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Passive Immunization of Channel Catfish (*Ictalurus punctatus*) against the Ciliated Protozoan Parasite *Ichthyophthirius multifiliis* by Use of Murine Monoclonal Antibodies

TIAN LONG LIN,† THEODORE G. CLARK, AND HARRY DICKERSON*

Department of Medical Microbiology, College of Veterinary Medicine, University of Georgia, Athens, Georgia 30602

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Fish acquire immunity against the ciliated protozoan parasite *Ichthyophthirius multifiliis* following sublethal infection. The immune response includes the elaboration of humoral antibodies against a class of abundant surface membrane proteins referred to as immobilization antigens (i-antigens). Antibodies against these proteins immobilize the parasite in vitro, suggesting a potential role for the i-antigens in protective immunity. To test this hypothesis, passive immunization experiments were carried out with naive channel catfish, *Ictalurus punctatus*, using immobilizing murine monoclonal antibodies (MAbs). Fish were completely protected against lethal challenge following intraperitoneal injection of 20 to 200 μg of MAb. Although fish succumbed to infection at lower doses, palliative effects were observed with as little as 2 μg of antibody. In experiments in which animals were challenged at various times following inoculation, an inverse relationship between parasite load and serum immobilizing activity was seen. Of seven MAbs which conferred protection, all were immunoglobulin G class antibodies. The only immobilizing MAb that failed to protect was an immunoglobulin M antibody that was absent from surface mucosa as determined by enzyme-linked immunosorbent assay. The implications of these findings for the development of a vaccine against *I. multifiliis* and immunity against surface pathogens of fish are discussed.

Ichthyophthirius multifiliis is a ciliated protist that infects the skin and gill epithelia of virtually all species of freshwater fish. It is an obligate parasite with a life cycle that consists of fish-associated and free-swimming stages referred to as trophonts and theronts, respectively (17, 18). *I. multifiliis* is one of the most virulent protozoan pathogens of teleosts and poses a serious threat for the rapidly growing aquaculture industry (21). The repertoire of chemotherapeutic agents available for treatment is limited, as many are carcinogens and cannot be used in fish produced for human consumption. In addition, chemicals added to the water usually require multiple treatments, as most are effective only on the free-swimming stages and not on the feeding trophont within the fishes' skin (7).

Because of these limitations, immunoprophylaxis would ultimately be a safer and more economical alternative to postinfection treatment. Fish acquire immunity against *I. multifiliis* following sublethal infection, and sera and mucus from immune fish immobilize live parasites in vitro (3, 4, 7–9, 20, 28, 36, 37). Immobilizing antibodies are postulated to play an important role in protective immunity, and we have focused our studies on the antigens that stimulate their production with the idea that these may be candidates for the development of protective vaccines (10).

The antigens of *I. multifiliis* that elicit immobilizing antibodies have been purified by affinity chromatography with monoclonal antibodies (MAbs) and comprise a family of abundant phosphatidylinositol (GPI)-anchored cell surface proteins with molecular masses ranging between 40 and 70 kDa (10, 12, 15, 23). They are analogous to well-characterized surface proteins (referred to as immobilization antigens or i-antigens) found in the free-living ciliates *Paramecium* and *Tetrahymena* species (2, 33). The i-antigens of *I. multifiliis* are the major targets of the humoral immune response in fish (8, 12, 14, 37, 38).

In this report we describe passive immunization experiments demonstrating that immobilizing murine MAbs confer protection against *I. multifiliis*. These findings provide a strong argument that immobilizing antibodies produced by actively immunized fish play an important role in protection and suggest that purified i-antigens could be used as subunit vaccines against this obligate parasite.

Although MAbs have been tested for passive immunoprophylaxis and immunotherapy against microbial pathogens in mammals and other vertebrate species (1, 5, 30, 31, 35), very few studies on their use in fishes have been published (29). Our results raise the possibility that mouse MAbs could serve as therapeutic agents in fish for the treatment of *Ichthyopthirius* infection.

MATERIALS AND METHODS

Parasite isolation and growth. Three different isolates (representing two serotypes) of *I. multifiliis* were used in these experiments. The first isolate, designated G1, was described in a previous study (23). The second isolate, G3, was cloned from a single tomont obtained from an infected brown trout (*Salmo trutta*). The third isolate, G5 (serotypically related to G3), was isolated from an infected channel catfish (*Ictalurus punctatus*) and is currently maintained by serial passage on channel catfish fingerlings as described elsewhere (32).

Membrane protein extraction and i-antigen purification. Theront membrane proteins were solubilized in 1% (vol/vol) Triton X-114 (Sigma Chemical Co., St. Louis, Mo.) at 0°C and isolated by phase separation at 37°C as described previously (14, 23). The G3 i-antigen was purified from membrane proteins by affinity chromatography with MAb G3-61 as described for the G1 i-antigen (23). Membrane protein and i-antigen samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (immunoblotting). Briefly, samples were run in 10% acrylamide gels under reducing conditions and stained with Coomassie blue (14) or silver nitrate (23). For Western blots, proteins resolved by SDS-PAGE were electrophoretically transferred to nitrocellulose and probed with rabbit antisera against affinity-purified G1 i-antigen (15).

* Corresponding author. Mailing address: Department of Medical Microbiology, University of Georgia College of Veterinary Medicine, D. W. Brooks Dr., Athens, GA 30602. Phone: (706) 542-5794. Fax: (706) 542-5771. Electronic mail address: Dickerson.H.@calc.vet.uga.edu.

† Present address: Fujian Academy of Agricultural Sciences, Fuzhou, Fujian, People's Republic of China.

Experimental fish. Channel catfish (*I. punctatus*) fingerlings (20 to 25 g) were obtained from a local hatchery. Prior to experiments, fish were treated with a formalin-malachite green mixture to remove external parasites and maintained in a flowthrough system. Animals were fed daily with commercial trout starter feed. Water temperature was maintained at 23 to 25°C, and water quality (NH_3, NO_2, and pH) was monitored with standard test kits.

Production of immobilizing MAbs. Production of immobilizing MAbs against *Ichthyophthirius* strain G1 was described previously (23). Immobilizing MAbs were produced in the current study against strain G3 by similar procedures. Briefly, hybridomas were prepared from BALB/c mice immunized with theront membrane protein fractions, screened for immobilizing MAbs, and cloned three times by limiting dilution. MAb class and subclass were determined by enzyme-linked immunosorbent assay (ELISA) using rabbit anti-mouse immunoglobulin (Ig) class and subclass-specific antisera (Sigma). To produce ascites fluids, 10^7 antibody-producing hybridoma cells were inoculated intraperitoneally (i.p.) into BALB/c mice primed with pristane (Aldrich Chemical Co., Milwaukee, Wis.). Ascites fluids were harvested 10 to 14 days later and stored at −70°C. MAbs were purified from ascites fluids with a commercial kit (E-Z SEP; Pharmacia Biotech, Piscataway, N.J.), and the purity was confirmed by analysis on SDS-polyacrylamide gels stained with Coomassie blue (14). Ig concentrations were determined by measuring the A_{280}.

Characterization of MAb binding by ELISA. (i) Blocking assay. Assays were carried out using MAb G3-61 coupled to alkaline phosphatase (G3-61AP) and unlabeled MAbs G3-27, G3-74, and 10H3. To prepare the conjugate, 3.5 mg of purified G3-61 was mixed with 2.5 mg of alkaline phosphatase (Sigma) and coupled with glutaraldehyde as described previously (19). A 96-well ELISA plate (Micro Test III; Becton Dickinson and Co.) was coated with 50 μl of *I. multifiliis* G3 membrane protein at a concentration of 5 μg/ml in phosphate-buffered saline (PBS; 136 mM NaCl, 2.7 mM KCl, 2.7 mM KCl, 8 mM Na_2HPO_4) by incubation at 4°C overnight, and the remaining protein-binding sites were blocked with 2% (wt/vol) bovine serum albumin (BSA) in TBST (20 mM Tris-HCl, 500 mM NaCl, 0.1% [vol/vol] Tween 20, pH 7.5). The plate was washed three times with TBST and reacted with MAb ascitic fluids (G3-27, G3-74, or 10H3) diluted 1:10 in 2% BSA–TBST for 1 h 22°C. MAb 10H3 against *I. multifiliis* G1 and 2% BSA (no MAb) served as negative controls, and unconjugated G3-61 served as a positive control. After three washes with TBST, the G3-61AP conjugate (diluted 1:2,000 in 2% BSA in TBST) was added to the wells and incubated as above. After washing, the enzyme substrate *p*-nitrophenylphosphate (alkaline phosphatase substrate kit; Bio-Rad) was added to the wells. After 30 minutes, the optical densities at 405 nm were measured on a 96-well plate ELISA reader.

(ii) Sandwich ELISA. To attach MAb to a 96-well plastic ELISA plate, 50 μl of ascites fluid diluted 1:500 in PBS was placed in wells (four wells per MAb) and incubated at 4°C overnight. Nonspecific protein-binding sites were blocked with 2% BSA in TBST. After washing three times in TBST, 50 μl of G5 membrane protein (5 μg/ml) was added to each well and incubated at 22°C for 1 h. (To prepare soluble membrane antigens, live *I. multifiliis* G5 theronts were solubilized in 1% [vol/vol] Tween 20 [Sigma] in Tris-buffered saline and centrifuged at 10,000 × *g* for 30 min. The supernatant was transferred to a small beaker and incubated overnight at 4°C with gentle stirring.) After incubation of antigen with the attached MAbs, the wells were washed five times with TBST and then incubated for 1 h at 22°C with MAb G3-61AP diluted 1:2,000 in 2% BSA–TBST. After final washes, the enzyme substrate *p*-nitrophenylphosphate was added and color development measured at 405 nm as described above.

Immobilization assays. Antibodies were tested for the ability to immobilize live *I. multifiliis* theronts in vitro as described previously (15).

Passive immunization of fish and parasite challenge. Passive transfer experiments were carried out with immobilizing and nonimmobilizing MAbs. Channel catfish fingerlings were injected i.p. with 0.5 mg of purified murine Ig in 0.2 ml of PBS (MAbs against G3) or ascites fluid containing an equivalent amount of Ig (MAbs against G1). In groups that received immobilizing MAb 10H3, fish were also inoculated with ascites fluid diluted to 20 and 2 μg of Ig. Fish were challenged with parasites homologous in serotype to the MAb with which they were injected. Experimental groups consisted of 10 fish, and most experiments were carried out in replicates of two or more. Each group was challenged with 2,000 theronts per fish in 38-liter aquaria and observed at least twice daily for 30 days until fish died or recovered from infection (16). Infected fish died at 5 to 10 days after challenge. Comparison of percent survival between groups of fish injected with immobilizing or nonimmobilizing MAbs (IgG) was made by a *z* test with the Yates correction applied with the SigmaPlot program (Jandel Scientific Software, San Rafael, Calif.). Water temperature was maintained at 23 to 24°C. Aquaria were equipped with biological filtration, and water quality was monitored daily.

Determination of minimum protective dose of MAb. Channel catfish fingerlings were placed into four groups (20 fish per group), three of which were injected i.p. with immobilizing MAb G3-27 (IgG1) at a dose of 200, 20, or 2 μg of MAb per fish. The fourth group was injected with 0.2 ml of PBS. All four groups were challenged with 5,000 theronts per fish 24 h after injection and placed in separate 38-liter aquaria. Parasites visible with the naked eye in the skin of infected fish were counted over the entire surface of the body at day 5 postchallenge. Mortalities were recorded in each group until all fish died or recovered. The differences in the median values of the days to death were analyzed by a Kruskal-Wallis one-way analysis of variance on ranks, and pairwise multiple comparisons of groups were performed by the Student-Newman-Keuls method using the SigmaStat statistical program (Jandel Scientific Software).

Duration of protection by immobilizing MAb. Channel catfish fingerlings were placed into four groups (14 fish per group) and injected i.p. with immobilizing MAb G3-27 at a dose of 0.5 mg of Ig per fish. Fish in groups 1 through 4 were injected sequentially at 10-day intervals (i.e., fish in group 2 were injected 10 days after injection of fish in group 1, fish in group 3 were injected 10 days after fish in group 2, and fish in group 4 were injected 10 days after fish in group 3). All four groups were challenged simultaneously 24 h after injection of group 4. This inoculation scheme allowed fish to be challenged at 30, 20, 10, or 1 day after injection of MAb. At the time of challenge, four fish from each group were removed for blood collection and their sera were tested for immobilizing activity. The remaining 10 fish were challenged with 5,000 theronts per fish in separate aquaria. Trophonts in the skin were counted with the naked eye at 5 days postchallenge, and mortalities were recorded daily until all fish died or recovered from infection. The medians of the days to death were compared by statistical analysis as above.

Detection of MAb in fish sera and mucus. Channel catfish fingerlings were injected i.p. (0.5 mg of Ig per fish) with one of the three following MAbs: 10H3 (IgG1), 9C9 (IgM), and G3-27 (IgG1). Each MAb was injected into four fish. An additional group of four fish was injected intravenously with MAb 9C9. To collect mucus, fish were anesthetized with 100 to 200 ppm of tricaine methane sulfonate (Argent Chemical Laboratories, Redmond, Wash.) and each animal was gently wiped over both lateral surfaces with a cotton swab. Each mucus-saturated swab was squeezed out into 0.2 ml of ice-cold PBS. The PBS solution was centrifuged at 14,000 × *g* for 1 min, and the supernatant was collected for testing. Serum and mucus samples collected on days 1 and 6 were tested by ELISA for the presence of murine MAb. Fifty microliters of *I. multifiliis* (strain G1 or G3) membrane protein (20 μg/ml) was added to wells of a 96-well ELISA plate and incubated overnight at 4°C. Plates were blocked with 10% (wt/vol) nonfat milk in TBST. After washing three times with TBST, serum or mucus samples were added to the wells and incubated at 22°C for 1 h. After washing in TBST, rabbit anti-mouse IgG or IgM antiserum conjugated to alkaline phosphatase (Sigma) was added to the wells at the manufacturer's suggested working dilution and incubated at 22°C for 1 h. Substrate color development was carried out as described above.

RESULTS

Passive immunization with MAbs. MAbs produced in this laboratory have been used to identify and characterize the immobilization antigens of two different *I. multifiliis* serotypes, designated G1 and G3. As determined in this study, serotype G3 contains a single i-antigen of ~55 kDa as judged by affinity chromatography, Western blotting, and ELISA (Fig. 1 and 2). The i-antigens of serotype G1 were previously characterized by this laboratory and found to consist of two antigenically related polypeptides of 48 and 60 kDa (23). Immobilizing MAbs against the G1 and G3 serotypes are specific and immobilize only cells of the homologous strain in vitro.

To determine whether immobilizing MAbs could protect naive fish against *Ichthyophthirius* infection, passive immunization studies were carried out with the G1 and G3 isolates. The degree of protection afforded by MAbs (measured as survival) is shown in Table 1. Of the eight immobilizing MAbs used in these studies, seven conferred a high degree of protection following lethal challenge (138 fish survived of a total of 140 challenged). The only immobilizing MAb that failed to protect was an IgM class antibody, designated 9C9 (discussed further below). In contrast, of the six control (nonimmobilizing) MAbs used, none provided any measurable degree of protection. These included two MAbs (A1-4 and G3-140) which appear to recognize i-antigens of the G1 and G3 serotypes in Western blots (data not shown).

Although it was clear that passively immunized fish could survive lethal challenge (Table 1), it was of interest to determine the extent to which such fish were parasitized. Animals were challenged 24 h following injection with either 0.5 mg of MAb G3-27 or the same amount of control (nonimmobilizing) MAb G3-140 or G3-74. As shown in Table 2, of the 18 fish injected with MAb G3-27, 17 appeared completely free of trophonts, whereas fish injected with MAbs G3-140 and G3-74 all had large numbers of parasites on their surface.

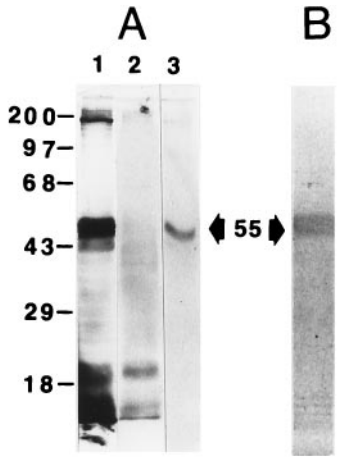

FIG. 1. Affinity purification of the i-antigen of serotype G3. i-antigen was purified by affinity chromatography with immobilizing MAb G3-61 coupled to Sepharose 4B (see Materials and Methods). (A) Protein samples resolved by SDS-PAGE were transferred to nitrocellulose paper and probed with rabbit polyclonal antiserum against affinity purified i-antigens of *Ichthyophthirius* serotype G1 i-antigen. This antiserum has been shown to cross-react in Western blots with the i-antigens of heterologous parasite strains (15). Lane 1, protein fraction applied to the column (10 μg of detergent-solubilized theront membrane protein); lane 2, unbound column flowthrough fraction (10 μg); lane 3, eluted antigens (~0.5 μg). The ~55-kDa i-antigen is marked. Protein standards (molecular mass in kilodaltons) are indicated to the left. (B) Affinity purified G3 i-antigen (~0.5 μg) was resolved by SDS-PAGE and stained with silver nitrate.

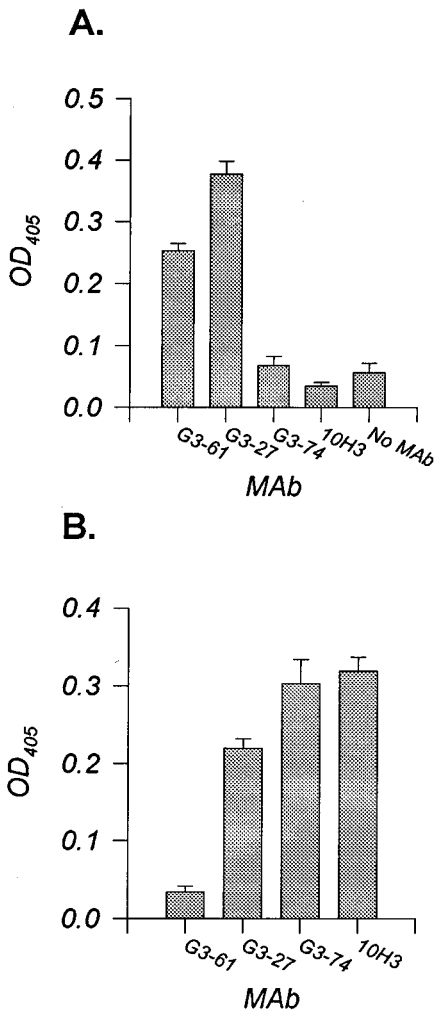

FIG. 2. Analysis of MAb binding by ELISA. (A) Sandwich ELISA. Individual wells of 96-well ELISA plates were coated with MAbs against *I. multifiliis*. These included immobilizing MAbs against the G3 serotype (G3-61 and G3-27), a control antibody which binds an irrelevant 14-kDa antigen in G3 (G3-74), and an immobilizing MAb against the heterologous serotype G1 (10H3). Plates were reacted with detergent-solubilized *I. multifiliis* membrane protein, followed by MAb G3-61 coupled to alkaline phosphatase. Signals were detected at a wavelength of 405 nm (OD, optical density). Note that G3-61 and G3-27 appear to bind the same antigen. (B) Direct ELISA. Individual wells of 96-well plates were coated with *I. multifiliis* membrane protein and incubated with MAb G3-61, G3-27, G3-74, or 10H3. After washing, samples were reacted with the G3-61–alkaline phosphatase conjugate used in panel A. As expected, unlabeled G3-61 blocked binding of the G3-61 conjugate. In contrast, MAb G3-27 interferes only slightly with MAb G3-61 binding, suggesting that these antibodies recognize independent epitopes on the same antigen.

Minimum protective dose. To determine the minimum dose of MAb required for passive protection, groups of fish were injected i.p. with various amounts of purified MAb G3-27 and challenged. At the highest dose (200 μg), 95% of fish survived, while at lower doses all fish became infected and died (Fig. 3). Nevertheless, groups injected with the lower doses (20 and 2 μg), showed a significant difference ($P < 0.05$) in days to death compared both with each other and with the control group injected with PBS alone. A similar dose-dependent effect was seen with MAb 10H3, which in the range of 20 to 2,000 μg gave complete protection against lethal challenge with the G1 serotype while 2 μg failed to protect (data not shown).

Duration of MAb protection. To determine the duration of protection afforded by MAbs, naive fish injected with MAb G3-27 were challenged at various times after inoculation. As shown in Fig. 4, no trophonts were visible on the surface of fish challenged 1 day after antibody administration. Nevertheless, fish challenged 10, 20, and 30 days following passive immunization all became infected, with the number of parasites on each fish being directly proportional to the length of time between inoculation and exposure. The number of parasites per fish in the group challenged at 30 days was significantly higher ($P < 0.05$) than that in groups challenged at 1, 10, and 20 days. Although all fish died in the groups challenged at more than 1 day following immunization, a significantly greater ($P < 0.05$) median days to death was seen in fish challenged at 10 and 20 days after MAb inoculation than in controls (data not shown). There was no significant difference ($P < 0.05$) in median days to death between controls and fish challenged at 30 days post-MAb inoculation. Finally, when immobilizing MAb titers in the sera from these fish were measured (Fig. 4), a significant difference ($P < 0.05$) was seen between titers in each group, and a proportional decrease in immobilizing activity occurred with increasing susceptibility of fish to infection.

Detection of immobilizing MAbs in mucus and serum. As indicated above (Table 1), all immobilizing MAbs that conferred passive protection were IgG isotypes. The only MAb which failed to protect was 9C9, an IgM-class antibody. To test whether IgM was present in cutaneous mucus (that is, the site of infection) at the time of challenge, mucus samples were assayed by ELISA for the presence of MAbs at 1 and 6 days after i.p. inoculation. As shown in Fig. 5, MAb 9C9 could not be detected at levels above background in either serum or mucus at 1 day postinjection. In contrast, the IgG1 isotypes 10H3 and G3-27 were readily detectable in both mucus and serum, although the levels in mucus were below that required for immobilization of live theronts in vitro (not shown). Interestingly, the kinetics of turnover of MAbs 10H3 and G3-27 appeared to be more rapid in mucus than in serum. At present,

TABLE 1. Characterization of murine MAbs against *I. multifiliis*

MAb[a]	Isotype	In vitro immobilization of parasite	Passive protection as % survival (no. live/total no.)[b]
10H3	IgG1	Yes	100 (60/60)
3H12	IgG3	Yes	80 (8/10)
8E11	IgG1	Yes	100 (10/10)
6A11	IgG1	Yes	100 (10/10)
5A8	IgG1	Yes	100 (10/10)
9C9	IgM	Yes	0 (0/20)
3B8	IgG3	No	0 (0/40)
B2-11	IgG1	No	0 (0/10)
1G1	IgG3	No	0 (0/20)
A1-4	IgM	No	0 (0/10)
G3-27	IgG1	Yes	100 (30/30)
G3-61	IgG1	Yes	100 (10/10)
G3-135	IgG1	Yes	ND
G3-140	IgG1	No	0 (0/20)
G3-74	IgG1	No	0 (0/20)

[a] MAbs 10H3 through A1-4 are against *I. multifiliis* G1; MAbs G3-27 through G3-74 are against *I. multifiliis* G3.

[b] Channel catfish were challenged with *I. multifiliis* 24 h after i.p. inoculation of MAb. Parasites were homologous in serotype to the MAb with which fish were injected. Negative controls consisted of 30 naive fish, and positive controls were 19 actively immunized animals. Controls were challenged with same number of parasites as passively immunized fish. All naive fish and fish injected with nonimmobilizing MAbs died at 5 to 10 days following challenge. Actively immunized fish all survived. The difference in percent survival between fish injected with immobilizing MAbs (IgG) or nonimmobilizing MAbs was significant ($P = 0.00$) as calculated by the z test with the Yates correction factor. ND, not done.

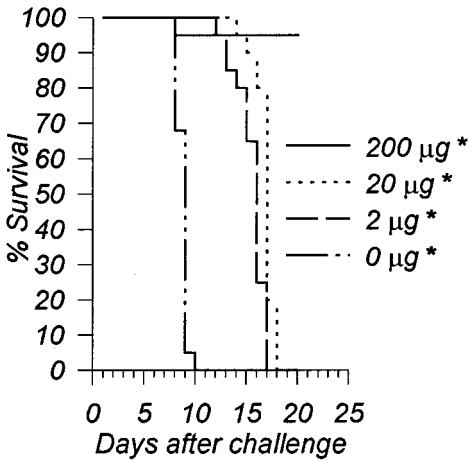

FIG. 3. Survival and days to death of channel catfish passively immunized with MAb. Fish were injected i.p. with immobilizing MAb G3-27 at doses of 200, 20, or 2 μg per fish. Control fish received PBS only. All groups were challenged 24 h after inoculation with infective *I. multifiliis* theronts. The majority (95%) of fish immunized with 200 μg of G3-27 survived infection. Fish injected with the lower doses all died following challenge. *, significant difference ($P < 0.05$) in median days to death between groups as calculated by Kruskal-Wallis analysis of variance and pairwise multiple comparison by the Student-Newman-Keuls method.

it is unclear whether this is an artifact arising from sampling or represents the kinetics of mouse antibody translocation and breakdown in mucus.

DISCUSSION

The results presented here demonstrate that mouse MAbs against *Ichthyophthirius* i-antigens protect naive fish against infection, thus providing strong support for previous arguments regarding the role of i-antigens in active immunity and the potential use of these proteins as protective immunogens.

On the basis of the number of detectable antigens in Western blot analyses, i-antigens appear to be the most predominant targets of the humoral response to the parasite (37). In addition, immobilizing antibodies have been localized to the surface mucosa of actively immune fish (10, 37). Although it would seem intuitively obvious that antibodies would have to be present at the site of infection for them to have an effect, the results of passive immunization experiments with MAb 9C9 (an IgM-class antibody) provide direct evidence for this. As shown in Fig. 5, 9C9 was not present in cutaneous mucus following i.p. injection, and while it strongly immobilized cells in vitro, it failed to confer passive protection. The inability of MAb 9C9 to reach the host surface could be due to size exclusion (mouse IgM is a pentameric 900-kDa molecule) or rapid turnover (compared with mouse IgG). Preliminary studies in this laboratory have suggested that both possibilities may be true since following intravenous injection, MAb 9C9 was present at low levels in serum and completely absent from mucus 24 h after immunization (22). It should be mentioned in this regard that we have been unable to protect naive fish against *I. multifiliis* by passive immunization with serum antibodies from convalescent fish (37) and presume that this is also due to an inability of such antibodies to reach the fish surface. Fish Igs are large tetrameric molecules (~700 kDa) and are much closer in size to mammalian IgM than IgG (6). Furthermore, studies with passively injected ^{125}I-labeled fish Ig have shown that such antibodies are excluded from mucosal surfaces of the marine teleost *Archosargus probatocephalus* (Sheepshead) because of factors other than metabolic turnover (26). There is considerable evidence that fish maintain a local secretory mucosal immunity in their skin (25–27, 34). The fact that antibodies against *I. multifiliis* are present in the mucus of actively immune fish, together with the results reported here, suggests that this secretory immunity plays an important role in host resistance. Indeed, while little is known about the nature of mucosal immunity against microbial pathogens in fish, *I. multifiliis* offers a useful model for studying this phenomenon.

Because sera and mucus from immune fish immobilize live parasites in vitro, one of the mechanisms first postulated to play a role in protection against *I. multifiliis* was immobilization of invading parasites by antibody (9, 20, 36). This idea has nevertheless been called into question by experiments in which the fate of parasites on actively immune fish was assessed by in

TABLE 2. Passive immunization of channel catfish with MAbs

MAb[a]	Immobilizing activity	Infection following challenge with *I. multifiliis*[b]	
		No. of fish infected/ no. exposed	No. of parasites per fish[c]
G3-27	Yes	1/18	0.0 (0.0, 0.0)[d]
G3-140	No	19/19	136.0 (22.5, 208.7)
G3-74	No	20/20	75 (44.0, 141.0)

[a] MAbs are against *I. multifiliis* G3.

[b] Fish (20 g each) were inoculated i.p. with 0.5 mg of purified MAb and challenged 24 h later.

[c] Parasites visible to the naked eye as white spots were counted over the entire surface on both sides of test animals in each group. Values are medians with quartiles in parentheses (25th and 75th, respectively).

[d] Significantly different ($P < 0.05$) from values for fish injected with nonimmobilizing MAbs as calculated by Kruskal-Wallis analysis of variance and pairwise multiple comparison by Dunn's method.

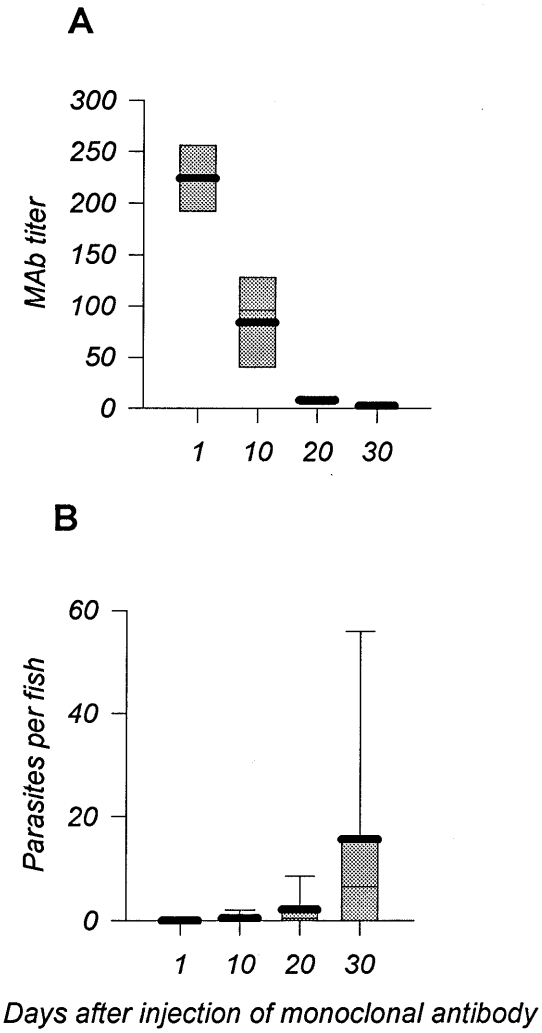

FIG. 4. Duration of MAb activity in passively immunized channel catfish. (A) Serum samples were collected at days 1, 10, 20, and 30 following i.p. inoculation of MAb G3-27 (0.5 mg per fish). Antibody titers (measured as immobilization of theronts in vitro) are shown for each time point. (B) Channel catfish were challenged at days 1, 10, 20, and 30 after i.p. inoculation of MAb G3-27. The number of trophonts (counted in the skin of fish at 5 days after exposure) are shown for each time point. In both panels the lower and upper ends of the grey boxes define the 25th and 75th percentiles, respectively, with a heavy line at the median and error bars defining the 10th and 90th percentiles.

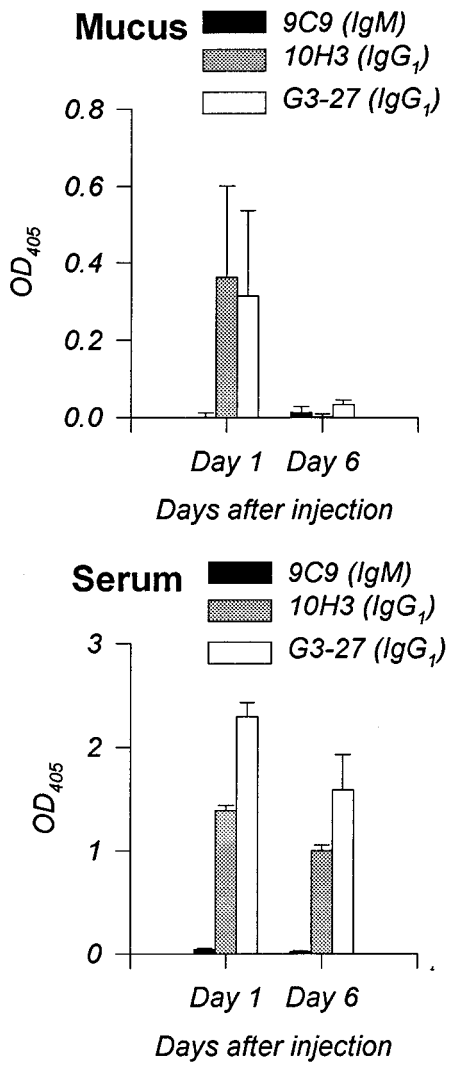

FIG. 5. Detection of immobilizing MAbs in mucus and serum. Mucus and serum samples were collected at days 1 and 6 following i.p. inoculation of immobilizing MAb 9C9, 10H3, or G3-27 (0.5 mg per fish). MAbs in mucus and sera were assayed by ELISA using plates coated with *I. multifiliis* membrane protein and rabbit anti-mouse conjugate (see Material and Methods). Signals were detected at a wavelength of 405 nm (OD, optical density). Bars indicate the relative amount of MAb present in mucus or serum at each time point (mean ± standard error; $n = 4$).

situ mapping (13). In these studies it was observed that theronts placed on tail fins of immunized carp (*Cyprinus carpio*) left the fish within 2 h of challenge. Because theronts could leave and were thus motile, it was argued that immobilizing antibodies could not play a role in this phenomenon. Despite this, we find that while the concentrations of immobilizing MAbs in the mucus of passively immunized fish are usually below the threshold for immobilization, such fish are fully protected. Furthermore, recent work from this laboratory has shown directly that immobilizing MAbs injected into *I. multifiliis*-infected fish can induce parasites to leave the host, indicating that the same mechanisms are at play in both passive and active immunity (11). The paradox of how antibodies that cause immobilization in vitro stimulate motility in vivo (that is, premature exist from the host) remains to be solved, with one possibility being that low levels of antibody induce an avoidance response by the parasite (11).

Different *I. multifiliis* serotypes have been shown to exist in nature on the basis of immobilization (15). In passive immunization experiments carried out with heterologous strains, protective immunity appeared to be serotype specific as well (11). Nevertheless, actively immune fish are protected against different serotypes (24), suggesting either that nonimmobilizing epitopes common to the i-antigens of different strains play a role in protection or that other protective antigens exist. In this regard, rabbit polyclonal antisera against affinity purified i-antigens of serotype G1 cross-react on Western blots with the i-antigens of all parasite isolates tested thus far (10, 15). Furthermore, i-antigens are the predominant cross-reacting polypeptides in Western blots probed with antisera from actively immune fish (37). Thus, it is clear that the i-antigens of different serotypes have shared antigenic determinants. We are currently cloning i-antigen genes from different serotypes and hope to identify unique and conserved i-antigen epitopes by

sequence analysis and epitope mapping. The number of immobilization serotypes of *I. multifiliis* that occur in nature is unknown. To date we have found five different serotypes (designated A to E) among eight isolates. Two isolates belong to serotype C, and three isolates belong to serotype D. We have not found mixed infections in naturally occuring outbreaks and do not yet know whether cross-reactive immunity plays a role in nature. Nevertheless, epidemiologic questions such as these are of obvious interest for vaccine development and are currently being investigated.

In summary, passive immunization of channel catfish with mouse MAbs suggests that the i-antigens of *I. multifiliis* are protective antigens and as such are primary candidates for use in a subunit vaccine against this virulent parasite. Moreover, the efficacy of immobilizing MAbs themselves in preventing and eliminating *I. multifiliis* infection points the way toward their use as therapeutic agents in fish.

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