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Purification and Partial Characterization of Immobilization Antigens from *Ichthyophthirius multifiliis*

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ABSTRACT. *Ichthyophthirius multifiliis*, a parasitic ciliate of freshwater fishes, was found to have surface antigens (Ag) which elicited immobilizing antibodies (Ab) when injected into rabbits. An effort was made to purify and characterize these Ag (referred to as immobilization Ag) because of their potential role in protective immunity in fishes. Mice immunized with theront cilia were used for production of immobilizing monoclonal antibodies (MAb). Hybridomas were screened by indirect immunofluorescent light microscopy and immobilization of live parasites. Six hybridomas producing immobilizing MAb were cloned. Immobilizing MAb were used to affinity purify Ag solubilized with Triton X-114 and Na deoxycholate. Two membrane protein Ag of approximately 48 and 60 kDa were identified. Immobilizing MAb failed to react with these Ag on Western blots and, conversely, MAb that reacted with the Ag on Western blots did not immobilize live organisms. These results suggest that immobilization required native conformational epitopes which were altered by Western blotting procedures. Individual MAb reactive on Western blots recognized both the 48- and 60-kDa proteins indicating the presence of common epitopes. Affinity purified Ag elicited immobilizing antisera when injected into rabbits, mice, and channel catfish.

Key words. Antisera, immunofluorescence, monoclonal antibody, parasite, protective immunity, surface membrane proteins, Western blot.

THE free-living ciliates *Paramecium* and *Tetrahymena* become immobilized by antibodies (Ab) that react with surface proteins referred to as immobilization antigens (i-Ag) [9, 18, 19, 21, 22, 27]. In *Paramecium*, the i-Ag are large glycosylated proteins varying in size from 251 to 300 kDa [18, 21]. The i-Ag of *Tetrahymena* are smaller, non-glycosylated proteins ranging from 30 to 58 kDa [1, 9, 27]. Different i-Ag are produced under various environmental conditions making them a useful system for studying the regulation of expression of surface Ag genes [17, 18, 20, 24]. Their function, however, remains unknown [1, 19].

Immobilization has also been described in the taxonomically related ciliate *Ichthyophthirius multifiliis* (order Hymenostomatida), a parasite of freshwater fishes. Sera from fishes which survive infection immobilize the organism in vitro [6, 13]. Immobilizing Ab from fishes react with surface membrane proteins [5]. In a previous study it was found that antisera from rabbits inoculated with membrane protein fractions of the parasite also immobilized the parasite and reacted with a prominent 43-kDa membrane glycoprotein on Western blots [8]. The gel-purified (and denatured) 43-kDa Ag, however, failed to elicit immobilizing antisera when injected into rabbits [8].

We sought to identify unequivocally the i-Ag of *I. multifiliis* because of their potential role in protective immunity [5, 13]. Possible approaches to this problem included the purification of large quantities of native protein for the production of antisera. This was obviated by the fact that *I. multifiliis* is an obligate parasite and difficult to grow in quantities sufficient for these purposes. Monoclonal antibody (MAb) production provided an alternative method to identify the Ag in question.

In this paper we describe six murine MAb that immobilized both the infective (theront) and feeding (trophont) stages of *I. multifiliis*. Immobilizing MAb recognized two antigenically related membrane proteins of approximately 48 and 60 kDa. Conformational epitopes of these surface Ag appeared to be crucial for immobilization of the parasite. Antigens purified by affinity chromatography elicited the production of immobilizing antisera.

MATERIALS AND METHODS

***Ichthyophthirius multifiliis* culture.** An *I. multifiliis* culture was isolated from an infected fish obtained at a local pet store and maintained by serial passage on channel catfish (*Ictalurus punctatus*) [8]. Theronts and trophonts (previously referred to as tomites and trophozoites, respectively) were collected as described [8].

Production of MAb. Hybridomas were prepared by standard procedures [16, 23]. Balb/c mice were injected intraperitoneally (ip) twice and intrasplenically once with 50 μg and 10 μg, respectively, of ciliary protein [see 8]. Three days after the intrasplenic injection spleen cells were fused with SP2/0-Ag-14 myeloma cells (American Type Culture Collection, Bethesda, MD). Culture supernatant fluids were screened for Ab against parasites by indirect immunofluorescent assay (IFA) and enzyme-linked immunosorbent assay (ELISA). Positive cells were cloned 3× by limiting dilution and their supernatant fluids tested by immobilization and Western blotting. The MAb class and subclass were determined by Ouchterlony gel diffusion tests using rabbit anti-mouse immunoglobulin class and subclass specific antisera (Sigma Chemical Co., St. Louis, MO). To produce ascites fluid, 1×10^6 antibody-producing hybridoma cells were inoculated ip into Balb/c mice primed with pristane (Aldrich Chemical Co., Milwaukee, WI). Ascites fluids were harvested 10–14 days later.

Preparation of ascites fluids and antisera. Ascites fluids containing serum Ab were produced in Balb/c mice immunized with trophonts, ciliary membrane proteins, and affinity-purified proteins. Two inoculations (50 μg cell and membrane protein or 10 μg affinity-purified protein) were given ip with Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FICA) (Sigma) at 3-wk intervals. A third inoculation was given without adjuvant. After 4 days 1×10^6 SP2 cells were injected ip and the ascites fluids were collected.

Antisera against affinity-purified proteins were produced in New Zealand White rabbits and channel catfish. In rabbits, two inoculations of 50 μg each were given subcutaneously (with FCA and FICA, respectively) in multiple dorsal-lumbar sites at 3-wk intervals. Blood was collected from ear veins two weeks after the last injection. Fish weighing 15–20 g were injected ip with 5 μg of protein in FCA or FICA at 3-wk intervals. Blood was collected from caudal veins/arteries after 3 weeks.

Affinity-purified Ab were prepared from mouse ascites fluids and rabbit antisera using MAb affinity-purified antigen coupled to cyanogen bromide-activated sepharose columns [14].

Ascites fluids and sera were heat inactivated at 56° C for 30 min and stored at −70° C.

Immobilization assays. Immobilization assays were performed as described previously [6]. End-point titers were the

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inverse of the highest dilution in which all of the organisms were immobilized.

To assay for immobilization inhibition, Ab solutions were adjusted to concentrations 4× higher than those of end-point titers (e.g. if an end-point titer was 16, then the titer used in the test would be 4). Fifty microliters of Ab was incubated with various Ag in 50 μl of phosphate buffered saline (PBS: 136 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4) for 1 h at 37° C. The following Ag were tested: MAb affinity-purified protein (1.25 μg), total membrane protein fractions (10 μg), and membrane protein fractions depleted of protein by passage over MAb affinity columns (10 μg). Live theronts (10^3 in 10 μl) were added and incubated for 1 h at room temperature (RT: 23–25° C). The cells were examined for immobilization every 15 min with a dissecting microscope.

Enzyme-linked immunosorbant assay. Cilia or freeze-thawed cell lysates were suspended in 10 mM Na-acetate (pH 7.0) and adjusted to protein concentrations of 50 μg/ml. Fifty microliters was added to each well of 96-well plates (Falcon 3911 Microtest III, Becton Dickinson, Oxnard, CA) and dried under vacuum. Protein binding sites were blocked with 1% (w/v) bovine serum albumin (BSA) in PBS. Antibodies diluted in 1% BSA/PBS were added and incubated at RT for 1 h. The plates were washed 3× with 0.05% (v/v) Tween 20 (Sigma) in PBS (TPBS). Goat anti-mouse IgA,M,G peroxidase conjugate (Sigma) diluted 1:500 in 1% BSA/PBS, was added and incubated as above. Plates were washed 3× with TPBS and once with distilled water before the addition of the o-phenylenediamine chromogenic enzyme substrate. Antibody binding was detected by measuring absorbance at 492 nm with an ELISA plate reader (Bio-RAD Model 2550, Bio-Rad Laboratories, Richmond, CA).

We used ELISA to compare the amounts of specific Ag in ciliary membranes, axonemes, or aqueous supernatant fluids following Triton X-114 extraction. Fractions were adjusted to protein concentrations ranging from 20 μg/ml to 0.156 μg/ml in 10 mM Na-acetate (pH 7.0) and 50 μl was dispensed into six replicate wells of a microtiter plate, dried, and blocked with 1% BSA. Monoclonal antibody 10H3 ascites fluid (diluted 1:1,000), SP2 ascites fluid (diluted 1:1,000), or mouse antiserum (diluted 1:3,000) were added to duplicate wells and Ab binding detected as described above.

Indirect immunofluorescent microscopy. Indirect immunofluorescent microscopy was done as described previously with modifications [8]. Theronts were fixed by incubation at 0° C for 30 min in 1.8% formaldehyde (v/v) in 25 mM Na acetate (pH 7.5). Two thousand formalin-fixed cells were spread onto individual 2.5 × 7.5 cm glass slides and air dried. Slides were immersed in −20° C acetone for 3–5 min and dried. Forty-eight squares were drawn on each slide in waterproof ink. Ascites fluid (25 μl) diluted 1:1,000 in PBS or undiluted culture supernatant fluids were added to each square and incubated in a humidifed box at RT for 1 h. The slides were washed 3× with PBS and then covered with affinity-purified goat anti-mouse IgG fluorosceinisothiocyanate (FITC) conjugate (Sigma, St. Louis, MO) diluted 1:100. After 1 h at RT the slides were washed, and the theronts were mounted under a coverslip in a drop of 50% glycerol in PBS. Theronts were examined with a Zeiss microscope using epifluorescence and were photographed using Kodak (Rochester, NY) Panatomic X film.

Membrane protein extraction. Theront membrane proteins were solubilized in 1% (v/v) Triton X-114 (Sigma, St. Louis, MO) at 0° C and isolated by phase separation at 37° C [3]. The procedure was performed as previously described except that the extraction was carried out at pH 8.4 rather than pH 7.5 [8].

Purification of immobilization antigens by affinity chromatography. Purification and coupling of MAb to cyanogen bromide-activated sepharose 4B (Sigma) were done following standard protocols [14]. Five milliliters of parasite membrane protein (2–5 mg/ml) solubilized in 1% (v/v) Triton X-114 (Sigma) in 10 mM Tris-HCl buffer (TB, pH 8.4) was combined with 5 ml of 4% (w/v) sodium deoxycholate (DOC, Fisher Scientific, Pittsburgh, PA) in TB and incubated for 1 h at 0° C. Insoluble protein aggregates were removed by centrifugation at 100,000 *g* for 1 h at 4° C. Two to five milliliters of solubilized membrane protein was applied to affinity columns containing 5 ml of immobilized MAb 10H3 or 9C9 (5 mg MAb/ml). The columns were washed with 0.5% DOC in TB, followed by TB without DOC. The bound Ag was eluted with 0.05 M diethylamine (pH 11.5). Fractions were collected and neutralized in 0.2 ml of 1 M glycine-HCl (pH 5.0). Protein concentration was monitored by absorbance at 280 nm with a DU-64 Spectrophotometer (Beckman Instruments, Fullerton, CA). Purity and antigenicity were analyzed by polyacrylamide gel electrophoresis and ELISA. Fractions containing purified proteins were pooled, dialyzed in TB and stored at −70° C.

Table 1. Characteristics of MAb reacting to *Ichthyophthirius*.

MAb	ELISA[a]	Immobilization[b]	Western blot	Ig type
10H3	+	20,480	−[c]	IgG_1
3H12	+	20,480	−	IgG_3
8E11	+	20,480	−	IgG_1
9C9	+	20,480	−	IgM
5A8	+	5,120	−	IgG_1
6A11	+	5,120	−	IgG_1
3B8	−	−	+[d]	IgG_3
3E5	−	−	+	IgG_1
A1-4	+	−	48, 60[e]	IgM
B7-5	−	−	+	IgG_1
B2-11	−	−	+	IgG_1
B8-15	+	−	48, 60	IgG_3
T4-2	+	−	48, 60	ND[f]
T5-6	+	−	48, 60	IgG_1
A4-10	+	−	48, 60	ND
A3-14	+	−	48, 60	ND
T3-15	+	−	48, 60	ND

[a] Trophont lysates and affinity purified protein were used as Ag.
[b] Highest titer of ascites fluid causing immobilization.
[c] Does not react.
[d] Reacts with proteins other than immobilization Ag.
[e] Protein sizes in KDa.
[f] Not determined.

Gel electrophoresis and Western blotting. One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was done using standard methods [15]. Affinity-purified Ag and membrane proteins deplete and replete with these Ag were run with and without 2-mercaptoethanol in sample buffers. Proteins were resolved in 10% gels and visualized by staining with Coomassie Blue [8] or silver nitrate [26]. Protein sizes were estimated by comparing migration with high molecular weight protein standards (Bethesda Research Labs, Gaithersburg, MD) and pre-stained protein standards (Bethesda Research Labs and Sigma).

To perform Western blots, 5 μg of affinity-purified Ag or 14 μg of ciliary protein were run on mini-gels (Bio-Rad, Richmond, CA). The resolved proteins were electrophoretically transferred to 0.45 μm nitrocellulose filters [25]. Protein binding sites were blocked with 1% BSA in 0.05% Tween 20, 10 mM Tris-HCl, 150 mM NaCl, pH 7.5 (TTBS). Filters were incubated with MAb or polyclonal Ab (diluted 1:3,000 in TBS with 1% BSA) for 1 h at RT, washed 3× with TTBS, and incubated for 1 h with goat anti-mouse or goat anti-rabbit IgG alkaline phospha-

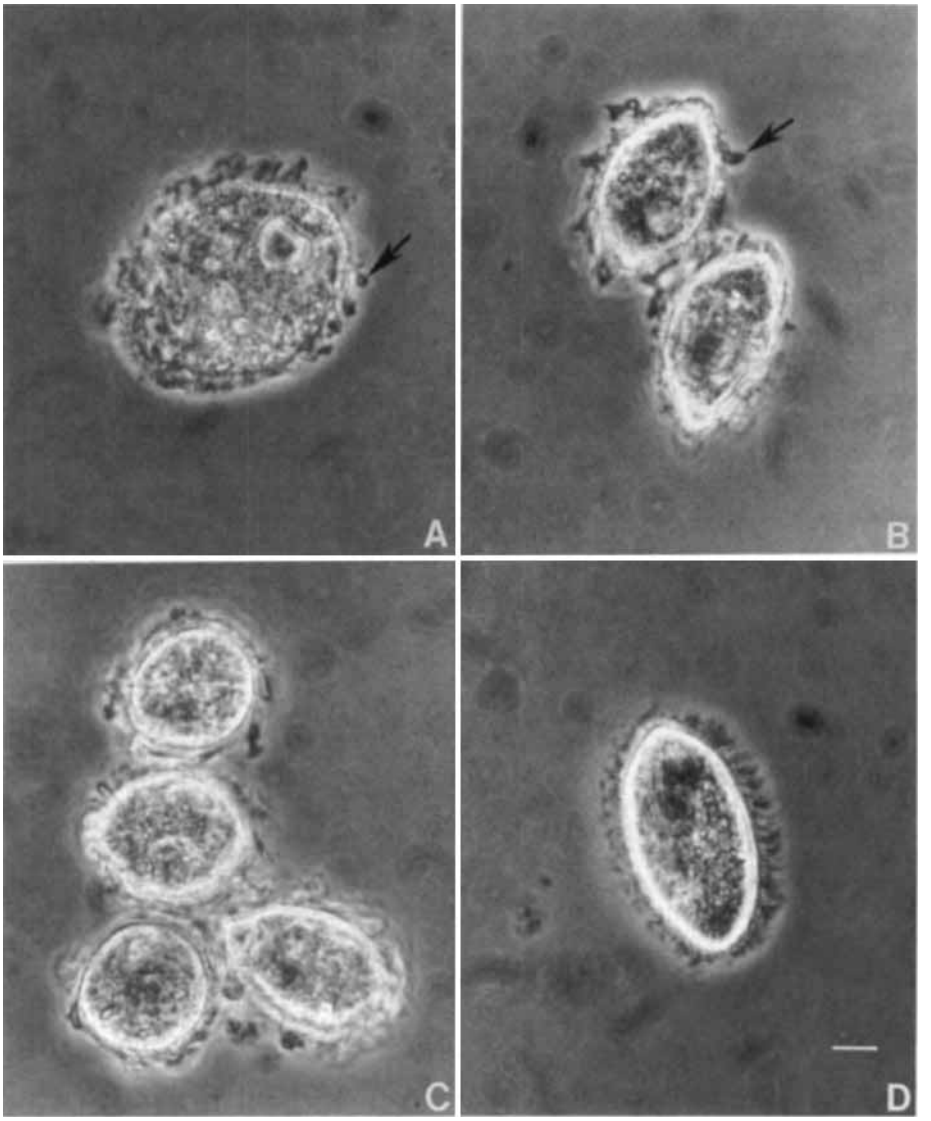

Fig. 1. Immobilization of *I. multifiliis* theronts. Theronts were incubated for 30 min in ascites fluids (1:10,000) containing (**A–C**) immobilizing MAb 10H3 or (**D**) non-immobilizing anti-*I. multifiliis* MAb 3B8. Arrows indicate agglutination of cilia at the tips (compare to normal cilia in D). Bar = 10 μm.

tase diluted 1:1,000 (Promega, Madison, WI). Filters were washed with TTBS and incubated with the substrate nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP, Promega) at RT.

Detection of sugar residues. Affinity-purified Ag or membrane protein fractions were resolved by SDS-PAGE in 10% gels and transferred electrophoretically onto nitrocellulose filters. After blocking with 0.1% Tween 20, the filters were incubated with a concanavalin A biotin conjugate (Sigma) diluted 1:1,000. Filters were washed 3× and incubated in avidin-HRP (Sigma) diluted 1:1,000. Concanavalin A binding was detected by incubation with 4-chloro-1-naphthol substrate at RT.

Similar assays were done using a glycan differentiation kit (*First Choice,* Boehringer Mannheim, Indianapolis, IN) containing five different lectins coupled to digoxigenin. Lectin binding was detected immunologically with sheep anti-digoxigenin conjugated with alkaline phosphatase.

Determination of protein concentration. Protein concentrations were estimated by the Bradford Coomassie Blue binding assay (Bio-Rad) using ovalbumin as standard or by absorbance at 280 nm.

RESULTS

Production of immobilizing MAb. Balb/c mice were inoculated with cilia isolated from *I. multifiliis* theronts to produce sera which immobilized both theront (invasive) and trophont (feeding) forms of the parasite. These mice were used for the production of immobilizing MAb. Hybridomas were tested for the secretion of Ab by indirect immunofluorescence, ELISA, and immobilization. Of a total of 52 hybridomas secreting anti-*I. multifiliis* Ab, six independent cell lines were cloned which produced immobilizing MAb. The characteristics of these MAb are listed in Table 1. Theronts and trophonts immediately stopped swimming when incubated with MAb. Within 1 min immobilized organisms began to stick together, and by 5–10 min they had formed large aggregates. The parasites became immobilized in ascites fluids at titers as high as 10,000 (Fig. 1). All of the organisms within a well were typically immobilized to the same

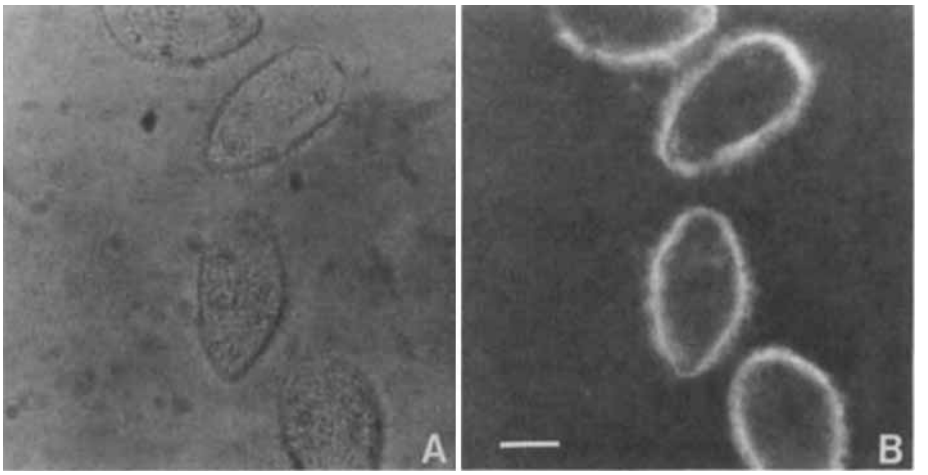

Fig. 2. Indirect immunofluorescent staining of *Ichthyophthirius* theronts. Cells were fixed in 1.8% formalin and incubated with undiluted MAb 10H3 culture supernatant fluid followed by goat anti-mouse FITC conjugate (1:100). Theronts were examined by (**A**) light or (**B**) fluorescence microscopy. Bar = 20 μm.

degree. Surface cilia appeared to be fused at the tips (Fig. 1A–C). Organisms incubated in ascites fluid containing non-immobilizing anti-*I. multifiliis* MAb appeared normal (Fig. 1D). The free-living ciliate *Tetrahymena thermophila* (American Type Culture Collection, Gaithersburg, MD) was not affected when incubated in immobilizing anti-*I. multifiliis* MAb.

Localization of immobilization antigens. Immobilization of live cells and surface fluorescence of fixed theronts (Fig. 2) indicated that immobilization Ag were diffusely located on the surface of the organism. The Ag were found to be enriched in membranes of theront cilia, the primary surface structures of the organism. This was determined by the fractionation of cilia into detergent and water-soluble components using the amphoteric detergent Triton X-114. Detergent-soluble fractions (containing hydrophobic integral membrane proteins), water soluble fractions (containing hydrophilic proteins), and insoluble ciliary axonemes were reacted with immobilizing MAb to test for the presence of Ag by ELISA (Fig. 3). The amount of MAb signal was approximately 100-fold higher in cilia membrane proteins than in the other fractions. Signals in axonemal or aqueous phase Ag could only be detected when relatively large amounts of protein (>1 μg) were coated onto ELISA plates.

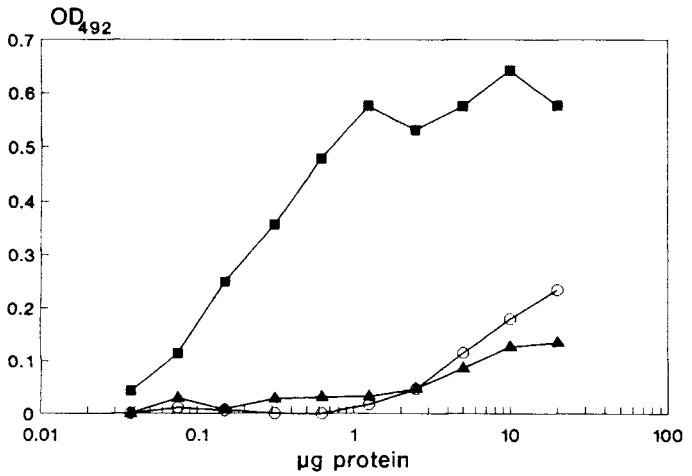

Fig. 3. Localization of immobilization Ag in ciliary membrane proteins by ELISA. Cilia were treated with Triton X-114 as described in the text and 96-well microtiter plates coated with various concentrations of membrane proteins (■——■), aqueous phase proteins (▲——▲), or axonemes (O——O). The plates were reacted with immobilizing MAb 10H3 ascites fluid (1:1,000), followed by goat anti-mouse horseradish peroxidase (1:500). SP2 ascites fluid was used as a negative control (results not shown). Enzyme substrate was added and absorbance at OD_{492} measured.

Purification and partial characterization of MAb-purified antigens. Immobilizing MAb were used to purify Ag by affinity chromatography. Membrane proteins solubilized in Triton X-114 and Na deoxycholate were loaded onto MAb 10H3 (IgG_1) or MAb 9C9 (IgM) sepharose 4B affinity columns. After washing the columns, the bound Ag were eluted with 0.05 M diethylamine (Fig. 4). Bound and unbound fractions were analyzed by ELISA and SDS-PAGE. MAb 10H3 and 9C9 as well as the four other immobilizing MAb reacted in ELISA tests only with the affinity purified fractions. The affinity-purified Ag were identified as two proteins of approximately 48 and 60 kDa (Fig. 4). They migrated independently under both reducing and non-

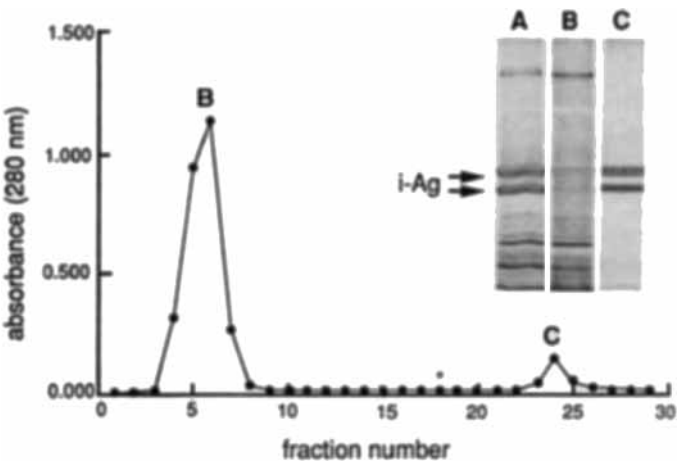

Fig. 4. Affinity purification of *Ichthyophthirius* immobilization Ag. *Ichthyophthirius multifiliis* membrane proteins solubilized in Triton X-114 and Na deoxycholate were run on a sepharose 4B column containing immobilizing MAb 10H3 and collected in 1.5 ml fractions. When protein could no longer be detected in wash buffers, 50 mM diethylamine (pH 11.5) was added at the point indicated by the asterisk. **A**. Membrane proteins; **B**. unbound membrane proteins in pass-through fractions; and **C**. eluted Ag were resolved on a 10% non-reducing SDS-polyacrylamide gel and visualized by staining with silver nitrate. The 48- and 60-kDa immobilization Ag are indicated by the arrows. Protein sizes were determined by comparison of migration with protein standards. (see Materials and Methods and Fig. 5).

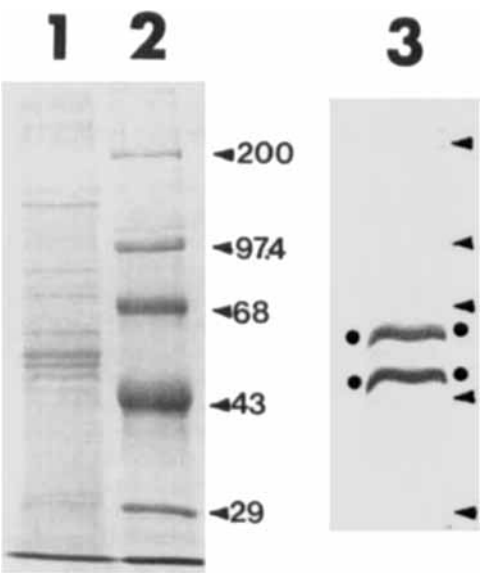

Fig. 5. Western blot of *Ichthyophthirius* cilia. Cilia solubilized in Laemli sample buffer (~14 μg protein in each lane) were resolved by reducing SDS-PAGE and stained with Coomassie Blue (lane **1**) or electroblotted onto nitrocellulose filter paper and probed with rabbit antisera (diluted 1:2,000) against affinity purified immobilization Ag (lane **3**). Goat anti-mouse IgG coupled to alkaline phosphatase (diluted 1:8,000) was used to detect antibody binding. The position of the immobilization Ag are indicated by the dots on either side of lane 3. Cilia were also probed with rabbit preimmune sera which did not react (results not shown). Lane **2**. The bands represent the following molecular weight standards: myosin, 200 kDa; phosphorylase b, 97.4 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; and carbonic anhydrase, 29 kDa. The arrowheads to the right of lane 3 show the positions of the same standards after transfer to nitrocellulose.

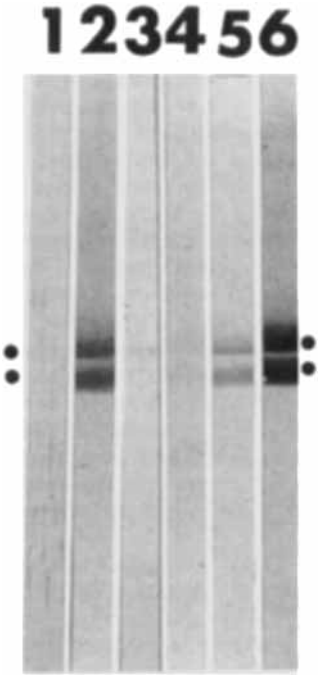

Fig. 6. Western blot of *Ichthyophthirius* immobilization antigens. Affinity-purified immobilization Ag (~0.5 μg) in lanes 2–6 and *Tetrahymena thermophila* whole cells (~4 μg protein) in lane 1 were resolved by reducing SDS-PAGE and blotted onto nitrocellulose filter membranes. The filters were cut into strips and the lanes probed individually with the following Ab diluted 1:3,000: lanes **1**, **2**. mouse polyclonal antisera against affinity-purified immobilization Ag; lane **3**. SP2 ascites fluid; lane **4**. ascites fluids containing non-immobilizing MAb 3B8; lane **5**. non-immobilizing MAb T4-2; and lane **6**. non-immobilizing MAb T3-15. Goat anti-mouse IgG conjugated to alkaline phosphatase (diluted 1:8,000) was used to detect Ab binding. The positions of the 48- and 60-kDa immobilization Ag are indicated with dots on the right and left sides of the figure. Sizes were estimated by comparison with migration of known protein standards (see Fig. 5).

reducing conditions. These proteins were prominent in cilia and membrane protein fractions resolved by SDS-PAGE and stained with Coomassie Blue or silver nitrate (Fig. 4, 5). Attempts to demonstrate carbohydrate on the Ag gave somewhat equivocal results. Weak signals were consistently seen with the lectins concanavalin A and *Galanthus nivalis* agglutinin (both of which recognize terminal mannose groups). These were at levels, however, which made adequate photographic reproduction impossible. Glycosylation of the proteins was therefore considered to be minimal.

Immobilizing antisera with titers as high as 1:500 were produced in rabbits, mice, and channel catfish following the injection of affinity-purified Ag. Rabbit and mice antisera reacted specifically with the 48- and 60-kDa proteins on Western blots (Fig. 5, 6). Catfish antisera were not tested on Western blots.

We tested indirectly whether membrane Ag other than the 48- and 60-kDa proteins also elicited immobilizing Ab. Immobilizing antisera produced by the injection of mice with whole organisms were preabsorbed with various Ag and retested for immobilization. Immobilization was completely eliminated by preabsorption of sera with the 48- and 60-kDa affinity-purified proteins, but was not affected by preabsorption with membrane fractions from which these Ag had been depleted. These results indicate that the 48- and 60-kDa proteins are the primary immobilization Ag of *I. mutifiliis*.

Immobilizing MAb (including 10H3 and 9C9 which were used to purify the Ag) did not react with the 48- and 60-kDa proteins on Western blots. Conversely, a number of MAb recognizing the affinity-purified antigens on Western blots did not immobilize live parasites (see Table 1, Fig. 6). No MAb were found that did both. On the other hand, polyclonal antisera produced against affinity-purified proteins immobilized parasites and bound to both the 48- and 60-kDa proteins on Western blots (Fig. 5, 6).

DISCUSSION

Fishes recovering from *I. multifiliis* infection produce antisera which immobilize the parasite in vitro. It has been postulated that immobilization in vivo contributes to protective immunity [5, 6, 13]. Immobilizing MAb were produced in mice in an attempt to purify and characterize the Ag involved in this response. Six independently cloned cell lines secreting immobilizing MAb (five IgG isotypes and one IgM) were established from 52 hybridomas secreting anti-*I. multifiliis* Ab. These MAb immobilized theronts (the free-swimming and infective stage) as well as trophonts (fish-associated feeding stage). Immobilization was caused by the paralysis of cilia which appeared to be fused at their tips (Fig. 1A, B). This is analogous to the immobilization of *Paramecium* where Ab against the predominant surface Ag induce the fusion of ciliary membranes [2, 11, 18].

The specific Ag responsible for immobilization were two membrane proteins of approximately 48 and 60 kDa. The criteria for their identification as immobilization Ag were as follows. First, the two proteins were co-purified by affinity chromatography using two different immobilizing MAb (10H3 and 9C9). In addition, they reacted in ELISA with four other in-

dependently cloned immobilizing MAb. It is unlikely that these MAb were reacting with minor contaminants, since only the 48- and 60-kDa proteins were detectable in silver-stained gels of affinity-purified Ag fractions. Second, preincubation with the 48- and 60-kDa Ag absorbed out immobilizing activity from antisera against whole-cell Ag. Immobilization did occur when the same antisera were pre-absorbed with protein fractions depleted of affinity-purified Ag. Third, and most importantly, affinity-purified Ag elicited immobilizing antisera when injected into rabbits, mice and channel catfish. Taken together, these results argue strongly that the 48- and 60-kDa proteins are the primary immobilization Ag of *I. multifiliis*. Finally, a 1.2 kb *I. multifiliis* cDNA has been recently characterized in our laboratory which encodes a predicted protein identical in its N-terminal amino acid sequence with the mature 48-kDa i-Ag [7]. This cDNA contains a long open reading frame and specifies a protein having a periodic structure that is highly reminiscent of the i-Ag of both *Tetrahymena* and *Paramecium*. This finding further suggests that the 48- (and 60-) kDa proteins are bona fide i-Ag.

The sizes reported here for the *I. multifiliis* immobilization Ag should be considered estimates. Variations of as much as 3,000 Da (on SDS-PAGE) were observed with protein samples taken from the same isolate at different times. Moreover, the recently identified cDNA corresponding to the 48-kDa Ag mentioned above encodes a deduced protein of M_r 39,429, substantially smaller than that predicted on the basis of SDS gel analysis [7].

Immobilizing MAb did not react with the 48- and 60-kDa Ag on Western blots. Western blotting often alters MAb binding sites, especially those consisting of conformational determinants [12]. We therefore believe that secondary or tertiary protein configurations are necessary for immobilization. It was found that several non-immobilizing MAb reacted with the 48- and 60-kDa Ag on Western blots (Fig. 6). These MAb presumably bind to epitopes which are not denatured under the conditions of SDS-PAGE and which are not involved in immobilization. In this regard, immobilizing and non-immobilizing MAb will be useful for mapping the molecular topology of immobilization Ag. Indeed, these studies are of direct relevance to vaccine development since presentation of native Ag appears to be critical for stimulating the protective immune response of fishes against *I. multifiliis* [4].

The 48- and 60-kDa Ag appear to be related proteins since they have common or similar epitopes which were recognized on Western blots by individual MAb (Fig. 6). This is supported by the fact that MAb-affinity chromatography resulted in the simultaneous purification of both Ag. It is assumed that the proteins are not covalently associated since they appeared as separate bands on SDS-PAGE in the absence of reducing agents.

The 48- and 60-kDa antigens were enriched in ciliary membrane fractions and appear to be integral membrane proteins based on their solubility in the amphoteric detergent Triton X-114. These results are consistent with earlier immunoblotting studies where it was found that Ab from *I. multifiliis*-immune channel catfish also reacted with membrane protein Ag of cilia [5]. While we believe the small amount of Ab binding detected in ciliary axonemes in the present study was the result of incomplete extraction by detergent, it is also possible that it represents a true distribution of Ag in this location (possibly anchored to submembraneous structures).

Ag did not partition in the aqueous phase during Triton X-114 extraction of cilia (Fig. 3). In addition, cilia washed with low salt solutions did not release detectable Ag (data not shown). This does not eliminate the possibility, however, that soluble Ag are shed or secreted by live organisms. Indeed, preliminary data suggest that this does in fact occur. Antibodies against the 48- and 60-kDa proteins reacted on Western blots with similarly-sized proteins found in supernatant fluids of theronts and tomonts (unpubl. results). Studies are in progress to determine how Ag released from live parasites may relate to the purified membrane Ag described here.

In a previous study, we found that *I. multifiliis* membrane protein fractions elicited immobilizing antisera in rabbits and speculated that a predominant 43-kDa glycoprotein was responsible [8]. The proteins described in the present study are obviously different. There are two major Ag instead of one, and they differ in molecular mass. Moreover, antisera from the previous study did not immobilize the organisms used here. It appears that the isolates used in the two studies expressed different immobilization Ag. It is not yet clear whether this was due to the expression of completely different genes or alleles of the same gene that had undergone sequence divergence. It should be mentioned that the use of different *I. multifiliis* isolates was necessary in the two studies because strains become senescent over time (2–3 yr) and cryopreservation of live organisms has not yet been successful.

The immobilization Ag of *I. multifiliis* appear analogous in several respects to surface Ag of the free-living ciliates *Tetrahymena* and *Paramecium*. They are similar in size to the 30- to 58-kDa immobilization Ag of *Tetrahymena* [1, 9, 27] as well as to a 40- to 45-kDa surface membrane Ag of *Paramecium tetraurelia* [10]. As in these ciliates, the immobilization Ag of *I. mutlifiliis* are major surface proteins. Immobilization of free-living ciliates has no obvious biological significance since the organisms are unlikely to encounter immobilizing Ab in their natural environment. Antibody-induced immobilization has been used primarily as a phenotypic marker for the study of genes encoding ciliate surface proteins [17, 18, 24, 27]. *Ichthyophthirius multifiliis*, on the other hand, elicits and presumably encounters specific immobilizing Ab in the host [6, 13]. Indeed, immobilizing MAb interfere directly with the establishment of infection. We have found that MAb injected intraperitoneally into fish confer passive protection against parasite challenge (Lin & Dickerson, unpubl. results). Whether this immunity is caused by immobilization in vivo or by other mechanisms remains to be determined. Nevertheless, the surface Ag to which these Ab bind appear to be important in the establishment of infection.

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Trypanosoma cruzi Infection of BSC-1 Fibroblast Cells Causes Cytoskeletal Disruption and Changes in Intracellular Calcium Levels

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ABSTRACT. The disruption of vimentin and actin filaments of host BSC-1 fibroblast cells by *Trypanosoma cruzi* was investigated using a mouse monoclonal anti-vimentin antibody and rhodamine phalloidin, respectively. Indirect immunofluorescence microscopy demonstrated that infection of BSC-1 cells by *T. cruzi* caused disruption of both cytoskeletal components. The disruption was greater as infection progressed. Mechanisms other than mechanical ones may play a role in the disruption since disrupted cytoskeletal elements were well removed from the parasites. In the determination of intracellular calcium concentrations using Fura-2 AM, infected and uninfected cells both showed an initial increase in intracellular calcium levels. At later times of infection (3 to 5 days), intracellular calcium levels of infected cells were significantly lower than those of control cells. There was no specific localization of intracellular calcium in the infected host cells as determined by image analysis.

Key words. Actin, amastigotes, indirect immunofluorescence, microtubules, vimentin.

TRYPANOSOMA cruzi is an obligate intracellular parasite which causes Chagas' disease or South-American trypanosomiasis [7, 12, 19]. *Trypanosoma cruzi* has a complex diphasic life cycle comprising infection of vertebrate hosts and transmission by arthropod vectors, particularly by blood-sucking reduviid bugs of the subfamily Triatominae. It has three distinct morphological forms in its biological cycle, namely the amastigotes (spheromastigotes), epimastigotes and trypomastigotes [12]. It is the amastigotes or replicative forms which are present intracellularly in host cells of mammals, particularly muscle cells. These amastigotes multiply by binary fission in the cytoplasm. After extensive multiplication inside the host cell, amastigotes transform into non-dividing trypomastigotes approximately 24 h prior to cell lysis and parasite release [42].

Different approaches have been used in an attempt to understand the mechanisms involved in the pathogenicity of *T. cruzi* to host cells. One approach has been to analyze the surface properties of the different developmental stages of the parasite life cycle involved in host-parasite interactions [31] to elucidate the mechanism of parasite entry into the host cell [1, 25, 29].

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