Immunological Dissimilarity in Protein Component (Dynein 1) Between Outer and Inner Arms Within Sea Urchin Sperm Axonemes

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ABSTRACT The 0.5 M KCl-treatment solubilizes the outer arms from sea urchin sperm axonemes. ~30% of A-polypeptide, corresponding to dynein 1 in SDS-polyacrylamide gel, was solubilized by this treatment (as SEA-dynein 1). Electron microscopic observation indicated that the extracted axonemes lacked the outer arms in various degrees. The SEA-dynein 1 was purified and an antiserum against it was prepared in rabbits. The specificity of antiserum to dynein 1 was determined by immunoelectrophoresis and Ouchterlony's double-diffusion test. The anti-dynein 1 serum inhibited ATPase activity of purified SEA-dynein 1 by 95%. By the indirect peroxidase-conjugated antibody method, the loci of SEA-dynein 1 within the intact, salt-extracted and mechanically disrupted axonemes were determined to be the outer arms: deposition of electron-dense materials which represents their localization was detected at the distal ends of the outer arms, in the case of intact axonemes. The 5–6 cross-bridge was hardly decorated. No decoration was seen in the salt-extracted axonemes lacking all the outer arms. In disrupted axonemes, which consist of single to several peripheral doublets, electron-dense materials were deposited only at the outer arms.

~73% of axonemal ATPase activity sensitive to antiserum was solubilized by repeated salt-extractions. One-half of A-polypeptide (SEA-dynein 1 located at the outer arms) was contained in the pooled extracts. The extracted axonemes contained another half of A-polypeptide (SUA-dynein 1 supposed to locate at the inner arms) and retained 31% of axonemal ATPase activity that was almost resistant to antiserum. Solubilized SUA-dynein 1 was immunologically the same as SEA-dynein 1. This result indicates that *in situ* SUA-dynein 1 did not receive anti-dynein 1 antibodies, coinciding with the result obtained for salt-extracted axonemes lacking all the outer arms by the enzyme-antibody method mentioned above. These observations suggest the immunological dissimilarity in dynein 1 between outer and inner arms but do not tell us that the inner arms do not contain dynein 1.

The peripheral nine doublet microtubules of sperm axonemes bear two parallel rows of projections, called outer and inner arms (1). The sea urchin and fish sperm axonemes contain two types of ATPase: dynein 1 and dynein 2 (11, 18, 23). The major ATPase, dynein 1, locates at the outer arms, the distal ends of which correspond to the enzymatically essential portions (22).

One major area of controversy in eukaryotic flagellar motility at present is the question of whether the outer and inner arms are identical in protein component and function. From the standpoint of morphology, the arms differ from one another in structural configuration as observed in thin cross-sections or negatively stained images of cilia and flagella (2, 28). With respect to extractability, they also differ such that the outer arms are solubilized from axonemes by salt-extraction, while the inner arms remain in place (14). Since the outer arms have 60–65% of the total axonemal ATPase activity, whereas the inner arms are responsible for only some residual activity, the dynein 1 located at the outer arms has a higher enzymatic activity than the one located at the inner arms (14). From the evidences that (*a*) anti-Fragment 1A antibodies bind only to the outer arms (22), (*b*) they inhibit the movement of reactivated spermatozoa (8, 9, 21, 25), and (*c*) ATP-induced tubule

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extrusion of trypsinated axonemes is suppressed by them (15), we assume that the two members of a pair of arms differ immunologically from one another.

In contrast to the above observations suggesting a dissimilarity, Gibbons has inferred that the outer and inner dynein arms are equally liable to induce sliding (10). This speculation has been reinforced by Gibbons and Gibbons (6), and by Hata et al. (12). Although they have not actually shown that the inner arms contain dynein 1 or ATPase, observations that (*a*) salt-treatment removes the outer arms completely from axonemes, (*b*) the salt-extractable fraction contains one-half of the A-polypeptide, corresponding to dynein 1 in SDS-polyacrylamide gel, and (*c*) residual axonemes contain another half of the polypeptide admit an assumption that the latter A-polypeptide would be derived from dynein 1 located at the inner arms (14).

The point of controversy is, therefore, the identity of the A-polypeptide in the outer arms and the one remaining in the salt-extracted axonemes. In the present experiments, we prepared an antiserum directed against dynein 1 extracted from the outer arms and investigated the localization of the other half of A-polypeptide. Instead of using a low-ionic-strength extract of dynein 1 which seemed to contain both types of A-polypeptide, salt-extractable dynein 1 that contained a half of it was used as a starting material for obtaining a purified antigen. To locate dynein 1 by immunoelectron microscopy, the enzyme-antibody method was chosen, because peroxidase-conjugated IgG was supposed to exhibit a better permeability with respect to the axonemes because of its smaller molecular size.

MATERIALS AND METHODS

Materials

The spermatozoa were obtained from the sea urchin *Anthocidaris crassispina* and used immediately or within one to three months after glycerination.

0.5 M KCl Treatment of Axonemes

Axonemes were prepared from the glycerinated spermatozoa by the method of Ogawa et al. (21). The axonemes obtained from 50 ml of packed spermatozoa were suspended in 100 ml of the 0.5 M KCl-extracting solution (10 mM Tris-HCl buffer, pH 7.8, containing 0.5 M KCl, 5 mM $MgSO_4$, 0.2 mM EDTA and 0.1 mM dithiothreitol). After being held for 30 min at room temperature, the suspension was centrifuged at 12,000 *g* for 15 min to obtain the 0.5 M KCl-extractable dynein 1 (abbreviated as SEA-dynein 1) as a supernatant and the EDTA-extractable ATPase (abbreviated as EDTA-dynein 1) as a precipitate. For purifying the SEA-dynein 1, the supernatant was concentrated after dialysis against TEM (10 mM Tris-HCl buffer, pH 8.3, containing 0.2 mM EDTA and 0.1% 2-mercaptoethanol) and subjected to successive chromatography on Sepharose 4B and hydroxylapatite columns originally used for purifying dynein 1 from a low-ionic-strength extract of whole axonemes (19). The EDTA-dynein 1 was solubilized by a low-ionic-strength dialysis of the extracted axonemes. For purifying it, the precipitate was suspended in 100 ml of TEM and dialyzed against the same solution. After centrifugation at 50,000 *g* for 1 h, the supernatant was concentrated and subjected to successive chromatography on Sepharose 4B and hydroxylapatite columns (19). The residue of reextracted axonemes was thoroughly suspended in 100 ml of TEM by homogenization with a Teflon homogenizer.

Densitometric Tracing of A-Polypeptide in Gel

SDS PAGE was performed as described (20). Proteins were stained with Coomassie Blue, and gels were scanned with a Toyo Scanning Densitometer at 610 nm. The quantity of protein loaded on the gel was adjusted so that each sample contained total proteins originating from 39.5 μg of whole axonemes. Tracings of the scans were cut out and weighed to determine the relative areas of the peaks.

Anti-Dynein 1 Serum

The purified preparation of SEA-dynein 1 was used as an antigen. Before injection of antigen, one male rabbit was bled to obtain a sample of preimmune serum to be used for control experiments. The antigen (0.5–1 mg protein) in TEM was emulsified with an equal volume of complete Freund adjuvant (Difco Laboratories, Detroit, MI) and the emulsion was injected into the femoral muscles and the back of the neck. Immunoinjection was performed once every week for five consecutive weeks. Serum was stored at 4°C in the presence of 1% NaN_3. Immunospecificity of antiserum to dynein 1 was determined by conventional immunoelectrophoresis (29) and Ouchterlony's double-diffusion test.

ATPase Assay in the Presence of Antiserum

Crude IgG from serum was used for the inhibition test. To prepare it, 2 ml of the serum was subjected to successive ammonium sulfate fractionation both at 50% saturation and the pellet was dissolved in 1.8 ml of TBS (10 mM Tris-HCl buffer, pH 8.3, containing 0.15 M NaCl), followed by dialysis against TBS. The ATPase assay in the presence of crude IgG for obtaining the results shown in Figs. 4 and 5 was carried out by the method described in a previous paper (20).

Enzyme-Antibody Method

The localization of dynein 1 within the intact, salt-extracted and mechanically disrupted axonemes was examined by immunoelectron microscopy using the indirect peroxidase-conjugated antibody method described in the preceding paper (23). The former two specimens were obtained as described previously (22). The last one was prepared by disrupting Tritonated axonemes with repeated sucking-blowing in a Pasteur pipet, so that the axonemal peripheral doublet-microtubules appeared as opened or fragmented arrays.

Immunofluorescence Microscopy

The intact and salt-extracted axonemes were examined by indirect immunofluorescence microscopy as described in the previous paper (22).

Measurement of the Rate of ATP-dephosphorylation by Reactivated Spermatozoa

A suspension of freshly shed, stock sperm was prepared so that a 10-μl portion, diluted with 5 ml of 0.5 M NaCl, produced an optical density reading of 0.25 at 550 nm. A 15-μl portion of stock sample was added directly to 0.3 ml of extracting solution for an experiment with motile spermatozoa, or after breaking with a Thermomixer for an experiment with broken spermatozoa. Then, an indicated amount of anti-dynein 1 crude IgG was reacted with demembranated spermatozoa, and the mixture was kept for 10 min at 4°C. The whole solution was transferred to a reactivating solution (16 ml) in a vessel of pH -stat, and the ATP-dephosphorylation was assayed at 25°C. The ingredients of all solutions were the same as those of Brokaw and Simonick (5).

RESULTS

Properties of Antigen and Antiserum

COMPARISON OF THE QUANTITY OF A-POLYPEPTIDE FRACTIONATED BY SALT-TREATMENT: When the axonemes were treated with 0.5 M KCl as described above, three fractions having ATPase activity were obtained: SEA-dynein 1, EDTA-dynein 1, and the residue of reextracted axonemes. The electrophoretic patterns in the SDS polyacrylamide gel of whole axonemes and these fractions are shown in gels *a-d* of Fig. 1. Densitometric tracings of gels *b*, *c* and *d* showed that the relative intensities of the A-band in three fractions were 0.3, 0.6 and 0.1, respectively. Under the present condition, the quantity of A-polypeptide in the SEA-dynein 1 fraction was always smaller than that in the EDTA-dynein 1 fraction. In this connection, Mohri et al. (16) have reported that the outer arms within *A. crassispina* sperm axonemes were much more resistant to salt-treatment as compared to two other species of sea urchin collected in Japan.

ANTIGEN AND ANTI-DYNEIN 1 SERUM: The SDS-polyacrylamide gel electrophoretic pattern of the antigen is shown

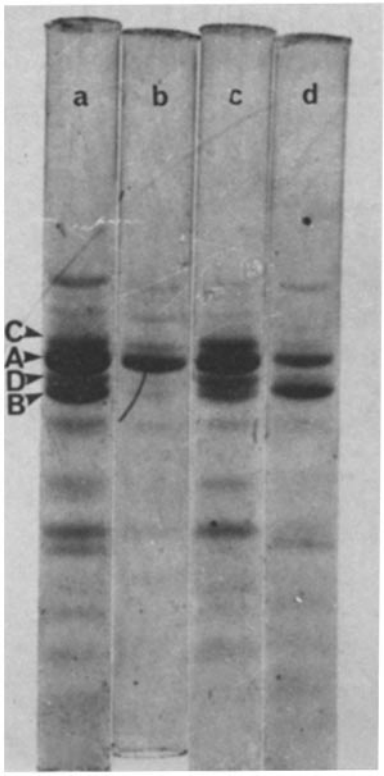

FIGURE 1 SDS PAGE at 3.5% acrylamide concentration. Gel *a*, whole axonemes (39.5 μg protein); gel b, SEA-dynein 1 (0.9 μg); gel c, EDTA-dynein 1 (12.3 μg); gel d, residue of reextracted axonemes (23 μg). Duration of electrophoresis was doubled to reveal the high molecular weight region of whole axonemes and the salt-extract. In four bands indicated by arrowheads, A- and D-polypeptides correspond to dynein 1 and dynein 2, respectively. Other proteins (C- and B-polypeptides) had no ATPase activity. An aliquot of each fraction was also used for the test of inhibition by anti-dynein 1 serum (see Fig. 5).

in Panel *A* of Fig. 2. One major A-polypeptide and a few minor bands immediately below it were resolved in the gel. Because dynein 1 was liable to degradation during purification, the latter polypeptides were its degradation products. The immunospecificity of the newly prepared anti-dynein 1 serum to SEA-dynein 1 was examined by immunoelectrophoresis as shown in Panel *B* of Fig. 2. The electrophoresed, crude preparations gave a single precipitin line vs. antiserum. Because of the aggregated features of intact dynein 1 (24), the profile in plate *a* showed a slightly tailed precipitin line. Dynein 1 gave rise to Fragment 1A with ATPase activity by trypsin digestion which was electrophoresed as a sharp band in the polyacrylamide gel not containing SDS (17). Thus, a sharp precipitin line was seen in trypsin-treated SEA-dynein 1 vs. antiserum (see plate *b*).

OUCHTERLONY'S DOUBLE-DIFFUSION TEST: Fig. 3 shows the double-diffusion test on agarose to demonstrate the immunological similarity between the SEA-dynein 1 and EDTA-dynein 1. Plates *a* and *b* are purified and crude specimens vs. anti-dynein 1 serum, respectively. Fragment 1A was used to assess the antigen determinant of antiserum. Precipitin lines completely fused, and no spur formed. This experiment shows that the antiserum directed against the SEA-dynein 1 can react with EDTA-dynein 1.

INHIBITION OF ATPase ACTIVITY BY ANTISERUM: The effects of anti-dynein 1 serum on the ATPase activity of both SEA- and EDTA-dynein 1 were examined. As shown in Fig. 4, the inhibition curves are the same, and 100 μl of IgG inhibited them by 95%. Fragment 1A with an activity of 41.3 mU was completely inhibited by the addition of 50 μl of IgG.

Similar experiments were carried out using the crude preparations of both enzyme fractions and the residue of reextracted axonemes (Fig. 5). In the presence of 100 μl of IgG, the residual ATPase activities were 36, 25, and 55%, respectively, suggesting that antiserum which was raised against SEA-dynein 1 can inhibit the ATPase activities of the other two ATPase fractions, based on extractability in various degrees. The inhibition test, taken together with the above immunological test, led us to conclude that ATPases which gave rise to A-polypeptide in the SDS-polyacrylamide gel were not immunologically distinguishable from one another, if they were solubilized from the axonemes.

Immunoelectron Microscopy

We previously prepared an antiserum directed against Frag-

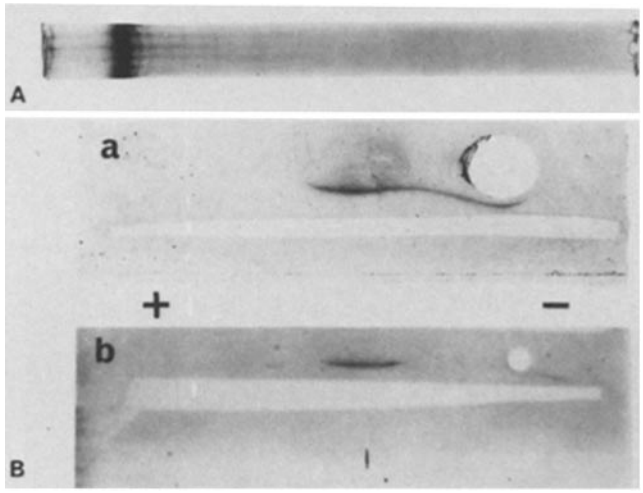

FIGURE 2 Antigen and antiserum. Panel *A*, SDS PAGE pattern of the purified SEA-dynein 1 (13 μg protein). 5% polyacrylamide gel was used and electrophoresis was terminated when the marker dye ran to the end of the gel. Panel *B*, immunoelectrophoretic pattern of anti-dynein 1 serum vs dynein 1. Plate *a*, crude SEA-dynein 1 (100 μg protein); plate *b*, trypsin-treated, crude SEA-dynein 1 (100 μg). Plates were incubated for 1 wk at 4°C. Precipitin line was stained with Coomassie Blue.

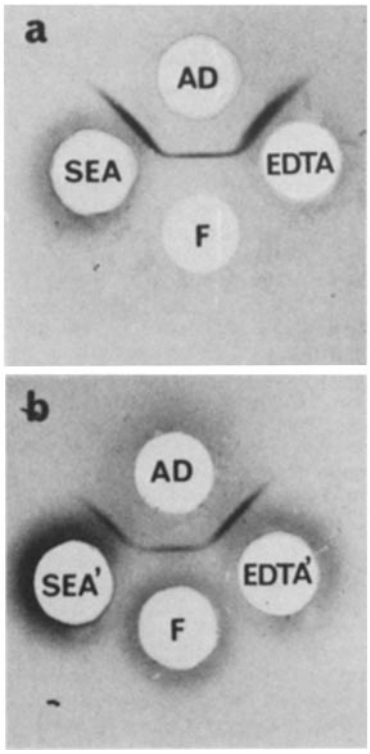

FIGURE 3 Ouchterlony's double-diffusion test. Well *AD*, anti-dynein 1 serum (20 μl); well *SEA*, purified SEA-dynein 1 (26 μg protein); well *EDTA*, purified EDTA-dynein 1 (12.3 μg); well F, Fragment 1A (13.7 μg); well *SEA'*, crude SEA-dynein 1 (158 μg); well *EDTA'*, crude EDTA-dynein 1 (474 μg). Plates were incubated for 2 d at 4°C. Precipitin lines were stained with Coomassie Blue.

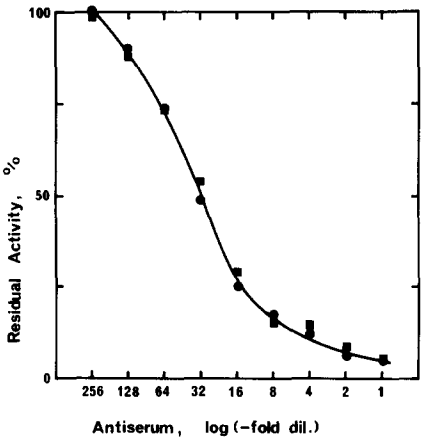

FIGURE 4 Inhibitory effect of anti-dynein 1 serum on dynein 1 ATPase activity. (●) SEA-dynein 1 with activity of 21.6 mU; (■) EDTA-dynein 1 with activity of 21.6 mU. One unit was defined as the amount of enzyme required to liberate one μmol Pi/min under the present experimental conditions. Purified preparations were used.

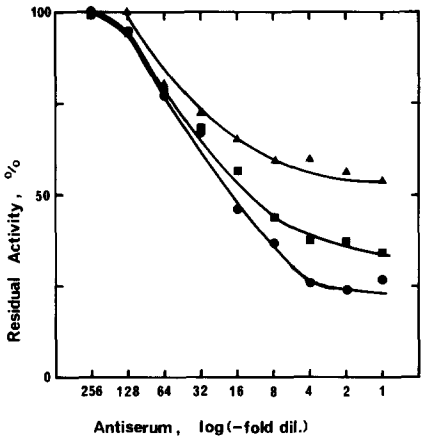

FIGURE 5 Effect of antiserum on three ATPase activities fractionated by salt-treatment. See gels *b–d* of Fig. 1 for samples. (■) crude SEA-dynein 1 (23.4 mU); (○) crude EDTA-dynein 1 (20.2 mU); (▲) residue of reextracted axonemes (16.5 mU).

ment 1A (20). Since a low-ionic-strength extract of dynein 1 was used as a starting material to obtain it, it might be a mixture of SEA-dynein 1 and another A-polypeptide giving dynein 1. In the present experiment, the localization of dynein 1 was determined by the use of the newly prepared antiserum directed only against the SEA-dynein 1.

DEMEMBRANATED INTACT AXONEMES: When axonemes were treated with anti-dynein 1 serum followed by peroxidase-conjugated anti-rabbit IgG, the reaction products of the peroxidase were detected between the outer doublet-microtubules, at the distal ends of the outer arms as shown in Fig. 6 *d–i*. On inspecting the electron micrographs, it was noted that decoration was quite rare on the outer arm projecting from the no. 5 doublet. When the antiserum was replaced with preimmune serum, no decoration was seen between the outer doublets as shown in Fig. 6 *a–c*. Accordingly, it was confirmed that the SEA-dynein 1 originated from the outer arms.

Fig. 7 *b* shows a longitudinal section of the axonemes treated with anti-dynein 1 serum. In contrast to the axonemes treated with preimmune serum (Fig. 7 *a*), one side of the outer doublets was roughly surfaced with electron-dense materials. The average minimum interval between them was ~20–30 nm, coinciding with that of previous ferritin labelings (16). The other side of them was not significantly different from the control one.

SALT-EXTRACTED AXONEMES: Although no significant decoration was observed on the inner arms of intact axonemes, there was a possibility that the antiserum and/or the peroxidase-conjugated IgG could not reach inside the axonemes because of the presence of the outer arms and/or the formation of IgG-outer arm complexes. To examine this possibility, we also processed axonemes as described above from which the outer arms had been extracted with 0.5 M KCl. As shown in Fig. 8 *d–f* in which axonemes lacked all the outer arms, no decoration with electron-dense materials was seen near the inner arms or inside the axonemes. The experimental axonemes were not distinguishable from the control ones as presented in Fig. 8 *a–c*.

The axonemes of the sea urchin *A. crassispina* appeared to be rather resistant to salt-extraction. Some of the outer arms remained intact in many cases. Sometimes, there were axonemes that retained all of them. Whenever the electron-dense materials were detected between the outer doublets, there were always outer arms that had not been removed by salt-extraction (data not shown). To confirm incomplete extraction of outer arms at mass scale, we examined the extracted axonemes, by

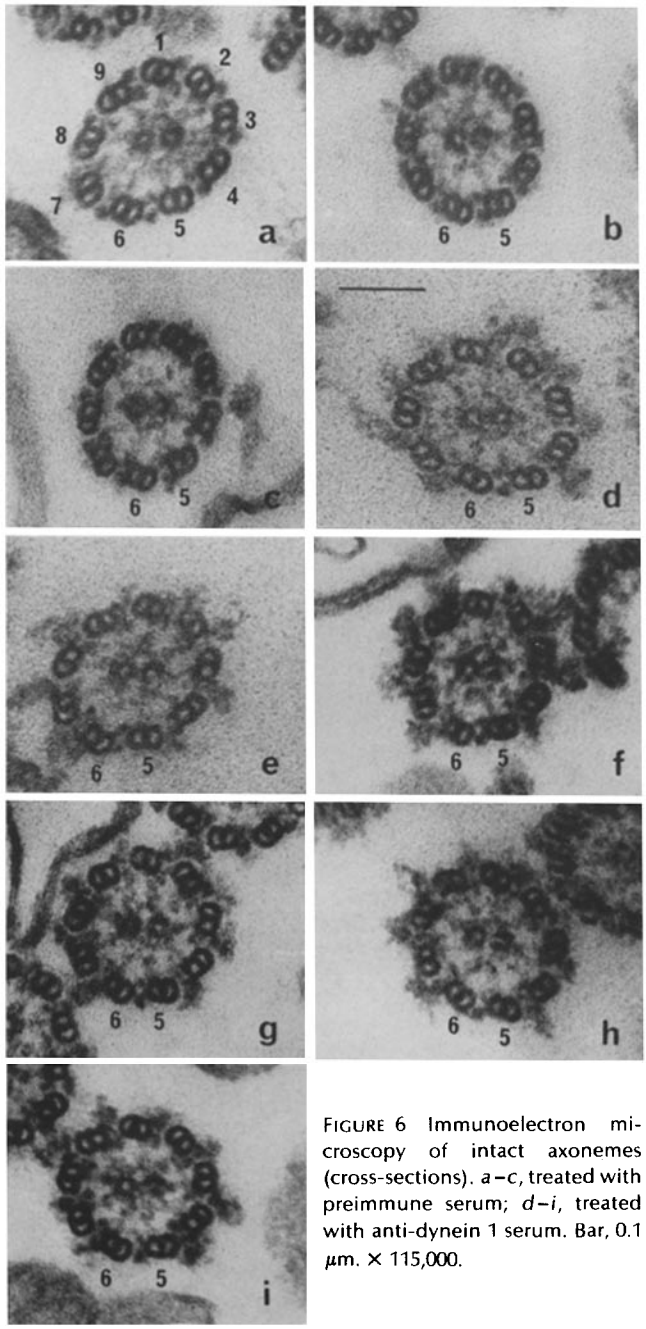

FIGURE 6 Immunoelectron microscopy of intact axonemes (cross-sections). *a–c*, treated with preimmune serum; *d–i*, treated with anti-dynein 1 serum. Bar, 0.1 μm. × 115,000.

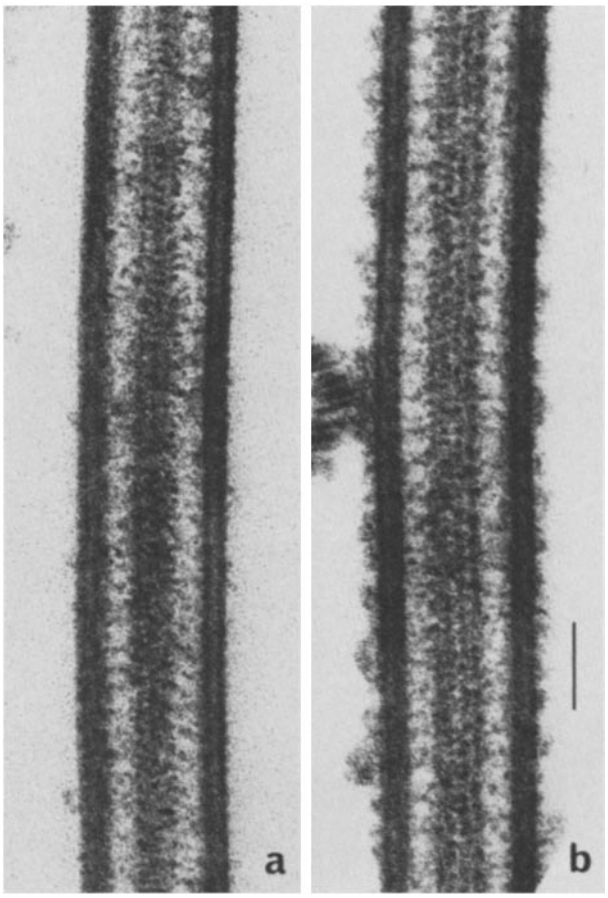

FIGURE 7 Immunoelectron microscopy of intact axonemes (longitudinal sections). *a*, treated with preimmune serum; *b*, treated with antiserum. Bar, 0.1 μm. × 115,000.

indirect immunofluorescence microscopy (Fig. 9). The axonemes pretreated with preimmune serum did not emit a significant fluorescence, while bright fluorescence was emitted from the intact axonemes pretreated with anti-dynein 1 serum. Extracted axonemes also emitted the fluorescence, but their intensity was clearly lower than that of intact axonemes. This probably reflects the fact that some of the outer arms remained unextracted. Therefore, even if the electron-dense materials were seen between the peripheral doublets, it does not always mean that the inner arms were labeled.

FRAGMENTED ARRAY OF OUTER DOUBLETS: The possibility that the inter-doublet links such as nexin interfere with the reaction between antibodies and inner arms should be examined. In the previous experiment (22), no ferritin particles were observed near the inner arms even when the 9 + 2 structures appeared as opened or fragmented arrays. While these axonemes were fragmented after or during the immunocytological processing, axonemes were mechanically disrupted in advance by the standard processing in the present experiment. Both arms remained in place (Fig. 10 *a* and *b*), and the electron-dense materials produced by peroxidase reaction were detected only in the vicinity of the outer arms, resulting in the morphologically longer appearances (Fig. 10 *c–f*). Since the appearance of the inner arms was not distinguishable from that of control ones, the inner arms were found not to be decorated with antibodies. Similarly, no decoration occurred at the inner arms when disassembled doublets of salt-extracted axonemes were used (compare Fig. 10 *g* and *h* with *i* and *j*).

Possible Function of Outer Arms in Flagellar Movement

From the above results, it was found that the anti-dynein 1 antibodies bound only to the outer arms. Using their immunological specificity, we may be able to learn the possible function of the outer arms in flagellar movement.

COMPLETE EXTRACTION OF OUTER ARMS: Because the outer arms within *A. crassispina* sperm axonemes were rather resistant to salt-extraction, a fraction of EDTA-dynein 1 as described above might be contaminated with SEA-dynein 1 of the remaining outer arms. Extraction of SEA-dynein 1 could be judged to be completed by preparing the axonemes no longer sensitive to antiserum. The repeated salt-extractions described in the legend of Fig. 11 gave rise to a satisfactory result. Combined extracts contained one-half of A-polypeptide, and fraction *2 x Extracted Axoneme* retained the other half. This separation is immunologically important, for all of the ATPase activities in whole axonemes inhibited by antiserum were extracted, indicating complete removal of outer arms. The A-polypeptide-giving protein retained in axonemes after repeated salt-extractions is now named SUA-dynein 1. Although EDTA-dynein 1 was a tentative name, SEA- and SUA-dynein 1 are the functional ones.

COMPARISON OF ATPase ACTIVITY OF IN SITU SEA- AND SUA-DYNEIN 1: ~ 73% of the axonemal ATPase activity was suppressed by antiserum that inhibited the ATPase activity of SEA-dynein 1 up to 95%. Thus, *in situ* SEA-dynein

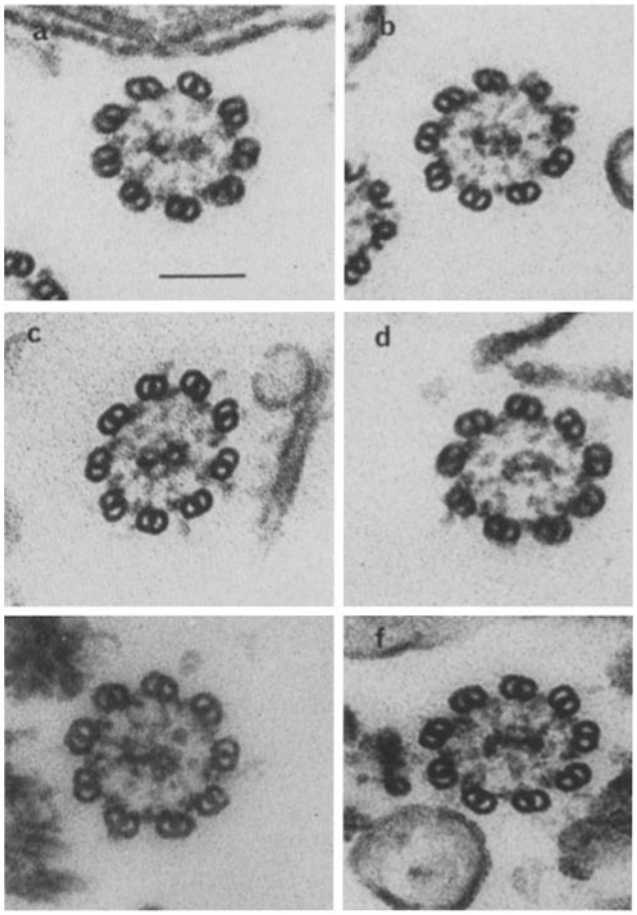

FIGURE 8 Immunoelectron microscopy of salt-extracted axonemes. *a–c*, treated with preimmune serum; *d–f*, treated with antiserum. Bar, 0.1 μm. × 115,000.

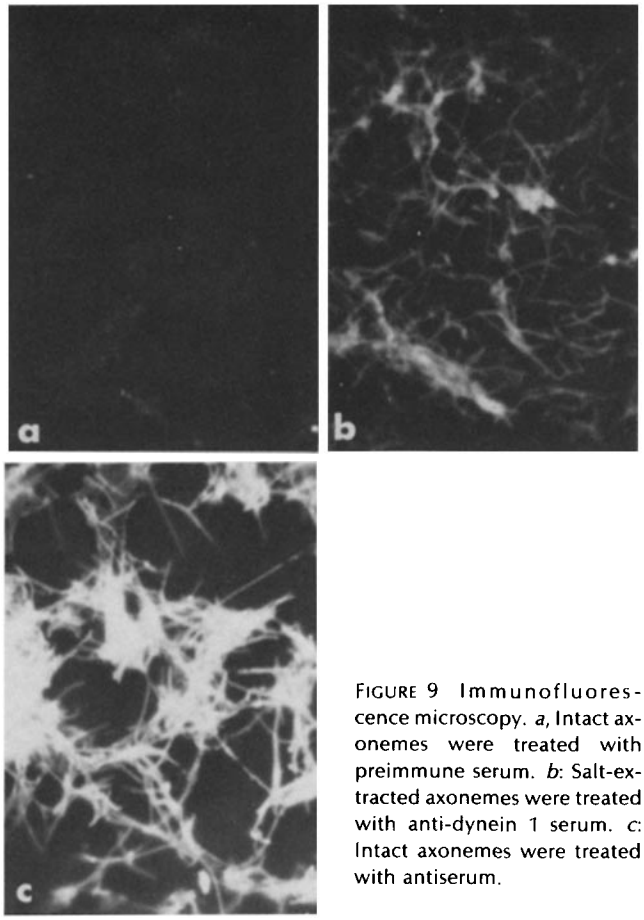

FIGURE 9 Immunofluorescence microscopy. *a*, Intact axonemes were treated with preimmune serum. *b*: Salt-extracted axonemes were treated with anti-dynein 1 serum. *c*: Intact axonemes were treated with antiserum.

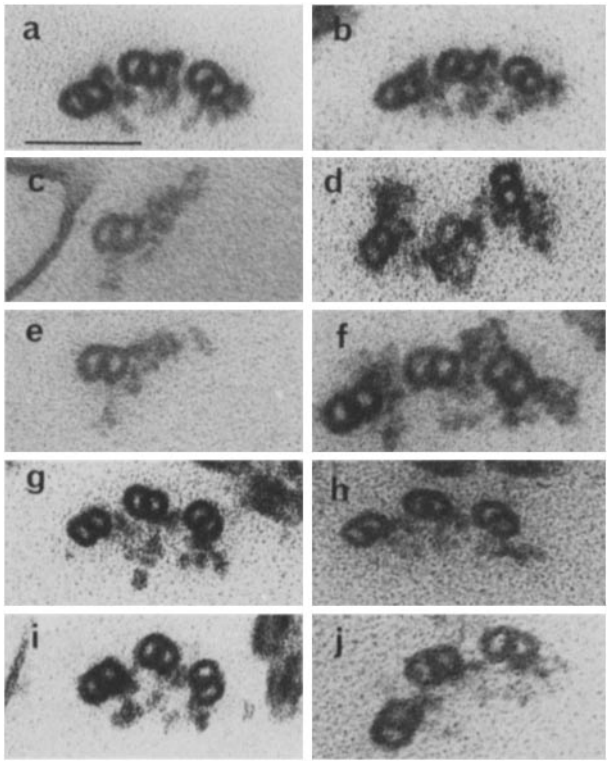

FIGURE 10 Immunoelectron microscopy. Fragmented arrays of outer doublet microtubules were treated with preimmune serum (*a* and *b*) or antiserum (*c*–*f*). The salt-extracted axonemes were also fragmented and treated with preimmune (*g* and *h*) or anti-dynein 1 serum (*i* and *j*). Bar, 0.1 μm. × 150,000.

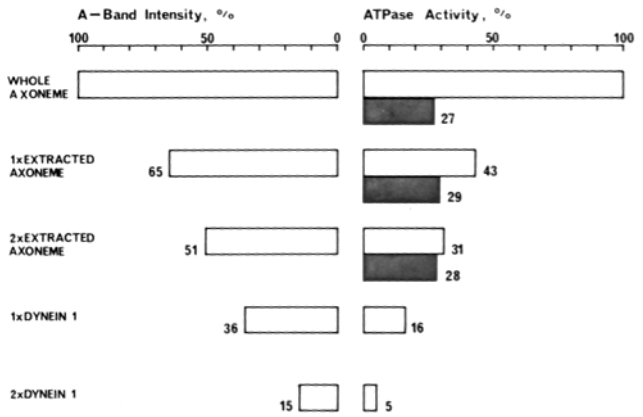

FIGURE 11 Relationship of A-polypeptide and ATPase activity fractionated by repeated salt-extractions. Axonemes were prepared from 10 ml of freshly shed spermatozoa. They were suspended in 10 ml of salt-extracting solution, and extraction was continued for 30 min at room temperature. The precipitate (*P-1*) and the supernatant (as *1 x Dynein 1*) were separated by centrifugation. *P-1* was again suspended in 10 ml of salt-extracting solution (0.2-ml portion was taken out as *1 x Extracted Axoneme*). The precipitate (*P-2*) and the supernatant (as *2 x Dynein 1*) were separated. *P-2* was suspended in 10 ml of TEM (0.2-ml portion was taken out as *2 x Extracted Axoneme*) and dialyzed against the same solution. After the content was centifuged, the supernatant (*Low Extract*) was obtained. A 50-μl portion of each fraction was subjected to SDS-PAGE and the quantity of A-polypeptide contained in it was determined by densitometric tracing of stained gels. Another aliquot was mixed with antiserum, and the mixture was kept overnight at 4°C. The ATPase assay was performed as described in a previous paper (20). The amount of A-polypeptide and ATPase activity of each fraction were expressed as relative values against those in whole axonemes. The ATPase activity in the shaded graph was obtained in the presence of antiserum. The ATPase activity of a fraction *Low Extract* has been inhibited by 55% of antiserum.

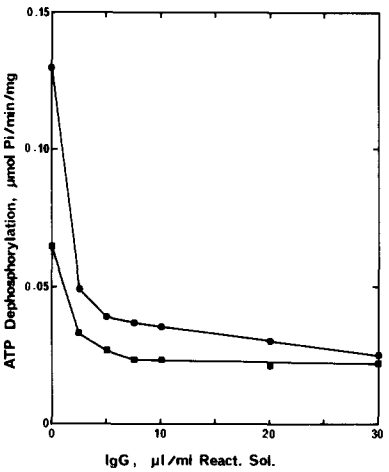

FIGURE 12 Effect of anti-dynein 1 serum on the rate of ATP-dephosphorylation by reactivated spermatozoa (●) and by homogenized ones (■).

1 was found to represent >73% of the total axonemal ATPase activity. The residual 27% of the activity would be represented by *in situ* SUA-dynein 1 and dynein 2. The ATPase activity of the fraction *2 x Extracted Axoneme* in the absence of antiserum corresponds to that of *Whole Axoneme* fraction in the presence of antiserum. This indicates that binding of antibodies to *in situ* SEA-dynein 1 does not affect the ATPase activity of *in situ* SUA-dynein 1.

EFFECT OF BLOCKADE OF IN SITU SEA-DYNEIN 1 ON MOVEMENT-COUPLED ATP-DEPHOSPHORYLATION: The rates of ATP-dephosphorylation of reactivated, motile, and

broken spermatozoa were 0.13 and 0.065 μmol Pi/min per mg protein, respectively, under the oligomycin-free condition. The rate in motile spermatozoa (*Vi*) pretreated with anti-dynein 1 serum decreased with the use of increasing amounts of this antiserum. The rate in nonmotile spermatozoa (*Vhi*) also decreased on pretreatment with this antiserum but less markedly than in motile ones (*Vi*) (see Fig. 12). By addition of 30 μl of antiserum per ml of reactivating solution, both *Vi* and *Vhi* decreased to a level of 0.03 μmol Pi/min per mg protein. Phase-contrast microscopic observations of specimens taken out of the pH-stat showed that all spermatozoa stopped propagating bends. The difference between *Vi* and *Vhi*, interpreted as movement-coupled ATP-dephosphorylation (4), became nearly zero with an increasing amount of antiserum. Because the antibodies bound specifically to *in situ* SEA-dynein 1 and because their binding did not affect the ATPase activity of *in situ* SUA-dynein 1, the inhibition was due to the blockade of the outer arms by them.

DISCUSSION

The anti-dynein 1 serum used in the present work was directed against dynein 1 constituting the outer arms. Thus, even if the inner arms consisted of dynein 1, the immunological specificity of new antiserum to the protein component of the outer arms would be superior to that of the previous anti-dynein 1 and anti-Fragment 1A sera (20, 21), antigens of which were purified from a low-ionic-strength extract of axonemes containing protein components of both arms. Taking into consideration Ouchterlony's double-diffusion test and ATPase inhibition by antiserum, the antigen determinant would be the essential site affecting activity and would be similar to that of anti-Fragment 1A serum (20) but distinct from that of the previous anti-dynein 1 serum (21). The reason why the latter anti-dynein 1 serum did not inhibit the enzymatic activity of dynein 1 remains obscure. It may be due, in part, to the fact that since antigen determinants of dynein 1 would be so many that recognition of a specific site is determined by chance, the characteristics of antisera differ from preparation to preparation.

Anti-dynein 1 serum blocked ATPase activity of SEA-dynein 1 by 95%. However, the inhibition was less evident with the crude enzyme preparation than with the purified one (compare Figs. 4 and 5). Then, the question arises whether antiserum-resistant ATPase activity in the crude SEA-dynein 1 fraction was derived from another unidentified ATPase or expressed by dynein 1 which had been complexed with proteins after extraction so that antibodies could not bind to it. The former possibility is connected with the evidence of Huang et al. (13) that the outer arms of *Chlamydomonas* flagellar axonemes contain two different ATPases. According to the evidence of Bell et al. (3) on sea urchin sperm axonemes, A-polypeptide of dynein 1 can be resolved into two electrophoretically distinct Aα and Aβ bands. Since these bands were separated in the presence of SDS, it is uncertain whether native dynein 1 is constructed from Aα and Aβ subunits or whether dynein 1 is not one species but a mixture of dynein 1Aα and dynein 1Aβ, each with ATPase activity, such as in *Chlamydomonas*. If the outer arms in sea urchin sperm also contain two dynein 1 species, the high resistance of the crude SEA-dynein 1 fraction would be interpretable on the basis that our purification process concentrated either of them which was used as an antigen as a result. The second possibility is based on the fact that since antiserum inhibited 73% of the axonemal ATPase activity all of which was solubilized by repeated salt-extractions, the resistance of the crude dynein 1 preparation was regained after extraction. Minor protein components might interfere with antibody binding to dynein 1.

A pair of arms within *Chlamydomonas* flagellar axonemes has been demonstrated to consist of different proteins, on the basis of genetic analysis (13). Chemical and functional equivalences have been shown between the outer and inner arms of sea urchin sperm axonemes on the basis of chemical dissection (6, 14). The present work, together with the previous report (22), has tried to demonstrate the immunological dissimilarity in protein component (dynein 1) between the two members of a pair of arms, since a major area of controversy in the eukaryotic flagellar motility at present is the question of whether they are identical in protein component and function. In contrast to the previous ferritin-conjugated IgG, the present peroxidase-conjugated IgG was supposed to exhibit a better permeability with respect to the axonemes because of its smaller molecular size. Thus, the failure of the binding of anti-dynein 1 antibodies to the inner arms led us to conclude that the protein components constituting the outer and inner arms are immunologically different. Even in the case of disrupted axonemes where the permeability barrier against antibodies penetrating the axonemes could be neglected, no antibody binding occurred at the inner arms, whereas the outer arms were clearly decorated. We here emphasize the immunological dissimilarity in protein components (dynein 1) between the two members of a pair of arms.

The salt-treatment of axonemes followed by SDS polyacrylamide gel electrophoresis of each fraction, on the other hand, appears to indicate that the inner arms also contain immunologically similar dynein 1. Repeated salt-extractions (see Fig. 11) solubilized one-half of A-polypeptide (SEA-dynein 1), leaving the other half in the residual axonemes (SUA-dynein 1). Our immunoelectron microscopy did not clarify the localization of SUA-dynein 1. When extracted axonemes were treated with a low-ionic-strength solution (residue of reextracted axonemes of Fig. 5) or when an extract was obtained from them (a *Low Extract* fraction of Fig. 11), their ATPase activities were partially inhibited by antiserum. Accordingly, it can be concluded that, in contrast to the solubilized one, *in situ* SUA-dynein 1 hardly receives antibodies. Its location may be the inner arms as speculated by Gibbons (10).

Then, the question arises as to how *in situ* SUA-dynein 1 does not receive antibodies. In some ciliary and flagellar axonemes, the paired arms of the no. 5 doublet are connected with the extraprojection from the no. 6 doublet. Although the presence of such structure was not clearly resolved in sea urchin sperm axonemes (see Fig. 6 *a–c*), the outer arm projecting from the no. 5 doublet hardly received the anti-dynein 1 (present result) and anti-Fragment 1A (22) antibodies. The inner arms are also connected with an interdoublet link and do not receive antibodies. These connectors may protect dynein 1 against antibodies. It is not clear in our electron micrographs whether in opened arrays of the doublet the link was detached from the inner arms. If these arms actually contain dynein 1, the connectors seem to be candidates for regulatory proteins on dynein 1 and flagellar movement, for they compete with the antibodies on the catalytic or regulatory site on the dynein 1 molecule.

Reactivated spermatozoa lacking the outer arms retain a 0.5 (6) or 0.4 (8) beat frequency compared to control ones. If the outer and inner arms function equivalently in the swimming of

reactivated spermatozoa, it is difficult to interpret our present result that anti-dynein 1 serum inhibited their motility of them and did not affect the enzymatic activity of *in situ* SUA-dynein 1. Anti-Fragment 1A serum inhibited ATP-driven tubule extrusion (26, 27) of trypsinated axonemes (15). Since the specific activity of *in situ* SEA-dynein 1 measured threefold that of *in situ* SUA-dynein 1, it also is not easy to understand how two types of dynein 1 with different specific activities produce the same sliding power. Although the possibility that the steric hindrance of outer arms occurred by complexing with IgG exerts a severe effect on the "walking along" of inner arms on doublets remained to be ruled out, the experiment with anti-dynein 1 serum which interacted with dynein 1 without inhibiting ATPase activity showed that such hindrance is not enough to suppress the "walking" of inner arms, for its inhibitory effect on beat frequency (sliding ability) was less evident than that of antiserum which inhibited ATPase activity (21). However, we may be able to interpret this disputable point on the basis of the assumptions that (*a*) although inner arms have a sliding ability intrinsically the same as that of outer arms, they do not function in normal movement, and (*b*) that upon removal of outer arms, they start to function.

We express our thanks to Professor Hideo Mohri of the University of Tokyo and Professor Eisuke Eguchi of Yokohama City University for the use of pH stat and electron microscopic facilities, respectively.

This work was supported in part by a grant-in-aid (No. 574300) from the Ministry of Education of Japan to K. Ogawa.

Received for publication 3 June 1981, and in revised form 21 October 1981.

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