

Effects of Antibodies against Tubulin on the Movement of Reactivated Sea Urchin Sperm Flagella

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ABSTRACT Antibodies binding to sea urchin flagellar outer-doublet tubulin have been isolated from rabbit sera by tubulin-affinity chromatography employing electrophoretically purified tubulin as the immobilized substrate. This procedure provides "induced" antitubulin antibody from immune sera and "spontaneous" antitubulin antibody from preimmune sera. These antitubulins were characterized in terms of their specificity, ability to bind to sea urchin axonemes, and effects on the motility of reactivated spermatozoa. Induced antitubulin antibody specifically reduced the bend angle and symmetry of the movement of demembrated reactivated spermatozoa without affecting the beat frequency. At identical concentrations, spontaneous antitubulin had no effect on motility. Affinity-purified induced antitubulins from three other rabbits all gave specific bend-angle inhibition, whereas their corresponding spontaneous antitubulins had no effect on the flagellar movement. The effects of antitubulin on microtubule sliding were examined by observing the sliding disintegration of elastase-digested axonemes induced by $MgATP^{2-}$. Affinity-purified induced antitubulin antibody, in quantities sufficient to completely paralyze reactivated flagella, did not inhibit microtubule sliding. The amplitude-inhibiting effect of induced antitubulin on reactivated spermatozoa may be caused by action on a mechanism responsible for controlling flagellar bending rather than by interference with the active sliding process. This is the first report of an antitubulin antibody having an inhibitory activity on microtubule-associated movement.

Eukaryotic cilia and flagella are microtubule bundles arranged, with supplementary structures, to produce repeated, propagated bending. The nine outer-doublet tubules slide against each other; this sliding is directly mediated by ATP and by dynein cross-bridges (21, 42). The frequency of oscillatory sliding is directly related to the ATP concentration (3, 23) and is probably also related to kinetic properties of dynein ATPase sites.

The active sliding process can be observed in isolation from bending by utilizing demembrated flagellar axonemes that have been briefly digested with trypsin (42) or elastase (6). When $MgATP^{2-}$ is added to these preparations, the axonemes disintegrate. Disintegration can be directly observed with dark-field light microscopy and can be seen to occur by sliding extrusion of doublet microtubules (6, 42). It remains to be determined how the active sliding is translated into waves of bending and how the amplitude and wavelength of these waves are determined. Structures other than microtubules and dynein—such as the radial spokes, connections to the central pair tubules, and nexin interdoublet links—have been proposed as

fulfilling this function, perhaps by selectively impeding sliding and forcing the axoneme to bend locally (43).

As part of a general exploration of antibodies as probes of flagellar movement, the present study examines antibodies against the major structural protein of flagella, tubulin. Other workers have raised antibodies to tubulin and used them in a variety of studies, including visualizing cytoplasmic cytoskeletons (16, 45), assaying for the presence of tubulin in various species (10), comparing the structural cross-reactivity among tubulins from various sources (17), measuring the synthesis of tubulin (26), and observing lymphocyte capping (18). In some cases, induced antitubulins were able to precipitate tubulin in immunodiffusion assays; in other situations, antitubulins were detected by immunofluorescence (14) or by radioimmunoassay (32), but no precipitation was observed.

In the course of our study we detected low levels of spontaneous antitubulin antibodies in the normal sera of all of the rabbits we have examined (1). Our observations confirm the results of Karsenti et al. (25), who found low-level, nonprecipitating antitubulins in a variety of nonimmune mammalian

sera. These spontaneous antitubulins in indirect immunofluorescence were able to stain vinblastine-induced paracrystals but gave only diffuse and indistinct staining of cultured mouse cells under conditions where induced antitubulins gave distinct staining of cytoplasmic microtubules.

In the present study, we report in detail the preparation, purification, and characterization of antitubulin antibodies from the preimmune and immune sera of one rabbit. We have examined the effects of antitubulin antibodies on the movement of reactivated sea urchin sperm flagella by measuring effects on beat frequency and wave amplitude and the active sliding of outer-doublet microtubules in flagella that have been briefly digested with elastase. We show that the effects on flagellar movement of induced antitubulins from four rabbits are remarkably similar.

MATERIALS AND METHODS

Materials

Concentrated spermatozoa from *Strongylocentrotus purpuratus* were obtained as described previously (33). For experiments with reactivated spermatozoa, a stock sperm suspension was prepared by dilution with 1–2 vol of cold 0.5 M NaCl. The concentration of the sperm suspension was adjusted until a 10- μ l portion, diluted into 5.0 ml of 0.5 M NaCl, produced an optical density reading of 0.24–0.26 at 550 nm (8). For preparations of axonemes and axonemal proteins, the spermatozoa were washed once with seawater and then either used at once for preparation of axonemes or stored with 50% glycerol at -15°C (34). The preparation of the major flagellar ATPase, dynein 1, purified from low ionic strength extracts of flagellar axonemes, was the same as that used in our previous experiments (33).

Preparation of Tubulins

Outer-doublet microtubule protein was obtained from axonemes by the se-

quential solubilization procedure of Stephens (41). First, axonemes were extracted twice with 0.6 M KCl, 10 mM Tris-Cl, 0.1 mM dithiothreitol (DTT), pH 8.0, at room temperature for 30 min to remove most of the dynein and central-pair tubulin. The samples were centrifuged at 20,000 g for 30 min; the resulting pellets were extracted at room temperature with 10 mM Tris-Cl at pH 8.0 for 30 min to remove minor components and then centrifuged at 20,000 g for 30 min. The pellets were then extracted with 1 mM Tris-Cl, pH 8.0, at 37°C for 30 min and centrifuged at 20,000 g for 30 min; the pellet was reextracted with the same buffer at 45°C and centrifuged at 20,000 g for 30 min. The supernates from both of these 1-mM Tris-Cl extractions contained outer-doublet tubulin. A third tubulin preparation was obtained from the pellet remaining after the second outer-doublet extraction. This pellet was suspended in distilled water, dialyzed against water, and lyophilized; it was labeled "residue tubulin." The three tubulin preparations were shown to have ATPase activities of <0.01 , as compared with an activity of 1.48 (micromoles of phosphate liberated per milligram of protein per minute) for the combined 0.6 M KCl extracts. All three tubulin preparations were dialyzed against distilled water, lyophilized, and shown to be pure by SDS polyacrylamide electrophoresis, which showed only tubulin and no dynein bands. For some of the immunodiffusion assays, outer-doublet tubulin was alkylated by carboxymethylation (12) with recrystallized iodoacetic acid.

The tubulin used to inject rabbit r2 (Table I) and used as the immobilized absorbent in all affinity chromatography was purified by preparative gel electrophoresis. Outer-doublet tubulin was electrophoresed in 6% polyacrylamide continuous SDS gels. One gel from each electrophoresis run was stained to determine the relative mobility of the tubulin band. The tubulin band was sliced out of each gel, homogenized in 8 M urea, 0.36 M Tris-Cl, pH 8.6, and extracted at 37°C overnight. The acrylamide was pelleted by centrifuging the mixture at 20,000 g for 30 min, and the supernate was saved and concentrated. The supernate contained only tubulin, as shown by reelectrophoresing a portion of the supernate (Fig. 1 b). Some of this electrophoretically purified material was reduced and carboxymethylated (12) and electrophoresed in 7% polyacrylamide discontinuous SDS-urea gels, which revealed only the α - and β -tubulin bands (Fig. 1 c).

Immunizations

Because tubulin is a highly conserved protein in a wide range of eukaryotes (30), SDS was routinely used to dissolve the antigen in an attempt to provide a maximum of denatured, "new" determinants to the rabbits' immune systems.

TABLE I
Injection Schedules for the Rabbit Sera

Rabbit	Antigen preparation	1st Injection	2nd Injection	3rd Injection	4th Injection	Bleeding	Ouchterlony
1	OD tubulin SDS PAGE	Day 0 0.48 mg 0.1% SDS CF tp,im,sc	Day 16 0.31 mg 0.1% SDS CF sc	Day 50 0.48 mg 0.1% SDS No adjuvant im	Day 78 0.36 mg 0.1% SDS No adjuvant im	Days 85, 92, 100	Negative
2	OD tubulin SDS PAGE	Day 0 0.72 mg 0.1% SDS CF tp,im,sc	Day 16 0.31 mg 0.1% SDS CF sc	Day 33 0.31 mg 0.1% SDS No adjuvant sc	Day 50 0.18 mg 0.1% SDS No adjuvant im	Days 65, 71, 78	Positive
3	Residue tubulin	Day 0 5.0 mg 0.05% SDS CF tp,im,sc	Day 24 4.1 mg 0.1% SDS No adjuvant ip	—	—	Day 38	Positive
4	Residue tubulin	Day 0 6.0 mg 0.1% SDS CF tp,im,sc	Day 14 1.8 mg 0.1% SDS No adjuvant sc	Day 31 3.0 mg 0.1% SDS No adjuvant im	Day 59 1.5 mg 0.1% SDS No adjuvant im	Days 66, 73	Positive
5	Axonemes, extracted	Day 0 20.0 mg 0.1% SDS CF tp,im,sc	Day 14 8.0 mg 0.1% SDS No adjuvant sc	Day 31 3.0 mg 0.1% SDS No adjuvant im	—	Days 46, 52	Positive

Adult female New Zealand White rabbits were used in all cases. Ouchterlony immunodiffusion was performed with whole sera against purified alkylated outer-doublet tubulin.

OD, outer-doublet; SDS PAGE, tubulin eluted from SDS polyacrylamide gel slices; CF, complete Freund's adjuvant; tp, rear toepad; im, femoral intramuscular; sc, subcutaneous along the back; ip, intraperitoneal.

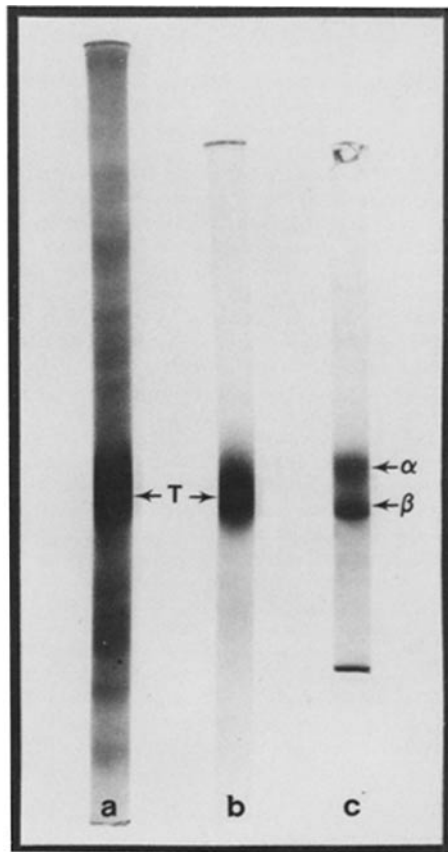


FIGURE 1 Polyacrylamide gel electrophoresis. (a) Whole *S. purpuratus* axonemes solubilized in 0.1% SDS on a continuous SDS, 6% polyacrylamide gel. (b) Electrophoretically purified outer-doublet tubulin (60 μ g) used as an antigen and as an immunoabsorbent. (c) Electrophoretically purified tubulin after carboxymethylation on a discontinuous urea-SDS, 7% polyacrylamide gel.

The use of SDS during immunization was similar to procedures of Lazarides (28). Complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) was used. The sera used in most of the studies reported in this paper were derived from one rabbit, r2, which was injected with electrophoretically purified outer-doublet tubulin. In addition, other rabbits were injected with different preparations of tubulin, as summarized in Table I. Rabbit r1 was also injected with electrophoretically purified outer-doublet tubulin but failed to produce precipitating antibodies; rabbits r3 and r4 were injected with residue tubulin; and rabbit r5 was injected with whole axonemes that had been extracted twice with 0.6 M KCl and once in low-ionic-strength buffer (34) to remove most of the dynein. A polyacrylamide gel of this material showed only a heavy tubulin band and a faint band of high molecular weight. This high-molecular-weight material may represent unextracted dynein or aggregates of tubulin.

Anti-bovine serum albumin was obtained by injecting 2 mg of antigen (fraction V, Sigma Chemical Co., St. Louis, Mo.) emulsified with complete Freund's adjuvant into the rear toepads and femoral muscles of an adult female New Zealand White rabbit and boosting with 2 mg of bovine serum albumin (BSA) subcutaneously 2 wk later. The serum collected 1 wk after the booster injection strongly precipitated BSA in immunodiffusion assays and was used in these experiments.

Anti-dynein 1 serum was the same as that used in our previous experiments (33). Anti-fragment A serum was the gift of Dr. Kazuo Ogawa (National Center for Biological Sciences, Okazaki, Japan) (35).

All sera were routinely stored at 4°C in 1% sodium azide.

Purification of Immunoglobulins

IgG was purified from rabbit sera by twice precipitating with 33% ammonium sulfate, at room temperature, followed by DEAE-cellulose chromatography at 4°C in 0.01 M phosphate as described by Garvey et al. (19). Purity was confirmed by 12% polyacrylamide SDS gel electrophoresis, which revealed only the expected bands for the heavy and light chains of IgG.

Preparation and Purification of Fab Fragments

Monovalent Fab fragments were obtained from immune and preimmune sera (from r2) by a limited papain cleavage (19). Proteolysis was accomplished with ~1 mg papain (Sigma Chemical Co., two times recrystallized) per 100 mg IgG at 37°C for 12 h. Cleavage was performed at an antibody concentration of ~8 mg/ml. The reaction was stopped with an excess of iodoacetamide. Fraction I Fab fragments were purified by chromatography over a carboxymethylcellulose column with a step-gradient of acetate. The peak eluting at 0.01 M acetate was concentrated and shown to not contain any crystallizable fragment of immunoglobulin (Fc) activity as assayed by goat anti-rabbit IgG heavy chain sera (Cappel Laboratories Inc., Cochranville, Pa.), but it was precipitated by another antiserum specific for Fab (Cappel Laboratories Inc.).

Tubulin-affinity Chromatography

Tubulin-affinity chromatography was employed to purify antitubulin antibodies for subsequent experiments. The procedure used to couple electrophoretically purified outer-doublet tubulin to Sepharose was similar to that recommended by Pharmacia (40). Cyanogen bromide-activated Sepharose 4B was hydrated in 1 mM HCl. To this was added electrophoretically purified outer-doublet tubulin in 0.1 M carbonate/bicarbonate, 0.5 M NaCl, pH 9.2, buffer. Coupling was accomplished at 4°C for 24 h. The slurry was washed with the carbonate/bicarbonate buffer. Unreacted active groups were blocked by treatment with an excess of 0.5 M ethanolamine, pH 9.6, at room temperature for 3 h. The product was alternately washed with pH 9 borate and pH 4 acetate buffers; both of these buffers also contained 0.5 M KCl. Several identical tubulin-affinity columns were prepared in this way—each with a bed volume of 10 ml containing ~1 mg of electrophoretically purified outer-doublet tubulin covalently linked to the Sepharose. Chromatography was performed at 4°C. IgG fractions obtained from sera after two ammonium sulfate precipitations were dialyzed against buffer I containing 0.1 M NaCl, 20 mM Tris-Cl, 2 mM EGTA, and 0.01% Na₂S₂O₅, pH 7.5, and were slowly (<10 ml/h) run into the tubulin-affinity columns in the same buffer. The columns were continued in buffer I until the major, nonretained absorbance (at 280 nm) peak was fully eluted. The columns were then washed with several column volumes of buffer II containing 0.6 M NaCl, 20 mM Tris-Cl, 2 mM EGTA, and 0.01% Na₂S₂O₅, pH 7.5, to wash off nonspecifically aggregating material. Final elution was effected by a single pH stepdown with buffer III, which was buffer II brought to pH 2.2 with HCl.

Recovered antibody fractions were pooled and concentrated by ultrafiltration over an Amicon PM-10 membrane (Amicon Corp., Lexington, Mass.), dialyzed against appropriate buffers for subsequent experiments, and stored in 0.01% Na₂S₂O₅ at 4°C. Special care was taken throughout so that no cross-contamination between immune and preimmune samples occurred, i.e., separate affinity columns and different Amicon membranes were employed throughout. The amount of protein in these antibody preparations was measured, and the dilution of preimmune and immune antitubulins was adjusted to give equivalent antibody concentrations.

Radioiodine Binding Assays

Tubulin-affinity purified antibodies—intact antibodies and Fab fragments from rabbit r2—and anti-BSA immunoglobulins were radioiodinated (sodium ¹²⁵I from New England Nuclear, Boston, Mass.) in isotonic phosphate with the chloramine T method (24). Specific activities for each of the five antibody preparations (counts per minute of ¹²⁵I per microgram of protein) were: r2 immune, 64,548; r2 preimmune, 61,012; r2 immune Fab, 307,704; r2 preimmune Fab, 106,722; and anti-BSA, 99,702. To evaluate antibody binding to axonemes, equivalent quantities of the immune and preimmune antitubulins (0.042 μ g each), Fab fragments (0.028 μ g each), or anti-BSA (0.150 μ g) were mixed with various dilutions of axonemes in phosphate-buffered saline (PBS). Anti-BSA was used to measure any nonspecific sticking of iodinated antibodies to the axonemes. Unlabeled BSA (2 mg/ml final concentration) was added to all tubes as a carrier protein. Alternatively, gelatin (2 mg/ml final concentration) was substituted for BSA as an unlabeled carrier. Results obtained with either carrier were essentially identical. The final volume per reaction tube was adjusted to 250 μ l, and the tubes were incubated overnight at 4°C with slow end-over-end agitation. After incubation, the tubes were centrifuged at 1,400 g for 10 min, and 125 μ l of the supernate was micropipetted into a clean tube and counted for counts per minute of ¹²⁵I on a Nuclear Chicago gamma counter (this number is referred to as S). The remaining half-supernatant plus pellet was also counted (P). The percent of counts residing in the pellet, then, was calculated as 100 \times (P - S)/(P + S). This figure was adjusted for zero-level binding by performing the identical procedure on samples containing the same amounts of ¹²⁵I antibody, carrier, and axoneme-free buffer. Maximum possible levels of binding were determined by mixing the labeled antibodies with 10% (final concentration) TCA and BSA (3.3 mg/ml,

final concentration) and incubating at 4°C overnight. The pellets, after centrifugation, were counted as described above to obtain the 100%-TCA-precipitable counts. Results are expressed as percent of TCA precipitable counts of ¹²⁵I in the pellets after subtracting zero-level backgrounds, which never exceeded 10%.

Immunofluorescence of Spermatozoa

Whole spermatozoa from *S. purpuratus* were allowed to settle on coverslips and were demembrated with 1% Triton X-100, stabilized in 20 mM 2-(*N*-morpholino)ethane sulfonic acid (MES), 4% polyethylene glycol (PEG), 70 mM NaCl, 1 mM EGTA, 0.5 mM MgCl₂, and 2.5 mM GTP, pH 6.9 (38), incubated with tubulin-affinity purified antibodies, and observed by indirect immunofluorescence (27) with fluorescein-conjugated goat anti-rabbit IgG (Miles Laboratories, Inc., Elkhart, Ind.).

Observations of Motility of Reactivated Spermatozoa

Demembrated spermatozoa of *S. purpuratus* were prepared and reactivated with extraction and reactivation solutions with the same compositions as those used previously (4), with an ATP concentration of 0.15 mM in the reactivation solution. A 10-μl portion of a standardized stock sperm suspension was mixed into 1.0 ml of extraction solution. After 60 s, a 10-μl portion of extracted spermatozoa was added to 1.0 ml of reactivation solution containing an aliquot of antibody preparation and gently mixed. A drop of these spermatozoa was immediately observed by dark-field microscopy. Observations, frequency measurements, and photographs were made as described previously (33). All work was carried out at 18°C.

Bend angles were measured by protractor from the negatives on the screen of a microfilm reader. The bend-angle measurement was routinely made as near as possible to halfway along the length of the flagellum. Principal and reverse bend angles (20) were measured independently and then averaged.

Observations of Microtubule Sliding

A portion of the stock sperm suspension was homogenized for 30 s with a Vortex mixer (Scientific Products Co., Div. American Hospital Supply Corp., McGaw Park, Ill.) to obtain axonemal fragments. Demembrated axonemes were prepared by mixing 10 μl of this homogenized sperm suspension with 0.5 ml of Triton extraction solution (9), and were then digested by adding 1.5 μg of elastase (Calbiochem-Behring Corp., San Diego, Calif.) and incubating for 15 min at 18°C. The mixture was then diluted with 2.5 ml of ATP-free reactivation solution (6) containing 5 μg/ml chicken ovinhibitor (Sigma Chemical Co.). These elastase-digested axonemes were stored at 0°C for subsequent experiments and were stable over an entire day.

For each experiment, 50 μl of elastase-digested axonemes were mixed with 5 μl of freshly demembrated, intact spermatozoa and 35 μl of reactivation buffer containing an antibody preparation. After incubation for 30 min at 18°C, 10 μl of reactivation solution containing 1.5 mM MgATP²⁻ was added to produce a final MgATP²⁻ concentration of 0.15 mM. The preparations were then observed and photographed by dark-field microscopy. All experiments were carried out at 18°C.

Other Procedures

Standard methods included: Ouchterlony immunodiffusion (39) and immunoelectrophoresis (19), continuous SDS polyacrylamide gel electrophoresis (44), as modified by Gibbons et al. (22), discontinuous SDS-urea polyacrylamide gel electrophoresis (13, 46), as described by Ludueña and Woodward (30), determination of protein by the method of Lowry (2, 29), and assay for ATPase activity by measurement of inorganic phosphate (15).

RESULTS

Immunizations

Sera from rabbits injected with tubulins were assayed for antitubulin activity by immunodiffusion against outer-doublet tubulin. Rabbits r2-r5 all produced tubulin-precipitating sera; rabbit r1 was one of several rabbits in which immunization was unsuccessful. Details of these immunizations are given in Table I. We have attempted various sea urchin tubulin immunizations in 17 rabbits; of these, five (r2-r5 and one other) tested positive.

Purification of Antitubulins by Affinity Chromatography

Initial tubulin-affinity chromatography experiments were performed with whole, unfractionated immune (2 ml) and preimmune (2 ml) r2 sera. With preimmune serum, the low pH elution yielded a relatively small amount of protein. However, when the same preimmune serum was preconcentrated three fold, and identical volumes of the two samples were again chromatographed, a quantity of preimmune antibody comparable to immune antibody was eluted. These early experiments with whole sera demonstrated the ability of the tubulin-affinity columns to selectively absorb antibodies. Electrophoresis on 12% polyacrylamide SDS gels showed that several serum components, including albumin, were totally absent from the eluted samples, which contained only the heavy and light chains of IgG.

To obtain the antitubulins used in this study, tubulin-affinity chromatography was carried out with ammonium sulfate-precipitated IgG fractions from immune and preimmune sera; ~10 mg of protein from each serum was loaded on affinity columns (10-ml bed volume, ~1 mg of tubulin covalently linked). Ultraviolet absorbing peaks of comparable size were obtained, as shown in Fig. 2 *a*. Similar elution patterns were obtained with immune and preimmune monovalent Fab fragments from r2 sera (Fig. 2 *b*).

Material Recovered from Tubulin-affinity Chromatography Is IgG

Immuno-electrophoresis of affinity-purified immune and preimmune material showed the eluted samples to be exclusively IgG because no class of antibody other than IgG was detected by specific goat anti-rabbit antibodies to IgG, IgM,

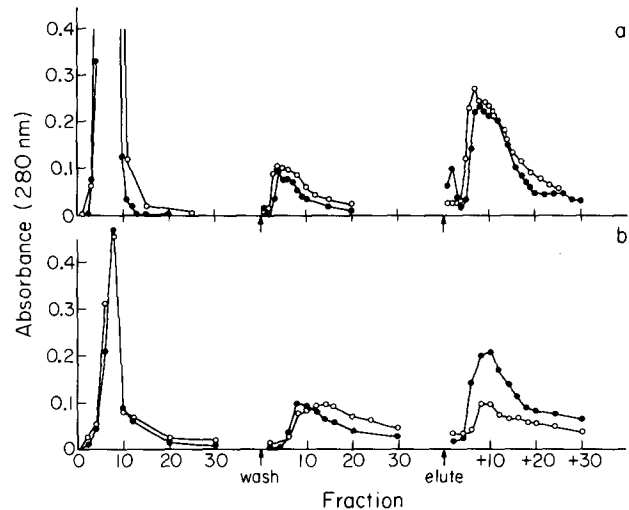


FIGURE 2 Tubulin-affinity chromatography of ammonium sulfate precipitates of sera. Each column had a bed volume of 10 ml to which ~1 mg of electrophoretically purified tubulin was covalently linked. Columns were run at <10 ml/h, 50-drop fractions were collected, and absorbance was measured at 280 nm. (a) 10 mg of immune globulin (solid circles) compared with 10 mg of preimmune globulin (open circles). (b) 3 mg of immune monovalent Fab fragments (solid circles) compared with 3 mg of preimmune monovalent Fab fragments (open circles). Similar patterns were obtained with all of the samples chromatographed.

and IgA (Cappel Laboratories Inc.) (results not shown). The homogeneity of the purified material was also examined by electrophoresis of samples on 12% polyacrylamide SDS gels. The material retained on the affinity columns electrophoresed as two bands that ran identical to IgG heavy and light chains.

Characterization of the Antitubulins

Ouchterlony immunodiffusion assays were performed to test the ability of serum preparations to precipitate various axone-mal proteins. The figures shown in this paper were obtained with preparations isolated from r2 sera; similar results were noted with antitubulins from r2-r5. Fig. 3 *a* shows that immune antitubulin serum precipitated a component present in a crude 0.6 M NaCl extract of axonemes (well 3). It also precipitated purified outer-doublet tubulin (well 2) but did not precipitate dynein (wells 1 and 4). Fig. 3 *b* shows that whole antitubulin immune serum (well 4) formed precipitin lines against a solution of alkylated outer-doublet tubulin (similar results were obtained with other preparations of tubulin, alkylated or na-

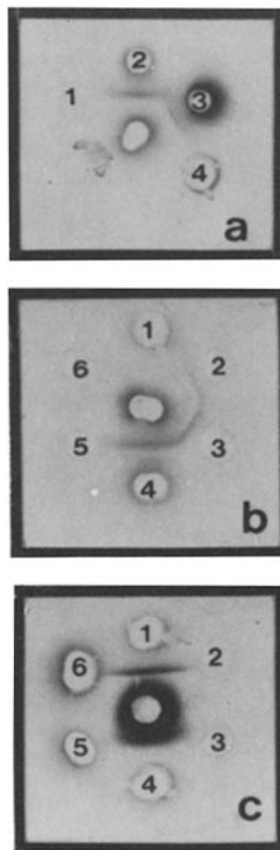


FIGURE 3 Ouchterlony immunodiffusion assays. Samples were allowed to diffuse ~48 h at room temperature, after which they were dialyzed with PBS and stained with Coomassie Blue. (a) Well 1, purified dynein; well 2, alkylated tubulin; well 3, 0.6 M NaCl extract of axonemes; well 4, purified dynein; center well, ammonium sulfate precipitated immune antibody. (b) Well 1, whole preimmune serum; wells 2 and 3, tubulin-affinity-purified antibody from immune serum (0.56 mg/ml); well 4, whole immune serum; wells 5 and 6, tubulin-affinity-purified antibody from preimmune serum (0.56 mg/ml); center well, alkylated outer-doublet tubulin (3 mg/ml). (c) Well 1, anti-dynein 1; wells 2 and 3, affinity-purified antibody from immune serum (0.56 mg/ml); well 4, anti-fragment A; wells 5 and 6, affinity-purified antibody from preimmune serum (0.56 mg/ml); center well, dynein 1.

tive; lyophilized, alkylated outer-doublet tubulin redissolved at 3 mg/ml in 0.5 M KCl was used in this study because of its homogeneity and its solubility). Affinity-purified antitubulin (wells 2 and 3) formed a precipitin line that was identical to the main line formed by the whole immune serum. Neither the preimmune serum (well 1) nor the affinity-purified preimmune antitubulin (at the same protein concentration as the immune samples, wells 5 and 6) formed a detectable precipitin line against tubulin under these conditions. The immunodiffusion shown in Fig. 3 *c* indicates that neither of the affinity-purified antitubulins precipitated dynein. No spurs in the precipitin lines formed by anti-dynein 1 (well 1) and by anti-fragment A (well 4) occurred toward the antitubulins. This further indicates that the antitubulins used in these experiments did not cross-react with dynein 1.

Besides the immunodiffusion results, several lines of evidence indicate that the antibodies used in these studies were specific for tubulin. Specificity was enhanced by isolating all the antitubulins by affinity chromatography employing electrophoretically purified outer-doublet tubulin. This purification scheme is similar to that used by Fuller et al. (16) in their preparation of "monospecific" antitubulins. Indirect immunofluorescence of chick embryo fibroblasts showed that the affinity-purified immune antitubulin, but not the preimmune antibody, strongly stained cytoplasmic microtubules (results not shown). Immunoglobulin-affinity chromatography employing ammonium sulfate-precipitated antibodies from immune and preimmune sera covalently coupled to Sepharose and then exposed to a whole cytoskeletal extract of chick fibroblasts specifically retained α - and β -tubulins, as revealed by two-dimensional electrophoresis (Elias Lazarides, personal communication).

Antitubulins Bind to Axonemes

To examine the binding to axonemes of affinity-purified antitubulins from immune and preimmune sera, we devised a simple radiobinding assay, taking advantage of the easily sedimented axonemes. The results in Fig. 4 are expressed as percent of the TCA-precipitable counts of ^{125}I in the pellet after subtraction of the zero-level backgrounds. All of the antitubulins (from r2), both intact antibodies and monovalent Fab fragments from both immune and preimmune sera, bound to axonemes. Minimal trapping of ^{125}I anti-BSA was observed, indicating that the binding by the antitubulin preparations was caused by more than nonspecific association of iodinated antibodies with axonemes. When DEAE-purified unfractionated IgG from normal serum (iodinated to approximately the same specific activity as the affinity-purified fractions) was tested, no binding was observed, again demonstrating an absence of nonspecific antibody trapping by the pellets. The differences in binding between the whole immune antibodies and the whole preimmune antibodies is significant and appears to reflect an approximately 100-fold lower affinity for the axonemes by the preimmune antitubulins. The ^{125}I Fab fragments were assayed for binding at a different protein concentration than the intact antibodies, and, therefore, amounts of binding by the intact antibodies and by the Fab fragments cannot be compared. No significant difference in binding between immune Fab fragments and preimmune Fab fragments was shown by this assay.

Indirect immunofluorescence was performed to further demonstrate the binding of antitubulin antibodies to axonemes. When chick fibroblasts were stained, the affinity-purified im-

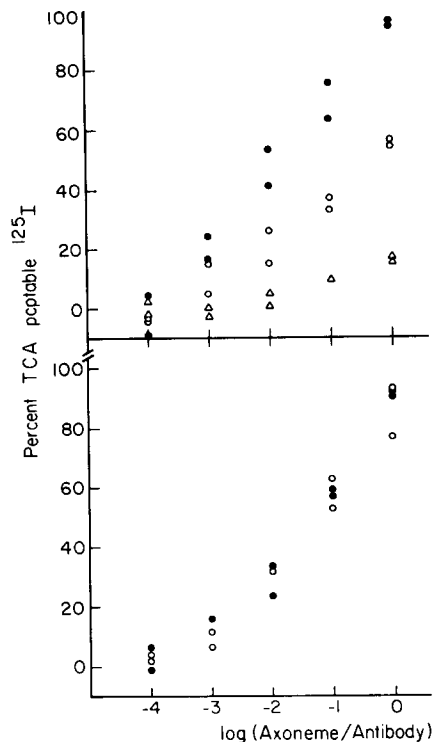


FIGURE 4 Radiobinding assay. Upper panel, binding to axonemes of tubulin-affinity-purified intact antibodies from immune (solid circles) and preimmune (open circles) sera (0.042 μg each). Precipitation of ^{125}I anti-BSA (triangles) is also shown to demonstrate that nonspecific trapping of antibodies by the pelleting axonemes was minimal. Lower panel, binding to axonemes of tubulin-affinity-purified Fab fragments from immune (solid circles) and preimmune (open circles) sera (0.028 μg each). In both figures, results are shown as percentages of TCA-precipitable counts ^{125}I in pellets vs. the log of the dilution of axonemes (arbitrary units).

immune antitubulin allowed the characteristic striking visualization of cytoplasmic and spindle microtubules, whereas the affinity-purified preimmune antitubulin, at the same concentration, gave a weak, diffuse staining pattern (25). However, when the antitubulins were used to visualize demembrated spermatozoa, as shown in Fig. 5, both the immune and preimmune antitubulins stained the flagella but not the heads.

Effects of Antitubulins on the Motility of Reactivated Spermatozoa

The effects on the motility of reactivated flagella were examined by briefly demembrating spermatozoa, mixing an aliquot of the demembrated spermatozoa into a reactivation solution containing 0.15 mM ATP and a known quantity of the affinity-purified immune or preimmune antitubulin, and examining the spermatozoa by dark-field light microscopy. Because we observed a rapid initial inhibition of beat frequency by sodium azide at the concentrations used in some of these experiments, controls treated with appropriate volumes of dilution buffer containing azide were also measured. Fig. 6 shows the effects of affinity-purified immune and preimmune antitubulins (from r2) on the frequencies and bend angles of individual spermatozoa as a function of time. The antibodies were at a final concentration of 15 $\mu\text{g}/\text{ml}$ in the reactivation solution. Neither of the antibodies affected the beat frequencies when compared with the effects of the dilution buffer alone. However, the immune antitubulin selectively inhibited the

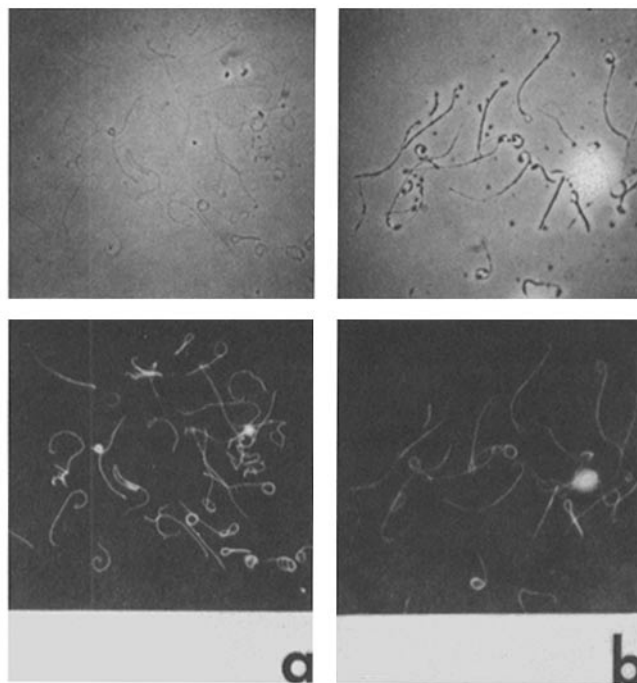


FIGURE 5 Phase-contrast (top) and indirect immunofluorescence (bottom) micrographs of *S. purpuratus* sperm that had been demembrated with 1% Triton X-100 and then stabilized (see Materials and Methods). (a) Sperm incubated with affinity-purified antitubulin from immune serum (0.25 mg/ml). (b) sperm incubated with affinity-purified antitubulin from preimmune serum (0.25 mg/ml). Photographs were taken at the same exposure and developed under the same conditions for immune and preimmune samples.

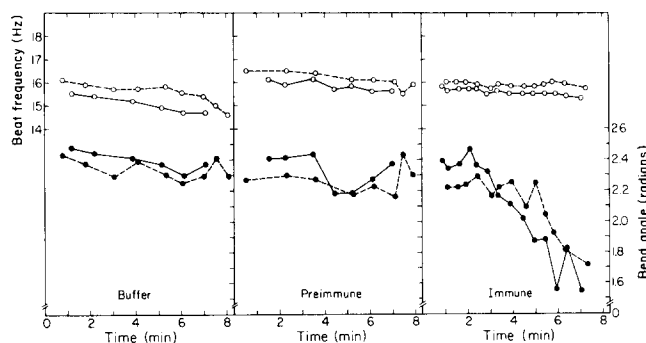


FIGURE 6 Effects of buffer, affinity-purified r2 preimmune antitubulin, and affinity-purified r2 immune antitubulin on beat frequencies (open circles) and bend angles (solid circles) of individual reactivated spermatozoa as a function of time. Both antitubulins were added to reactivation solutions to a final concentration of 15 $\mu\text{g}/\text{ml}$. An equivalent volume (90 μl) of buffer was added to 1 ml of reactivation solution to obtain the first set of curves (the sodium azide contributed by this quantity of buffer, 1.4 mM, was able to inhibit the beat frequency by $\sim 13\%$ when compared with untreated spermatozoa). Each curve represents one spermatozoon. Similar data with r2 sera were obtained from measurements of >30 spermatozoa. Data from the most complete records were selected for this figure. Demembrated spermatozoa were added to reactivation solution at time zero.

bend angle of the flagella, whereas the preimmune antibodies did not affect the amplitude.

To see whether these specific amplitude effects were shared by antitubulins isolated from other immune sera, another pair

of affinity-purified antitubulins from immune and preimmune sera (from r4) was examined, as shown in Fig. 7. Again, there was no effect on beat frequencies by either antibody preparation, and amplitude was selectively inhibited only by the immune antitubulin. However, these results were obtained with lower concentrations, $\sim 2.5 \mu\text{g/ml}$, of immune and preimmune antibodies. The effects of these antitubulins are also shown in the photographs in Fig. 8.

In addition to the two pairs of affinity-purified antitubulins already discussed (from r2 and r4), three more antitubulins (Table I) were examined for their effects. Two of these three precipitated tubulin in immunodiffusion assays (r3 and r5). Each of the five pairs was tested by adding affinity-purified antibody to a concentration of $10 \mu\text{g/ml}$ in the reactivation solution. After incubation of demembrated spermatozoa in the reactivation solution plus antibody mixture for 10 min, samples of the spermatozoa were photographed by dark-field microscopy, and average values of beat frequencies and bend angles were obtained. Fig. 9 compares each pair of preimmune and immune antitubulins. None of the immune and preimmune antitubulins affected beat frequencies. As before, the preimmune antitubulins did not alter bend angles. All of the four antitubulins that gave positive immunodiffusion results significantly inhibited amplitudes, with each immune antitubulin having a different magnitude of amplitude effect under these conditions. Immune antitubulin from rabbit r5 was particularly effective, and, under the conditions of these experiments, $20 \mu\text{g/ml}$ was able to completely paralyze the flagella after a 10-min exposure. With all of the immune antibodies that affected amplitude, the symmetry of the waveform was simultaneously affected, as illustrated in Fig. 8.

Effects of Antitubulins on Microtubule Sliding

Observations of the effects of antitubulins on microtubule sliding were carried out with the most potent antitubulin preparation (from r5), which was able to completely inhibit the movement of demembrated flagella. Elastase-digested axonemes were mixed with demembrated, undigested spermatozoa so that, in the same field of observation, effects on axoneme disintegration and effects on reactivated movement

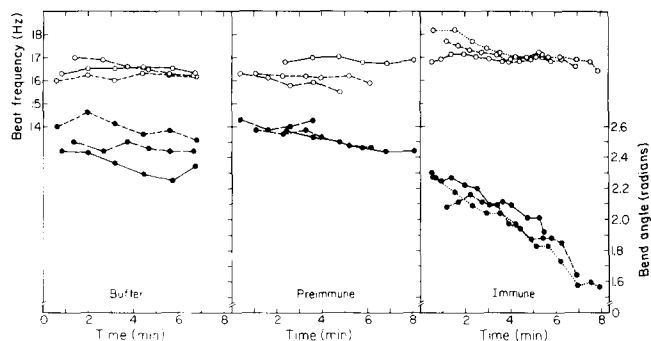


FIGURE 7 Effects of r4 antitubulins on reactivated flagellar motility, as in Fig. 6. Both antitubulins were added to reactivation solutions to a final concentration of $2.5 \mu\text{g/ml}$. An equivalent volume ($5 \mu\text{l}$) of buffer was added to 1 ml of reactivation solution to obtain the first set of curves. Each curve represents one spermatozoon. Similar data with r4 sera were obtained from measurements of >120 spermatozoa. Data from the most complete records were selected for this figure. Demembrated spermatozoa were added to reactivation solution at time zero. Open circles, beat frequency; closed circles, bend angle.

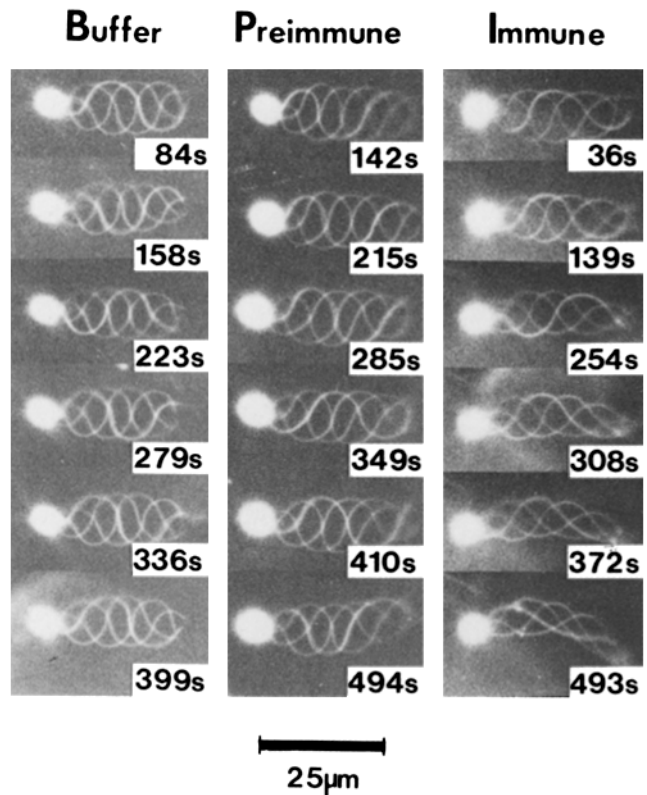


FIGURE 8 Examples of photomicrographs of the spermatozoa measured in Fig. 7. The spermatozoon treated with buffer is plotted with a broken line in Fig. 7, the preimmune-treated spermatozoon by a solid line in Fig. 7, and immune-treated spermatozoon by a dotted line in Fig. 7.

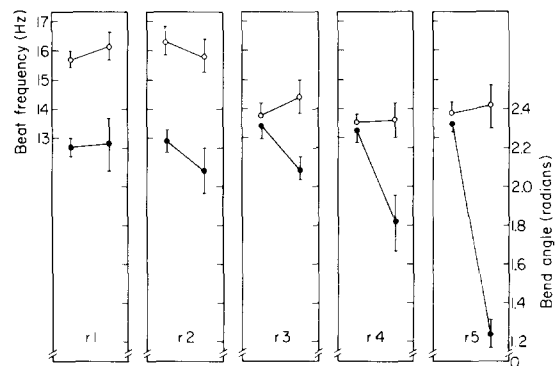


FIGURE 9 Comparison of affinity-purified antitubulins from preimmune and immune sera from five different rabbits (r1-r5; see Table I). Demembrated spermatozoa were incubated for 10 min with $10 \mu\text{g/ml}$ affinity-purified antibody, and then several different spermatozoa were photographed. The open circles are averaged results for beat frequencies; the solid circles are averaged bend angles. SD are also shown for each averaged value. Sample sizes ranged from 6 to 13 spermatozoa. The first point of each pair of points joined by a line represents the value obtained from the preimmune antibody; the second point is the value from the immune antibody.

could be observed. A further control was provided by the use of anti-fragment A, which was able to completely paralyze reactivated flagella (33) and which also totally inhibits MgATP^{2-} -mediated microtubule sliding in trypsin-treated axonemes (31). The results illustrated in the photographs in this paper were all obtained with identical samples of elastase-digested axonemes and demembrated spermatozoa.

Fig. 10 *a* and *b* illustrates results obtained after 30-min incubation with affinity-purified antitubulin from immune r5 serum. The undigested spermatozoa, which still have intact flagella attached to heads, have been completely inhibited and are motionless. The digested axonemal fragments, which have been completely detached from heads by the combination of homogenization and elastase digestion, are almost completely disintegrated, as indicated by the scarcity of intact axonemal fragments and the presence of curled tubules and other recognizable axonemal debris (Fig. 10 *a*). These photographs were taken with an antitubulin concentration (28 $\mu\text{g}/\text{ml}$) that was just sufficient to give good inhibition of motility at the relatively

high axoneme and sperm concentration required to obtain these photographs. Experiments with lower axoneme and sperm concentrations, giving antibody/axoneme ratios up to five times greater, also showed almost complete disintegration of the axonemes. We also observed preparations in which MgATP^{2-} was added by diffusion from the edge of the coverslip and confirmed that the disintegration of axonemes after incubation with antitubulin occurred by sliding extrusion of microtubules.

Fig. 10 *c* and *d* illustrates the results obtained with control experiments omitting antitubulin or with an affinity-purified antitubulin preparation from preimmune r5 serum (at 28 $\mu\text{g}/$

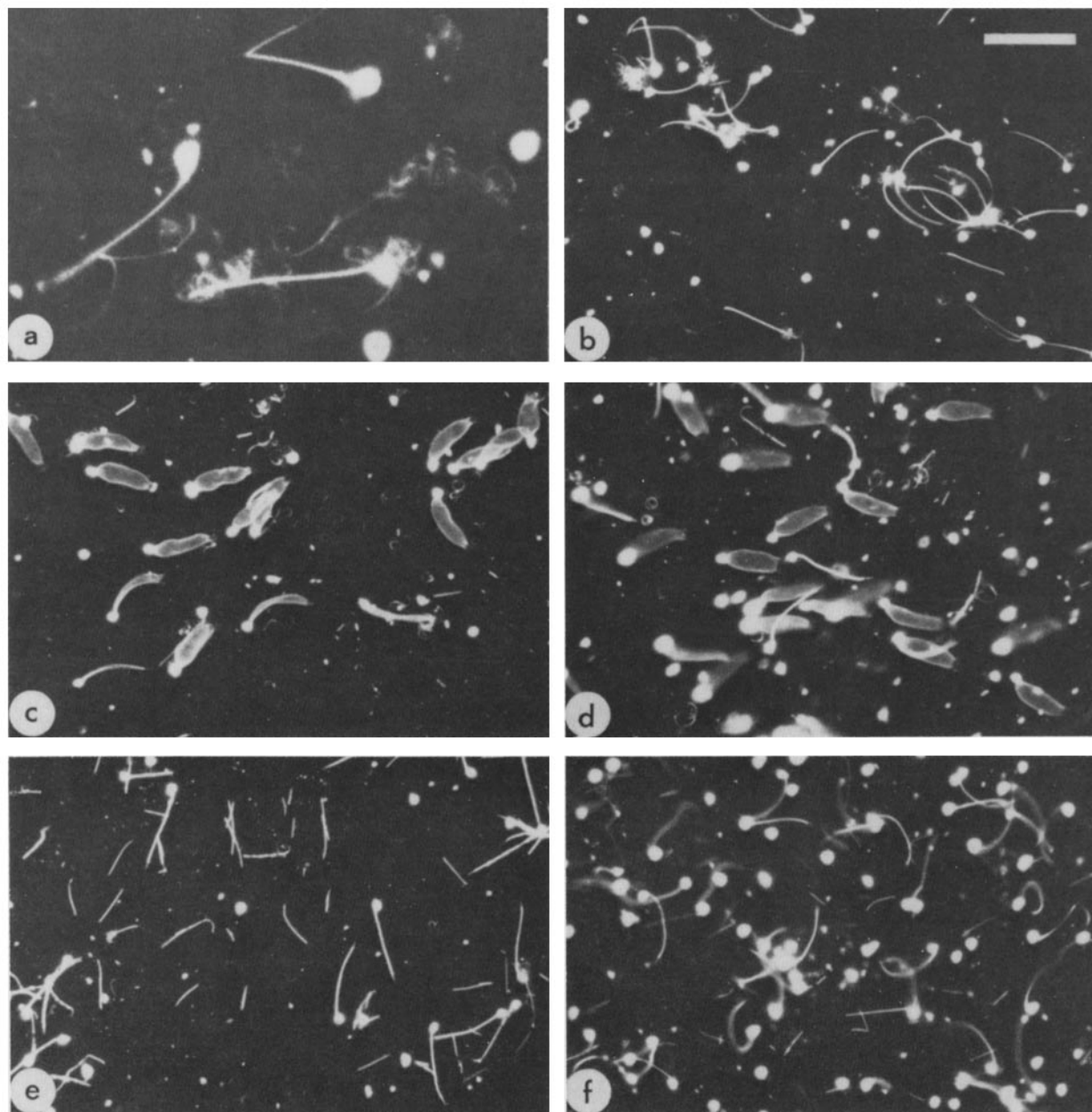


FIGURE 10 Dark-field photomicrographs of mixtures of demembrated spermatozoa and elastase-digested axonemal fragments. Sperm and axoneme concentrations were identical for each sample; apparent differences in concentration are caused by nonuniform dispersion of the samples under the coverslips. (*a* and *b*) After incubation with affinity-purified immune antitubulin (28 $\mu\text{g}/\text{ml}$) and addition of ATP. *a* has been enlarged 2.5 times, as compared with the other photomicrographs. (*c*) After incubation with buffer and addition of ATP. (*d*) After incubation with affinity-purified preimmune antitubulin (28 $\mu\text{g}/\text{ml}$) and addition of ATP. (*e*) After incubation with antifracture A and addition of ATP. (*f*) After incubation with immune antitubulin (28 $\mu\text{g}/\text{ml}$), without addition of ATP. All are taken with 0.25-s exposures. Bar, 50 μm , except in *a*, where it represents 20 μm .

ml), which does not inhibit motility. In this case, in addition to axonemal disintegration, addition of ATP caused reactivation of the motility of the intact demembrated spermatozoa, and the photographs show the flagellar beat envelopes of spermatozoa that have their heads attached to the microscope slide. In these cases and in Fig. 10 *b*, there are a few axonemes that do not disintegrate.

Fig. 10 *e* illustrates the results obtained after addition of ATP to preparations incubated with antibody against a tryptic fragment of dynein 1 (35), showing that both beating of intact spermatozoa and disintegration of axonemal fragments are inhibited by this antibody (31). This experiment was carried out with 10 μ l/ml of the same preparation used for earlier studies of the effect of the antibody on the motility of these spermatozoa (33); this concentration was just sufficient to give almost complete inhibition of bending and disintegration after a 30-min incubation.

Fig. 10 *f* illustrates the appearance of the suspension in an antitubulin experiment before the addition of ATP. Intact axonemal fragments can be seen in addition to the intact spermatozoa.

Additional experiments of the same type were carried out at lower ATP concentrations to determine the minimum concentration required for disintegration. The minimal concentration of MgATP^{2-} for beating and disintegration was usually in the range of 5–10 μ M (4, 20); essentially normal disintegration was observed with 8.3 μ M MgATP^{2-} in the absence of antitubulin. After incubation with 30 μ g/ml antitubulin, weak disintegration was observed with 25 μ M MgATP^{2-} , and essentially normal disintegration was observed with 33 μ M MgATP^{2-} . A similar increase in the minimum MgATP^{2-} concentration required for disintegration was observed under conditions where flagellar beating is inhibited by CO_2 in the presence of 0.1 M NaHCO_3 at pH 8.4 (C. J. Brokaw, unpublished observations).

DISCUSSION

Our immunization procedures using SDS-denatured antigen were successful in producing antitubulin antibodies that precipitated tubulin in immunodiffusion assays. From all our attempts at immunization, no pattern emerges for "good" antigen purification or for a successful injection protocol. It is possible that some of the serum samples that tested negative by immunodiffusion nevertheless contained "induced" antitubulins that could have been captured by tubulin-affinity chromatography. However, the affinity-purified postinjection serum tested in r1 (Fig. 9) that did not precipitate tubulin did not affect the flagellar waveform. Tubulin-affinity chromatography served as a useful method for purifying induced antitubulins from immune sera.

In contrast, all of the preimmune rabbit sera we have tested by tubulin-affinity chromatography contain "spontaneous" antitubulins. Our finding of spontaneous antibodies to tubulin confirms the results of an earlier study by Karsenti and co-workers (25) in which they also used tubulin-affinity chromatography to isolate antitubulins from a variety of nonimmune mammalian sera. They also found that their spontaneous antibodies did not precipitate tubulin nor did they give efficient immunofluorescent staining of cytoplasmic microtubules. Similarly, we find that spontaneous antitubulin antibodies purified by affinity chromatography do not precipitate tubulin in immunodiffusion assays as compared with precipitation by equivalent amounts of induced antitubulin, and that spontaneous antibodies do not strongly stain chick fibroblast microtubules by indirect immunofluorescence. We have further differen-

tiated these two types of antitubulins on the basis of presence or absence of a specific inhibition of flagellar motility.

We have demonstrated that the antitubulins from both immune and preimmune sera are IgG-type antibodies and have used affinity chromatography to ensure that these antibodies are specific for tubulin. Immunodiffusion assays showing only a single precipitin line between purified antitubulin immunoglobulin and crude axonemal extracts and the demonstration of fluorescent staining of tubulin paracrystals by Karsenti et al. (25) provide additional evidence that these antibodies are specifically directed against tubulin. The unique effect of antitubulin antibodies on the amplitude of reactivated flagella and their lack of effect on the disintegration of elastase-digested axonemes further demonstrate their clear difference from antidynein antibodies.

Measurements of binding of ^{125}I -labeled antitubulins to demembrated axonemes demonstrate that both induced and spontaneous antitubulins bind to axonemes but that the spontaneous antibody appears to have a much lower affinity for the axonemes. Furthermore, Fab fragments from both sera also bind strongly, suggesting that the binding of both spontaneous and induced antitubulins is true antibody binding and not a recognition of tubulin by some other portion of the antibody (e.g., the Fc portion). Indirect immunofluorescence also demonstrates that both antitubulins are able to bind demembrated sperm tails but that only induced antitubulin decorates chick fibroblast cytoplasmic microtubules. We interpret these immunofluorescence data to reflect our immunization and antitubulin purification procedures. Sea urchin tubulin-affinity chromatography purifies those antibodies from both immune and preimmune sera that bind to sea urchin flagella. The sea urchin tubulin determinants to which the spontaneous antitubulins bind may not be available in sufficient quantity on chick microtubules. In addition, the SDS denaturation of sea urchin tubulin during immunization apparently increases the antigenicity of determinants to which only the induced antitubulins are able to respond. These additional sites may be present on both species' tubulin, allowing the induced antitubulin to react with chick microtubules.

Effects of Antitubulins on Movement

This paper reports two motility-related properties of induced antitubulin antibodies: the specific inhibition of bend amplitude in reactivated flagella and the lack of inhibition of microtubule sliding in elastase-digested axonemes. The specific inhibition of amplitude was shared, to different degrees, by all four of the induced antitubulins and was not obtained with the spontaneous antitubulins found in normal sera. This combination of properties—amplitude inhibition without inhibition of sliding—was found previously with CO_2 (5, 7); it has also been shown that CO_2 inhibition of intact echinoderm spermatozoa leaves them in a state of low stiffness, referred to as a relaxed state (37). A specific inhibition of bend amplitude, without inhibition of beat frequency, has also been reported for demembrated sea urchin spermatozoa treated with *N*-ethylmaleimide (11). Our results suggest that all of these cases of amplitude-inhibition may represent effects on microtubules.

In contrast to these results, previous studies with antibodies against dynein and its tryptic fragment have shown inhibition of both beat frequency and bend amplitude (33). Beat frequency is strongly influenced by the concentration of MgATP^{2-} (3, 23) or the competitive inhibitor, MgADP^- (36), which are known to directly affect the action of dynein. Antibody against dynein fragment A, at a concentration that completely inhibits

beating, also completely inhibits the sliding disintegration of axonemes digested with elastase or trypsin. Antibodies against dynein or dynein fragment A are likely to act by interfering with the $MgATP^{2-}$ -mediated detachment of cross-bridges between flagellar microtubules, leading to a rigorlike state in which stably attached cross-bridges resist both the sliding that must occur during normal bending and the sliding disintegration of digested axonemes.

Amplitude-inhibiting agents, such as antitubulins, CO_2 , and *N*-ethylmaleimide might act by interfering with the attachment of cross-bridges to sites on the B-tubules. The cross-bridges would then not be able to generate sufficient force to bend the flagellum and produce normal movement, but even a small number of actively working cross-bridges might be sufficient to overcome the lesser forces needed for axonemal disintegration, as long as the axoneme remains in a relaxed state without stable cross-bridges. Alternatively, these amplitude-inhibiting agents may act on control mechanisms that are required to convert active sliding forces into oscillatory bending and bend propagation, without inhibiting the active force-producing mechanism at all. In this case, the central-pair microtubules might also be a target on which antitubulins and the other amplitude-inhibiting agents are acting. Other possibilities, such as an increased stiffness caused by binding of antitubulin to microtubules, can be imagined; however, this would imply different modes of action for antitubulin and for CO_2 .

Spontaneous antitubulins were not found to inhibit flagellar beating, and in other experiments (not shown) we were unable to detect any effect of affinity-purified immune monovalent Fab fragments on the movement of the reactivated flagella. Binding to axonemes was demonstrated with all of these antibody preparations, but in the case of the spontaneous antitubulins, the binding affinity was sufficiently low to explain the inability to observe any effect on flagellar motility. At this stage, these experiments do not provide any additional information about the way in which antitubulin antibodies cause inhibition of flagellar bend amplitude.

So far, each of the three types of antibody preparations—anti-fragment A, antidynein, and antitubulin—has given a distinct pattern of inhibition of flagellar motility. However, only in the case of our antitubulins has it been shown that the specific pattern of inhibition is common to antibody preparations from several different immunized animals, and only in the case of antitubulins has specific inhibition of a single parameter of movement—amplitude, in contrast to a combination of effects on amplitude and frequency—been demonstrated. Antitubulins may be particularly useful probes for further studies of the function of microtubules in flagellar movement.

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