

## Surface antigen cross-linking triggers forced exit of a protozoan parasite from its host

(immunotherapy/ciliate/catfish/*Ichthyophthirius*/GPI-anchor)

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**ABSTRACT** We used the common fish pathogen *Ichthyophthirius multifiliis* as a model for studying interactions between parasitic ciliates and their vertebrate hosts. Although highly pathogenic, *Ichthyophthirius* can elicit a strong protective immune response in fish after exposure to controlled infections. To investigate the mechanisms underlying host resistance, a series of passive immunization experiments were carried out using mouse monoclonal antibodies against a class of surface membrane proteins, known as immobilization antigens (or i-antigens), thought to play a role in the protective response. Such antibodies bind to cilia and immobilize *I. multifiliis* *in vitro*. Surprisingly, we found that passive antibody transfer *in vivo* caused rapid exit of parasites from the host. The effect was highly specific for a given *I. multifiliis* serotype. F(ab)<sub>2</sub> subfragments had the same effect as intact antibody, whereas monovalent Fab fragments failed to protect. The activity of Fab could, nevertheless, be restored after subsequent *i.p.* injection of bivalent goat anti-mouse IgG. Parasites that exit the host had detectable antibody on their surface and appeared viable in all respects. These findings represent a novel instance among protists in which protective immunity (and evasion of the host response) result from an effect of antibody on parasite behavior.

The parasitic ciliate *Ichthyophthirius multifiliis* has significant impact on commercial aquaculture, and it is well-known to amateur fish enthusiasts as the causative agent of “Ich,” or “white-spot” (1, 2). Aside from its practical importance, it offers a unique model for the study of host–parasite interactions (3). *Ichthyophthirius* invades the epithelium of the skin and gills and readily kills fish maintained in closed aquatic systems. Nevertheless, fish can develop a strong acquired immunity against the parasite after exposure to controlled infections (for review, see ref. 4). Indeed, recent work has suggested that a novel mechanism of protection may underlie host resistance (5).

Studies with actively immune carp have shown that parasites can invade epithelium, but ≈80% disappear within 2 hr of infection with little or no trace. Based on these findings, it was concluded that immunity results from rapid, premature exit of parasites from the host (5). Although this conclusion was intriguing, a mechanism that could account for this phenomenon was lacking. Furthermore, previous studies (4) have supported a more conventional model in which parasites are either blocked from entry, or killed *in situ*, by antibodies against a defined class of surface membrane proteins known as immobilization antigens, or i-antigens (6, 7).

The i-antigens comprise a family of highly abundant, Glycosyl Phosphatidylinositol (GPI)-anchored membrane proteins (8, 9) that have been actively studied in the free-living ciliates *Paramecium* and *Tetrahymena* in connection with antigenic switching at the cell surface (10, 11). Antibodies

directed against these proteins bind cilia and prevent cell movement. The i-antigens of *Ichthyophthirius* are immunodominant in fish (C. Xu & T.-L.L., unpublished data), and the response to natural infection includes the production of both serum and mucus antibodies that immobilize all stages of the parasite *in vitro* (4). Clearly, the presence of immobilizing antibodies within host tissues is inconsistent with observations that parasites can invade and then escape the epithelium of actively immune fish.

To address this question, a series of passive immunization experiments have been carried out using mouse mAbs against the i-antigens of *Ichthyophthirius*. Rather than immobilizing the parasite *in vivo*, we found that such antibodies caused the rapid exit of *I. multifiliis* from the host. The effect was highly specific for a given *I. multifiliis* serotype and required antigen cross-linking on the parasite surface.

### MATERIALS AND METHODS

**Parasite Cultures.** *I. multifiliis* (serotype G3) was isolated from an infected brown trout (*Salmo trutta*) obtained from a local hatchery. Parasites were cultured from a single cloned tomtom and maintained in the laboratory on juvenile channel catfish (*Ictalurus punctatus*) as described (12). A independent isolate, designated G4, was obtained from an outbreak of *I. multifiliis* in the channel catfish holding facility at the University of Georgia. This isolate was cloned and maintained as above. Based on immobilization assays with polyclonal mouse and catfish antisera, G4 was found to have the same serotype specificity as a previously characterized strain, designated G2 (4, 13).

**mAbs.** BALB/c mice were immunized with a detergent-soluble membrane protein fraction from *I. multifiliis* theronts (6), and hybridomas were prepared as described (6). Hybridoma supernatants were screened by ELISA for the production of antibodies against the membrane protein fraction, and positive cells were cloned three times by limiting dilution. Supernatant fluids were then rescreened for their ability to immobilize *I. multifiliis* theronts using standard assays (6, 14). Four cell lines tested positive in these assays, and one (designated G3-61) was used for the production of immobilizing mAbs. A cell line that produced nonimmobilizing mAbs (designated G3-74) was used for the isolation of control antibodies.

For mAb purification, BALB/c mice were primed with pristine (Aldrich) and inoculated with  $1 \times 10^7$  antibody-producing cells. Ascites fluids were harvested 10–14 days later, and antibodies were isolated using a commercial purification kit (E-Z SEP—B ascites) as described by the manufacturer (Middlesex Sciences Inc., Foxborough, MA). Protein concen-

Abbreviations: i-antigen, immobilization antigen; GPI, Glycosyl Phosphatidylinositol.

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trations were determined spectrophotometrically (15). mAb isotypes were determined by ELISA using rabbit anti-mouse immunoglobulin (class and subclass) specific antisera (Sigma). Although a complete description of mAb specificities is not reported here, mAb G3-61 was found by affinity chromatography and SDS/PAGE to bind a predominant 55-kDa membrane antigen in the G3 serotype (not shown). In previous experiments (4), this same polypeptide was shown to react with a monospecific polyclonal rabbit antiserum that recognizes the i-antigens of a number of different *I. multifiliis* strains. The control antibody, mAb G3-74, was found to react exclusively with a 14-kDa membrane polypeptide on Western blots.

**Host Infection and Passive Immunization.** For whole body infections, juvenile channel catfish (10–20 g) were placed in jars containing 800–1000 ml of carbon-filtered water and exposed to *I. multifiliis* theronts at an infective dose of 5000 parasites per fish for 24 hr. For tail fin infections (unless otherwise noted), fish were anesthetized with tricaine methanesulfonate (100–200 mg/liter), and their tails were placed in a trough containing 50 ml of infectious theronts at a concentration of 500 parasites per ml for 5 min. After exposure to parasites, animals were rinsed briefly with carbon-filtered water and placed in 38-liter aquaria. For passive immunizations, mAbs were brought to final concentration of 2.5 mg/ml in PBS containing 136 mM NaCl, 2.7 mM KCl, 8 mM Na<sub>2</sub>PO<sub>4</sub>, and 1.5 mM KH<sub>2</sub>PO<sub>4</sub> and injected into the peritoneal cavity of fish using a 1-ml syringe fitted with a 25 gauge needle.

**Generation of Proteolytic Subfragments.** After initial purification (see above), mAbs were subjected to molecular sieve chromatography on Sephacryl S-200 (Pharmacia), and the antibody-containing fractions were digested under standard conditions with either pepsin or papain for the production of F(ab)<sub>2</sub> and Fab subfragments, respectively (15). Intact antibody and contaminating Fc fragments were removed from protein digests by affinity chromatography on protein-A agarose (Boehringer Mannheim) (15). Before loading on the column, antibody-containing solutions were adjusted to 3.3 M NaCl, 1.5 M glycine, and 0.1 M sodium borate (pH 8.9). Eluted fractions were passed over the column a second time, and the resulting Fab and F(ab)<sub>2</sub> subfragments were concentrated by addition of saturated ammonium sulfate to a final concentration of 60% (wt/vol). Precipitates were then dissolved in PBS and dialyzed against the same buffer before freezing at –80°C. Proteolytic subfragments were judged to be >98% pure by SDS/PAGE. Immobilizing activities of Fab and F(ab)<sub>2</sub> preparations were determined by *in vitro* assay (6). At roughly equivalent concentrations, the immobilization activity of F(ab)<sub>2</sub> fell within two doubling dilutions of intact antibody, whereas the activity of Fab was ≈500-fold less.

**Immunofluorescence Microscopy.** Parasites that had exited fish following passive antibody transfer were fixed for 10 min with 4% formalin in 0.5× PBS. Cells were then washed extensively in 0.5× PBS and reacted for 1 hr with a 1:50 dilution of fluorescein isothiocyanate-labeled goat anti-mouse IgG (Sigma) in 0.5× PBS. Unbound antibody was removed by successive washes in 0.5× PBS. Cells were observed using an Olympus BH-2 phase contrast microscope equipped with fluorescence optics and photographed with Kodak Tri-X film.

## RESULTS

### Effect of Immobilizing mAbs on Infected Channel Catfish.

A panel of mouse mAbs developed in this laboratory has been used to characterize the i-antigens of *Ichthyophthirius* (4, 6). The specific mAb used in this study, designated G3-61, is an IgG<sub>1</sub> isotype that strongly immobilizes all stages of *Ichthyophthirius* (strain G3) *in vitro* and binds the predominant 55-kDa i-antigen expressed by the G3 serotype. To determine the effect of mAb G3-61 on parasites *in vivo*, juvenile channel catfish were exposed to *I. multifiliis* infections and maintained

in aquaria for 3 days to allow parasites to develop. Fish were then divided into two equal groups and injected i.p. with either 0.5 mg of purified mAb G3-61 or 0.5 mg of the control antibody, mAb G3-74, which binds a 14-kDa *Ichthyophthirius* membrane protein and completely lacks immobilization activity *in vitro*. When examined 24 hr after immunization, mAb G3-61-injected fish had no visible parasites, whereas control animals were heavily infected and subsequently died. The appearance of immunized fish is shown in Fig. 1. By subjecting test animals to localized infections of their tail fins, trophonts (the host-associated stage of *I. multifiliis*) could be visualized by phase contrast microscopy within the epidermis (Fig. 1), and the kinetics of the antibody effect could be determined. As shown in Fig. 2, parasites were found to rapidly disappear from mAb G3-61-injected fish beginning ≈3 hr after inoculation and were essentially gone by 12 hr.

To determine whether mAb G3-61 was acting directly on parasites, experiments involving mixed infections with differ-

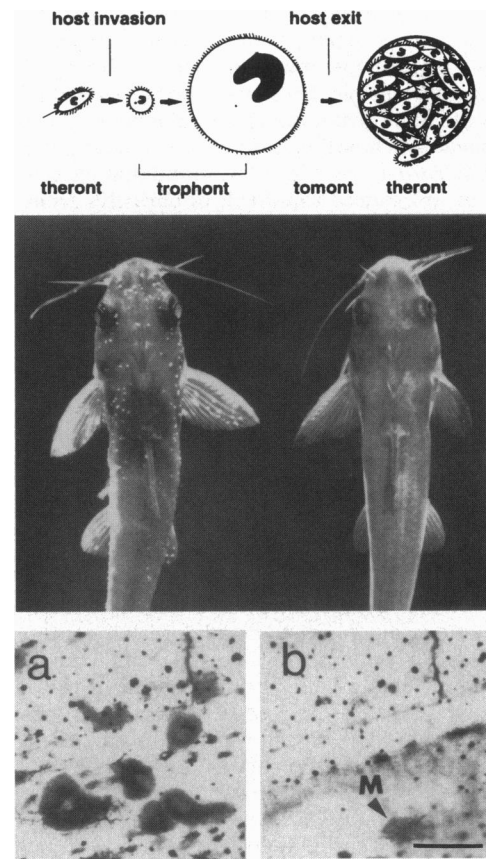


FIG. 1. Passive immunization of infected channel catfish. (Top) The life cycle of the parasite. Theronts swim through the water and invade the skin and gill epithelia of susceptible hosts. While on the fish they transform into trophonts that feed off host tissue. Over the course of the next 4–7 days (22°C), they grow large enough to become visible to the naked eye as individual white spots on the fish. In response to unknown cues, trophonts leave the host and again swim through the water (as tomonts). Parasites then attach to an inert support, encyst, and divide to form 100–1000 daughter cells (18–24 hr at 22°C), which finally escape the cyst to complete the life cycle. (Middle) Juvenile channel catfish were infected with *I. multifiliis* and, 72 hr later, injected i.p. with 0.5 mg each of either mAb G3-61 (Right) or mAb G3-74 as a control (Left). Note the absence of spots on the fish on the right. Photographs were taken 24 hr after passive immunization. Identical results were obtained with 28 fish in four separate experiments (control animals all died within 2 weeks of *I. multifiliis* infection). (Bottom) A region of the tail fin of a fish is shown in phase contrast before (a) and 24 hr after (b) passive immunization with 0.5 mg of mAb G3-61. For reference, seven trophonts are visible in a. M, melanophore. (×50; bar = 250 μM.)

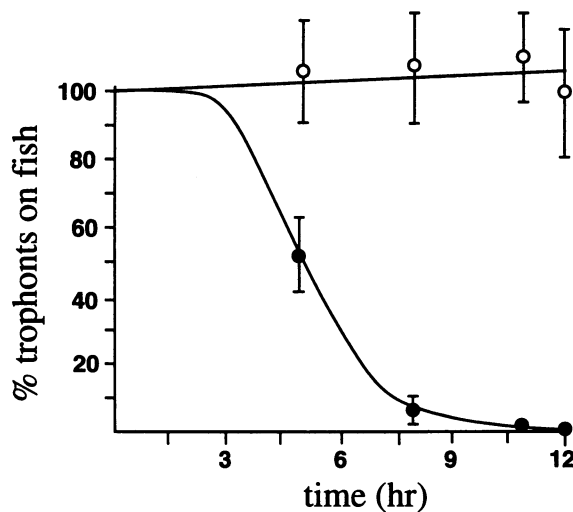


FIG. 2. Kinetics of parasite clearance. Six juvenile channel catfish were subjected to tail fin infections. After 3 days, animals were examined, and the number of parasites on the tails of each fish was counted. Animals were then divided into two equal groups (with an average of 149 trophonts per tail fin in each group) and injected i.p. with either 0.5 mg of mAb G3-61 (●) or 0.5 mg of mAb G3-74 (○). After immunization, parasite numbers were counted at periodic intervals over 12 hr. For quantitation, fish were anesthetized in tricaine methanesulfonate (100–200 mg/liter) and then covered loosely in plastic wrap (with the tail fin exposed). Animals were then placed on a glass slide, and the tail fins were examined under phase contrast optics using a  $\times 4$  objective mounted on an Olympus CK2 inverted microscope. Fish were returned to the water within 10 min to ensure recovery from the anesthetic. The increase in parasite numbers seen on control animals is consistent with a previous study, suggesting that trophonts divide on the fish (16) (see also Tables 1 and 2).

ent *I. multifiliis* serotypes were carried out. Fish were simultaneously infected on their upper and lower tail fins with the G3 strain (upper tail fin) and with a heterologous serotype designated G4 (lower tail fin). The G4 serotype is phenotypically indistinguishable from G3 at the light microscope level but expresses different i-antigens (4) and is not immobilized by mAb G3-61. As shown in Table 1, although G3 trophonts were completely cleared after passive antibody transfer, mAb G3-61 had no observable effect on the G4 strain.

#### Antigen Cross-Linking Is Required for Parasite Clearance.

In experiments using proteolytic subfragments of mAb G3-61, bivalent F(ab)<sub>2</sub> was found to have the same effect as intact antibody, whereas monovalent Fab failed to elicit parasite clearance (Table 1). Interestingly, the lack of an effect by Fab correlated with its inability to immobilize the parasite *in vitro*. This was not due to an absence of antigen binding, since [as demonstrated previously with *Paramecium* (17, 18)] the activity of Fab could be restored to within two doubling dilutions of intact antibody by subsequent addition of bivalent goat anti-mouse IgG to immobilization assays (data not shown). Interestingly, goat antibody could restore the activity of Fab *in vivo* as well. Administration of 0.5 mg of goat anti-mouse IgG to infected animals 9 hr after injection with Fab resulted in >80% reduction in the number of parasites present on fish (Table 1).

**Forced Exit of *Ichthyophthirius* in Response to Antibody.** In the previous experiments, obvious signs of cell lysis did not accompany parasite clearance, and the possibility that trophonts simply left the host in response to mAb G3-61 was considered. This possibility was difficult to verify by direct (microscopic) observations of trophont behavior, because prolonged anesthesia killed the fish. Moreover, parasites normally begin to mature and exit the host within the time frame of these experiments (19, 20), and it would have been impos-

Table 1. Effect of mAbs on parasite load

Serotype	Antibody preparation	Avg. no. parasites per tail	
		0 hr	24 hr
G3*	G3-61	28 $\pm$ 5 <sup>†</sup>	0
G4*	G3-61	35 $\pm$ 3 <sup>†</sup>	71 $\pm$ 27
G3	F(ab) <sub>2</sub>	39 $\pm$ 44 <sup>‡</sup>	0
G3	Fab	49 $\pm$ 44 <sup>‡</sup>	76 $\pm$ 67
G3	Fab + G $\alpha$ M	254 $\pm$ 238 <sup>§</sup>	50 $\pm$ 29
G3	G3-74 + G $\alpha$ M	101 $\pm$ 14 <sup>†</sup>	141 $\pm$ 22

Mixed infections with different *I. multifiliis* serotypes (\*) were carried out by applying theronts of the G3 and G4 strains to small regions (10–20 mm<sup>2</sup>) on the upper and lower tail fins of fish. Polypropylene centrifuge tubes (15-ml conical tubes with their bottoms cut off) were pressed firmly against the upper and lower parts of the fin, and cell suspensions containing 500 theronts per ml were added to each tube (G3 on the upper fin and G4 on the lower fin). Preparations were incubated for 5 min to allow parasites to establish on the host before the suspensions were withdrawn. Fish were then rinsed briefly in H<sub>2</sub>O and returned to aquaria. Fish were injected with 0.5 mg of the listed antibody preparation 72 hr after initial exposure to the parasite. Tail fins were examined by phase contrast microscopy, and trophonts were counted just before (0 hr) and 24 hr after passive immunization. As was evident in Fig. 1, parasite numbers increase on the control animals during the time course of these studies (16). The effect of goat anti-mouse IgG (G $\alpha$ M) was examined in separate experiments in which the secondary antibody was administered 9 hr after mAb injection. The greater number of parasites seen in the Fab + G $\alpha$ M group reflects a higher initial infective dose (2500 theronts per ml). \*Mixed infections on the same tail fin.

<sup>†</sup>Three fish  $\pm$  SD.

<sup>‡</sup>Four fish  $\pm$  SD.

<sup>§</sup>Five fish  $\pm$  SD.

sible to know for any given trophont whether it had exited the fish on its own or had been driven off by antibody. Indirect studies, nevertheless, provided clear evidence on this point. Infected channel catfish were injected with either mAb G3-61 or the control antibody, mAb G3-74, and parasites were harvested from the water 24 hr after immunization. As shown in Table 2, nearly 80% of tomonts recovered from G3-61-injected fish had initiated cell division at the time they were harvested, compared with only 14% in the case of the control group. Furthermore, tomonts collected from G3-61-injected

Table 2. Forced exit of parasites from fish

Antibody preparation	% Tomonts dividing	Average no. of theronts per tomont	Total estimated tomonts
G3-61	78.9%*	65.7*	577
G3-74	14.2% <sup>†</sup>	116.0 <sup>†</sup>	262

Ten juvenile channel catfish were exposed to whole-body infections. After 72 hr, animals were divided into equal groups and injected i.p. with either 0.5 mg of mAb G3-61 or the same amount of mAb G3-74 as a control. Immediately after mAb injection, each group was placed in a separate beaker with 1.5 liters of H<sub>2</sub>O. Fish were removed 24 hr later, and representative samples of tomonts (\*, <sup>†</sup>) were placed individually into the wells of 96-well plates. Parasites were examined under a dissecting microscope, and the percent undergoing division was determined. Tomonts in the plates were maintained for an additional 24 hr, after which all had completed division. Individual wells were pooled, and resulting daughter cells (theronts) were counted with a hemocytometer. The average number of theronts per tomont was then calculated for each representative group. Finally, theronts present in the beakers (48 hr after immunization) were harvested by centrifugation at 1000  $\times$  g for 2 min in oil centrifuge tubes (IEC HN-SII centrifuge) and counted as above. Estimates of the total number of tomonts that had left the fish were made by dividing total theronts (beakers plus 96-well plates) by the average number of theronts per tomont calculated for each group.

\*Based on 204 tomonts.

<sup>†</sup>Based on 148 tomonts.

fish gave rise to only half the number of daughter cells than did those from controls (Table 2). The number of theronts generated per tomont is directly proportional to tomont size, which in turn is a function of the length of time parasites stay on the fish; the sooner they exit, the smaller they are (21). Finally, estimates of the total numbers of parasites in the water revealed that more than twice as many had exited mAb G3-61-injected fish than had left the controls. In further experiments in which the actual numbers of parasites in the water were counted (rather than inferred), we consistently found 2–10 times the number of tomonts exiting G3-61-injected fish than the controls. As shown in Fig. 3, parasites obtained from these fish were motile, and virtually all had mouse antibody bound to their surface.

## DISCUSSION

Taken together, the results described here argue strongly that parasites are forced to exit the host in response to antibody and provide the molecular basis for previous observations by Cross and Matthews (5), who showed that infective theronts can invade the epithelium of actively immune carp, but appear to exit the host prematurely. In this regard, the ability of immobilizing antibodies to trigger, what is essentially a cell motility phenomenon, might seem paradoxical. Nevertheless, immobilization *in vitro* requires a threshold of antibody concentration, and we presume the stimulus for premature exit occurs at antibody levels that are below that threshold. Interestingly, treatment of the free-living ciliate *Paramecium* with concentrations of i-antigen antibody that are subthreshold for immo-

bilization results in a classical avoidance response (22). Ciliates in general are exquisitely sensitive to chemical gradients, and their response [directional swimming or chemokinesis (23)] is controlled through electrophysiological properties of the ciliary/plasma membrane (24). While a mechanism of immunity involving avoidance behavior would tie a problem in immunoparasitology to observations on cell motility dating back almost a century (25), other possibilities could explain this phenomenon as well. For example, antigen cross-linking could activate (or reverse) the normal developmental switch that regulates the transition between (fish-associated) trophont and free-swimming (tomont and theront) stages of the parasite.

Regardless of the precise mechanism involved, it is clear that mAb G3-61 acts directly on the parasite and that antibody binding alone is not sufficient to cause the effect. Thus, forced exit almost certainly reflects a physiological response by the parasite (leading to a change in establishment behavior), rather than a passive process such as simple detachment resulting from competition between mAb G3-61 and some hypothetical ligand within the host. The i-antigens are GPI-anchored proteins (8, 9), and while their biological function is unknown, it is conceivable that they play a role in signal transduction. While such proteins are restricted to the outer leaflet of the plasma membrane, it should be mentioned that antibody-mediated clustering of GPI-anchored proteins in mammalian T-cells promotes lymphocyte activation through a pathway that appears to involve tyrosine kinases of the Src family (26).

To our knowledge, a mechanism of protective immunity involving an effect of antibody on parasite behavior would be extremely novel. Indeed, we know of only one possible analogy to this situation in nature, namely, immunity in rats to the metazoan *Trichinella spiralis*. In this case, antibodies mediate rapid expulsion of infectious organisms from the host by mechanisms that appear to involve both host and parasite functions (27, 28).

While the results presented here indicate that forced exit plays a role in protective immunity against *Ichthyophthirius*, other mechanisms may contribute to host resistance as well. Substantial numbers of parasites may, in fact, be killed on the fish after antibody binding, and indirect evidence has suggested that more than a single mechanism may be at play in acquired immunity (29, 30). Nevertheless, the ability of mAb G3-61 to passively protect fish against *I. multifiliis* is noteworthy in at least two further respects. First, the degree of protection afforded in this case is extraordinarily high (essentially 100%) and rivals that for any host-parasite system known. Second, because trophonts are confined to the epidermal layer of the skin and gills, mouse antibodies (in this case an IgG<sub>1</sub>) must reach the surface of fish in order for them to be effective. This is significant with respect to the immune system of teleosts, since fish immunoglobulin (which is tetrameric) is highly restricted in its ability to reach the mucosal surfaces of experimental animals after i.p. injection (31). While it is known that antibodies are present at the surface of fish (that is, in mucus) (32, 33), the origin of such antibodies is poorly understood.

Finally, since tomonts that exit fish in response to antibody appear viable, the effect described here may spare both the parasite and the host and could be viewed equally as a mechanism of host evasion (by the parasite) and protective immunity (for the host).

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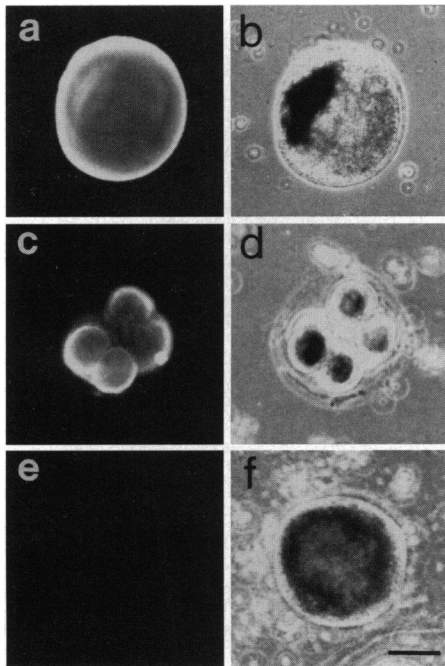


FIG. 3. Antibody binding to the surface of *Ichthyophthirius*. Paired fluorescence (*a*, *c*, and *e*) and phase contrast micrographs (*b*, *d*, and *f*) of *I. multifiliis* tomonts are shown. (*a–d*) Cells of the G3 serotype harvested from water surrounding infected fish 12 hr after passive immunization with 0.5 mg of mAb G3-61. Parasites were fixed and then reacted with fluorescein isothiocyanate-labeled goat anti-mouse IgG. The tomont in panels *c* and *d* had encysted and undergone two divisions. A total of 30 tomonts were examined, and all had detectable antibody bound to their surface. (*e* and *f*) A tomont of the G4 serotype (as a negative control) that had been reacted sequentially with mAb G3-61 (5  $\mu$ g/ml in PBS), followed by fluorescein isothiocyanate-labeled goat anti-mouse IgG as above. Note the lack of antibody staining in *e* (consistent with the known absence of cross-reacting i-antigens on the surface of the G4 strain). ( $\times 98$ ; bar = 100  $\mu$ M.)

1. Matthews, R. A. (1994) in *Parasitic Diseases of Fish*, eds. Pike, A. W. & Lewis, J. W. (Samara, Dyfed, U.K.), pp. 17–42.
2. Dickerson, H. W. & Dawe, D. L. (1995) in *Fish Diseases and Disorders*, ed. Woo, P. T. K. (CAB International, Wallingford, U.K.), pp. 181–227.
3. McCallum, H. I. (1982) *Parasitology* **85**, 475–488.
4. Clark, T. G., Lin, T. L. & Dickerson, H. W. (1996) *Annu. Rev. Fish Dis.* **5**, 113–131.
5. Cross, M. L. & Matthews, R. A. (1992) *J. Fish Dis.* **15**, 497–505.
6. Lin, T. L. & Dickerson, H. W. (1992) *J. Protozool.* **39**, 457–463.
7. Clark, T. G., McGraw, R. A. & Dickerson, H. W. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 6363–6367.
8. Capdeville, Y., Cardoso de Almeida, M. L. & Deregnacourt, C. (1987) *Biochem. Biophys. Res. Commun.* **147**, 1219–1225.
9. Ko Y.-G. & Thompson, G. A., Jr. (1992) *J. Protozool.* **39**, 719–723.
10. Preer, J. R., Jr. (1986) in *The Molecular Biology of Ciliated Protozoa*, ed. Gall, J. G. (Academic, London), pp. 301–339.
11. Smith, D. L., Berkowitz, M. S., Potoczak, D., Kruse, M., Raab, C., Quinn, F. & Doerder, F. P. (1992) *J. Protozool.* **39**, 420–428.
12. Noe, J. G. & Dickerson, H. W. (1995) *J. Parasitol.* **81**, 1022–1024.
13. Dickerson, H. W., Clark, T. G. & Leff, A. A. (1993) *J. Eukaryot. Microbiol.* **40**, 816–820.
14. Clark, T. G., Dickerson, H. W., Gratzek, J. B. & Findly, R. C. (1987) *J. Fish Biol.* **31**, 203–208.
15. Harlow, E. & Lane, D. (1988) *Antibodies: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
16. Ewing, M. S., Ewing, S. A. & Kocan, K. M. (1988) *J. Protozool.* **35**, 549–552.
17. Eisenbach, L., Ramanathan, R. & Nelson, D. (1983) *J. Cell Biol.* **97**, 1412–1420.
18. Barnett, A. & Steers, E., Jr. (1984) *J. Cell Sci.* **65**, 153–162.
19. MacLennan, R. F. (1942) *J. Exp. Zool.* **91**, 1–13.
20. Ewing, M. S., Lynn, M. E. & Ewing, S. A. (1986) *J. Protozool.* **33**, 388–391.
21. Canella, M. F. & Rocchi-Canella, I. (1976) *Ann. Univ. Ferrara Sez. III* **3**, 1–510.
22. Preer, J. R., Jr. (1959) *J. Immunol.* **83**, 276–283.
23. Van Houten, J. & Preston, R. R. (1988) in *Paramecium*, ed. Görtz, H.-D. (Springer, New York), pp. 282–300.
24. Machemer, H. (1988) in *Paramecium*, ed. Görtz, H.-D. (Springer, New York), pp. 185–235.
25. Jennings, H. S. (1906) *Behavior of the Lower Organisms* (Columbia Univ. Press).
26. Brown, D. (1993) *Curr. Opin. Immunol.* **5**, 349–354.
27. Appleton, J. A. & McGregor, D. D. (1984) *Science* **226**, 70–72.
28. Bell, R. G., Appleton, J. A., Negrao-Correa, D. A. & Adams, L. S. (1992) *Immunology* **75**, 520–527.
29. Houghton, G. & Matthews, R. A. (1990) *J. Fish Dis.* **13**, 269–280.
30. Leff, A. A., Yoshinaga, T. & Dickerson, H. W. (1994) *J. Fish Dis.* **17**, 429–432.
31. Lobb, C. J. & Clem, L. W. (1981) *Immunology* **127**, 1525–1529.
32. Bradshaw, C. M., Richard, A. S. & Sigel, M. M. (1971) *Proc. Soc. Exp. Biol. Med.* **136**, 1122–1124.
33. Lobb, C. J. (1987) *Dev. Comp. Immunol.* **11**, 727–738.