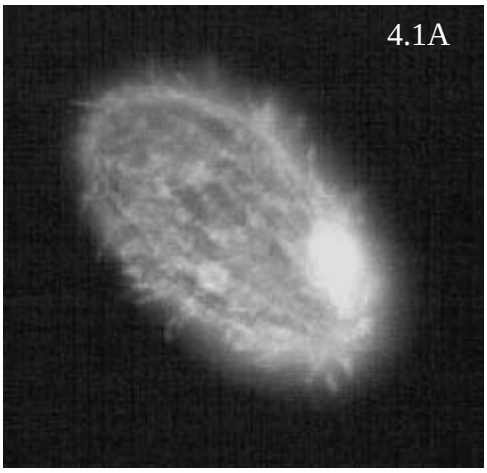
Figure 4.1. **Immunofluorescent analyses of recombinant *Tetrahymena* expressing *I. multifiliis* i-antigens on cell surface.** *T. thermophila* strain CU522 was transformed with the plasmid pBICH3 carrying the encoding sequence of *I. multifiliis* G5 i-antigen gene. The fragments *btu1/MTT5'- IAG52A[G5]* was used to construct inducible expression transformants, and the resulting transformant was named MTT.G5A. The transformant was grown to log phase, and then cadmium chloride were added to MTT.G5A at a concentration of 5 μg/ml to induce expression of *I. multifiliis* i-antigen. Cells were fixed, and probed with rabbit antibody against *I. multifiliis* G5 i-antigen. The cell shown in **4.1A** was transformants MTT.G5A induced with cadmium for 6 hours; the cell in **4.1B** was before induced with cadmium.


4.1A

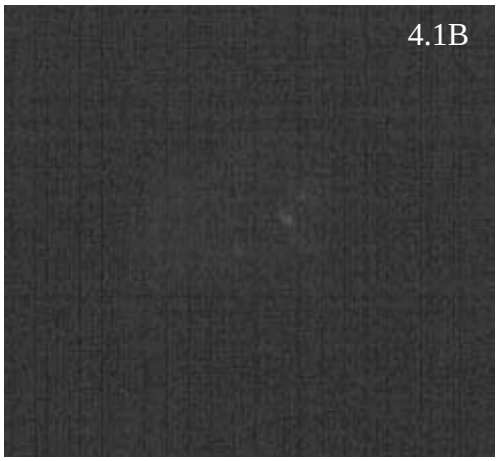
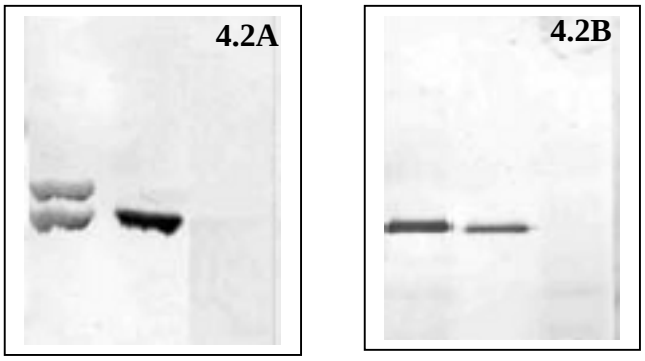
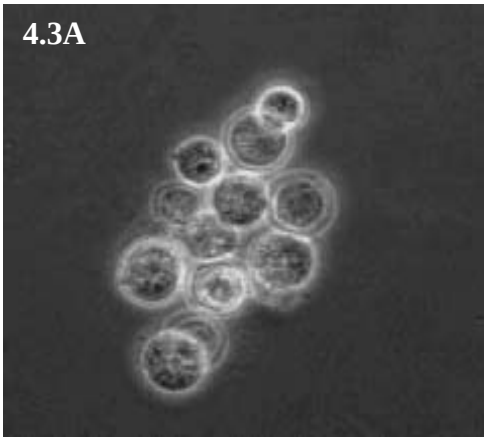

4.1B

Figure 4.2. Western blot analysis of expression of *I. multifiliis* i-antigens in *T. thermophila*. The recombinant *T. thermophila* were grown to the log phase, and then cadmium chloride was added at the concentration of 5ug/ml to induce the expression of *I. multifiliis* i-antigens. Each lane was loaded with 5×10^4 cells except for lane 1. The blot **4.2A** had membrane proteins of *I. multifiliis* NY1 (lane 1), MTT.G1 cells induced for 6.5 hours by cadmium chloride (lane 2), and MTT.G1 cells right before adding Cadmium chloride (lane 3). The blot **4.2B** had purified i-antigen of *I. multifiliis* G5 (lane 1), MTT.G5A cells induced for 6.5 hours by cadmium chloride (lane 2), and MTT.G5A cells right before adding Cadmium chloride (lane 3). The blot **4.2A** was probed with mAb 10H3 (serotype A), and blot **4.2B** was probed with mAb G3-61 (serotype D).



Lane: 1 2 3 1 2 3

Figure 4.3. Immobilization of *T. thermophila* over-expressing *I. multifiliis* i-antigens by mouse monoclonal immobilizing antibodies. The expression of *I. multifiliis* i-antigens on the surface of *T. thermophila* was confirmed by immobilization test using serotype-specific mouse monoclonal immobilizing antibodies. MTT.G1 cells were immobilized by serotype A mAb 10H3 up to 1:320 (4.3A), and MTT.G5.1 cells were immobilized by serotype D mAb G3-61 up to 1:160 (4.3B). No cross-reaction was observed.


4.3A

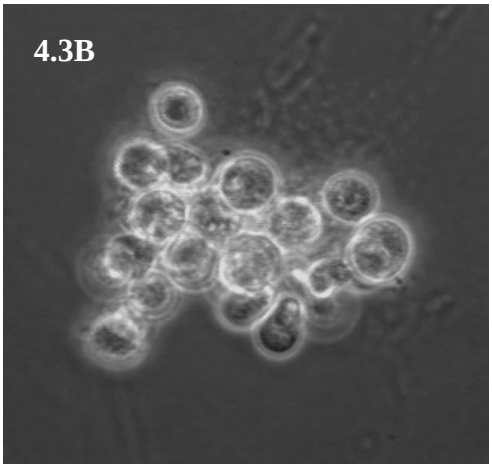
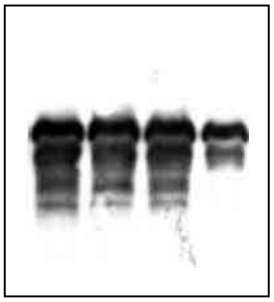

4.3B

Figure 4.4. Stability of recombinant i-antigens produced by *T. thermophila.*

The recombinant *T. thermophila* MTT.G1 was grown to the log phase, and then induced with cadmium chloride (5ug/ml) for 12 hours. Induced MTT.G1 cells were transferred into 10 mM Tris-HCl (pH7.5) buffer and kept at room temperature. At different time points, a sample of starved cells was collected. All samples were resolved on 10 % SDS-PAGE. Each lane was loaded with 5 x 10^4 cells. Western blot was probed with rabbit antibody against *I. multifiliis* i-antigen. The result showed that the recombinant i-antigen protein present in MTT.G1 cells up to 17 days without significant degradation.



0h 4h 4d 17d

Time of starving

Figure 4.5. Analysis of affinity purified recombinant i-antigen expressed by recombinant *T. thermophila*. Recombinant *I. multifiliis* G1 i-antigens produced by *T. thermophila* were purified by affinity chromatography using rabbit antibody against *I. multifiliis* G5 i-antigen. The reduced protein samples were resolved by 10 % SDS-PAGE and stained with Coomassie Bright Blue. The membrane associated recombinant i-antigen (Lane 1) was purified from T.G1 membrane protein solubilized in 1% (v/v) Triton X-114, and the secretory form i-antigen (Lane 2) was purified from the cell culture medium of T.sG1.

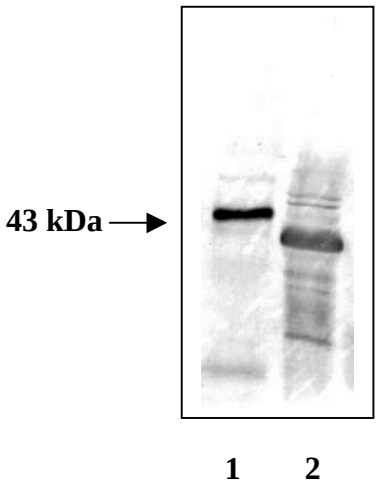
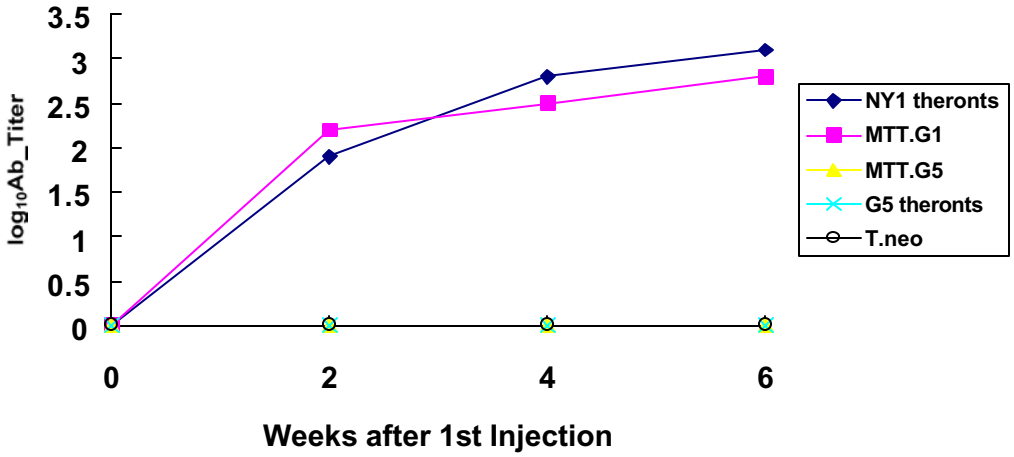

43 kDa
1
2

Figure 4.6. Serum immobilizing antibody response. Serum samples were collected from immunized channel catfish at various time points, and the immobilization assay was used for determining serum immobilizing antibody levels against: (4.6A) *I. multifiliis* isolate NY1 (serotype A), and (4.6B**)** *I. multifiliis* isolate G5 (serotype D). The end point titers are the inverse of the highest dilution in which all of *I. multifiliis* live theronts were immobilized. Channel catfish immunized with NY1 theronts (5000 cells per fish) or MTT.G1 (10^6 cells per fish) developed high serum immobilizing serum antibody titers against *I. multifiliis* NY1. Fish immunized with G5 theronts (5000 cells per fish) or MTT.G5A (10^6 cells per fish) developed high serum immobilizing serum antibody titers against *I. multifiliis* G5. Fish sera did not immobilize heterologous *I. multifiliis* cells. The antibody titer maintained 640 to 1280 at week 6 when the challenge was performed.



4.6A. Serum immobilizing antibody response against *I. multifiliis* NY1.

4.6B. Serum immobilizing antibody response against *I. multifiliis* G5.

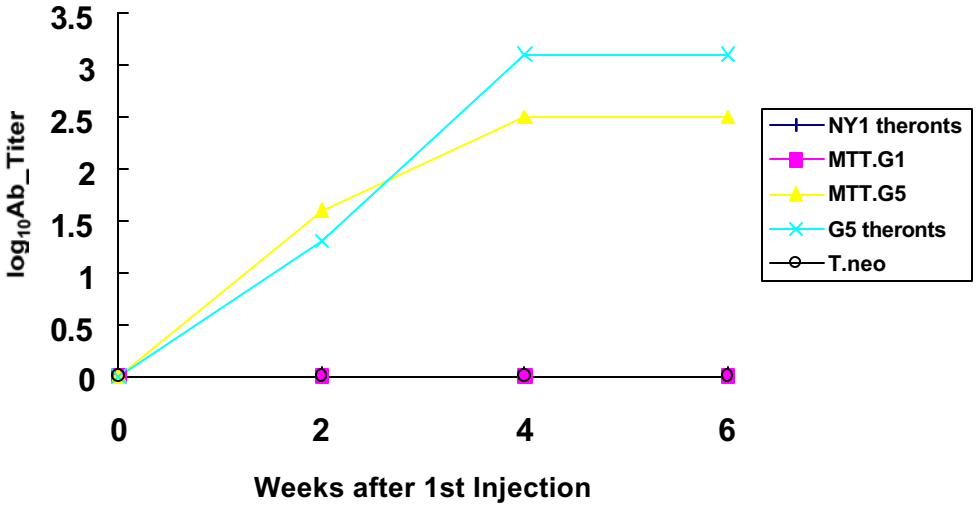


Figure 4.7. Vaccination trial 1. Channel catfish were immunized with (1)*Tetrahymena* live cells expressing i-antigen on cell surface, T.G1 live cell (10^6 cells per fish), (2) *Tetrahymena* live cells expressing secretory form i-antigen, T.sG1 live cell (10^6 cells per fish), (3) purified membrane associated i-antigen produced by *Tetrahymena*, T.G1 i-antigen (10 µg per fish), (4)) purified secretory form i-antigen produced by *Tetrahymena*, T.sG1 i-antigen (10 µg per fish), (5) *Tetrahymena* live cell not producing i-antigen, T.neo live cell (10^6 cells per fish), (6) Bovine serum albumin(BSA, 10 µg per fish).

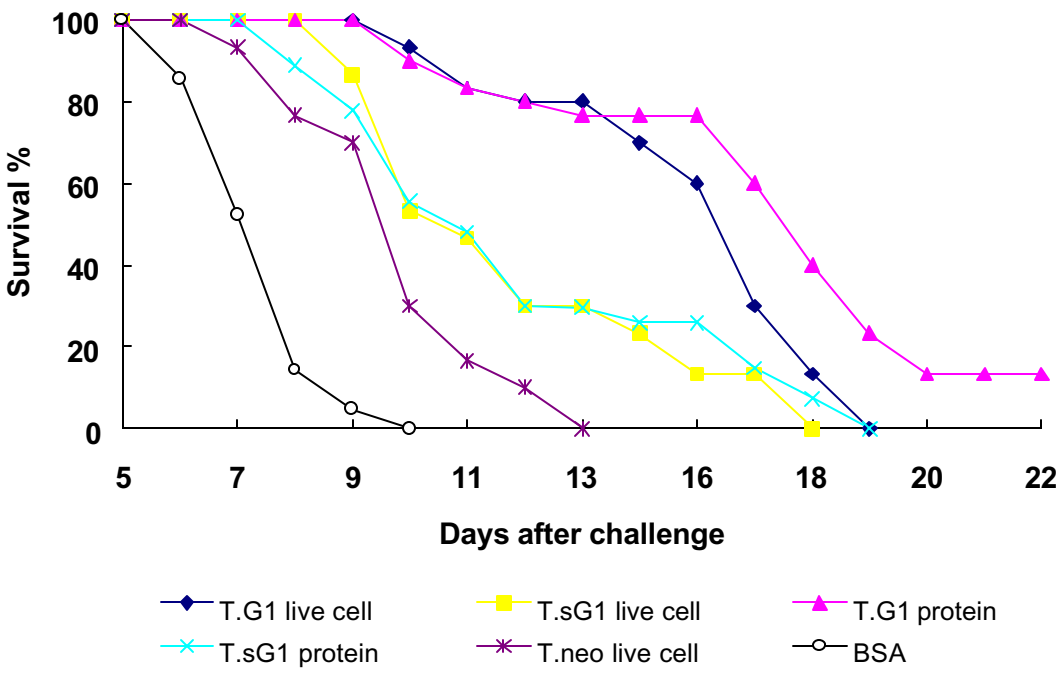
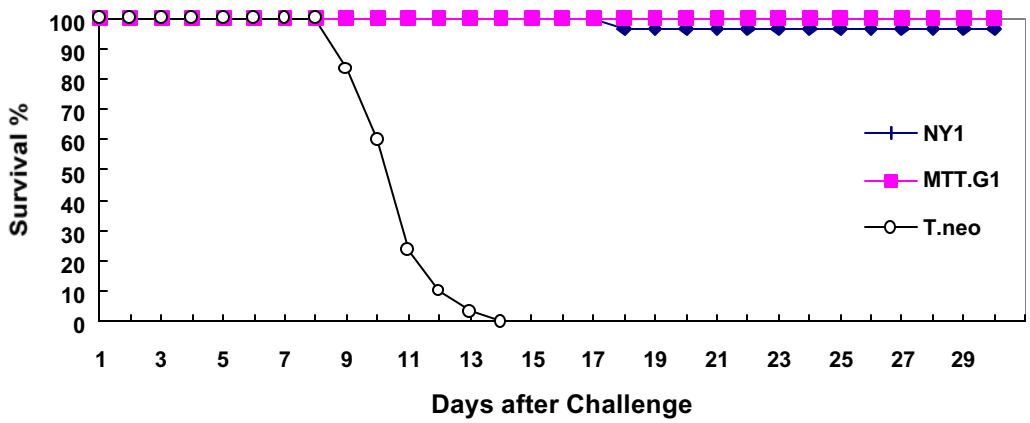

100
80
60
40
20
0
Survival %
5
7
9
11
13
16
18
20
22
Days after challenge
T.G1 live cell
T.sG1 live cell
T.G1 protein
T.sG1 protein
T.neo live cell
BSA

Figure 4.8. Vaccination trial 2. Channel catfish in test groups were injected i.p. with live cells (10^6 cells per fish) of MTT.G1 or MTT.G5A. Fish injected i.p. with *I. multifiliis* live theronts (5000 cells per fish) were used as the positive controls, and fish injected i.p. with T.neo were used as the negative controls. Groups of 30 immunized fish were challenged with live theronts of *I. multifiliis*. The survival percentage was recorded daily.



4.8A. Challenged with I. multifiliis isolate NY1

4.8B. Challenged with I. multifiliis isolate G5

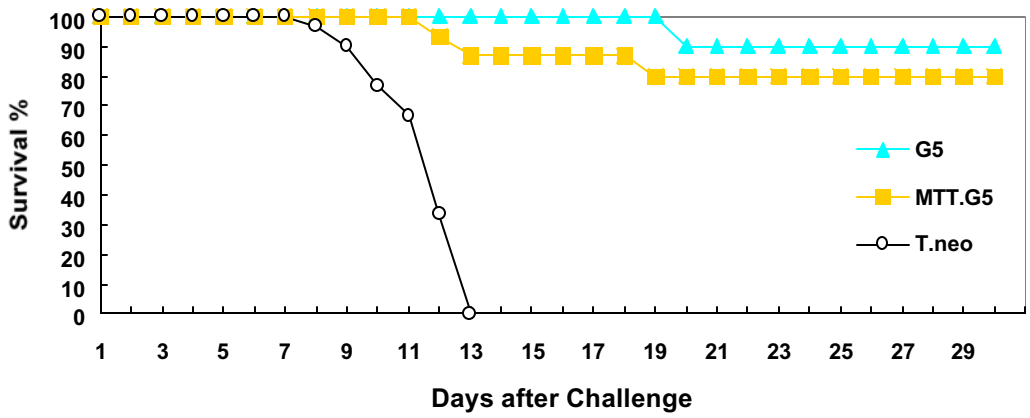


Table 4.1. The genes encoding the i-antigens of *I. multifiliis* serotype A and D.

Gene name	Product (i-antigen)	Name of Transformant	*I. multifiliis* Serotype	GenBank id	Reference
IAG48[G1]	48 kDa (Full-length)	T.G1	A	AF140273	(6)
C19/IAG48[G1]	48 kDa (C-deleted)	T.sG1	A	N.A.	(4)
IAG52A[G5]	52 kDa	MTT.G5A	D	AF324424	

These genes were used to transform *T. thermophila* cells by biolistic bombard. The gene *IAG48[G1]* was used to target the full-length 48 kDa i-antigen of *I. multifiliis* serotype A on the cell surface of *T. thermophila*. The gene *C19/IAG48[G1]* is derived from *IAG48[G1]* and lacks the coding region for a 19-peptide at the C-terminus that contains the putative GPI addition site. The *C19/IAG48[G1]* was used to express the secretory 48 kDa i-antigen. The gene *IAG52-1[G5]* encodes the 52-kDa i-antigen of *I. multifiliis* serotype D.

Table 4.2. Vaccination trial 1.

Immunogen[a]	Dose[b]	No. of fish Challenged	Survival %	MDD ± SD	Serum Ab before challenege
T.G1 live cell	$1x10^6$ cells	30	0	15.97 ± 2.91	1:20
purified T.G1 i-antigen	10 µg + CFA / IFA	30	13.3	16.35 ± 3.47	1:10
T.sG1 live cell	$1x10^6$ cells	30	0	12.30 ± 3.02	1:20
purified T.sG1 i-antigen	10 µg + CFA / IFA	27	0	12.11 ± 3.33	0
T.neo live cell	$1x10^6$ cells	30	0	10.30 ± 2.07	0
BSA	10 µg + CFA / IFA	21	0	7.57 ± 1.03	0

[a] Immunogens are: (1)*Tetrahymena* live cells expressing i-antigen on cell surface, T.G1 live cell, (2) *Tetrahymena* live cells expressing secretory form i-antigen, T.sG1 live cell, (3) purified membrane associated i-antigen produced by *Tetrahymena*, T.G1 i-antigen, (4)) purified secretory form i-antigen produced by *Tetrahymena*, T.sG1 i-antigen, (5) *Tetrahymena* live cell not producing i-antigen, T.neo live cell, (6) Bovine serum albumin(BSA).

[b] All fish were immunized twice with the same dose at an interval of 14 day and all fish were challenge with live theronts of *I. multifiliis* NY1 (15000 cells per fish).

Table 4.3. Vaccination trial 2.

Immunogen[a]	Dose[b]	No. of fish Challenged	Challenged with	Survival % [c]	MDD ± SD[d]	Serum Ab before challenge
NY1 theronts	$5x10^3$ cells	30	5000 NY1 theronts	96.7	18	1:1280
MTT.G1	$1x10^6$ cells	30	5000 NY1 theronts	100	N.A.	1:640
T.neo	$1x10^6$ cells	30	5000 NY1 theronts	0	11.0 ± 1.3	0
G5 theronts	$5x10^3$ cells	30	15000 G5 theronts	90	20	1:1280
MTT.G5.1	$1x10^6$ cells	30	15000 G5 theronts	80	14.7 ± 3.4	1:640
T.neo	$1x10^6$ cells	30	15000 G5 theronts	0	11.6 ± 1.4	0

[a] Fish were immunized with *Tetrahymena* live cells over-expressing *I. multifiliis* i-antigens, MTT.G1 or MTT.G5.1 by injection i.p.. Positive control fish were immunized with *I. multifiliis* live theronts by injection i.p..

[b] All fish were immunized twice with the same dose at an interval of 14 days.

[c] Statistical analyses by z-test showed that: there was not a significant difference in the survival percentage between (1) the group injected with MTT.G1 cells and the group injected with NY1 theronts ($p = 0.99$), or (2)) the group injected with MTT.G5A cells and the group injected with G5 theronts ($p = 0.47$); but there were significant differences in the survival percentage between the test (or positive control) groups and the negative control groups ($p = 0.00$).

[d] Statistical analysis by one-way ANOVA indicated that there was a significant difference ($p < 0.01$, Student-Newman-Keuls Method) in the MDD among fish in test and control groups.

CHAPTER 5

OLIGODEOXYNUCLEOTIDES CONTAINING A CPG MOTIF ENHANCE PROTECTIVE IMMUNITY ELICITED BY A PROTEIN SUBUNIT VACCINE AGAINST *ICHTHYOPHTHRIUS MULTIFILIIS*[1]

[1]Wang, Xuting and Harry W. Dickerson. To be submitted to *Journal of Fish Diseases.*

ABSTRACT

A vaccine comprised of the purified immobilization antigen (i-antigen) protein of *Ichthyophthirius multifiliis* induces protective immunity in juvenile channel catfish when administered with Freund's adjuvant. In this study, we tested a CpG oligodeoxy-nucleotide (ODN) for the ability to stimulate humoral responses to i-antigen, and to elicit protective immunity. The results showed that a subunit i-antigen vaccine administered with CpG ODN conferred comparable immunity to that seen with i-antigen vaccine administered with Freund's adjuvant.

Keywords: *Ichthyophthirius multifiliis,* channel catfish (*Ictalurus punctatus*), immobilization antigen, immunization, subunit vaccine, Immunostimulatory oligodeoxynucleotides, CpG, adjuvant.

INTRODUCTION

Ichthyophthirius multifiliis (Fouquet) is the most pathogenic ciliated protozoan parasite of fish. It is the etiological agent of "white spot" disease and infects a wide range of freshwater fish. Morbidity in farmed fish species such as channel catfish (*Ictalurus punctatus*) often reaches 100%(8). Current strategies for the control and treatment of the parasite are dependent upon the use of chemicals such as formalin. These compounds kill only the free-swimming infective (theront) and replicative (tomont) stages, but are ineffective against the fish-associated (trophont) stage (8). Also, chemical treatments impose additional stress on infected fish, which may increase mortality. Some of the most effective chemicals, such as malachite green, are prohibited from use on fish intended for human consumption.

Prophylactic immunization against *Ichthyophthirius* would be a practical alternative to chemical treatment, based on the fact that fish surviving infection develop immunity to subsequent parasite challenge (4, 7, 8, 10, 15). We have shown that surface proteins purified from *I. multifiliis*, referred to as immobilization antigens, confer protective immunity to juvenile channel catfish(19). These purified proteins initiate protective immunity only when presented in context with an appropriate inflammatory response such as that stimulated with adjuvants like Freund's adjuvant (2), X. Wang and H. W. Dickerson, unpublished data). Freund's complete adjuvant (FCA) is widely used in research, but often causes severe inflammatory lesions that result in chronic granulomas, abscesses, and tissue damage. Injected intraperitoneally, it causes peritonitis and adhesions. Because of these adverse side-effects, an alternate adjuvant is needed that elicits an appropriately modulated inflammatory response with minimal

tissue damage and that is easy to administer in aquaculture (preferably orally or by immersion).

Immunostimulatory oligodeoxynucleotides containing the cytosine-guanine nucleotide motif (CpG ODNs) have promise as adjuvants for both DNA and protein vaccines. In mammalian systems, CpG ODN stimulate cytokine production by cells of the innate immune system, and act as potent B-cell mitogens when used at high concentration *in vitro* (12). *In vivo*, CpG ODNs at low concentration induce strong antibody responses against co-administered antigens. In goldfish, CpG ODNs have recently been shown to stimulate antibody production against a model antigen (β-galactosidase) suggesting that the receptors for bacterial DNA are evolutionalarily conserved (11). The low toxicity of CpG ODNs relative to Freund's (21) makes them an attractive choice as potential adjuvants in fish vaccines. In this study, we sought to determine whether CpG ODNs would serve as effective adjuvants when administered in combination with *Ichthyophthirius* i-antigen protein.

MATERIALS AND METHODS

Antigen and adjuvants. The antigen used in this study was the i-antigen from the *I. multifiliis* G5 isolate. The protein was affinity purified with monoclonal antibody as described previously, and solubilized in phosphate buffered saline (PBS, 135 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.5) (14, 20).

Immunostimulatory oligodeoxynucleotide containing CpG motif (CpG ODN) (ODN 1990) and a negative control oligodeoxynucleotide (non-CpG) (ODN 1982) were supplied by Dr. Heather Davis (Loeb Research Institute, University of Ottawa, Canada).

Vaccines were prepared as described previously (3). Each ODN was dissolved in TE (10mM Tris 1mM EDTA buffer, pH 7.0), and added to protein antigen solution in a volume less than 10% of the total final volume. Antigen solution with ODN was combined with mineral oil (incomplete Freund adjuvant, Sigma, USA) at a 1:1 (v/v) ratio and emulsified to achieve a final i-antigen concentration of 100 μg/mL and ODN concentration of 200 μg/mL. The emulsion was used immediately for immunization.

Experimental fish. Channel catfish (*Ictalurus punctatus*) fingerlings (10-15 g) with no previous history of exposure to *I. multifiliis* were obtained from a local hatchery. Prior to immunization, fish were treated initially with formalin (25 ppm) to remove any external parasites and kept in a flow-through system until use. Fish were fed daily with commercial trout starter feed (Purina, USA). Water temperature was maintained at 20-23 ^{0}C, and water quality (NH_3, NO_2, and pH) monitored daily with standard test kits.

Propagation and collection of parasites.

The *Ichthyophthirius multifiliis* G5 isolate used in this study has been characterized previously (6, 13), and is currently maintained by serial passage on juvenile channel catfish (*Ictalurus punctatus*) (16). Five heavily infected fish were placed in a beaker containing 3 L carbon-filtered water, and trophonts were dislodged from their skin. Free-swimming trophonts were collected using a 200-mesh sieve, then transferred into 100 mL of carbon-filtered water and allowed to develop into theronts (18–20 h at 22 °C). Theronts were passed through a 400-mesh sieve, collected by centrifugation at 1000 *g* for 2 minutes, and washed once in 50 mL of carbon-filtered water. Cells were used immediately or pellets were flash frozen by rapid immersion in liquid nitrogen and stored at –70 °C.

Vaccination procedure. Channel catfish were distributed into four 38-liter aerated aquaria with conditioned biological filtration at a density of 35 fish per aquarium. Fish were immunized by intraperitoneal injection using a 1 mL syringe fitted with a 26 gauge needle. Each fish was given 0.1 mL vaccine emulsion. Fish in group 1 were immunized with i-antigen with CFA (complete Freund's adjuvant, positive control). Fish in group 2 were immunized with i-antigen with CpG ODN. Fish in group 3 were immunized with i-antigen with non-CpG ODN (CpG ODN negative control). Fish in group 4 were immunized with BSA (bovine serum albumin) with CpG ODN (protein negative control).

Challenge. Fish were challenged by surface exposure to 15,000 theronts/fish following a standard protocol (9). Mortalities were recorded daily in each group until all fish died or recovered from infection.

Collection of cutaneous mucus and serum samples. Serum and cutaneous mucus samples were collected from six channel catfish in each group at weekly intervals for five weeks after immunization. Fish were anesthetized with 100-200 ppm MS-222 (tricaine methane sulfonate, Argent Chemical Laboratories, Redmond, WA) and gently wiped over both lateral surfaces with cotton swabs. Each mucus-saturated swab was soaked in 0.1 mL of ice-cold PBS. The PBS-mucus solution was centrifuged at 14,000xg for 5 min at 4 ^{0}C and the supernatant collected. Mucus samples were normalized to a protein concentration of 100 µg/mL, supplemented with 1% BSA, and stored at –80 ^{0}C until use. Blood samples were taken from anesthetized fish immediately after mucus sample collection. Bleeding, preparation, and heat inactivation of sera were carried out as described previously (5).

Immobilization assays. Assays were carried out to determine serum immobilizing antibody level as previously described（5）with minor modification. Briefly, serum samples were prepared in 96-well microtitre plates as a series of doubling dilutions in 50% PBS. Each well contained 50 µL diluted serum. Live theronts (100~200) in 50 µL of carbon-filtered water were added to each well and incubated at room temperature (RT, 20-22 ^{0}C). Immobilization was determined by observation under a dissection microscope, and titers were expressed as the inverse of the highest dilution in which all of theronts were immobilized after incubation for 30 minutes at RT. Pre-immune serum samples were used as negative controls.

Detection of mucus antibodies by ELISA. To absorb antigen to plates, individual wells of ELISA plates (Falcon 3911Microtest III, Becton Dickinson, USA) were filled with 50 µL of purified i-antigen (20 µg/mL) in 25 mM sodium acetate buffer (pH 7.5) and incubated overnight at 4 ^{0}C. Control wells were coated with 2% (w/v) BSA. Non-specific protein binding was blocked by overnight incubation at 4 ^{0}C with 100 µL of 5% (w/v) BSA in Tris buffered saline with Tween 20 (TBST; 20 mM Tris-HCl, 50 mM NaCl, 0.05% v/v Tween 20, pH 7.5). Mucus samples (50 µl per well) were added to the plates and incubated for 1 hour at RT. Following three washes with TBST, mouse mAb anti-catfish Ig coupled to alkaline phosphatase (1:1000 dilution in 2% w/v BSA in TBST) was added (50 µL/well) and incubated for 1 hour at RT. After three washes with TBST, substrate p-nitrophenylphosphate was added, and plates were incubated for 1 hour in the dark at RT. The reaction was stopped with 4 N NaOH and plates were read at 405 nm with ELISA reader.

Statistical Analyses. Differences in the mean days to death (MDD) among groups were calculated by the one way analysis of variance (ANOVA). Differences in the proportion of survivors among groups were calculated by the z test. All calculations were done using the SigmaStat statistical software (Jandel Scientific Software, CA, USA).

RESULTS

Effect of adjuvants on humoral immobilizing antibody responses

Serum and mucus samples were collected from six immunized channel catfish in each group at various time points to determine the level of the humoral antibody against the immobilization antigen.

Antibody responses against the i-antigen were measured by immobilization of live parasites incubated in diluted, heat-inactivated sera. Channel catfish immunized with i-antigen in combination with CFA or i-antigen in combination with CpG ODN had high serum antibody titers against the i-antigen (Figure 5.1). The titers ranged from 160 to 640. Specific antibody was detected at week 2, reached the highest level at week 3, and maintained a high level through week 5, the last point at which sera were collected. Fish immunized with i-antigen with non-CpG ODN produced serum immobilizing antibodies with a titer of 1:80 at week 2, but no antibodies were detected at week 4. Fish injected with BSA with CpG ODN did not produce serum immobilizing antibodies.

Mucus antibodies against the i-antigen were not at a sufficiently high level to immobilize live parasites, but were detected by ELISA, which is consistent with our previous studies (19). As seen in Figure 5.2, fish immunized with either i-antigen with

CFA or i-antigen with CpG developed specific mucus antibodies against the *Ichthyophthirius* G5 i-antigen.

Effect of adjuvants on protective immunity.

On day 36 after injection, 30 fish in each of the four treatment groups were challenged by surface exposure to 15,000 live theronts per fish. We found that all negative control fish injected with BSA with CpG or i-antigen with non-CpG became heavily infected by 6 days and died within 13 days after challenge. In contrast, fish injected with i-antigen with CFA or i-antigen with CpG developed visible signs of infection at only 10 days and had a qualitatively lower parasite load. Forty percent of fish immunized with i-antigen with CFA survived, and 33.3 % of fish immunized with i-antigen with CpG ODN survived (Figure 5.3). Protection resulting from a single injection with i-antigen and either CFA or CpG ODN were comparable, but was less than that seen following two-injections of i-antigen in Freund's adjuvants (72 %, see (20)).

Statistical analysis using the z-test showed that there was no significant difference in the survival percentage between fish immunized with i-antigen with CFA and fish immunized with i-antigen with CpG ($P = 0.787$). There was a significant difference, however, between the survival of groups vaccinated with i-antigen with CFA or CpG and that of groups injected with i-antigen with non-CpG or BSA with CpG ($p<0.001$). The mean-days-to-death (MDD) of fish that died in groups immunized with i-antigen with CFA or CpG was significantly greater ($p < 0.001$, Kruskal-Wallis test ANOVA) than the MDD of fish immunized with i-antigen with non-CpG or BSA with CpG (Table 5.1). The statistical analysis showed that oligodeoxynucleotides containing CpG motif provided the same degree of immune stimulation as CFA.

DISCUSSION

Vaccines have become an important tool in the prevention of fish diseases in aquaculture. While many immunogens are effective alone, particularly those (such as LPS) that elicit innate immunity by themselves, most protein antigens require the use of adjuvants. Some adjuvants are thought to act by creating depots at the site of inoculation from where the antigen is slowly released and presented to the immune system over a prolonged period of time. Some adjuvants (such as CpG ODN) are non-specific immune activators that can be used to augment immune responses in an antigen-specific fashion when included in vaccines. Other adjuvants have both these properties, such as Freund's complete adjuvant in which the mineral oil acts as an antigen depot and the mycobacterial cell wall components act as a non-specific immune activators (21).

Adjuvants play a role in determining the type of immune response generated, which is essential for successful vaccination. A strong cell-mediated immunity associated with a Th1 type immune response, is necessary for the control of intracellular pathogens whereas strong humoral immunity, associated with both Th1 and Th2 type immune responses, appears to be essential for the control of extracellular pathogens.

Complete Freund's adjuvant has been an important model adjuvant commonly used in animal research, as it augments both humoral and cellular immune responses to a wide range of antigens. However, its side effects in fish include fecundity problems, growth reduction and melanisation and adhesions in the peritoneal cavity causing downgrading at the time of harvest. Thus, the search for alternative effective adjuvants is currently an important area in fish vaccine research.

In mammals, CpG ODNs are known to promote Th1 type immune responses with the secretion of IFN-γ, TNF-α and IL-12 cytokines, opsonizing antibodies, such as those of the IgG2a isotype, and strong CTL induction. They can be used ether in combination with mineral oil or water.

Specific in vivo cell-mediated immune responses in fish have been shown by allograft rejection, graft-versus-host reaction (GVHR) and delayed hypersensitivity reaction (DTH). In vitro studies also showed specific cell-mediated cytotoxicity against allogeneic target cells. Recently, two types of catfish alloantigen-dependent cytotoxic T cells were cloned from PBL from a fish immunized in vivo and stimulated in vitro with the allogeneic B cell line 3B11. Their recognition and cytotoxic mechanisms were characterized. The first type of CTL (group I) shows strict alloantigen specificity, i.e., they specifically kill and proliferate only in response to 3B11 cells. The second type (group II) shows broad allogeneic specificity, i.e., they kill and proliferate in response to several different allogeneic cells in addition to 3B11 (23). These *in vivo* and *in vitro* experiments strongly suggest the presence of cytotoxic T cells in fishes.

Many studies have confirmed the presence of functional homologues of mammalian cytokines in fish. A number of fish cytokine genes have been sequenced, including interleukin-1b, transforming growth factor-b, fibroblast growth factor (FGF) and several chemokines. Also, it is clear from hybridization studies using mammalian cytokine probes that a number of other cytokine genes are likely to be present in fish, such as interferon and erythropoietin (17).

Mammalian interleukin-1 (IL-1) has been postulated as an immune adjuvant. It has a wide range of biological activities centrally involved in the genesis and

maintenance of immune and inflammatory responses. Yin and Kwang demonstrated that the C-terminal of 162 amino acids of carp IL-1β precursor did enhance the antibody response to a specific bacterial antigen. In contrast to the side-effects observed in mammals caused by IL-1, such as high fever, no obvious physiological side-effcts were observed as a consequence of injecting the recombinant IL-1β polypeptide into carp. These results reveal the potential application of fish IL-1β in the role of immuno-adjuvant in fish vaccination (22).

The activation of innate immune responses by genomic DNA from bacteria and several non-vertebrate organisms represents a novel mechanism of pathogen recognition. The CpG-dependent mitogenic activity of DNA from the protozoan parasite *Babesia bovis* for bovine B lymphocytes has been demonstrated (1). Recently, activation of macrophages by DNA from the protozoan parasites *B. bovis, Trypanosoma cruzi*, and *T. brucei* was tested. The result showed that DNA from *Escherichia coli* and all three parasites stimulated B-lymphocyte proliferation and increased macrophage production of interleukin-12 (IL-12), tumor necrosis factor alpha (TNF-α), and nitric oxide (NO) (18). The recognition of protozoan DNA by B lymphocytes and macrophages may provide an important innate defense mechanism to control parasite replication and promote persistent infection.

Our results show that oligodeoxynucleotides containing CpG motif provide the same degree of immune stimulation as Freund's adjuvant without the obvious side effects, and indicate that CpG ODN may be a safe and highly effective adjuvant for fish vaccines.

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Figure 5.1: Serum Antibody Response. The serum samples were collected from immunized channel catfish at various time points and serum antibody levels were determined by an immobilization assay. The end point titers are the inverse of the highest dilution in which all of *I. multifiliis* live theronts were immobilized. Channel catfish immunized with i-antigen with CFA or i-antigen with CpG had high serum immobilizing serum antibody titers ranging from 160 to 640 beginning 2 weeks after the first injection. Fish injected with i-antigen with non-CpG developed serum immobilizing antibody. The antibody titer reached up to 1:80 at week 2, but decreased rapidly by week 4. Fish injected with BSA with CpG didn't produce immobilizing antibody.

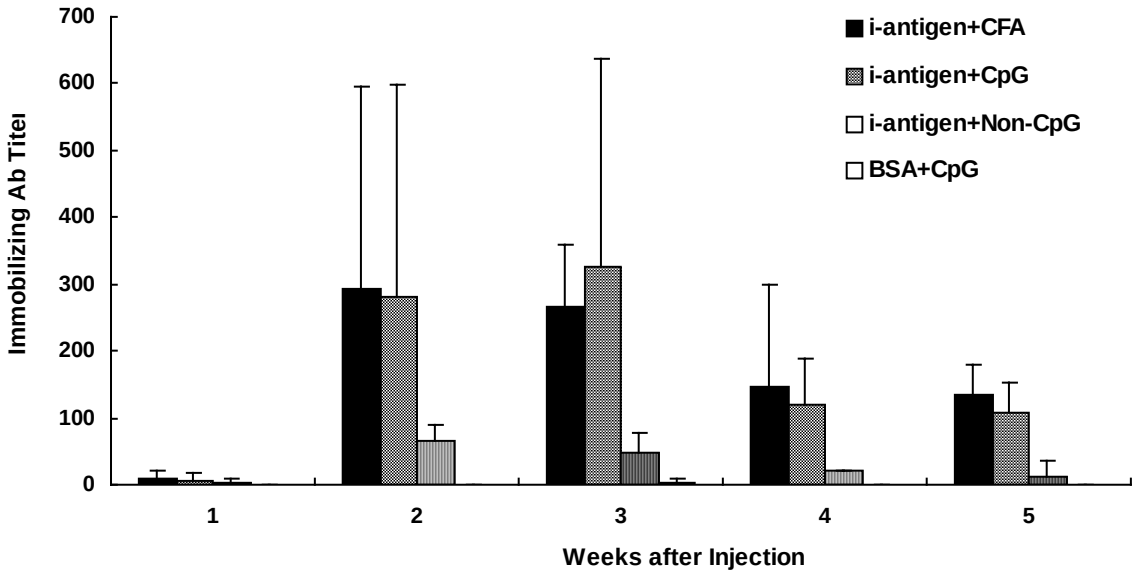
700
600
500
400
300
200
100
0
Immobilizing Ab Titer
1
2
3
4
5
Weeks after Injection
i-antigen+CFA
i-antigen+CpG
i-antigen+Non-CpG
BSA+CpG

Figure 5.2: Mucus antibody response. Mucus samples were collected from immunized channel catfish at various time points and mucus antibody levels determined by enzyme-linked immunosorbant assay (ELISA). Naïve fish mucus samples and 2 % BSA served as negative controls. Positive control were immune fish sera. Fish immunized with either purified G5 i-antigen with CFA or i-antigen with CpG had specific mucus antibodies against G5 i-antigen started from week 2 after immunization.

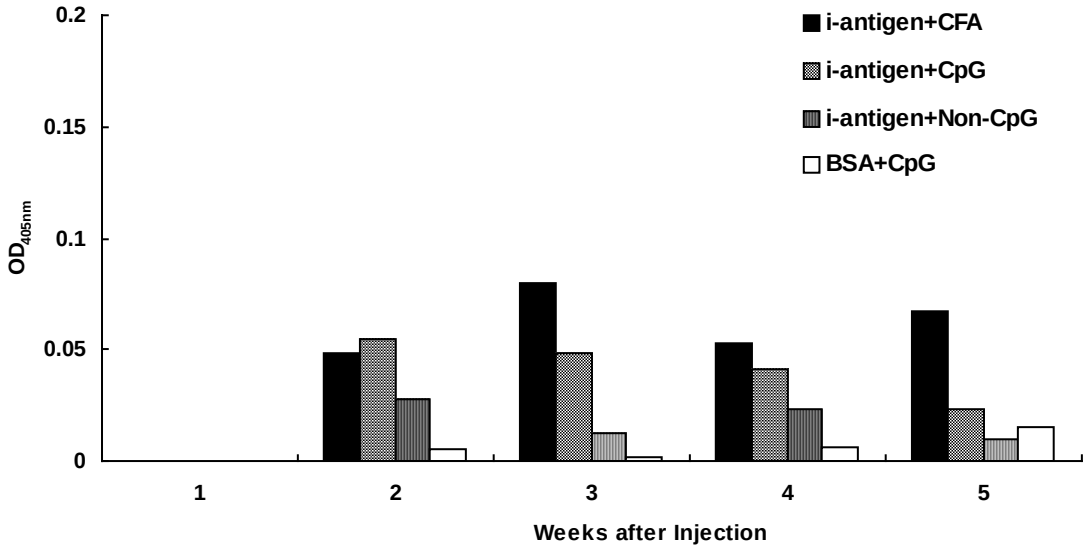
0.2
0.15
0.1
0.05
0
OD405nm
i-antigen+CFA
i-antigen+CpG
i-antigen+Non-CpG
BSA+CpG
1
2
3
4
5
Weeks after Injection

Figure 5.3: Protection efficacy. All fish were challenged with 15000 live *I. multifiliis* theronts per fish at day 36 after the first injection. Forty percent of fish (n=30) immunized with 10 µg of affinity purified G5 i-antigen with Freund's complete survived, and 33.3 % of fish (n=30) immunized with i-antigen with CpG survived. Negative control fish injected with i-antigen with non-CpG or BSA with CpG died by day 13 following challenge.

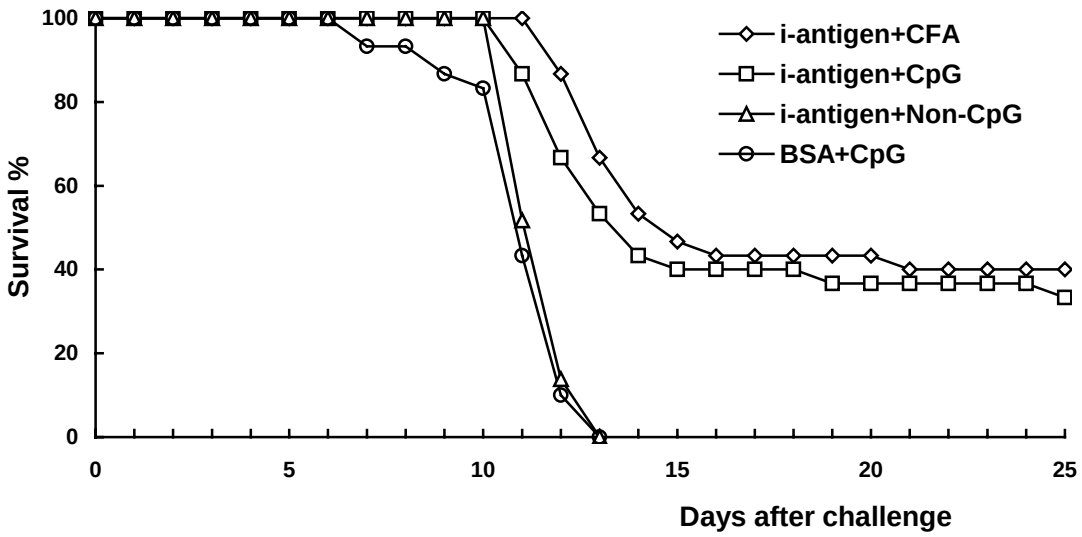
100
80
60
40
20
0
Survival %
0
5
10
15
20
25
Days after challenge
i-antigen+CFA
i-antigen+CpG
i-antigen+Non-CpG
BSA+CpG

Table 5.1. Statistical analysis of challenge experiment.

Vaccine [a]	No. of challenged [b] / No. of survived	Survival %	MDD ± SD [c]
i-antigen with CFA	30 / 12	40.0	13.8 ± 2.1
i-antigen with CpG	30 / 10	33.3	13.4 ± 3.1
i-antigen with non-CpG	29 / 0	0	11.7 ± 0.7
BSA with CpG	30 / 0	0	11.1 ± 1.5

[a] Catfish were injected ip with protein antigens or live theronts as described in Materials and Methods.

[b] Fish were challenged at a dose of 15000 theronts / fish by surface exposure.

[c] MDD stands for mean days to death, and SD stands for standard deviation. The MDD of fish that died in groups immunized with i-antigen with CFA or CpG was significantly greater ($p < 0.001$, Kruskal-Wallis test ANOVA) than the MDD of fish immunized with i-antigen with non-CpG or BSA with CpG.

CHAPTER 6

GENETIC IMMUNIZATION OF CHANNEL CATFISH (*ICTALURUS PUNCTATUS*) AGAINST *ICHTHYOPHTHIRIUS MULTIFILIIS*[1]

[1]Wang, Xuting and Harry W. Dickerson. To be submitted to *Journal of Fish Diseases.*

ABSTRACT

Injection of channel catfish with surface immobilization antigens (i-antigens) of *Ichthyophthirius multifiliis* elicits protective immunity against lethal challenge. Because *I. multifiliis* is an obligate parasite, we are attempting to develop a DNA-based vaccine to provide a more easily produced vaccine. To construct the DNA vaccines, the full-length *I. multifiliis* 55 kDa i-antigen gene, and its truncated forms lacking the coding sequence for either the N-terminal signal peptide or the C-terminal GPI anchor addition site were placed under the control of a CMV promoter in the plasmid pcDNA3.1. These DNA vaccines were injected intramuscularly into channel catfish fingerlings. Immunized fish were challenged with live parasites. Survival in all groups injected with the i-antigen gene constructs was higher than that of negative controls (pcDNA3.1/LacZ). Fish produced serum antibody recognizing i-antigen on Western blot. This is the first demonstration of a DNA vaccine containing i-antigen gene eliciting a serum antibody response and conferring a low degree of protective immunity against *I. multifiliis.*

Keywords: *Ichthyophthirius multifiliis*, channel catfish (*Ictalurus punctatus*), immobilization antigen, immunization, DNA vaccine, genetic immunization.

INTRODUCTION

Ichthyophthrius multifiliis (Fouquet) is the most pathogenic ciliated protozoan parasite of fish. It is the etiological agent of "white spot" disease and occurs world-wide. Morbidity in enclosed water systems with farmed fish species such as channel catfish usually reaches 100% because numbers of parasites increase by orders of magnitude with each round of infection (6).

The immobilization antigen (i-antigen) purified from *I. multifiliis* G5 isolate confers strong protection against a lethal challenge with the homologous strain when injected into juvenile channel catfish in Freund's adjuvant (20). Because *I. multifiliis* is an obligate parasite that can only be grown in relatively small numbers, it is necessary to develop substitutes for the i-antigen-based subunit vaccine. One alternative is a DNA vaccine, which has gained wide attention as a means for the control of infectious diseases in mammals (8, 19). Such vaccines have enormous potential in the field of aquaculture, and recent attempts to immunize salmonids with DNA-based vaccines against infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia (VHS) have met with success (1, 15). In this study, we sought to determine whether genetic immunization of channel catfish with DNA constructs containing i-antigen genes from *I. multifiliis* elicit protective immunity.

MATERIALS AND METHODS

DNA vaccines. An i-antigen gene from the *I. multifiliis* G5 isolate was modified to replace the ciliate specific glutamine UAA and UAG codons (which in most of eukaryotes are termination codons) with channel catfish preferred codons CAA and

CAG, respectively. Additionally, two truncated i-antigen genes were obtained by deleting the coding sequence for either the N-terminal signal peptide or C-terminal GPI addition site. These three versions of i-antigen gene were cloned into the eukaryotic expression vector pcDNA3.1(+) according to the manufacturer's procedure (Invitrogen, USA).

Experimental fish. Channel catfish (*Ictalurus punctatus*) fingerlings (10-15 g) with no previous history of exposure to *I. multifiliis* were obtained from a local hatchery. Prior to immunization, fish were treated with formalin (25 ppm) to remove any possible external parasites and maintained in a flow-through system. Fish were fed daily with commercial trout starter feed (Purina, USA). Water temperature was maintained at 20-23 ^{0}C, and water quality (NH_3, NO_2, and pH) monitored daily with standard test kits.

Propagation and collection of parasites. The *Ichthyophthirius multifiliis* G5 isolate used in this study has been characterized previously (3, 13), and is currently maintained by serial passage on juvenile channel catfish (*Ictalurus punctatus*) (17). Live theronts were obtained using previously described methods (5). Cells were used immediately or pellets were frozen in liquid nitrogen and stored at –70 °C.

Vaccination procedure. The DNA vaccines were diluted into PBS (135 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.5), and injected intramuscularly into the dorsal muscle of channel catfish at a volume of 50 µL per fish using a 1.0 mL syringe with a 32G needle.

In test 1, groups of 10 fish were vaccinated with either 10, 1 or 0.1 µg of plasmid DNA (pcDNA3.1) containing either the full-length, N-terminal or C-terminal deletion of the synthetic 55 kDa i-antigen gene. Two control groups were included: a negative control group injected with the pcDNA3.1 containing the lacZ gene, and a positive

control group vaccinated with 10 µg affinity purified i-antigen (21) in Freund's complete adjuvant and boosted with 10 µg i-antigen in Freund's complete adjuvant after 14 days.

In test 2, groups of 30 fish were vaccinated twice at a interval of 14 days with 1.0 µg of plasmid DNA (pcDNA3.1) containing different versions of i-antigen genes. Two negative control groups injected with either pcDNA3.1 or pcDNA3.1 containing the lacZ gene. A positive control group was immunized the same way as in experiment 1.

Challenge. Fish were challenged by surface exposure to 15,000 theronts per fish following a standard protocol (7). Aquaria were equipped with biological filtration, and water quality and temperature were monitored daily. Mortalities were recorded daily in each group until all fish died or recovered from infection.

SDS-PAGE and Western blotting. One-dimensional SDS-PAGE and Western blot analyses were done using standard protocols (14).

Detection of serum antibodies by ELISA. To absorb antigen to plates, individual wells of ELISA plates (Falcon 3911Microtest III, Becton Dickinson, USA) were filled with 50 µL of purified i-antigen (20 µg/mL) in 25 mM sodium acetate buffer (pH 7.5) and incubated overnight at 4 ^{0}C. Control wells were coated with 2% (w/v) BSA. Non-specific protein binding was blocked by overnight incubation at 4 ^{0}C with 100 µL of 5% (w/v) BSA in Tris buffered saline with Tween 20 (TBST; 20 mM Tris-HCl, 50 mM NaCl, 0.05% v/v Tween 20, pH 7.5). The serum samples were diluted 25 times in 20 mM Tris-HCl and added to the plates (50 µl per well). The plates were incubated for 1 hour at RT. Following three washes with TBST, mouse mAb anti-catfish Ig coupled to alkaline phosphatase (1:1000 dilution in 2% w/v BSA in TBST) was added (50 µL/well) and

incubated for 1 hour at RT. After three washes with TBST, substrate p-nitrophenylphosphate was added, and plates were incubated for 1 hour in the dark at RT. The reaction was stopped with 4 N NaOH and plates were read at 405 nm with ELISA reader.

Statistical Analyses. Differences in the mean days to death (MDD) among groups were calculated by the one way analysis of variance (ANOVA). Differences in the proportion of survivors among groups were calculated by the z test. All calculations were done using the SigmaStat statistical software (Jandel Scientific Software, CA, USA).

RESULTS

Serum antibody response

Channel catfish were intramuscularly injected with one of the three constructs, using different doses, 10 μg, 1 μg or 0.1 μg of plasmid DNA in PBS buffer. Two weeks later, two fish of each group were bled and the antisera were pooled and tested for the production of specific antibody by Western Blot and ELISA. SDS-PAGE was carried out under both reducing and non-reducing conditions, and Western blots were probed with pooled antisera from the different groups of fish. As shown in Figure 6.1 (Blot A), sera from fish vaccinated with constructs harboring the C-terminal deletion (lanes 4-6) reacted positively with the 55 kDa protein run under non-reducing conditions, as did sera from fish injected with the purified antigen itself (lane 12). In contrast, only weak signals were seen in equivalent blots of protein separated under reducing conditions, the one exception being with sera from fish injected with 10 μg of the full-length construct (Figure 6.1, Blot

B). These results suggest that antibodies against the product of the C-terminal deletion (and against the i-antigen itself) are directed primarily towards conformational epitopes, while those against the full-length protein are directed towards linear epitopes.

ELISA results (Figure 6.2) showed that the antibody response to the *I. multifiliis* i-antigen in DNA vaccinated fish were much lower than that in native i-antigen protein vaccinated fish, and the level of antibody against i-antigen in most of DNA vaccinated fish decreased over time after day 14.

Protection efficacy

Fish in test 1 were subjected to a standard challenge with live parasites 9 weeks after vaccination. The percent survival in all groups injected with the i-antigen constructs was higher than that in the negative control (LacZ) (Table 6.1). The relative survival percentages between test groups and negative control group were statistically significant by z-test ($p<0.01$).

In test 2, we used a larger size of groups (n = 30 fish), and fish were vaccinated twice at a 14-day interval with 1.0 μg of DNA. A negative control group injected with pcDNA3.1 was included to test for the non-specific protection induced by pcDNA3.1/lacZ. All fish were challenged on day 43. The result (Figure 6.3) showed that: 26.7 % of fish immunized with full-length i-antigen gene DNA construct survived, 23.3 % of fish immunized with N-terminal deletion gene DNA construct survived, and 13.3 % of fish immunized with C-terminal deletion gene DNA construct survived. All of negative control fish injected with either lacZ gene DNA construct or pcDNA3.1 died by day 23 following challenge. In contrast, 70 % of fish immunized with i-antigen protein survived.

Statistical analysis using Kruskal-Wallis test ANOVA showed that the median-days-to-death (MDD) of fish that died in groups immunized with i-antigen genes was significantly greater than the MDD of the infected control fish ($p < 0.05$). There is no significant difference in MDD between the groups immunized with i-antigen genes ($p = 0.236$).

DISCUSSION

DNA vaccines are at the forefront of human vaccine technology. They are also being developed against the major viral diseases of farmed fish. DNA vaccines have the following advantages: a) they remove the potential hazards associated with associated with "live" or attenuated pathogens; b) the manufacture of DNA vaccines is simple; c) DNA vaccines contain immunostimulant CpG motifs, so there is no need for adjuvant; d) DNA vaccines are very stable, making storage and transport very easy and less expensive.

The surface immobilization antigens of *I. multifiliis* elicit protective immunity. The genes of i-antigens have been cloned and characterized. Based on its deduced sequence, the 55 kDa i-antigen protein contains hydrophobic sequences at its N- and C-terminus that are presumed to target the protein to the plasma membrane (the N-terminal sequence acting as a signal peptide for ER localization, and the C-terminal sequence acting as a GPI-anchor cleavage and addition site). In addition the full-length gene, we made two alternate versions of the gene, one lacking the GPI-anchor addition sequence and the other lacking the N-terminal signal peptide. We tested these 3 versions of i-antigen genes based on the hypothesis that gene products might be processed differentially by the immune system following their expression in fish. In particular, the

product of the C-terminal deletion might give rise to a stronger humoral immune response since it would (in theory) be secreted from cells rather than be bound to the plasma membrane through a glycolipid anchor. The results showed that plasmid DNA containing the *I. multifiliis* i-antigen gene induced the specific antibody response against the expressed G5 i-antigen when injected into the dorsal muscle of channel catfish. Among these three constructs (full-length gene, gene without signal peptide sequence and gene without GPI addition site sequence), the full length gene is the most effective one to induce the antibodies against the linear epitopes on the G5 i-antigen.

In experiment 1, some fish survived in the group injected with the lacZ constructs. It might be interesting in light of recent evidence that methylated CpG motifs in bacterial DNA can stimulate both cytokine production and B-cell proliferation (12), and in some cases, can protect animals non-specifically against microbial pathogens (9). However, when we repeated with the larger group size, all fish died after challenge. The possible reasons may include: (1) Two batches of parasites used for challenge have different degree of virulence, which may lead to the fish in test 1 were under-challenged; (2) The density of fish in the aquaria in test 2 is 3 times higher than that in test 1. Even only a small number of immune fish were infected lightly in the first round infection, the cumulating of theronts from those infected fish in crowed aquarium is much fast, therefore, the subsequent infection is much heavier in test 2 and it overwhelmed the threshold of protection.

The low protection efficacy from test 2, together with the low serum antibody response, suggests that our efforts to increase the efficacy of DNA vaccines should be centered on targeting vector-encoded antigens to sites of immune induction (e.g., APCs

and high endothelial venules), and on co-administration of vectors encoding immunostimulatory substances such as cytokines, or B7.

DNA vaccines against *I. multifiliis* currently under development in our laboratories are being administered by conventional routes, including intramuscular injection and biolistic bombardment of the skin. While these routes are appropriate for laboratory testing (and could be used for commercial purposes as well), oral delivery represents an extremely attractive alternative for administration of DNA vaccines, particularly in aquaculture species. Based on recent experiments in mice, it may now be possible to administer genetic vaccines orally using biodegradable polymers.

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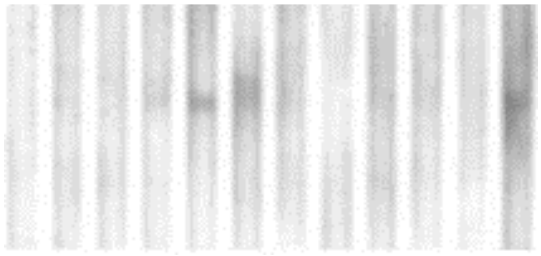
Figure 6.1. Western blot analysis of sera from vaccinated fish. The 55 kDa i-antigen from *I. multifiliis* G5 isolate was affinity purified and resolved by SDS-PAGE. Protein was then transferred to PVDF membrane and reacted sequentially with pooled sera from fish vaccinated with pcDNA3.1 DNA vaccine constructs followed by a monoclonal antibody the heavy chain of channel catfish Ig conjugate (alkaline phosphatase). On blot ***A***, lanes were probed with sera from fish immunized as follows: 1-3: 10, 1, 0.1 µg full-length gene construct, respectively; 4-6: 10, 1, 0.1 µg C-terminal deletion construct, respectively; 7-9: 10, 1, 0.1 µg N-terminal deletion construct, respectively; 10: 10 µg LacZ construct; 12: fish vaccinated with 20 µg affinity purified 55 kDa i-antigen (positive control). On blot ***B***, lanes were probed with sera from fish immunized as follows: 1: 10 µg full-length gene construct; 2: 10 µg C-terminal deletion construct; 3: 10 µg N-terminal deletion construct; 4: 10µg LacZ construct; 6: 20 µg affinity purified i-antigen. Naive fish serum was used as a negative control (blot ***A*** lane 11 and blot ***B*** lane 5).

Blot A
(non-reduced i-antigen)

1 2 3 4 5 6 7 8 9 10 11 12



Blot B
(reduced i-antigen)

1 2 3 4 5 6

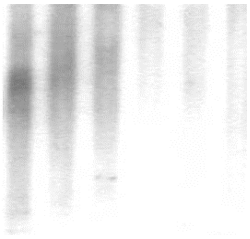


Figure 6.2. ELISA to determine serum antibody against G5 i-antigen. Serum samples were collected from immunized channel catfish at various time points and their antibody levels determined by enzyme-linked immunosorbant assay (ELISA). Naïve fish serum and 2% BSA served as negative controls. In Figure, "**F10**" stands for serum from fish immunized with **10** μg **full-length** gene DNA construct, "**C10**" stands for serum from fish immunized with **10** μg **C-terminal deletion** gene DNA construct, "**N10**" stands for serum from fish immunized with **10** μg **N-terminal deletion** gene DNA construct, and so on.

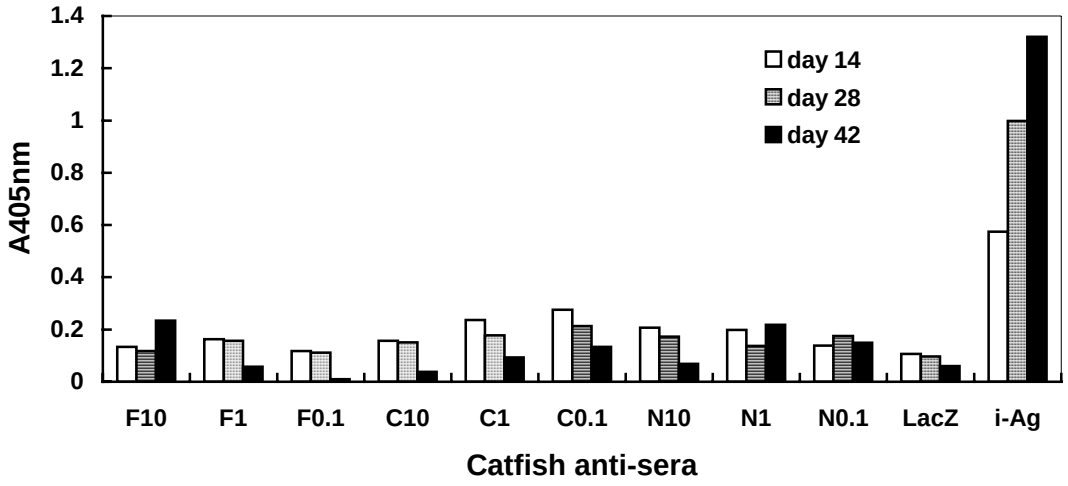

1.4
1.2
1
0.8
0.6
0.4
0.2
0
A405nm
day 14
day 28
day 42
F10
F1
F0.1
C10
C1
C0.1
N10
N1
N0.1
LacZ
i-Ag
Catfish anti-sera

Table 6.1. Challenge result of experiment 1.

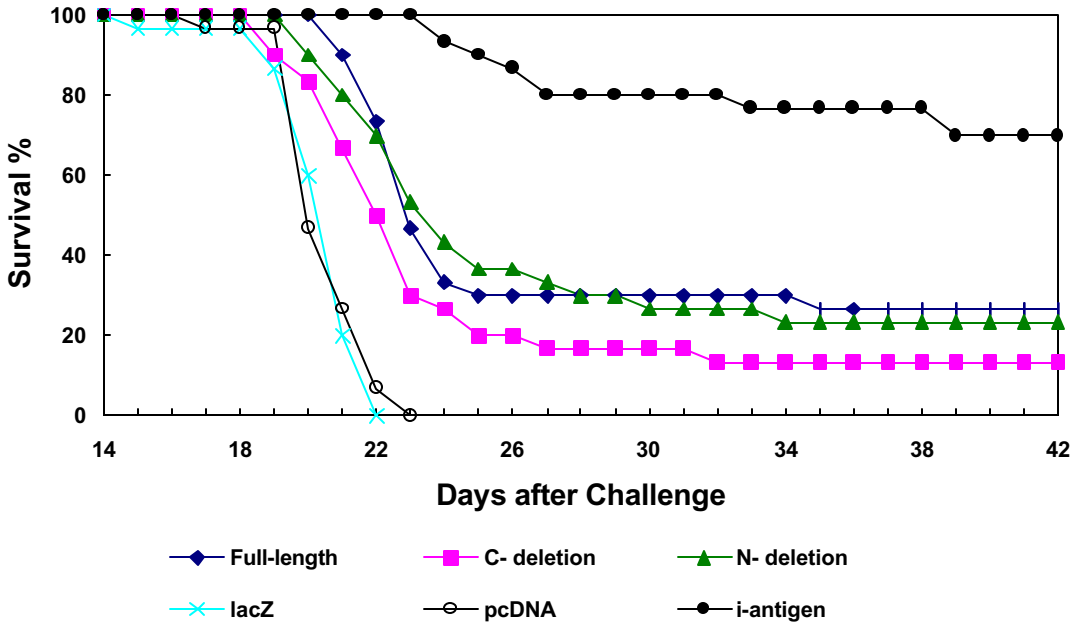
Vaccine[a]	Dose (μg)	No. of challenged[b]	No. of survived	RPS[c] (%)
Full-length	10	9	8	81.5
Full-length	1	10	9	83.3
Full-length	0.1	10	9	83.3
C-deletion	10	9	8	81.5
C-deletion	1	10	8	66.7
C-deletion	0.1	10	9	83.3
N-deletion	10	9	7	63.0
N-deletion	1	10	10	100
N-deletion	0.1	10	9	83.3
lacZ	10	10	4	0
i-antigen	20	10	10	100

[a] Catfish were injected im with DNA or protein antigens as described in Materials and Methods.

[b] Fish were challenged at a dose of 15000 theronts / fish by surface exposure.

[c] Relative percent survival; RPS = (1 - % mortality in vaccinated group / % in control group) x 100 (11). There was a significant difference in RPS between each test group and negative contrl group ($P<0.01$) by z-test.

Figure 6.3. Challenge result of experiment 2. Groups (n = 30 fish) were vaccinated twice at an interval of 14 days with 1.0 μg of DNA or 10 μg of i-antigen in Freund's adjuvant. All fish were challenged with 15000 live theronts per fish at day 43 after the first injection. Seventy percent of fish immunized with i-antigen protein survived, 26.7 % of fish immunized with full-length i-antigen gene DNA construct survived, 23.3 % of fish immunized with N-terminal deletion gene DNA construct survived, and 13.3 % of fish immunized with C-terminal deletion gene DNA construct survived. Negative control fish injected with either lacZ gene DNA construct or pcDNA3.1 died by day 23 following challenge.


100
80
60
40
20
0
Survival %
14
18
22
26
30
34
38
42
Days after Challenge
Full-length
C- deletion
N- deletion
lacZ
pcDNA
i-antigen

CHAPTER 7

CONCLUSIONS

Ichthyophthrius multifiliis (Fouquet) is the most pathogenic ciliated protozoan parasite of fish. It is the etiological agent of ichthyophthiriasis or "white spot disease" and infects a wide range of freshwater fish. Fish that survive infection acquire immunity to subsequent challenge and produce specific serum antibodies that immobilize the parasite *in vitro*. Studies in this laboratory have revealed a mechanism of protective immunity in which parasites exit the host prematurely in response to antibody binding. The target antigens for these protective antibodies are a class of highly abundant membrane proteins referred to as immobilization antigens, or i-antigens (i-Ag). The i-antigens exist as either one or two antigenically related polypeptides in the 40-60 kDa range that are bound to the plasma membrane through a glycolipid anchor. My research is focused on the biochemical and immuological characterization of i-antigens, and the results are directly related to the development of an effective vaccine.

Using immobilizing mouse monoclonal antibody as ligand, the i-antigen of *I. multifiliis* isolate G5 was purified to homogeneity by immunoaffinity chromatography. The affinity purified G5 i-antigen comprises a single peptide chain which has an approximate Mr of 55 kDa under reducing condition and 46 kDa under non-reducing condition as determined by SDS-PAGE. Its exact size by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry analysis is 44.3 kDa. The immunogenicity of this purified antigen was confirmed by inoculating rabbit and channel catfish to produce immobilizing antisera. When injected with Freund's

adjuvant, the G5 i-antigen confered strong protection in juvenile channel catfish against lethal challenge with the homologous strain. Fish injected with G5 i-antigen developed high titers of serum immobilizing antibodies. This is the first demonstration of a direct role for i-antigens in eliciting protective immunity suggesting that these proteins by themselves can serve as effective subunit vaccines against *I. multifiliis.*

The role of i-antigen in eliciting cross-protective immunity was explored. The i-antigens were purified from two isolates, NY1 and G5, representing the different immobilization serotypes (A and D, respectively). A vaccination trial with these purified i-antigens was carried out to test the efficacy of immunization against challenge with parasites of homologous or heterologous serotype. Three groups consisting of 70 channel catfish *(Ictalurus punctatus)* were immunized with G5 i-antigen, NY1 i-antigen, or bovine serum albumin (BSA). Fish immunized with i-antigens developed high titers of serum immobilizing antibodies whereas sera from control fish injected with BSA did not. Fish antibodies immobilized only parasites of homologous serotype and recognized only homologous i-antigen on Western blots under non-reduced condition. When challenged, 70% of fish immunized with G5 i-antigen survived G5 challenge and 33.3% of fish immunized with NY1 i-antigen survived NY1 challenge. All fish in control groups and groups immunized with i-antigen exposed to heterologous challenge died of *I. multifiliis* infection. This result indicates that i-antigens only confer protection against parasites of homologous serotype. Further, this experiment demonstrated difference in virulence between these two isolates.

Because *I. multifiliis* is an obligate parasite that can only be grown in relatively small numbers, its i-antigen genes have been introduced into a novel expression system,

the free-living non-pathogenic ciliate *Tetrahymena thermophila.* The recombinant i-antigens were either targeted to the cell surface in full-length form or secreted into the culture medium in a truncated form lacking GPI-anchor addition site. Also, the expression level of i-antigen was dramatically enhanced using an inducible promoter from the metallothionein gene of *T. thermophila*. To test the immunogenicity of the recombinant i-antigens, channel catfish were injected intraperitoneally twice at a 2-week interval with either live recombinant *Tetrahymena* cells expressing i-antigen or purified recombinant i-antigen proteins. Treated fish produced specific serum antibodies that immobilized live *I. multifiliis* parasites *in vitro* and recognized native i-antigens on Western blots. Moreover, 80-100% of fish immunized with live *Tetrahymena* over-expressing *Ichthyophthirius* i-antigens were protected from lethal change with parasites of homologous serotypes. These results indicated that recombinant i-antigens produced in *Tetrahymena* had the same antigenicity and immunogenicity as these protein produced by the parasite, and live cells of *Tetrahymena* over-expressing *Ichthyophthirius* i-antigens were effective vaccines against *I. multifiliis*.

Since complete Freund's adjuvant (CFA) has severe side effects in fish, alternative adjuvants are desired in vaccine research. A new adjuvant, oligodeoxynucleotides containing the dinucleotide CpG motif, was compared with CFA for the ability to induce antibody and protective immunity when administered with purified G5 i-antigen. Our results show that oligodeoxynucleotides containing the CpG motif provide the same degree of immune stimulation as Freund's adjuvant. The serum immobilizing antibody response in the fish injected with CpG ODN plus G5 i-antigen

follows the same kinetics as that in fish injected with CFA plus G5 i-antigen. The results indicate that CpG ODN is a safe and highly effective adjuvant for fish vaccines.

DNA vaccines containing the i-antigen genes were also evaluated. The i-antigen gene from *I. multifiliis* G5 isolate was modified to replace its ciliate specific code usages (UAA and UAG encoding glutamine instead of stop codons) with channel catfish preferred codons, CAA and CAG, respectively. Additionally, two truncated i-antigen genes were obtained by deleting the coding sequence for either the N-terminal signal peptide or C-terminal GPI addition site. These 3 versions of G5 i-antigen gene were cloned into the eukaryotic expression vector pcDNA3.1 and tested as DNA vaccines. DNA vaccines were injected intramuscularly into channel catfish fingerlings to evaluate their efficacy. Challenge with a lethal dose of live parasites showed that 13.3%-26.7% of fish injected with the DNA vaccines containing i-antigen genes survived, whereas all fish in the negative control group died. Also, fish produced specific serum antibody against G5 i-antigen. This is the first demonstration of DNA vaccines containing i-antigen genes eliciting serum antibody response and conferring low degree of protective immunity against *I. multifiliis.*

These results clearly indicate that i-antigens of *I. multifiliis* are protective antigens and good candidates for the development of vaccines against *I. multifiliis*.