Human Dynein and Sperm Pathology

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ABSTRACT   Human spermatozoa with normal structure and with different axonemal deficiencies (absence of axoneme, of arms, or of central structures) were studied by electron microscopy, SDS-polyacrylamide gel electrophoresis, and ATPase activity measurements.

Normal human sperm possess a complement of high molecular weight polypeptides with an electrophoretic migration similar to that of sea urchin and other mammalian sperm dyneins. Human high molecular weight bands are numbered one to four in order of increasing electrophoretic mobility; all of them are absent in spermatozoa that lack axoneme. The absence of doublet arms coincides with the absence of bands 2, 3, and 4; the absence of central structures coincides with a reduction in intensity of band 2. In the latter two abnormal conditions, band 1 has an increased intensity. The data are tentatively interpreted by attributing the polypeptides forming bands 3 and 4 to the arm structure, whereas band 2 is supposed to contain a mixture of polypeptides localized in the arms and in the central structures; these abnormal sperm contain modified polypeptides which gather in band 1.

Histochemical ATPase stainings indicate that this enzyme is localized mainly in the doublet arms and, to a minor extent, in the central structures.

The axonemal ATPase protein dynein was originally thought to consist of two polypeptide units with very high molecular weight (HMW) (7, 15). Subsequent studies by Bell et al. (5) distinguished eight electrophoretic bands in the characteristic HMW region of dynein from sea urchin sperm, corresponding to molecular weights of 300,000–350,000; Mabuchi et al. (16) found five slightly different bands in starfish sperm dynein, Warner et al. (22) found six HMW polypeptides in mollusc sperm and *Tetrahymena* cilia, and Huang et al. (11) found 10 HMW polypeptides in wild-type *Chlamydomonas*. These discrepancies may be caused in part by differences in electrophoretic techniques, but it is quite clear that dynein HMW polypeptides are highly variable as to number and electrophoretic properties among different organisms. With the publication of these results, a band-labeling problem arose: each author used a personal nomenclature (letters from A to D by Gibbons group, letters from A to C by Mabuchi group, numbers by Warner and Luck groups) so that a comparison among polypeptides from different organisms is very difficult. Because extractability and sedimentation properties of dynein proteins and electrophoretic properties vary in different organisms, we will follow Burns' suggestion (6) and retain separate nomenclatures until function and localization of each of the HMW components is clear. The problem of location is one of crucial importance in establishing the functional role of the polypeptides and ultimately the mechanism of axoneme contraction.

In this kind of study, specific extraction (8, 9, 13) and antibody-labeling approaches (17, 18) have been utilized.

Also, the study of structurally mutant axonemes with electrophoretic techniques has recently shed light on the polypeptide-structure relationship (11). The same rationale underlies our previously reported studies on the dynein polypeptides in animal species normally lacking specific axonemal structures (3).

The latter approach may be usefully extended to the study of human genetically inherited diseases, in which flagellar and ciliary motility are impaired because of the absence of axonemal components (2, 4). In this paper, we describe the HMW dynein polypeptides and ATPase activity of normal human spermatozoa and compare them with those of spermatozoa from three sterile individuals that showed *a*, complete absence of axoneme; *b*, absence of axonemal central structures ("9+0"); and *c*, absence of doublet arms.

MATERIALS AND METHODS

All sperm samples were obtained by masturbation. Only samples in which >80% of total cells appeared as spermatozoa were selected for further investigations. In control samples, >90% of total spermatozoa showed progressive motility.

Transmission Electron Microscopy

Semen samples obtained by masturbation and bioptic material of the olfactory epithelium were fixed in Karnovsky's fixative (12) for 1 h at 4°C, washed in 0.1

M cacodylate buffer, pH 7.2, overnight, postfixed in buffered 1% OsO₄ for 1 h at 4°C, dehydrated in a graded series of ethanols, and embedded in Epon. Sections cut with an LKB ultramicrotome (LKB Instruments, Inc., Rockville, Md.), mounted on copper grids, and stained with uranyl acetate and lead citrate were observed and photographed with a Philips EM 301 electron microscope.

Histochemistry

ATPase activity was detected by a modified Wachstein and Meisel medium, according to Abel et al. (1). Spermatozoa were fixed in 1.25% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 15 min and rinsed in the same buffer with 5% saccharose for 2 h; they were then incubated in a medium containing 75 mM Tris-maleate buffer, pH 7.2, 8 mM ATP, 8 mM MgSO₄, and 2 mM Pb (No₃)₂ for 45 min at 37°C, postfixed in 1% OsO₄, and embedded as mentioned above.

Scanning Electron Microscopy

Ejaculated spermatozoa washed with phosphate-buffered saline were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 30 min, rinsed in the same buffer, dehydrated through ethanols, Freon 113, and finally dried by the critical point method in a Bomar unit (The Bomar Co., Tacoma, Wash.), coated with evaporated gold-platinum, and observed under a Coates and Welter field emission scanning electron microscope.

Biochemical Methods

Immediately after liquefaction, ejaculates were centrifuged for 3 min in preweighed plastic tubes of a Beckman Microfuge (Beckman Instruments, Inc., Fullerton, Calif.). The supernate was discarded and carefully wiped off from the tube walls, and the sediment was suspended with 10 vol of a 2% solution of SDS in 0.9% NaCl, 0.01 M Tris-HCl of pH 7.5. After centrifugation as described above, the supernate was mixed with mercaptoethanol (10% final concentration) and with glycerol (10% final concentration) and placed for 3 min in a boiling water bath. These samples (SDS-extracts) could be stored at −30°C for several months without alteration of the electrophoretic pattern. Volumes of the SDS solution, mercaptoethanol and glycerol, were precisely measured with micropipettes (glycerol was pipetted after heating in a boiling water bath); therefore, different extracts could be assumed to correspond, with acceptable reproducibility, to same amount of sedimented sperm cells (~0.8 μg/10 μl). Electrophoresis was performed using the discontinuous high pH method of Laemmli (14). Separation gels (0.6 × 11 cm) contained 4% acrylamide; stacking gels contained 3% acrylamide. 50-μl aliquots of the SDS-extracts corresponding to ~4 mg of sedimented spermatozoa were applied to the gels, and electrophoresis was performed at 1-2 mA/gel. Staining was performed with 0.2% Coomassie Blue in 20% methanol and 7% acetic acid, and destaining was performed with 10-20% methanol and 7% acetic acid.

RESULTS

Submicroscopic Observations

As well known, the human sperm axoneme shows the classical 9+2 pattern, where arms, radial links, and central tubule projections are quite evident (Fig. 1). This basic structure is surrounded by nine accessory fibers and, around them, anteriorly by the mitochondrial sleeve and posteriorly by the fibrous sheath. The structure is exactly the same in the well known human syndrome named "round-headed" spermatozoa (21), which have no acrosome. We decided to use these abnormal spermatozoa to make sure that axonemal proteins were not degraded by acrosomal proteases (see Fig. 2 and Discussion). Previous researches (4, 19, 20) have demonstrated that an active role in motility is played only by the axoneme, whereas accessory fibers, sleeve, and sheath play an elastic, skeletal role. Three kinds of axoneme deficiencies have been studied by us.

COMPLETE ABSENCE OF AXONEME: We have examined a patient with this malformation affecting all the spermatozoa. Examined by scanning electron microscopy, the sperm appear of normal shape and length, the only peculiarity being the flat and unusually curved tail, as seen in scanning electron micrographs (Fig. 3). In section (Fig. 4), the tail region appears to be occupied simply by the mitochondrial sleeve and the fibrous sheath surrounding the classical bundle of nine accessory fibers. Axonemal microtubules are completely absent and the spermatozoa are immotile. The head region, including the acrosomal complex, is normal.

ABSENCE OF DOUBLET ARMS (KARTAGENER'S SYNDROME): This malformation also affects the total sperm population in an individual, presenting also the immotile cilia syndrome in the olfactory epithelium and "situs inversus".

Examined by scanning electron microscopy (Fig. 5), all spermatozoa appear normally shaped but have a straight tail and are all immotile. Sections show that the head (including the acrosome) and tail accessory structures (mitochondria, fibrous sheath, and accessory fibers) are normal. The axoneme is affected by the typical "Kartagener's" malformation in which arms are absent throughout the whole length of the nine doublets. The radial links and central tubules are normal (Fig. 6).

ABSENCE OF CENTRAL TUBULES (9 + 0 SYNDROME): Whereas the total population of spermatozoa in a patient is affected by this syndrome, the olfactory epithelium bears normal cilia (Fig. 9).

All the spermatozoa are immotile; only a small percent of them (one to two at most) presented feeble vibrations for a few seconds but did not generate waves. In scanning electron micrographs, they appear normally shaped but with a straight tail (Fig. 7). The head and neck regions, including the spermatid centrioles, are normal. The tail has a normal mitochondrial sleeve, fibrous sheath, and accessory fibers. The axoneme is normal in the doublet region, where arms and radial spokes are present, but has no central structures, lacking the two single tubules and their projections (Fig. 8).

ATPase Activity

The poor resolution of histochemistry in ATPase detection at the electron microscope level is well known. Our preparations of normal human sperm or round-headed human sperm show precipitates not only between the axonemal doublets, in the region of the arms, but also on the spoke heads and the projections of the central tubules (Fig. 10). In the deficient models studied by us, there was an absence of precipitate that correspond directly to the absent structures: the 9+0 model has ATPase activity only in the doublet region (Fig. 12), armless sperm only on radial links and around central tubules (Fig. 11).

Acrylamide Gel Electrophoresis

An acrylamide-gel electrophoretic analysis of dynein HMW polypeptides is presented in Fig. 13. Dynein bands from sea urchin and bull spermatozoa are shown in gels 1 and 2 and identified by letters according to Gibbons et al. (10) and Pallini and Gibbons,[1] respectively.

The relationship between sea urchin and mammalian dynein bands cannot, as yet, be established on the basis of their electrophoretic migration. From motile-human spermatozoa (gel 3), at least four major bands are visible: two upper closely spaced bands of comparable intensities (bands 1 and 2), which migrate, respectively, at the same rate as the A and D bands of sea urchin and as the J and K bands from bull sperm; one

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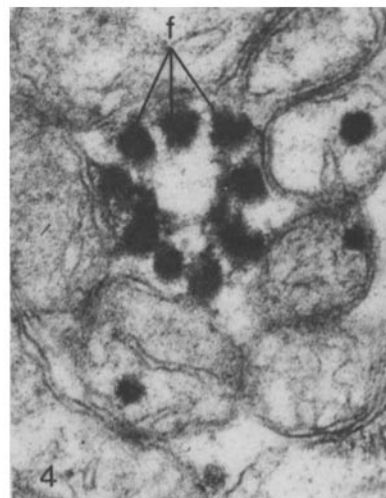
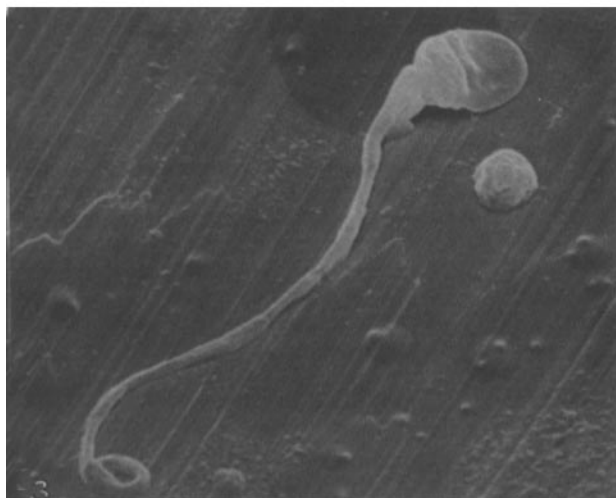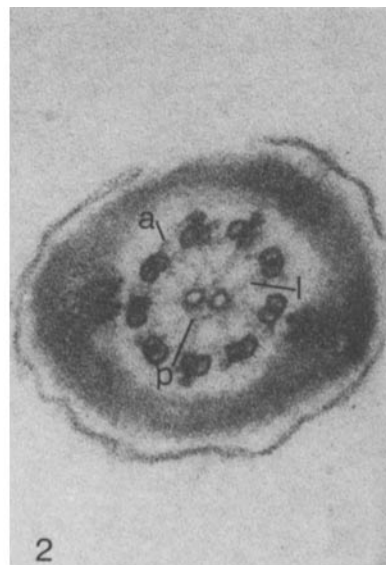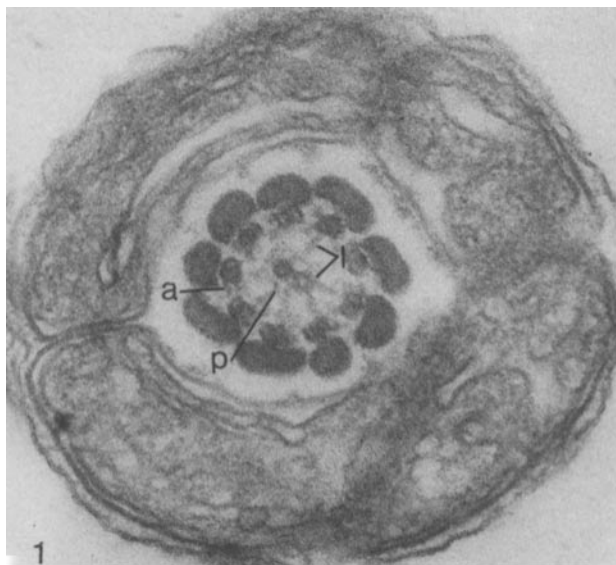[1] Pallini, V. and I. R. Gibbons. Manuscript in preparation.

FIGURE 1 Cross section of human sperm tail in the midpiece region. Doublet arms (a), radial links (l), and central tubules projections (p) are evident. × 75,000.

FIGURE 2 Cross sections of round-headed human sperm tail. Doublet arms (a), radial links (l), and central tubules projections (p) are evident. × 100,000.

FIGURE 3 Human spermatozoon lacking axoneme. Scanning electron microscopy, × 4,000.

FIGURE 4 Human spermatozoon lacking axoneme. Cross section of the tail in the midpiece region, in which only accessory fibers (f) are evident, whereas the basic 9+2 axoneme is missing. Cf. with Fig. 1. × 62,500.

diffuse band (band 3) in the position of the bull L and M doublet; and one intense band (band 4), which migrates faster than the others and which is comparable to the B band of sea urchin and to the N band of bull sperm.

Sperm from the patient affected by the round-headed, acrosomeless syndrome (gel 4) has an essentially normal complement of HMW bands; the only variation is manifested by a slight reduction of the intensity of band 2 and by a slight increase of the intensity of band 1. The same character has been found to occur in one out of 30 normal motile sperm samples we have examined in the course of this work; this sample derived from an individual of proved fertility. Therefore, this variation of the band pattern is caused either by differences that do not influence sperm function or by minor faults of the polypeptide extraction and electrophoretic procedures. The rest of the examined normal samples exhibited the

pattern of gel 3, with bands 1 and 2 of equal intensity; in some samples, two additional minor bands could be detected, one near band 3 and the other just below band 4.

Not even traces of bands were found in this region of the electrophoretic gels when axonemeless spermatozoa were examined (gel 5).

An analysis of the dynein bands from 9+0 and armless spermatozoa is presented in Fig. 14. A significant deviation of the pattern shown by 9+0 spermatozoa (gel 2) from that obtained from normal sperm (gel 3) consists of the reduction of the intensity of band 2 and of the markedly enhanced intensity of band 1. The identity of the bands from 9+0 spermatozoa is proven by the coelectrophoresis of this sample with normal human sperm (gel 5). The variation from the normal pattern of the 9+0 sample is far more marked than that occasionally found in motile spermatozoa, as can be easily
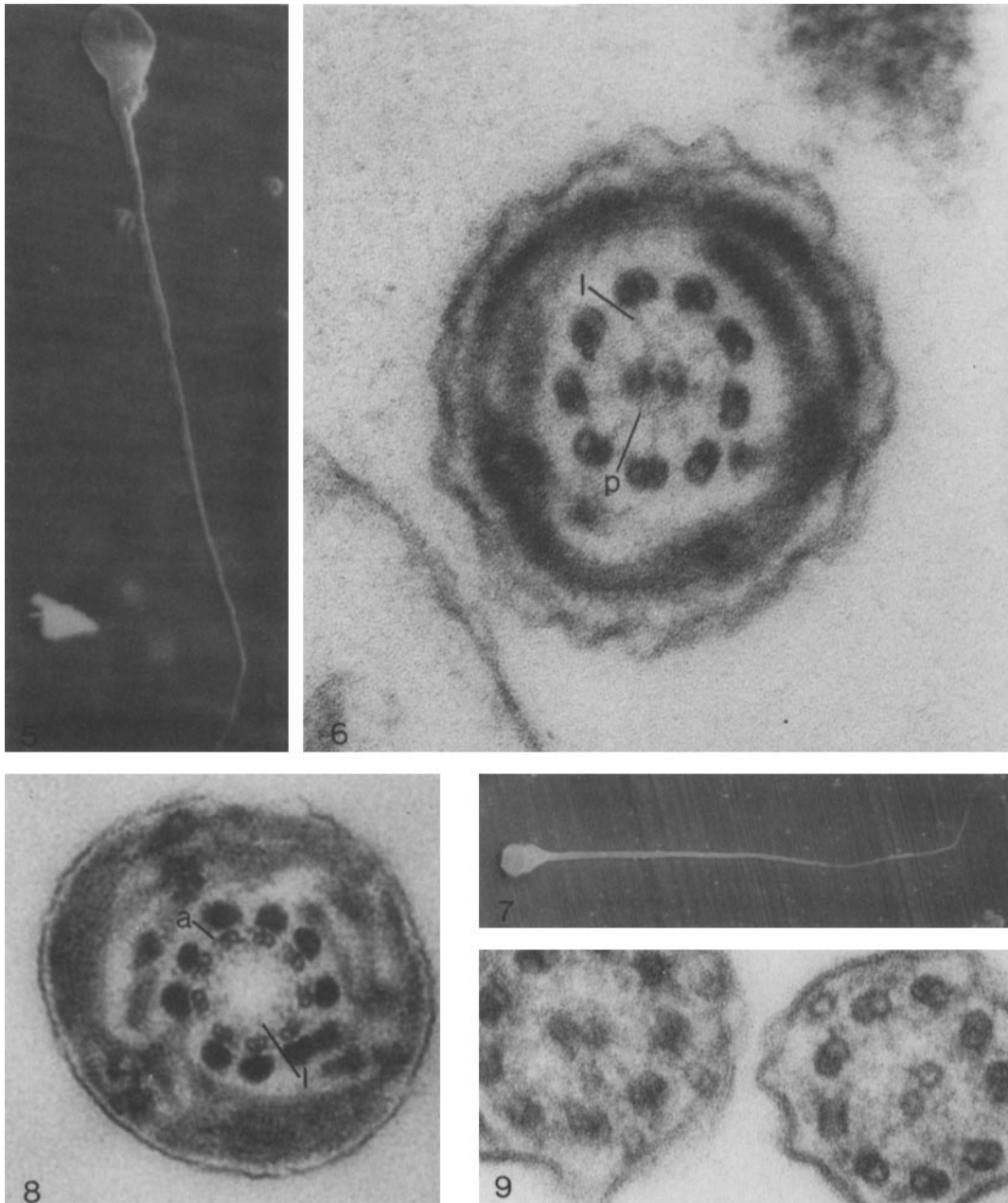
FIGURE 5   Kartagener's syndrome (armless) human spermatozoon. Scanning electron microscopy, × 3,000.

FIGURE 6   The same patient as in Fig. 5, cross section of the sperm tail. Radial links (*l*) and central tubules projections (*p*) are evident, doublet arms are missing. × 140,000.

FIGURE 7   9+0 axoneme syndrome, human spermatozoon. Scanning electron microscopy, × 2,000.

FIGURE 8   The same patient as in Fig. 7, cross section of the tail region. Doublet arms (*a*) and radial links (*l*) are evident. Central structures are missing. × 95,000.

FIGURE 9   The same patient as in Fig. 7, cross section of olfactory epithelium cilia. The 9+2 pattern is normal. × 140,000.

observed by comparing gel *2* of Fig. 14 and gel *4* of Fig. 13.

Armless spermatozoa (Fig. 14, gel *1*) were found to possess only one of the HMW dynein bands (band *1*) and its intensity is enhanced with respect to the electrophoretic pattern from normal sperm; only a diffuse staining is observed in the gel region corresponding to other bands (gel *1*). The identity of band *1* of this individual with band *1* of normal human sperm is proven by coelectrophoresis (gel *5*).

Three different ejaculates from the 9+0 and two different ejaculates from the Kartagener's patients were collected at time intervals of 2–3 mo and found to produce the typical electrophoretic patterns shown in Fig. 14.

DISCUSSION

Electrophoretic analysis of HMW bands in the dynein region demonstrates a species-specific pattern in mammalian spermatozoa. In this laboratory, we have observed that bull,[1] rat,

boar (Pallini et al., unpublished observations), and human sperm (present paper) all show different patterns. The common feature is the presence of three major bands which appear equally spaced in bull, whereas in human and rat sperm, the intermediate band is displaced upwards from the central position. Other minor bands are interposed among them. The present data do not provide enough information, such as which band corresponds to a particular ATPase in the axoneme, to allow a relationship to be established between bands of different mammalian species or between mammalian sperm bands and sea urchin sperm bands, the isoenzymic significance of which is now well established (10). The uppermost main bull and human bands appear similar to sea urchin A band, but this similarity may be limited to the electrophoretic migration of the polypeptide and may not be related to its function. The fastest migrating mammalian band (no. 4) migrates at a rate similar to that of the sea urchin B band. The intermediate major mammalian band (no. 2) is more difficult to compare with any of sea urchin polypeptides, having a different position for bull and human spermatozoa.

The major difficulty encountered in the study of mammalian dynein is a proteolytic cleavage of HMW polypeptides which occurs upon aging or treatment of sperm with Triton.[1] In the study of human sperm, freshly collected ejaculates were used and electrophoresis was performed without treatment with Triton. These precautions have ensured reproducible results in human and bull semen.[1] Moreover, because sperm proteases

FIGURE 13 High molecular weight polypeptides of sea urchin, bull, and human spermatozoa. Gel 1, sea urchin sperm flagella, 30 μg of total protein. Gel 2, SDS extract from ~4 mg of ejaculated, motile bull spermatozoa. Gel 3, SDS extract from 4 mg of normal human spermatozoa. Gel 4, SDS extract from 4 mg of acrosomeless, motile human spermatozoa (cf. Results). Gel 5, SDS extract from 4 mg of axonemeless, immotile human spermatozoa (cf. Results). Bands in the dynein region are labeled from A to D according to Pallini and Gibbons in bull[1] and from 1 to 4 in human spermatozoa.
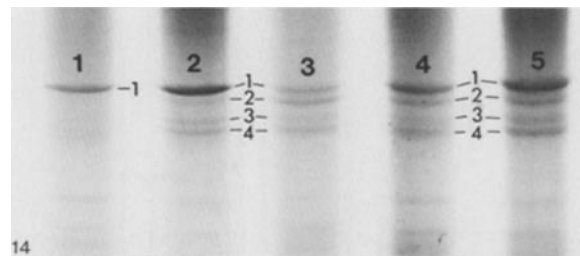
FIGURE 14 SDS gel electrophoresis on HMW polypeptide chains of human spermatozoa bearing different axoneme defects. Gel 1, armless axoneme; gel 2, 9+0 axoneme; gel 3, normal axoneme; gel 4, coelectrophoresis of normal and armless sperm; gel 5, coelectrophoresis of 9+0 and normal human spermatozoa. The dynein bands are labeled from one to four. SDS extracts from ~4 mg of cells for each cell type were used in the electrophoretic runs.
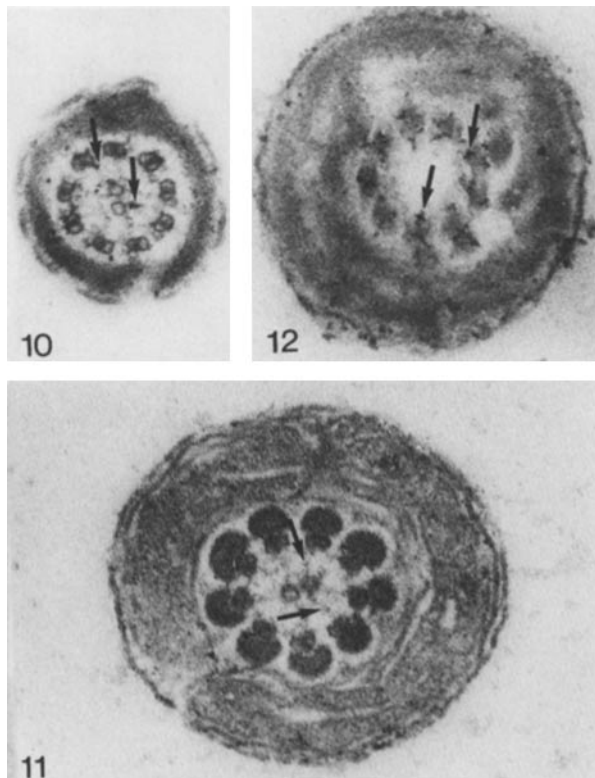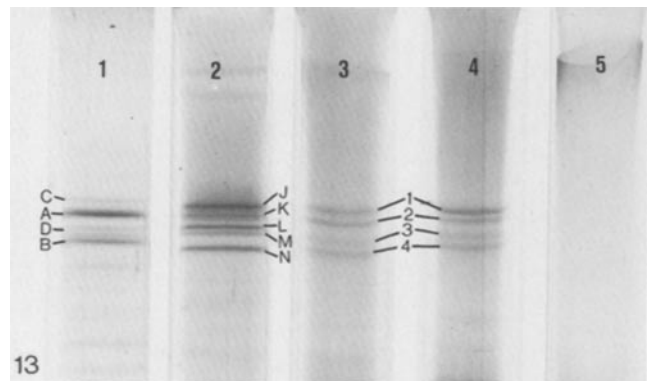

FIGURE 10 Cross section of a normal human sperm tail. ATPase activity (arrows) is present on the doublet arms, on the radial links, and around the central tubules. × 95,000.

FIGURE 11 Cross section of a Kartagener's syndrome (armless) human sperm tail. ATPase activity (arrows) is detectable only on radial links and around central tubules. × 95,000.

FIGURE 12 Cross section of a 9+0 human sperm tail. ATPase activity (arrows) is present only between the doublets. × 95,000.

are known to be packed in the acrosome, the finding of identical HMW band patterns in normal and acrosomeless spermatozoa greatly reduces the probability of proteolytic artifacts in the study of dynein polypeptides.

From an examination of the electrophoretic patterns in the axonemeless, 9+0, and armless (Kartagener's) spermatozoa, the following conclusions can be drawn: (a) HMW dynein chains are contained in the 9+2 axoneme and not in accessory structures. (b) Bands 2, 4 (close to the position of the sea urchin B band), and 3 (a minor one) are caused by polypeptides located in the arms because they are missing in armless and present in 9+0 axonemes. (c) Band 2 has a reduced intensity when central structures are absent and it appears to be missing where arms are absent. At least two interpretations of these results are possible; this band might consist of a mixture of different polypeptides localized in the arms and in the central structures with the more abundant component in the arms; alternatively, the same polypeptide might be predominantly localized in the arms and, to a lesser extent, in the central structures. (d) Band 1 (similar to the sea urchin sperm A band) shows an increased intensity both in 9+0 and armless axonemes. This effect is not caused by a failure of the electrophoretic method because the separation of the abnormally intense band 1 from band 2 is obtained in coelectrophoresis experi-

ments of normal with 9+0 and armless sperm polypeptides. The behavior of this electrophoretic component is, therefore, meaningful although unusual. It might indicate the occurrence in the matrix of abnormal human spermatozoa of mutant polypeptides of low electrophoretic mobility which are incapable of forming normal structures and which collect into a no. 1 band of enhanced intensity.

The absence or reduction of intensity of some electrophoretic bands in the dynein region is concomitant with a reduced amount of reaction product in the cytochemically detected ATPase. Whereas precipitates are visible between doublets and on central structures both in normal and in round headed spermatozoa, they are lacking in the doublet region in the armless sperm, and obviously are lacking in the central region when central tubules are absent.

In this paper, we have presented results that show that the electrophoretic mobilities of human sperm HMW peptides are similar to those of other mammalian sperm and of sea urchin sperm dynein. The peptides that comprise certain bands have been tentatively identified with structural features in the axoneme. We have demonstrated that ATPase activity is associated with the dynein arms and with structures in the central region of the axoneme. Further investigations of the properties of the HMW polypeptides discussed in this communication are necessary for a complete description of the system to be made.

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