A Monoclonal Antibody Against the Dynein IC1 Peptide of Sea Urchin Spermatozoa Inhibits the Motility of Sea Urchin, Dinoflagellate, and Human Flagellar Axonemes

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To investigate the role of axonemal components in the mechanics and regulation of flagellar movement, we have generated a series of monoclonal antibodies (mAb) against sea urchin (*Lytechinus pictus*) sperm axonemal proteins, selected for their ability to inhibit the motility of demembranated sperm models. One of these antibodies, mAb D1, recognizes an antigen of 142 kDa on blots of sea urchin axonemal proteins and of purified outer arm dynein, suggesting that it acts by binding to the heaviest intermediate chain (IC1) of the dynein arm. mAb D1 blocks the motility of demembranated sea urchin spermatozoa by modifying the beating amplitude and shear angle without affecting the ATPase activity of purified dynein or of demembranated immotile spermatozoa. Furthermore, mAb D1 had only a marginal effect on the velocity of sliding microtubules in trypsin-treated axonemes. This antibody was also capable of inhibiting the motility of flagella of *Oxyrrhis marina*, a primitive dinoflagellate, and those of demembranated human spermatozoa. Localization of the antigen recognized by mAb D1 by immunofluorescence reveals its presence on the axonemes of flagella from sea urchin spermatozoa and *O. marina* but not on the cortical microtubule network of the dinoflagellate. These results are consistent with a dynamic role for the dynein intermediate chain IC1 in the bending and/or wave propagation of flagellar axonemes.

INTRODUCTION

The active sliding of the peripheral doublet microtubules is responsible for ciliary and flagellar movement (Gibbons, 1981, 1989). Dynein arms that decorate each doublet play a motor role in this microtubule motion (Gibbons and Gibbons, 1972, 1973, 1976, 1979; Yano and Miki-Noumura, 1981). The attachment–detachment cycle between dynein and the adjacent doublet microtubule, and the variation in inclination of the globular heads of the dynein ATPase heavy chains triggered by

the binding and hydrolysis of ATP, results in the sliding of microtubules in the axoneme (Gibbons, 1981; Johnson, 1985).

Depending on the species studied, the outer dynein arm is made of multiple chains of 350–500 kDa: two chains for sea urchin and trout sperm flagella and three chains for *Chlamydomonas* flagella and *Tetrahymena* cilia. Associated with these heavy chains are two to five intermediate chains: three for sea urchin sperm flagella at 122, 96, and 76 kDa (with some variations in molecular mass according to species), five for trout sperm flagella at 85, 73, 65, 63, and 57 kDa, two for *Chlamydomonas* flagella at 78 and 69 kDa, and three for *Tetrahymena* cilia at 100, 85, and 70 kDa and four to eight smaller peptides between 5 and 25 kDa (Bell *et*

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al., 1979; Bell and Gibbons, 1983; Johnson, 1985; Gatti et al., 1989; Witman, 1989, 1992; King et al., 1990). Data obtained from monoclonal antibodies, ultraviolet/vanadate cleavage patterns (Lee-Eiford et al., 1986; Gibbons et al., 1989; King and Witman, 1989), and from DNA sequencing of the gene for the β-heavy chain of sea urchin dynein (Gibbons et al., 1991; Ogawa, 1991) suggest that the ATPase activity of the dynein molecule lays in the middle portion of the dynein globular head structure. Whereas the ATPase activity of the heavy chain dynein peptide is essential for the mechanochemical coupling involved in flagellar movement (Gibbons, 1981; Johnson, 1985), the intermediate and light chains of dynein have not yet been attributed any specific enzymatic activity nor a specific function in the sliding or bending of axonemal microtubules.

Experimental evidence indicates that the intermediate chains of outer dynein arms are in close proximity to the globular heads of the adjacent dynein arms (Goodenough and Heuser, 1989; Witman, 1989, 1992). King et al. (1991) provided evidence for a direct interaction between the 78 kDa intermediate chain of Chlamydomonas outer arm and the α-subunit of tubulin on subfiber A of doublet microtubules. Evidence for the importance of intermediate dynein peptides in assembly or attachment of outer dynein arms was provided by Mitchell and Kang (1991), who reported that the deletion of the 70-kDa intermediate dynein chain (ODA 6 mutants of Chlamydomonas reinhardtii) resulted in the deletion of dynein arms. Subsequent studies with revertants of ODA 6 mutants revealed that motility defects of these revertants do not correlate with deficits in the assembly of a specific dynein peptide because these revertants have normal outer arm structures. These results suggest that the 70-kDa intermediate chain may play a direct role in dynein arm function distinct from its role in the assembly process (Mitchell and Kang, 1993). The observations that the 21S outer arm dynein from sea urchin flagella dissociates at low ionic strength into a particle α containing the α-heavy chain and a particle β containing the β-heavy chain and the heaviest intermediate chain (IC1) and that only the β particle is capable of supporting the sliding of microtubules at a velocity even higher than that achieved by the whole 21S dynein particle are consistent with this hypothesis (Sale and Fox, 1988).

To investigate the role of axonemal components in the mechanics of flagellar movement, we have developed a series of monoclonal antibodies (mAb) against sea urchin (Lytechinus pictus) sperm axonemes. One of these antibodies, mAb D1, blocks the motility of demembranated spermatozoa by interacting with the IC1 peptide of the outer dynein arm. mAb D1 acts by modifying the shear angle and beating amplitude while having little effect on dynein ATPase. Furthermore, the same antibody also inhibits the motility of axonemes from human spermatozoa and from Oxyrrhis

marina, a 400 000 000-year-old species of dinoflagellate (Taylor, 1990).

MATERIALS AND METHODS

Preparation of Sea Urchin Axonemes

Sea urchin spermatozoa from L. pictus were collected in a plastic Petri dish at 4°C after injection of 0.5 M KCl into the intracoelomic cavity. Sperm suspensions were concentrated about twofold by centrifugation at 750 × g for 10 min at 4°C. The loosely pelleted sperm suspension was diluted with three volumes of demembranation medium (DM buffer) containing 0.15 M K acetate, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.1% Triton X-100, and 20 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 8.0. Heads and tails were detached after 12 strokes with a loose piston (type B) of a Dounce glass homogenizer. This suspension was diluted 10-fold with 0.15 M K acetate, 1 mM EGTA, 1 mM DTT, and 20 mM Tris-HCl, pH 8.0 (buffer A) and layered on 15% Percoll in buffer A. After centrifugation at 2500 × g for 10 min, the upper layer containing the axonemes were collected, pooled, and centrifuged at 10 000 × g for 20 min. The pelleted axonemes were resuspended in DM buffer and the same procedure was repeated. Finally, the axonemes were washed twice with buffer A and the final axonemal pellets were resuspended in specific buffers depending on the use.

Preparation of mAb D1

Purified axonemes suspended in 140 mM NaCl, 3 mM KCl, and 10 mM Na phosphate pH 7.4 (PBS) were injected in the intraperitoneal cavity of Balb/c mice (0.5 ml of a suspension at 1 mg/ml for each mouse). Similar injections were repeated four times at 3-wk intervals. Three days after the last injection, spleen cells from the two best responders were fused with myeloma Sp2/0 cells as described by Galfrè and Milstein (1981). mAbs in the hybridoma's culture media were screened for their capacity to inhibit the motility of sea urchin sperm models and to recognize proteins on nitrocellulose blots of axonemal proteins from sea urchin spermatozoa separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Demembranation and Reactivation of Sea Urchin Spermatozoa

Sea urchin spermatozoa were demembranated and reactivated according to the protocol of Brokaw (1985). This model system was used to screen hybridoma that produce antibodies capable of blocking sperm motility. Assays were run in 96-well ELISA plates with 150 μl of reaction mixture per well. In these conditions, >90% of the sperm models were highly motile for ≥1 h in the absence of antibody secreted by the hybridoma in the culture medium. The protocol of Cosson and Gagnon (1988) was used for the investigation of motility parameters. Under these conditions, >90% of sperm models were motile for >30 min at room temperature (21°C).

Analysis of Motility Parameters from Demembranated-Reactivated Sea Urchin Spermatozoa

Flagellar beat frequency, wave amplitude, curvature, and shear angles were investigated on freely motile sperm models using dark-field microscopy with a stroboscopic light of variable frequency (Chadwick-Hellmuth, El Monte, CA). Video recordings were obtained through a Panasonic (Secaucus, NJ) CCD video camera WV-F15E/S-VHS, a Hamamatsu video image processor (DVS 3000, Hamamatsu Photonics, Hamamatsu, Japan), and a Panasonic (AG7330/S-VHS) recorder. Direct photographs of reactivated sperm axonemes were also obtained using an Olympus OM2 camera after a 1-s exposure at a flash frequency of 4 Hz.

Measurements of the shear angle along the axoneme of freely motile sperm models were based on the method of Brokaw (1993) with the following modifications: the shear angle along the axoneme was defined as the angle between the local tangent of the curvature along the axoneme and a reference axis obtained from the head trajectory. This reference axis was chosen instead of the head axis because the latter is constantly reoriented as a result of its oscillation during the different bending phases within a cycle of beating as observed by Brokaw (1993) for *L. pictus*. The reference axis was obtained after accumulating 20 images of moving sperm models to be considered for shear angle analysis using the "trace function" of the DVS Hamamatsu video image processor, and after fitting a circle over the sperm trajectory, a tangent to this circle was traced, passing by the head–tail junction of the spermatozoon to analyze. The shear angle at every 1.5 μm along the axoneme was evaluated in radians; this angle was considered positive when the axoneme tangent was oriented toward the inside of the circular path fitting the sperm track accumulated by the Hamamatsu processor and negative when the tangent was oriented toward the outside of the circle. Similar analysis were performed at different time periods after incubation of sperm models with and without antibody. Representative time points were chosen to illustrate the effects of mAb D1.

The beating amplitude of flagellar movement was obtained from accumulation of images processed by the Hamamatsu processor. The amplitude of flagellar beating was defined as the half distance between the principal and reverse bends at a given position along the axoneme. The distances 13 ± 2 (mean ± SD) and 28 ± 2 μm from the head–tail junctions were selected for comparison purposes, and they represent approximately one and two thirds of the flagellar length, respectively. The curvature of flagellar bending at the same position on the axoneme was obtained by best fitting circles of different radius on single frame processed image by the Hamamatsu. The principal and reverse bends were previously defined elsewhere by Gibbons and Gibbons (1972), and the terms proximal and distal refer to the positions at 13 and 28 μm, respectively, away from the head–tail junction.

Sliding of Axonemal Microtubules from Sea Urchin Spermatozoa

Concentrated spermatozoa were first diluted 100-fold in sea water and then 40-fold in DM buffer (Cosson and Gagnon, 1988). After a period of 30 s, 5 μl of the suspension were added to 15 μl of reactivation buffer (Cosson and Gagnon, 1988) containing antibodies at the appropriate concentration. After 5 min of incubation at room temperature (22°C), 10 μl of trypsin at 1 μg/ml were added, followed by the addition, 2 min later, of 5 μl of 100 μM MgATP. An aliquot was immediately transferred onto a glass slide for observation either by dark-field microscopy with 25× objective or with oil immersion 40× or 100× objectives or by differential interference contrast with a 100× oil immersion objective coupled to a video equipment for higher magnification (5000× on video screen) analysis. The percentage of disintegrated axonemes was estimated from video records. The rate of sliding was obtained either from measurements of the length of microtubule slid between video frames or for statistical evaluation, from measurements of the period of time (number of video frames elapsed between the beginning and the end of curling of the axoneme [usually 300–350 frames]). Both methods yielded similar accuracy.

Demembranation and Reactivation of Oxyrrhis marina Flagella

The dinoflagellate *O. marina*, originally collected in the bay of Villefranche-sur-Mer, France, was grown as a monospecific culture in 50% sea water and fed with baker's yeast, as previously described (Cosson *et al.*, 1988). After 2 d of exponential growth, the dinoflagellates reached a stationary phase of growth. Cells were then concentrated by centrifugation at 1000× *g* for 5 min, immediately before experimentation. Four microliters of a concentrated cell suspension were

added to 96 μl of demembranation-reactivation medium containing 0.4 M sucrose, 0.15 M K acetate, 5 mM Mg acetate, 2 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 1 mM DTT, 0.5 mM ATP, 0.04% Triton X-100, and 20 mM Tris-HCl, pH 7.7. Control proteins or mAb D1 were added to this reactivation medium. Observations, by dark-field microscopy, with a stroboscopic light were recorded for 60 min. Under these conditions, in the absence of ATP, flagella of *O. marina* showed a complete arrest of movement within 1 min of Triton permeabilization, whereas in the presence of ATP, the flagella remained motile for >30 min at room temperature.

Isolation of O. marina Flagella

The isolation of *O. marina* flagella and their purification and biochemical properties have been described in details elsewhere (Godart and Huitorel, 1992). In brief, cells were concentrated and resuspended in deflagellation medium containing 0.4 M glucose, 0.15 M K glutamate, 5 mM Mg acetate, 2 mM EGTA, 1 mM DTT, protease inhibitors, and 20 mM Tris-HCl, pH 7.85. Calcium (20 mM final concentration) was added under agitation to separate flagella from the cell body. Flagella were then collected by centrifugation, demembranated with Triton X-100, and Triton-insoluble flagellar structures were isolated by centrifugation.

Demembranation and Reactivation of Human Spermatozoa

Human semen from normal volunteers were obtained by masturbation after 3 d of sexual abstinence. After a liquefaction period of 30 min, spermatozoa were purified by centrifugation on a Percoll gradient as described elsewhere (de Lamirande and Gagnon, 1992). Purified spermatozoa were demembranated and reactivated as previously reported (de Lamirande and Gagnon, 1986) except that 50 μM cAMP and 100 μg/ml of cAMP-dependent protein kinase (Sigma Chemical, St. Louis, MO) were added, and the MgATP concentration was lowered to 100 μM. The beat frequency of human sperm models was determined from video records played in slow motion.

Isolation of Outer Dynein Arms from Sea Urchin Sperm Axonemes

Washed sperm axonemes from *L. pictus* were adjusted to 3 mg/ml with cold 0.6 M NaCl, 4 mM MgSO$_4$, 1 mM EDTA, 1 mM DTT, and 10 mM N-2-hydroxyethyl piperazine N-2'-ethanesulfonic acid, pH 7.0. After an extraction period of 30 min at 4°C, the suspension was centrifuged at 35 000 × *g* for 20 min at 4°C to pellet the unextracted material. The supernatant was collected, concentrated by ultrafiltration on a YM 100 membrane (Amicon, Beverly, MA), and layered on a 5–20% continuous linear sucrose gradient in the same buffer. After centrifugation at 150 000 × *g* for 16 h, fractions were collected by puncturing the bottom of the tube and analyzed by SDS-PAGE. Their protein and ATPase activity contents were also determined.

Electrophoresis and Immunoblotting

Proteins were separated by SDS-PAGE according to Laemmli (1970). Electrotransfer of proteins from SDS-gels to nitrocellulose membrane was performed according to the method of Otter *et al.* (1987). Incubations with primary antibodies and secondary antibodies coupled to alkaline phosphatase were run in a Miniblotter 28 (Immunetics, Cambridge, MS) as described in details by the manufacturer.

Immunofluorescence

For immunolocalization of the mAb D1 antigen, sea urchin spermatozoa in sea water and *O. marina* in 50% sea water were diluted twofold with 4% paraformaldehyde on coverslips (9 mm in diameter). After a 6-min fixation period, they were then permeabilized, while fixation continued, by the addition of two volumes of 1% Triton X-

100 in PBS for another 6 min. The coverslips were then subjected to several washes with PBS, 0.1% Triton X-100, and 1% normal donkey serum in the same buffer. The specimen were then incubated with 10 μl of primary antibody (1–10 μg/ml) for 1 h at room temperature or overnight at 4°C. The coverslips were extensively rinsed with PBS and incubated with 10 μl of the appropriate secondary antibody 1/250 (donkey anti-mouse-dichlorotriazinyl amino fluoresceine and donkey anti-rat Texas red sulfonyl chloride, Jackson Laboratories, West Grove, PA) for 30 min at room temperature. After rinsing, the coverslips were incubated for 1 min with the Hoechst 33258 dye (Sigma, St. Louis, MO) at 5 μg/ml and mounted in 90% glycerol containing 20 mM Tris-HCl, pH 8, and 0.1% *p*-phenylenediamine as an antibleach. The observations were performed on a Zeiss (Thornwood, NY) Axiophot, using a 40×, 63×, or 100× Plan-Neofluor objective. Pictures were taken with a Zeiss camera and controller, after a 10- to 20-s exposure (1 s for Hoechst dye) on Kodak (Rochester, NY) T MAX 400 processed for this sensitivity.

Determination of ATPase Activity

ATPase activity of purified dynein preparations and of demembranated sea urchin sperm models was determined as previously described (Belles-Isles *et al.*, 1986). For demembranated spermatozoa, motility of control and antibody containing samples was evaluated by darkfield microscopy during the incubation for ATPase determination.

Protein Determination

Protein concentrations were determined by the method of Bradford (1976) using bovine gamma globulins as the standard.

RESULTS

Identification of the Antigen Recognized by mAb D1

mAb D1 originated from a mouse immunized with whole sea urchin (*L. pictus*) sperm axonemes and was selected for its capacity to inhibit the motility of sea urchin sperm models. To identify the axonemal antigen recognized by mAb D1, *L. pictus* axonemal proteins were separated by SDS-PAGE and blotted on nitrocellulose paper. mAb D1 recognized a single peptide with a molecular mass of 142 kDa (Figure 1). Proteins secreted from the Sp2/0 cells used in the fusion with spleen cells of immunized mice did not recognize any axonemal proteins, whereas the control IgM antibody obtained from a commercial source (Sigma Chemical, Cat. No. M2770) revealed a single band with a molecular mass of 115 kDa (Figure 1).

To further investigate which of the axonemal proteins was recognized by mAb D1, sea urchin (*L. pictus*) sperm axonemes were extracted with 0.6 M NaCl, a procedure known to release the outer dynein arms (Gibbons and Gibbons, 1972, 1979; Bell *et al.*, 1979). The antigen recognized by mAb D1 was extracted by this treatment and on immunoblots of axonemal proteins corresponded to the position of the first intermediate peptide chain of the outer arm dynein ATPase (Bell *et al.*, 1979) (Figure 2). To confirm this identification, the 0.6 M NaCl extract was separated on a 5–20% sucrose gradient. Analysis of ATPase activities and immunoblotting with mAb D1 of proteins from gradient fractions separated by SDS-
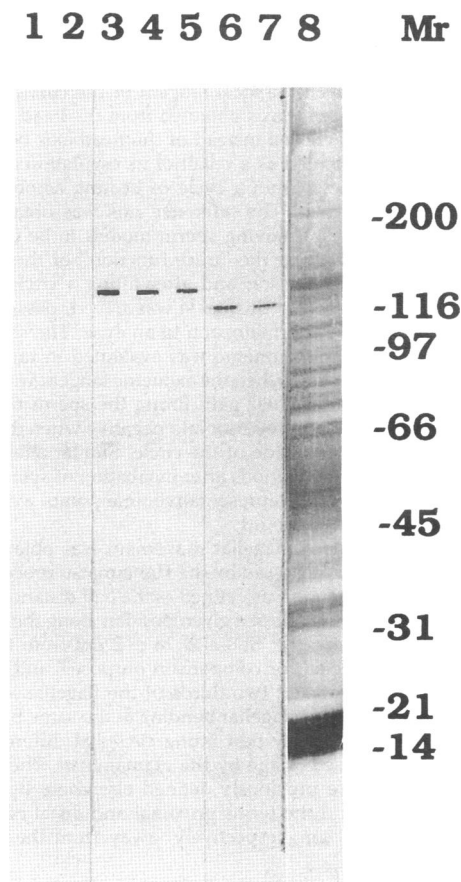

Figure 1. Immunoblots of axonemal sea urchin sperm proteins with mAb D1. Sea urchin (*L. pictus*) sperm axonemes were purified, separated by SDS-PAGE, and immunoblotted with 1, no primary antibody; 2, proteins from myeloma Sp2/0 used for the fusion with spleen cells from mice immunized with sea urchin sperm axonemes; 3–5, mAb D1 at 10, 2.5, and 0.5 μg/ml, respectively; 6 and 7, control IgM at 10 and 2.5 μg/ml; 8, Amido Black stained sea urchin axonemal proteins. Molecular weights of standards are indicated on the right.

PAGE revealed that the 142-kDa protein recognized by mAb D1 co-migrated with two heavy chains with masses >400 kDa and with the peak of ATPase activity (Figure 2), suggesting that the 142-kDa antigen recognized by mAb D1 was the heaviest intermediate chain (IC1) of the outer arm dynein ATPase.

Effects of mAb D1 on the Motility of Sea Urchin Sperm Models

Sea urchin (*L. pictus*) sperm models reactivated in the presence of mAb D1, at concentrations as low as 0.1 μg/ml, showed a progressive decrease in the percentage of motile spermatozoa over the 60-min reactivation period studied (Figure 3). In contrast, the incubation of control IgM (5 μg/ml), proteins secreted by Sp2/0 cells, or preimmune serum from mice immunized with sea urchin sperm axonemes had marginal or no effect on
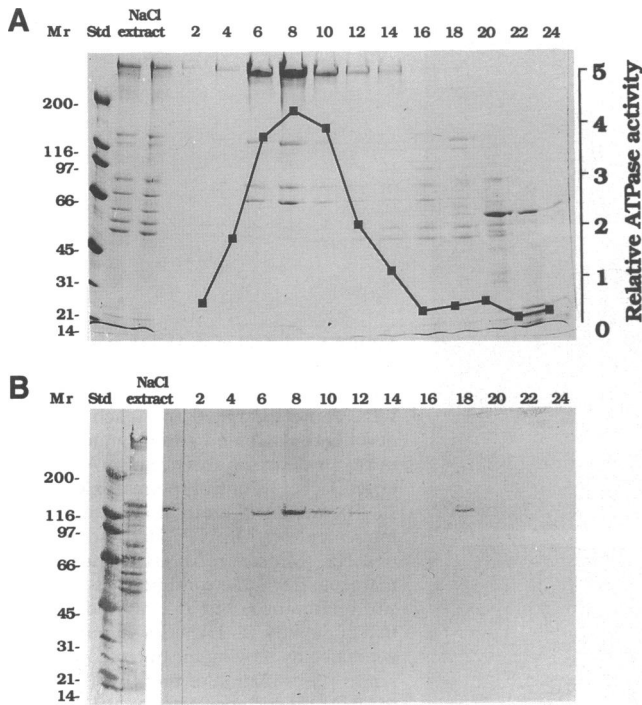
Figure 2. Immunoblots of sea urchin crude and purified outer arm dynein ATPase with mAb D1. Purified *L. pictus* axonemes were extracted with 0.6 M NaCl as described in MATERIAL AND METHODS. The extracts were centrifuged on a 5–20% sucrose gradient after which the fractions were analyzed by SDS-PAGE and immunoblotting, and ATPase activity was determined. (A) SDS-PAGE gels stained with Coomassie Brilliant Blue and ATPase activity of gradient fractions (the numbers refer to the fraction number). Molecular weights of standards are indicated on the left side. (B) Immunoblots of the same fractions as in A with mAb D1; the crude NaCl extract is included on the left side. Fraction one represent the bottom of the gradient.

Figure 3. Effect of mAb D1 on the motility of sea urchin spermatozoa. Spermatozoa (*L. pictus*) were demembranated and reactivated according to the protocol of Brokaw (1985) in the presence of proteins (30 μg/ml) secreted by Sp2/0 myeloma cell line (▲), preimmune serum (3 mg/ml) (△), control IgM (5 μg/ml) (●), mAb D1 (0.10 μg/ml) (□), mAb D1 (1 μg/ml) (■), and antiserum against sea urchin sperm axoneme (3 mg/ml) (○). Values shown are representative of those obtained in at least five other experiments.

motility. Higher concentrations of mAb D1 completely blocked axonemal motility within shorter time periods, similarly to the antiserum of mice from which the spleens were used to generate the clone D1 (Figure 3). Similar effects on motility were obtained with spermatozoa from three other species of sea urchin: *Arbacia lixula*, *Paracentrotus lividus*, and *Strongylocentrotus purpuratus*. The effects of mAb D1 on motility completely disappeared when the mAb was preincubated with a 0.6 M NaCl axoneme extract. This result further supported the observation that the mAb D1 acted by recognizing a peptide of the outer dynein arm.

Analysis of video records of *P. lividus* sperm models incubated with mAb D1 revealed decreases in the beating amplitude measured at 13 ± 2 and 28 ± 2 μm from the head–tail junction (Figure 4). The effects on the distal third of the axoneme was even greater than that depicted in Figure 4B because the distal section of the flagellum ended up completely immotile, whereas the proximal section still developed bending waves but of lower amplitude (Figure 5). However, because of the
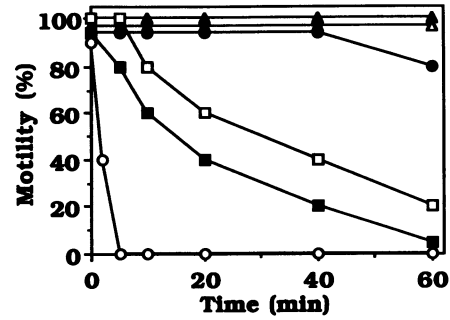
force generated from the incoming wave, the paralyzed distal end of the flagellum was displaced, thus showing an apparent beating amplitude. The decrease in amplitude of beating was associated with a decrease in flagellar bending (Figure 6). The curvature of the proximal
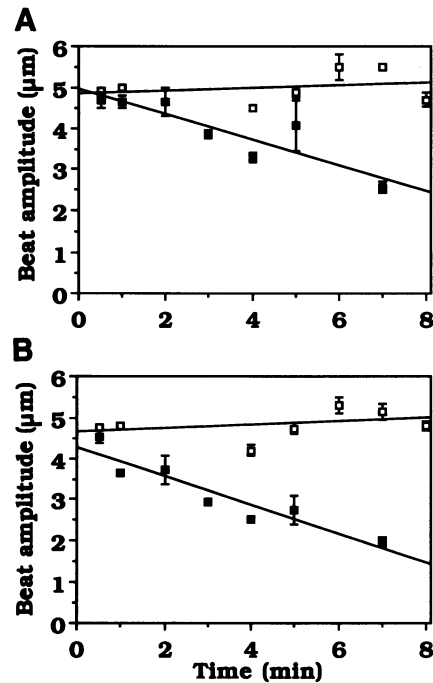

Figure 4. Beat amplitude of sea urchin spermatozoa incubated with and without mAb D1 as a function of time. Sea urchin (*P. lividus*) sperm models were incubated with and without mAb D1 (5 μg/ml). Measurements of beat amplitude at 13 (A) and 28 (B) μm from the head–tail junction were made from video records replayed and analyzed by the Hamamatsu processor. Controls without antibody (□) and mAb D1 (■). Values presented are means ± SEM for 8–15 measurements on different spermatozoa per time point.
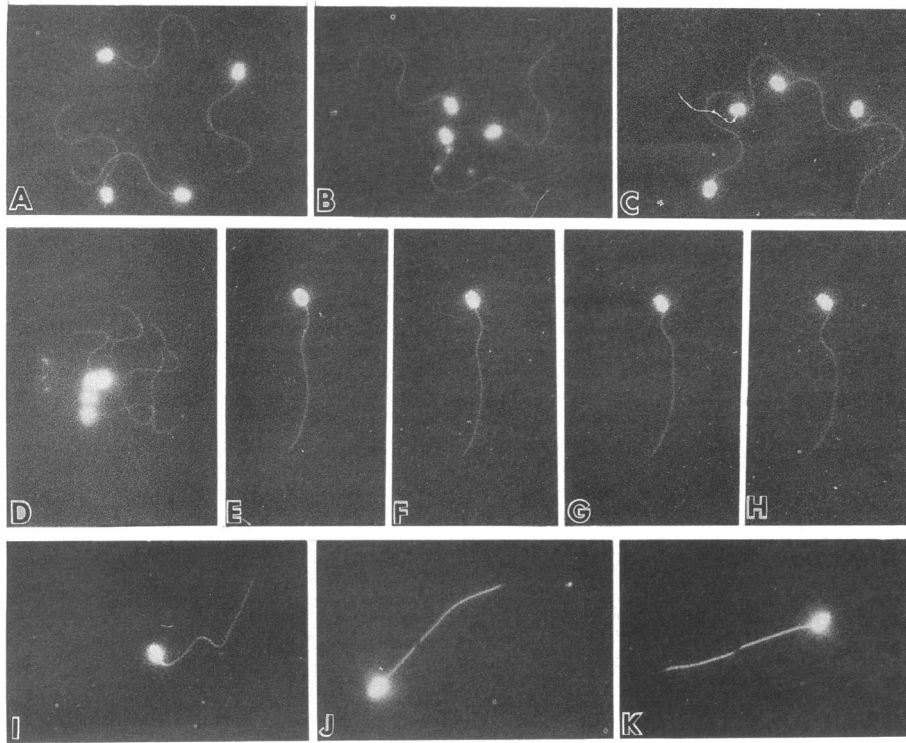
Figure 5. Effect of mAb D1 on the beating of flagella from sea urchin spermatozoa. Sea urchin (*P. lividus*) spermatozoa were demembranated and reactivated as described by Cosson and Gagnon (1988) in the presence or absence of mAb D1 (2 μg/ml). (Top) Control spermatozoa 3 (A), 11 (B), and 16 (C) min after initiation of movement by MgATP. Flash frequency = 4 Hz. (Middle) mAb D1 treated spermatozoa. (D) Same conditions as for control spermatozoa but at 2-min postinitiation of motility. (E–H) Series of four panels generated by moving the microscope stage 7 min postinitiation of motility. (Bottom) mAb D1 treated spermatozoa at 12 (I) and 13 (J and K) min postinitiation of motility.

principal bend measured at 13 μm from the head–tail junction was initially unaffected. The curvature of the proximal reverse bend at the same distance along the flagellum was reduced by half, similarly to that of the principal distal bend at 28 μm. On the other hand, the curvature of the distal reverse bend reached almost zero after 8 min (Figure 6).

Analysis of the shear angle along the whole axoneme of individual sperm models clearly indicated major modifications of the shear angle and an increase in
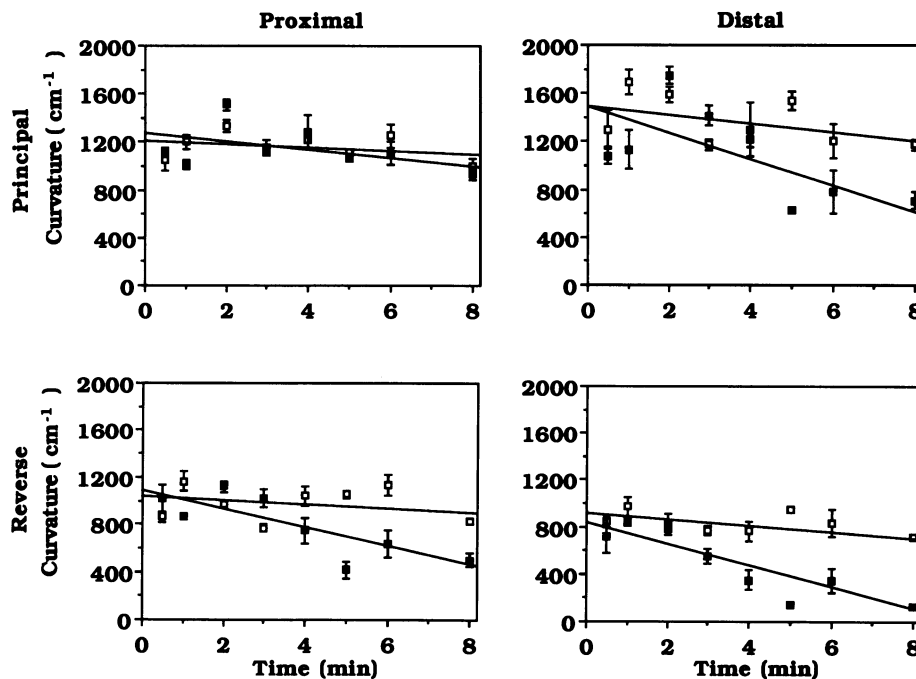

Figure 6. Effect of mAb D1 on the curvature of principal and reverse bends of sea urchin spermatozoa. Sea urchin (*P. lividus*) sperm models were incubated with (■) and without (□) mAb D1 (4.5 μg/ml). The curvature of principal and reverse bends at 13 (proximal) and 28 μm (distal) were measured as described in MATERIALS AND METHODS. Values represent mean ± SEM of 8–20 spermatozoa per time point.

asymmetry as the time of contact between mAb D1 and sperm models increased (Figure 7). A partial blockage of the distal flagellum became apparent after 7 min of contact, whereas at 8 min the axoneme showed a full paralysis of the flagellum from 15 μm to the tip of the axoneme. In contrast, control sperm models showed full propagation of the beating wave up to the tip of the flagellum even after 15 min of incubation. An apparent visual discontinuity was occasionally observed in several sperm models in the middle section of the flagellum (Figure 5), marking the beginning of the paralyzed distal end of the flagellum. However, this discontinuity of the diffracted light was only apparent because the distal portion was never physically separated from the proximal portion of the flagellum, thus trailing without active bending. Flagellar beat frequency was also decreased by mAb D1 but to a lesser extent. When the percentage of motile sperm models was decreased to <5% by the antibody, the beat frequency of the remaining motile spermatozoa was only reduced by 50% (Figure 8).

Effect of mAb D1 on Flagellar Dynein ATPase Activity and Sliding of Axonemal Microtubules

To determine whether mAb D1 directly affected dynein ATPase activity, demembranated-reactivated spermatozoa were incubated with different concentrations of mAb D1 and ATP hydrolysis was measured. The data indicated that ATPase activity coupled to flagellar beating was inhibited as the level of immobilized spermatozoa increased (Figure 9). On the other hand, when immotile demembranated spermatozoa or 0.6 M NaCl extracted and purified dynein ATPase were incubated with mAb D1, no inhibition of activity was observed despite the fact that the same purified soluble dynein ATPase was able to prevent the inhibitory action of mAb D1 on demembranated-reactivated spermatozoa (see above).

The effect of mAb D1 on the sliding of axonemal microtubules was also tested by incubating trypsinized sperm models under a low concentration of MgATP (14 μM) with and without antibody. Under these conditions, the beat frequency dropped from 3 ± 0.7 to 1.0 ± 0.1 Hz in the absence and presence of mAb D1, respectively. However, the sliding velocity was only slightly affected from 5.9 ± 0.2 μm/s (n = 21) for control to 5.0 ± 0.3 μm/s (n = 16) for mAb D1.
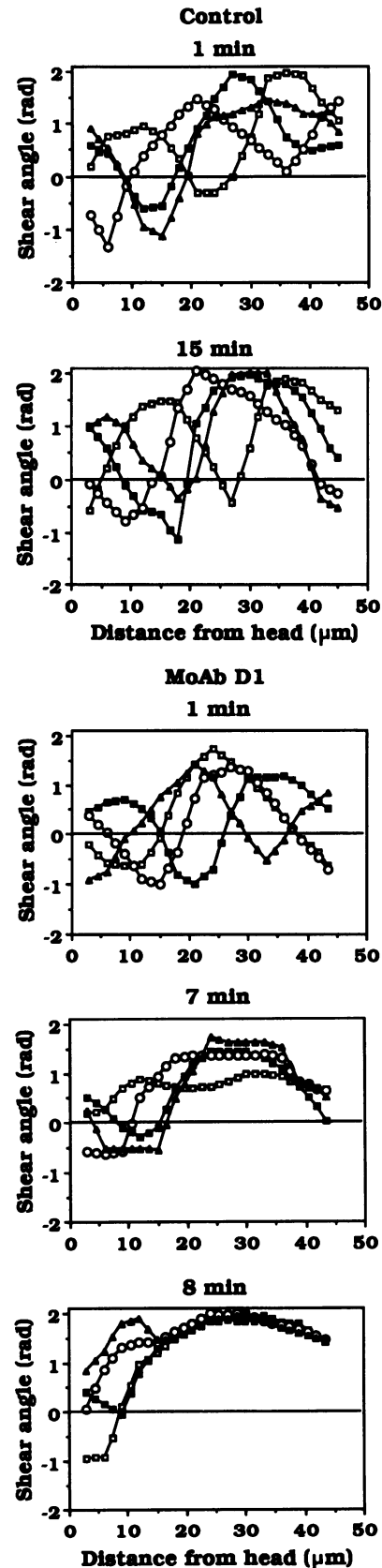
Figure 7. Effect of mAb D1 on the shear angle of sea urchin sperm flagella. Sea urchin (*P. lividus*) sperm models were incubated with and without mAb D1 (5 μg/ml) and analyzed by the Hamamatsu processor. Shear angles were measured on a representative spermatozoon on a sequence of four positions every 1.5 μm at 2 and 14 min for controls and at 1, 7, and 8 min for mAb D1. Tail positions 1 (□), 2 (■), 3 (○), and 4 (▲) are each spaced by 10 ms.
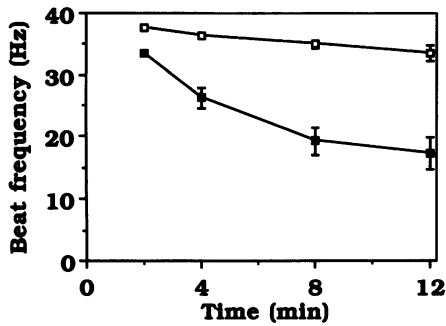

Control

1 min

15 min

Distance from head (μm)

MoAb D1

1 min

7 min

8 min

Distance from head (μm)

Figure 8. Effect of mAb D1 on the flagellar beat frequency. Sea urchin (*L. pictus*) spermatozoa were demembranated and reactivated with 1 mM MgATP in the presence of (■) and absence (□) of mAb D1 (5 μg/ml). Values presented are means ± SEM for 10–20 spermatozoa.

Effect of mAb D1 on Flagella from O. marina and Human Spermatozoa

To test whether the epitope recognized by mAb D1 on IC1 peptide could be present in axonemes of species only distantly related to sea urchins, mAb D1 was incubated with permeabilized *O. marina* and with demembranated-reactivated human spermatozoa. Although less potent than on sea urchin sperm motility, mAb D1 progressively blocked the motility of demembranated-reactivated human sperm models at 20 μg/ml. The antibody had little effect on flagellar beat frequency even when only 10% of the spermatozoa were motile (Figure 10). mAb D1 at 2 μg/ml also blocked the motility of the longitudinal and transversal flagella of *O. marina* (Figure 11). Furthermore, the pattern of inhibition observed with *O. marina* flagella was similar to that observed in sea urchin spermatozoa; mAb D1 decreased the beating amplitude of the proximal end of the flagellum and inhibited the wave propagation in the distal half of the longitudinal flagellum before the complete arrest of motility. However, with *O. marina*, a significant decrease in beat frequency was observed (Figure 11), and no evidence of apparent gap in the light diffracted by the flagellum was observed at the proximal end of the paralyzed section of the flagellum. Immunoblot analysis of *O. marina* proteins from purified flagella or from the whole Triton-insoluble cytoskeleton separated by SDS-PAGE revealed that the antibody recognized a major antigen with a molecular mass of 155 kDa (Figure 12).

Immunolocalization of IC1 in Sea Urchin Spermatozoa and in O. marina

Analysis by immunofluorescence of fixed sea urchin spermatozoa indicated that the antigen recognized by mAb D1 was distributed through the whole length of the axoneme similarly to the commercially available tubulin antibodies (Figure 13). However, whereas the
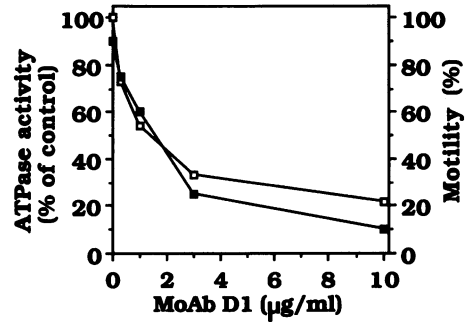

Figure 9. Effect of mAb D1 on dynein ATPase activity and sperm motility. Motile and immotile sperm *L. pictus* models and purified outer arm dynein ATPase (fraction 8 from sucrose gradient in Figure 2) were incubated with and without mAb D1 for ATPase activity determination. ATPase activity (□); sperm motility (■). The ATPase activity of immotile spermatozoa and of purified outer arm dynein ATPase were not affected by mAb D1.

staining was of uniform intensity for the latter antibodies, staining with mAb D1 was more intense in the proximal section of the axoneme. When *O. marina* was investigated under similar conditions, higher levels of fluorescence at the proximal end of the flagellum were followed by a gradient of decreasing intensity along the
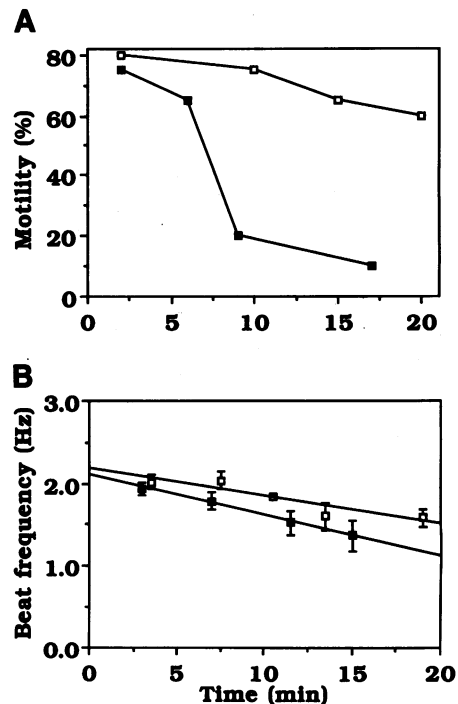

Figure 10. Effect of mAb D1 on motility of human spermatozoa. Human spermatozoa were purified, demembranated, and reactivated with 100 μM MgATP in the presence or absence of 20 μg/ml of mAb D1. (A) Motility in the presence (■) or absence (□) of mAb D1. (B) Flagellar beat frequency with (■) and without (□) 20 μg/ml of mAb D1. Values shown are representative of three other experiments.
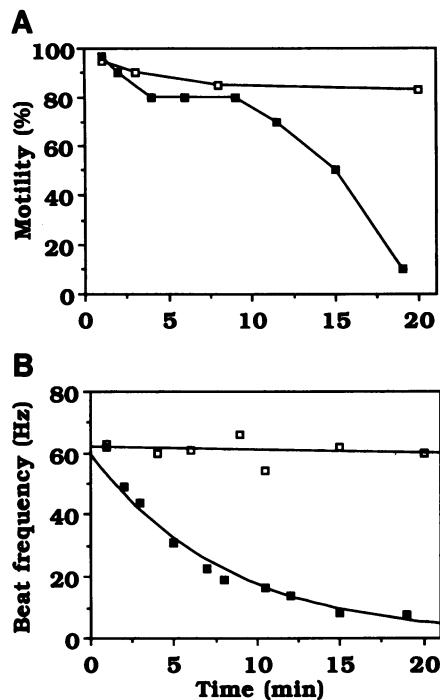
Figure 11. Effects of mAb D1 on the motility of flagella from *O. marina*. *O. marina* were demembranated and reactivated with 1 mM MgATP in the presence or absence of mAb D1. (A) Motility in the presence (■) or the absence (□) of 2 μg/ml of mAb D1. (B) Flagellar beat frequency with (■) and without (□) 2 μg/ml of mAb D1. Values shown are representative of three other experiments.

with sea urchin axonemes were without effect on sperm motility. Similarly, an unrelated commercial IgM antibody of unknown specificity, but which recognized an antigen with a molecular mass of 115 kDa in sea urchin axoneme, had no effect on sperm motility. Thus, the simple binding of IgM molecules to an axonemal antigen is not sufficient to block sperm motility. Furthermore, the observation that preincubation with a 0.6 M NaCl dynein extract prevented the action of mAb D1 is consistent with the conclusion that mAb D1 blocked motility by interacting with dynein IC1 peptides. The speed at which the inhibition of motility triggered by mAb D1 reached completion depended on the concentration of mAb D1 used. For all four sea urchin species investigated, as little as 0.1 μg/ml of mAb D1 was sufficient to block the motility of demembranated sperm models, within 10–15 min under the demembranation-reactivation conditions described by Cosson and Gagnon (1988).

axoneme. When the same axonemes were stained with the rat anti-tubulin antibody YL1/2, the staining was of equal intensity through the whole length of the axoneme. The cortical network of microtubules was well decorated with the anti-tubulin antibody, whereas it remained undetected with mAb D1.

DISCUSSION

mAb D1, selected for its capacity to inhibit flagellar motility at low concentrations, recognized only one polypeptide of 142 kDa in sea urchin sperm axonemes. Extraction of the outer dynein arm with 0.6 M NaCl and centrifugation of the high salt extract on sucrose gradient indicated that the 142 kDa peptide cosedimented with dynein ATPase activity. These results suggest that the 142-kDa protein, which had the same molecular mass as the first intermediate chain of dynein, is the heaviest intermediate chain (IC1) of the outer arm dynein on sea urchin sperm axonemes. This molecular mass of IC1 from *L. pictus* is higher than that reported for IC1 from other sea urchin species and corresponds to the mass of the IC1' peptide in other sea urchin species (Lee-Eiford *et al.*, 1986).

Proteins secreted by the myeloma cell line used for the fusion with spleen cells of the animal immunized
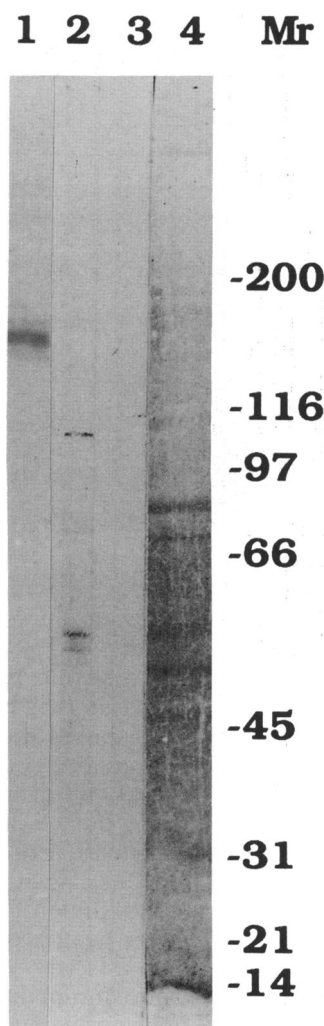

Figure 12. Immunoblots of *O. marina* proteins with mAb D1. *O. marina* were demembranated and the Triton-insoluble cytoskeleton was centrifuged, separated by SDS-PAGE, and immunoblotted with 1, mAb D1; 2, control IgM; and 3, no mAb. Amido Black staining of demembranated *O. marina* cytoskeletal protein transferred on nitrocellulose and molecular weights of standards are shown on lanes 4 and 5, respectively.
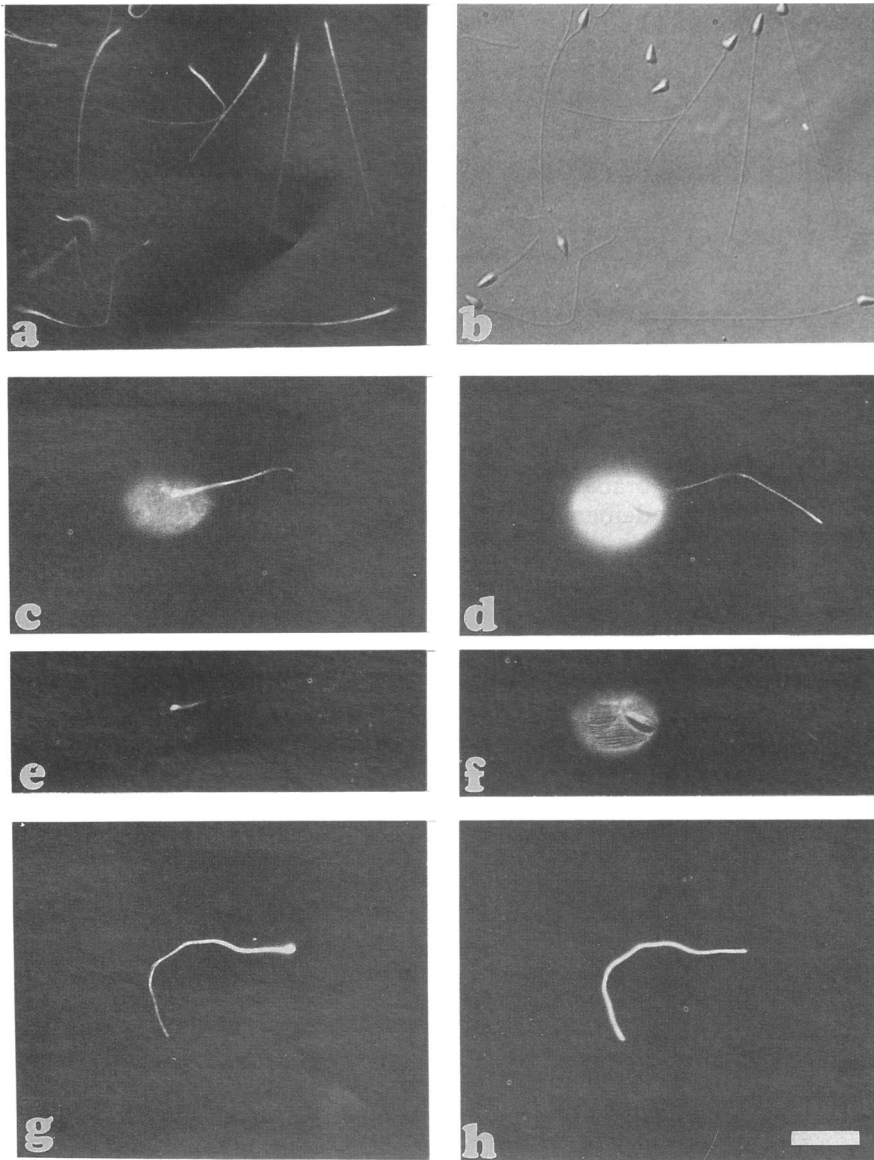
Figure 13. Immunofluorescence of sea urchin spermatozoa and *O. marina* with mAb D1. (a) Sea urchin (*P. lividus*) spermatozoa stained with mAb D1 and anti-mouse antibody-dichloro-triazinyl amino fluoresceine; (b) same field as in a but visualized by differential interference contrast microscopy; (c) whole *O. marina* stained as in a with mAb D1; (d) same field as c but stained with the anti-tubulin YL1/2 and anti-rat-Texas red sulfonyl chloride antibody after staining with mAb D1; (e and f) same pictures as c and d, respectively, but the photographic prints were exposed for the same short period of time to visualize and compare the staining of the cortical microtubules; (g) isolated flagellum of *O. marina* stained as in a with mAb D1; (h) the same field as in g but stained as in d with the anti-tubulin YL1/2. The bar represents 10 μm.

The mechanism by which mAb D1 interferes with the motility of sea urchin sperm models appears to involve an alteration of the amplitude of beating manifested by a decrease in bending, especially in the distal end of the flagellum that becomes paralyzed, whereas the proximal portion of the axoneme was still beating. However, this proximal portion was also affected but to a lesser extent and at a later time. Analysis of the shear angle along the whole axoneme clearly shows the progressive attenuation of the beating wave and stiffening of the distal flagellum, and the development of asymmetry as the time of contact with mAb D1 progresses. Flagellar beat frequency was also affected by mAb D1 but to a lesser extent. When nearly all spermatozoa became immobilized, the beat frequency was still at 50% of its normal value. When mAb D1, at twice

the concentration required to block flagellar motility, was added in the presence of axonemes incubated with trypsin, >90% of the axoneme slid over a period of 10–15 min, similarly to that of controls without antibodies. Because these results originated from stochastic events taking place over a relatively short period of time (a few seconds for full desintegration of the axoneme), microtubule sliding was investigated under conditions (14 μM Mg ATP) where the beat frequency of intact demembranated spermatozoa was decreased by two third from 3 to 1 Hz. Under these conditions, the sliding of microtubule doublets was marginally reduced from 5.9 to 5.0 μm/s in the presence of mAb D1. Thus, the sliding of microtubules is only slightly affected once the bending component of the axoneme is removed. These results are consistent with the observation that mAb D1

failed to inhibit the ATPase activity of purified outer dynein arms.

A predominent decrease in flagellar bending and a lack of effect on beat frequency has been reported for demembranated sea urchin spermatozoa incubated with either polyclonal anti-tubulin antibodies (Asai and Brokaw, 1980; Okuno et al., 1981) or monoclonal antibodies against α-tubulin (Asai et al., 1982). In both cases, the sliding of elastase-treated axoneme was unaffected. On the other hand, the antidynein polyclonal antibodies directed against a proteolytic fragment of dynein 1 from sea urchin sperm axoneme interferes with beat frequency, active sliding of axonemal microtubules, and bending and increases the resistance of axonemes measured under relaxing conditions (Gibbons et al., 1976; Ogawa et al., 1977; Okuno et al., 1981). In contrast to the dynein 1 fragment antibody, mAb D1 had no or little effect on purified dynein ATPase activity and sliding velocity in trypsinized axonemes.

In the flagella of the two other species studied, measurements of beat amplitude and shear angles are much more difficult to obtain and were not sought in the present study. As previously reported, the pattern of flagellar beating for human sperm models is irregular and in three dimensions (Chapeau and Gagnon, 1987). Because the two flagella beat in three dimensions and at an angle of 90° from each other, motility parameters for O. marina flagella are also difficult to analyze (Cosson et al., 1988). However, beat frequency measurements are reliable. The effects of mAb D1 on demembranated-reactivated human spermatozoa and on flagella of O. marina were somewhat different. In the first case, only a marginal effect on the beat frequency was observed, whereas in the latter case, the beat frequency was rapidly decreased to values as low as 15% of that of controls. Whether the presence in human sperm flagella of huge dense fibers attached to each doublet microtubule and of a fibrous sheath and the presence of a paraaxonemal structure in O. marina (Cachon et al., 1988) influence the effects of mAb D1 is presently unknown. Similarities between dynein intermediate chains from trout and sea urchin spermatozoa and Chlamydomonas flagella have been documented (King et al., 1990). These similarities were demonstrated on blots of axonemal proteins developed with Chlamydomonas dynein antibodies. However, the effects of these antibodies on the motility of flagella from the three species has not been reported. The observations that mAb D1 inhibits the motility of flagella from the primitive dinoflagellate O. marina, sea urchin spermatozoa, and human spermatozoa, covering a period of evolution of 400 000 000 years, indicate that the epitope recognized by mAb D1 is likely important and part of a mechanism fundamental to flagellar motility.

Immunoblots of proteins from the whole Triton-insoluble cytoskeleton and from purified axonemes of O. marina separated by SDS-PAGE revealed a single major band of antigen recognized by mAb D1. The molecular mass of 155 kDa for the antigen recognized was slightly higher than that of sea urchin spermatozoa, an observation often encountered in less evolved species. The antigen on human sperm axoneme recognized by mAb D1 has not yet been identified due to the limitation in loading sufficient axonemal proteins on SDS-polyacrylamide gels while avoiding overloading caused by the enormous amounts of proteins present in the fibrous sheath and auxiliary fibers surrounding the axoneme. Because of the presence of these auxiliary structures around the axoneme, axonemal proteins represent most likely <2% of the total proteins of human demembranated spermatozoa.

The decoration of sea urchin and O. marina flagella by mAb D1 and the absence of staining of the cortical microtubule network of the dinoflagellate suggest that the epitope recognized by mAb D1 is specific to proteins decorating microtubules involved in axonemal movement. The inhibition of axonemal motility initiated at the distal end of the flagellum, which, in some cases, upon the constant upcoming beating waves from the proximal flagellum, appears to result in the occasional fraying of the axonemal doublet microtubules. The reason for this initial paralysis of the distal section of the axoneme by mAb D1, whereas fluorescence data indicate higher fluorescent intensity in the proximal section of the axoneme, remains unexplained. This decrease in fluorescence as the distance to the sperm head increases may reflect an artefact caused by the method used in these immunofluorescence studies. However, it may also reflect a de novo situation where the concentration of mAb D1 antigens decreases along the axoneme. Even though the outer dynein arm must be present throughout the whole length of the axoneme, it is possible that a certain heterogeneity in its composition may exist along the axoneme. For example, it is conceivable that the IC1 peptide of the outer dynein arm recognized by mAb D1 may be one of the IC1 isotypes predominantly present in the proximal section of the axoneme, whereas a slightly modified IC1 isotype (but with a similar mass), not recognized by mAb D1, could be mainly concentrated in the distal portion of the flagellum. A similar heterogeneity in the composition and localization of inner dynein arms along the axoneme has recently been documented (Sale et al., 1989; Piperno and Ramanis, 1991; Smith and Sale, 1992).

Considering that mAb D1 behaves as a normal IgM with an apparent molecular mass of 950 kDa and a maximal diameter of 30 nm (Feinstein and Munn, 1969), the distance covered by one molecule is greater than that of two adjacent IC1 peptides on the same microtubule but smaller than that of two adjacent IC1 peptides on different microtubules. On the other hand, changes in conformation of IgM molecules have also been reported, where, upon binding of the antigen, especially a particulate antigen, the antibody molecule

takes a crab rather than a flat configuration. In this case, the span covered by one IgM molecule is only of the order of 15 nm (Feinstein and Munn, 1969), this would imply that only one dynein molecule interacts with the antibody. Whether mAb D1 interacts with one or two molecules of IC1 on the same microtubule remains to be elucidated.

As the time of contact between mAb D1 and demembranated sea urchin spermatozoa increased, occasional breaks became visible in the midsection of the sea urchin flagellum, the distal portion of the flagellum becoming completely immobilized, whereas the proximal portion remained functional, though with progressively decreasing amplitude until completely immobilized. These breaks were only apparent, probably reflecting a frayed section of the axoneme, likely resulting from the force exerted by the incoming wave on the paralyzed distal end of the axoneme. Although the possible presence of protease in the mAb D1 preparation may theoretically contribute to the occurrence of these breaks, the observation that mAb D1 action on motility was prevented by the addition of 0.6M NaCl extract containing the outer dynein arm is not consistent with this hypothesis. Similar apparent breaks or fraying in the axoneme have also been reported by other investigators (Brokaw, 1986, 1993; Kamiya and Okagaki, 1986).

The recent observation of King et al. (1991) on the direct interaction between the IC 78 kDa from Chlamydomonas and α-tubulin may provide clues on the role played by IC peptides. Several lines of evidence indicate that IC peptides are positioned on subfiber A in close proximity to the globular heads of adjacent dynein arms (Goodenough and Heuser, 1989; Witman, 1989, 1992; King et al., 1991). Because polyclonal antibodies against tubulins and monoclonal antibodies against α-tubulin interfere with sea urchin sperm motility by decreasing flagellar bend angle rather than beat frequency (Asai and Brokaw, 1980; Asai et al., 1982), we can hypothesize that mAb D1 blocks sperm motility by interfering with the interaction between IC1 and tubulin (possibly α-tubulin) on subfiber A. This interpretation would suggest that IC1 interaction with α-tubulin is important for motility and that IC1 may first be involved in bend propagation rather than in the sliding of microtubules.

ACKNOWLEDGMENTS

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