Virchows Arch [Pathol Anat] (1983) 399:265–275

Virchows Archiv A

The immotile cilia syndrome

Mice versus man

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Summary. When homozygous the recessive, pleiotropic, mutation *hpy* (hydrocephalic-polydactyl) produces post-natal hydrocephalus, complete sterility in males, and reduced reproductive performance in females. Because the fertility problems and the development of hydrocephalus could arise as consequences of defective flagella and ciliary axonemes, this mutant type might serve as a useful animal model for the immotile cilia syndrome. Ultrastructural defects seen in axonemes of flagella, and of cilia from the trachea, oviduct, and ependyma included: a deficiency of inner dynein arms (the most frequent defect); an absence of one or both central-pair tubules; extra central tubules; a displacement of one outer doublet and/or the central-pair tubules. Some axonemes showed more than one of these defects. The frequency of dynein-deficient axonemes in all three tissues was similar (about 35%) and fell within the range reported for human patients with the immotile cilia syndrome. On this basis, this mutant type might be considered as a useful animal model for such studies. There were no indications of situs inversus, nor was there a marked increase in respiratory problems. So *hpy/hpy* mice do not exhibit all of the clinical symptons characteristic of the human condition.

Key words: Axonemal defects – Immotile cilia syndrome – Mouse – Male – sterile mutant

Introduction

As is evident from the recent literature (e.g., Afzelius 1976 and 1979; Baccetti et al. 1979; Rott 1979; Sturgess et al. 1979) a variety of ciliary axonemal defects have been observed in patients presenting with chronic respiratory problems. Similar defects were also reported for sperm flagellar axonemes in such male patients who also exhibited reduced fertility or complete sterility. Not all of these cases had associated *situs inversus* and so could not

be classified as examples of Kartagener's syndrome – a previously recognized genetic disorder. Hence, following the suggestions of Afzelius (1976), Eliasson et al. (1977) and others, there is now a general agreement that these various conditions, including those diagnosed as Kartagener's syndrome, represent a spectrum of axonemal defects which can be grouped together under the common heading "immotile cilia syndrome". In view of the obvious limitations concerning investigations of ultrastructural and related aspects of genetic defects in human patients, possible animal models have attracted considerable interest. In this connection I have been studying the axonemal defects exhibited by mice homozygous for a recessive, pleiotropic, mutation *hpy* (hydrocephalic-polydactyl). Mutant males are completely sterile while females rarely produce more than three moderate-sized litters. Sterility in the male stems from an abnormal development of flagellar axonemes resulting in an absence of sperm tails (Bryan 1977a and 1981). Axonemal structures were present only in young spermatids and these showed various abnormalities; some resembling those reported for the immotile cilia syndrome in man. Because the reduced breeding performance of mutant females, and the development of hydrocephalus in both sexes, could result from defective ciliary action this mutant type might serve as a suitable animal model for the human condition. Accordingly, ultrastructural studies of cilia from various tissues were undertaken as part of an attempt to provide further support for this view. A detailed account of those studies has been presented elsewhere (Bryan 1983). The results of those studies together with others pertaining to spermiogenesis (Bryan 1977a, 1981 and continuing investigations), form the basis for this paper. As will be seen, certain of the findings for mice strongly parallel those described for the immotile cilia syndrome in man and, thus, provide a measure of support for the view expressed above.

Materials and methods

Animals of appropriate genotype were mated to provide mutant and nonmutant littermates. Offspring were examined for evidence of *situs inversus* first as neonates (the orientation of the milk-filled digestive tract is clearly visible through the body wall) and again when sacrificed. They were also checked at frequent intervals until sacrificed for indications of respiratory problems. Mutant and nonmutant littermates of each sex were sacrificed and samples of trachea, cerebral hemispheres abutting the lateral ventricles, oviduct, and testis, as appropriate, were removed and processed for light- and electron-microscopic studies. Living cell preparations for studies of ciliary activity were made as described previously (Bryan 1982). For electron microscopy, tissues were fixed and processed essentially as described by Bryan and Wolosewick (1973). In some instances the glutaraldehyde fixative was made 1% with respect to tannic acid. This modification was employed since the presence of tannic acid improves the electron microscope contrast of many sub-cellular components (see Kuhn and Engleman 1978).

Results

No indications of *situs inversus* were encountered in mutants from more than sixty litters examined to date. Neither did there appear to be any significant increase in the incidence of respiratory problems except, perhaps,

in the case of a few mutants which were allowed to survive to a relatively advanced age (about 1 year).

Because of variations in the nature of the principal axonemal defects observed in spermatids and in cilia from the different epithelial tissues, each type will be described separately.

Spermatids

In early spermatids (steps 1–3 of spermiogenesis) normal-appearing centriolar complexes bearing growing axonemes were seen in the course of their migration from the cell periphery to the distal pole of the spermatid nucleus (Fig. 1). Some of these axonemes showed a normal 9+2 organization while others lacked one or both members of the central-pair tubules (e.g., Fig. 2). The developing flagellar axonemes also appeared to be deficient in dynein. In some cross sections either no dynein arms seemed to be present or only the outer arms could be recognized. Too few axonemal profiles have been encountered, to date, to determine unequivocably whether or not the nexin links and radial spokes are completely normal. Spoke-like connections running from outer doublets to the central sheath can be seen in many axonemes but frequently they appeared somewhat distorted (e.g., Fig. 2). In older spermatids in which centriolar migration had been completed (steps 8–16) axonemal structures were rarely encountered and they were always abnormal. Defects ranged from a partial or complete absence of central-pair tubules to a complete absence of all axonemal components (only normal-appearing centriolar complexes were present). Evidence of attempted flagella formation was seen in only a very few spermatids. The example shown in Fig. 3 is highly exceptional in that a ring of outer dense fibers surrounds a few axoneme-derived microtubules. More typically, microtubules are completely absent and there is no predominant orientation of the outer dense fibers and associated mitochondria (Fig. 4).

Ciliated epithelia

All epithelia examined were well endowed with ciliated cells regardless of sex or phenotype (wild-type, *hpy/hpy* respectively). Moreover, living cell preparations of tracheal epithelium from *hpy/hpy* animals evinced high levels of ciliary activity. It was difficult to distinguish between samples from mutant and wild-type animals on the basis of the relative frequency of ciliated cells, or on the number or size of cilia per cell, or on the rate of ciliary beating. At the electron microscope level, the majority of cilia appeared to possess axonemes of normal ultrastructural appearance. No cells were seen in which all the cilia were abnormal. Instead, cilia with abnormal axonemes were intermixed with normal ones on a given cell (Fig. 5). No cilia were encountered in which signs of axonemal dismantling were evident. A common abnormality seen in cilia from all three epithelial types was a variation in the number of central microtubules. Numbers ranged from 0 to 5 with those axonemes containing three or more central

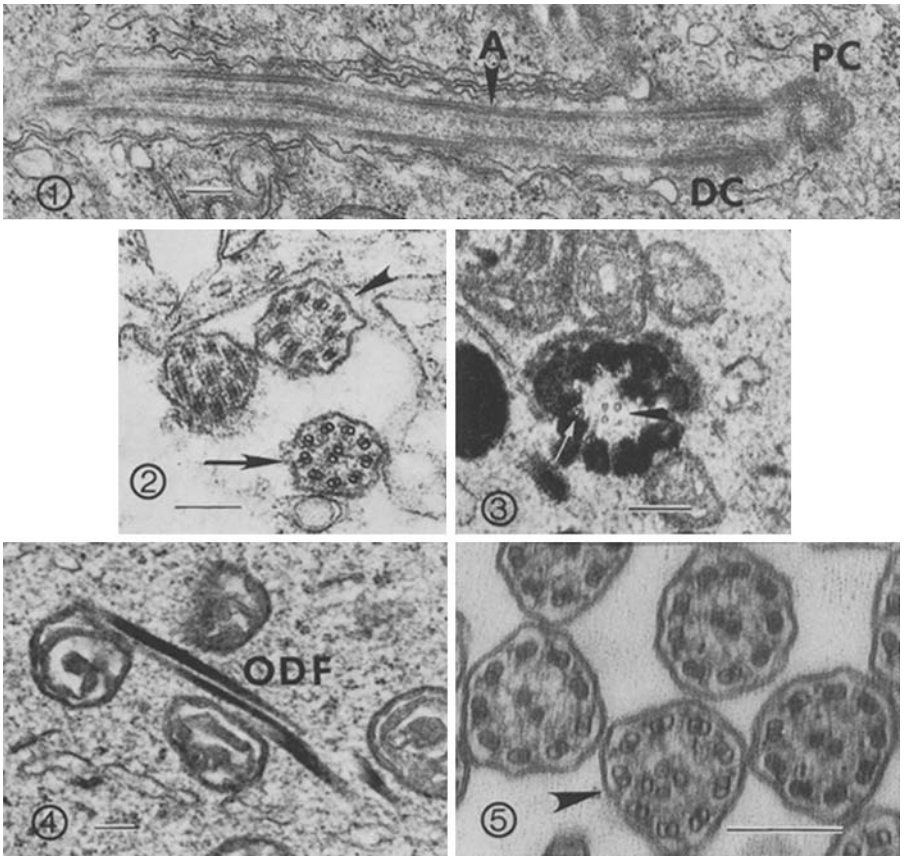

Fig. 1. A normal-appearing developing axoneme in a mutant early spermatid (*A* axoneme; *DC* distal centriole; *PC* proximal centriole). Magnification markers: 0.2 μm unless indicated otherwise

Fig. 2. Cross-sections of axonemes in mutant early spermatids; one (*arrowhead*) lacks both central tubules. The axoneme indicated by the arrow is deficient in dynein arms

Fig. 3. A partial flagellum. Note that singlet microtubules (*arrowhead*) and outer doublets (*white arrow*) are present within the ring formed by outer dense fibers

Fig. 4. Outer dense fibers and mitochondria in a more typical example of the abortive attempts at flagella formation (*ODF* outer dense fibers)

Fig. 5. Ciliary axonemes from the oviduct of a *hpy/hpy* female. One axoneme (*arrowhead*) contains 4 central tubules

tubules (Figs. 6, 7) being relatively more common in tissue samples from the trachea and oviduct. The frequency of axonemes exhibiting additional central tubules ranged from about 4% (oviduct) to about 24% (trachea). On the basis of the orientation of the central-pair tubules, cilia from a given cell from a non-mutant animal were aligned in the same general direction. In similar preparations from mutant animals, on the other hand, not

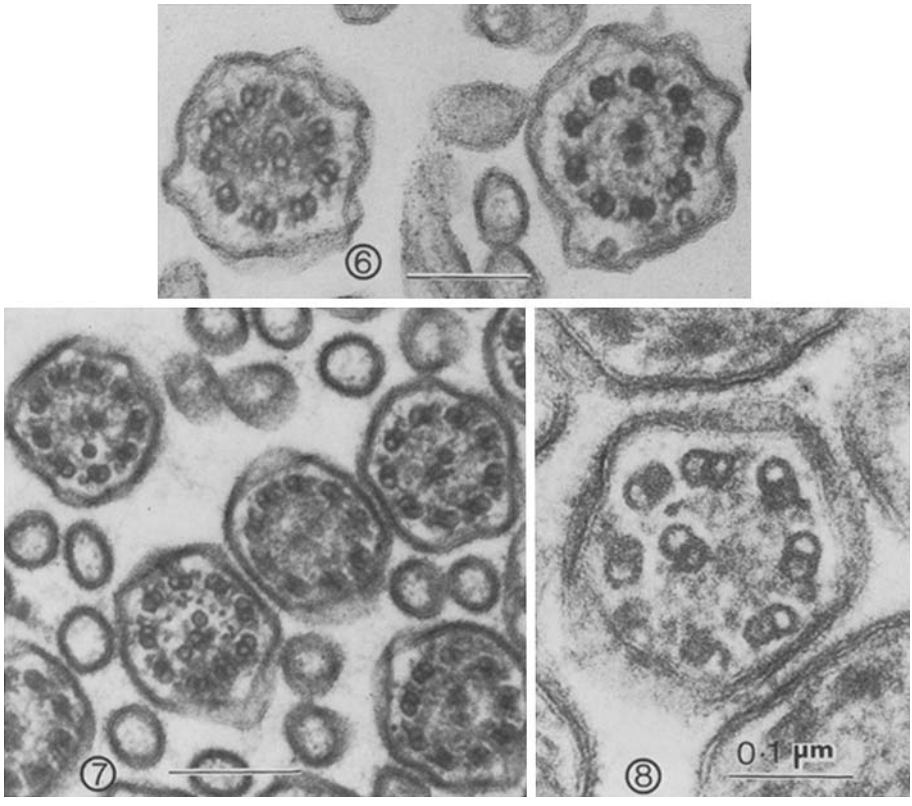

Fig. 6. Tracheal epithelium. Note that one axoneme has the typical 9+2 organization and the other is a 9+4

Fig. 7. Tracheal epithelium. Two axonemes each with three central tubules are visible. Note also the variation in orientation of the central-pair tubules (Glutaraldehyde-tannic acid fixation)

Fig. 8. Tracheal epithelium (glutaraldehyde-tannic acid fixation). Note that both central-pair tubules are missing and that one outer doublet has been displaced inwardly

all cilia from a given cell showed the preferred orientation; some appeared as if rotated through 45–90° (Fig. 7). Occasional axonemes in samples from trachea and ependyma showed a displacement of one of the outer doublets. Usually, the displaced member was located just within the ring formed by the remaining eight doublets; in the example shown in Fig. 8 the axoneme also lacks both central-pair tubules. In a few axonemes the central-pair complex was seen to be eccentrically located; in some instances this occurred together with the displacement of an outer doublet, in others not. All of the ciliary axonemes showing the normal 9+2 organization appeared to possess radial spokes. However, in some axonemes exhibiting increased numbers of central tubules or displaced doublets and/or eccentric central pair complexes, frequently the spokes appeared distorted or absent. The

situation with respect to the presence and normality of dynein arms is less clear. This is because the structural arrangement of the interior of numerous axonemes could not be easily interpreted. The appearance of especially the inner dynein arms (and radial spokes) was blurred by the presence of electron-opaque material lying in their vicinity. It was not clear whether or not this material might represent nexin links or poorly organized assemblages of dynein. Because of this uncertainty such axonemes were recorded as possessing inner arms. On this basis, both inner and outer arms were judged to be present in about 65% of the axonemes surveyed from tracheal, oviducal, and ependymal cilia. Outer dynein arms, on the other hand, could be clearly recognized in at least 95% of the axonemes from each of the three epithelial types; in some the outer arms appeared unusually prominent. Defective axonemes were not encountered in any of the comparable samples from nonmutant animals.

Discussion

It is obvious from the foregoing findings that while axonemes from ciliated cells and spermatids of mutants share certain defects there are fundamental differences between them. In early spermatids centrioles evince the typical ultrastructure and are capable of initiating axonemal development at the proper time (c.f., Fig. 1). Thus, the centrioles can be ruled out as causative agents for the observed axoneme dysgenesis. This is not surprising since, as is well known, functional centrioles are indispensable components of the mitotic apparatus. Hence, disabling centriolar defects resulting from a mutation would be expected to have lethal consequences for the organism (see also Duckett et al. 1979). Despite the indications of normal functional activity the resulting axonemal structures do not persist throughout spermiogenesis. Instead, they are completely degraded by the time the spermatids have advanced to step 8–9 of spermiogenesis (about 7 days after the onset of axoneme initiation) – with the result that only centriolar complexes and their associated flagellar neck-structures remain. An analogous situation has been reported for *Drosophila melanogaster* by Dybas et al. (1981). The mutation *ms (1)14* affects the stability of axonemes; they degenerate in spermatids of developmental ages comparable to those in mutant mice (i.e., equivalent to steps 8–14 of murine spermiogenesis). These abortive attempts at flagellar axoneme formation in *hpy/hpy* mice support the inference that such axonemes are unstable even though they may possess the requisite number of microtubular structures. Because of the paucity of even partially-assembled axonemes encountered in surveys of steps 5–8 spermatids the disassembly process must be accomplished relatively rapidly. To date, the evidence obtained does not provide a clear picture of this process so it is not yet possible to discriminate between alternative explanations for the instability. Thus, it is possible that the microtubules themselves are inherently unstable due to defects or deficiencies in their molecular components (tubulins, microtubule-associated proteins). On the other hand, it is also

possible that the unstable nature of the axonemes stems from defective radial spokes and/or nexin links (c.f., Fig. 2). Unfortunately, the very few instances in which late spermatids contain recognizable axoneme-derived microtubules (c.f., Fig. 3) lend themselves to either explanation. Therefore, clarification must await the outcome of various experimental approaches currently under investigation. In any event, the present findings for mouse spermatozoa are in marked contrast to those for man; the sperm tails were reported to be ultrastructurally normal except for a lack of dynein arms (e.g., Afzelius 1976). Although dynein arm deficiencies have been reported for both types of flagella axonemes, a lack of dynein obviously is not a primary cause of sterility in male *hpy/hpy* mice.

The most common of the abnormalities seen in ciliary axonemes from the three epithelial types examined was a deficiency in dynein arms. Although occasional axonemes appeared to lack both inner and outer arms, the most prevalent condition was an absence of inner arms. In this regard, the condition in *hpy/hpy* mice parallels that characteristic of many patients with the immotile cilia syndrome (e.g., Schneeberger et al. 1980; Afzelius 1981a). The other classes of ciliary abnormalities (described above) have not yet been seen in flagellar axonemes of *hpy/hpy* mice. It is not clear whether this is simply a consequence of the relative rarity of axonemes in seminiferous tubules of mutants or whether such effects are in fact restricted to cilia. However, axonemes with displaced central-pair complexes and outer doublets have been described for several patients exhibiting symptoms consistent with those of the immotile cilia syndrome (Sturgess et al. 1979). In all of those cases ciliary axonemes characteristically lacked radial spokes. As pointed out by these authors, an absence of radial spokes may abrogate the usual structural constraints imposed on axonemal components and, thereby, permit a displacement of outer doublets and/or central tubules to occur. Radial spokes were not always or uniformly delineated in axonemes of *hpy/hpy* mice which showed such defects (c.f., Fig. 8), so it is possible that the observed displacements may have arisen in this way.

In contrast to their counterparts in spermatids, ciliary axonemes in mutants clearly are stable structures, from which observation it might be inferred that there are site-specific differences in the manifestation of the expression of mutant genes affecting axonemal structure. The emerging molecular-level information indicative of multiple tubulins in cells is in agreement with such a conclusion. For example, four different α-subunits and two different β-subunits have recently been described for pig brain tubulin (Ponstingl et al. 1981; Krauhs et al. 1981) and a β-tubulin mutation which is testis-specific has been identified in *Drosophila melanogaster* by Kemphues et al. (1980). To some extent, such an interpretation is also supported by the present observation that an abnormality common to ciliary axonemes from all three epithelia – the presence of extra central tubules – has not been seen in spermiogenetic cells. A measure of additional support is furnished by the findings for a male-sterile patient reported by Baccetti et al. (1979). Spermatozoa from this patient were completely immotile and their

axonemes lacked central-pair complexes (tubules plus the central sheath). Cilia from the nasal epithelium, on the other hand, were completely normal; neither did this patient have a history of respiratory problems. In other words, the presence of defects in flagellar axonemes does not guarantee that ciliary axonemes will also be abnormal. The foregoing inference concerning the expression of the mutant gene would be further strengthened if clearcut examples of a reverse nature (abnormal cilia but functionally normal sperm tails) were known. Some possible cases have been described in the literature dealing with Kartagener's syndrome but, as pointed out by Rott (1979) and by Afzelius and Eliasson (1979), the matter of paternity was not well authenticated.

While the major defect encountered in axonemes from *hpy/hpy* mice (dynein arm anomalies) has been described for human patients with the immotile cilia syndrome, others (e.g., central-tubule anomalies) do not appear to be characteristic for this human condition. However, ciliary axonemes with anomalous numbers of central tubules have been reported for patients with retinitis pigmentosa (a condition stemming from the presence of a dominant mutation) by Fox et al. (1980). Similar anomalies also have been reported for a variety of nongene based conditions: asthma (Cutz et al. 1978), chronic sinusitis (Albegger 1978), smoking (Ailsby and Ghadially 1973). In other words, in man, certain ciliary axoneme abnormalities may be nonspecific responses rather than gene-induced conditions per se. Therefore, simply finding defects characteristic of the human immotile cilia syndrome in axonemes from *hpy/hpy* mice does not necessarily imply that this mutant type constitutes a good animal model for the human condition. It is necessary to show that all of the observed axonemal defects stem from the mutant state or to otherwise account for their presence. In this regard, it can be stated with assurance that axonemal defects, as described herein, were not encountered in extended surveys of cilia and flagella from nonmutant (+/+ and *hpy*/+) littermates. Since mutants and nonmutants were obviously raised together and subjected to identical conditions, environmental factors can be ruled out as causative agents. Neither were axonemal defects observed in tissue samples from a different male-sterile mutant in which the sperm flagella are functionally normal (Bryan 1977b). All of the foregoing observations argue strongly in favor of a genetic basis for the defects observed. However, it should be mentioned that the observed variations in the numbers of central tubules might conceivably stem from errors associated with an increase in the rate of ciliogenesis promulgated by the presence of dynein-deficient and presumably inactive cilia (for details see Bryan 1983). With this proviso it would appear reasonable to conclude that the major axonemal defects encountered in *hpy/hpy* mice are gene-induced. Next, there is the question of whether this mutant mouse exhibits any of the key clinical symptoms. In a recent article Afzelius (1981b) has listed four major criteria for the classification of human patients. They are: the presence of *situs inversus* in the patient or in a close family member, ciliary abnormalities (dynein arm deficiencies, abnormally oriented cilia),

little or no tracheobronchial clearance of mucus, and in males immotile spermatozoa of normal appearance. Unquestionably, mice homozygous for the *hpy* mutation do not meet all of these requirements. As reported above, spermatozoa of mutants completely lack flagella, no animals manifest *situs inversus,* and there is no obvious increase in the incidence of respiratory problems. At first glance, this latter finding is rather perplexing since the frequency of dynein-deficient ciliary axonemes is within the range (30–60%) reported for the immotile cilia syndrome (Afzelius 1981 a). Hence, it would appear logical to expect that, as is the case with human patients, mutant mice would also exhibit signs of respiratory distress stemming from reduced tracheobronchial clearance. Investigations of ciliary ultrastructure and of mucus clearance rates in two patients with another familial condition (Sturgess et al. 1980) are of interest in this regard since they provide a possible explanation. In these patients, ciliary axonemes possessed normal dynein arms but lacked central-pair tubules and also showed a displacement of an outer doublet (number one). Ciliary motility levels were judged to be about 10% of normal; mucus clearance was about 34% of normal in the prone position but fell to zero when the patients were upright. Thus, it is clear that when ciliary motility levels are marginal the effects of gravity can play a major role in determining the overall efficiency of the mucus-clearance process. So it is conceivable that the mucus clearance process in mice is relatively more efficient and, therefore, the observed level of dynein deficiency is less of an impediment than is a comparable level in man.

As reported above, the levels of dynein deficiency in oviducal and ependymal cilia were not markedly higher than those for tracheal cells. Hence, it does not appear likely that either the reduced breeding performance of mutant females or the development of hydrocephalus (in both sexes) can be primarily attributed to a lack of ciliary activity.

The predominant axonemal defect encountered in *hpy/hpy* mice (a deficiency of dynein arms) closely resembles that characteristic of the vast majority of human patients diagnosed as having the immotile cilia syndrome. In this regard, therefore, this mutant type may serve as a useful model for the human condition. However, in view of the findings concerning other axonemal defects which, as yet, have not been seen in immotile cilia syndrome patients, a fuller clarification of its status vis-a-vis the human condition must await further research.

It is clear from the literature that a relatively broad spectrum of axonemal defects has been encountered in different patients with the immotile cilia syndrome (e.g., Afzelius and Eliasson 1979; Afzelius 1981; Baccetti et al. 1979; Rott 1979; Sturgess et al. 1979). A similar heterogeneity may also exist in mice for, in addition to *hpy,* there are two other male-sterile mutations (*hop,* Johnson and Hunt 1971; *qk,* Bennett et al. 1971) which show similar flagella axoneme defects. Unfortunately, while spermiogenesis has been well studied in these mutants, comparatively little information is available concerning the nature and frequency of any ciliary axoneme

defects. However, it is quite probable that these mutant types may, in fact, represent additional examples of the murine equivalent of the immotile cilia syndrome.

Acknowledgement: The author wishes to thank the staff of the Central Electron Microscope Facility, University of Georgia, for photographic assistance.

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Accepted January 21, 1983