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ORIGINAL ARTICLE

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Immunohistochemical analysis of rat and human respiratory cilia with anti-dynein antibody: comparison between normal cilia and pathological cilia in primary ciliary dyskinesia

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Abstract Wistar Imamichi rat and human respiratory cilia were examined with anti-dynein antibody (AD2), which is specific for sea urchin sperm flagellar dynein. AD2-labelled fresh-frozen normal rat and human cilia stained clearly by immunofluorescence and the peroxidase-antiperoxidase (PAP) technique. On immunoelectron microscopy, AD2 labelled the outer dynein arms of normal human cilia. Paraffin-embedded normal human cilia also stained by immunofluorescence, although not always clearly. Neither the cilia of WIC-Hyd male rats, an animal model of Kartagener's syndrome, nor human cilia from patients with primary ciliary dyskinesia (PCD) reacted positively by the immunofluorescence or PAP technique. Western blots of normal rat cilia yielded a single band of about 450 kDa. In conclusion, AD2 recognizes the outer arm dynein heavy chains of healthy cilia and may be useful in diagnosing and classifying PCD light microscopically especially when only paraffin-embedded specimens are available. This approach may be of potential use for better defining and classifying PCD.

Key words Immunostaining · Primary ciliary dyskinesia · Kartagener's syndrome · Immotile cilia syndrome · WIC-Hyd rat

Introduction

Primary ciliary dyskinesia (PCD) is a congenital disease of ciliary function, commonly associated with flagellar dysfunction [14]. The typical clinical manifestations of PCD include recurrent sinopulmonary infection, situs inversus, otitis media and male sterility. PCD can also be associated with congenital heart disease, congenital deafness, skeletal malformations, schizophrenia, variable impairment of female fertility and hypoplasia or aplasia of the frontal sinus (and hydrocephalus especially in rats) [11, 14, 16]. Kartagener's syndrome and immotile cilia syndrome are included in this category.

Although measures of ciliary function (photometric measurements of ciliary beat frequency or radioisotopic measurement of mucociliary clearance) can contribute to making the diagnosis [4, 12], the mainstay of the diagnosis of PCD remains the ultrastructural appearance of cilia. In most cases, defective inner or outer dynein arms (or both) of the cilia are responsible for the impaired motility. The ATPase activity of the dynein arms is thought to be important for both ciliary motility and mucociliary transport [5].

In 1976, Afzelius first demonstrated ultrastructurally that the absence of dynein arms was the cause of the defective ciliary function underlying recurrent respiratory infections in PCD patients [1]. Specimens of patients suspected of having PCD may not have been properly embedded in blocks for electron microscopy prior to this date, leaving only paraffin-embedded specimens available.

In this study, we evaluated rat and human respiratory cilia immunohistochemically by three different methods using anti-dynein antibody (AD2), which is specific for sea urchin sperm flagellar dynein [20]. By comparing the normal cilia with the pathological cilia from WIC-Hyd male rats, the first experimental animal model of Kartagener's syndrome [16], and from human PCD patients, we attempted to detect the role of dynein in the pathogenesis of PCD and to establish a method of diagnosing or classifying PCD using paraffin-embedded specimens. In addition, dynein protein was evaluated by Western blotting.

Materials and methods

Ten normal Wistar Imamichi rats (1–10-weeks-old), ten WIC-Hyd male rats (1–2-weeks-old, kindly given by M. Koto, Chugai Phar-

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Table 1 Clinical characteristics of primary ciliary dyskinesia patients

Patient number	Age (years)	Sex	Sinusitis	Bronchiectasis	Situs inversus	Infertility	Otitis media	Others
1	34	M	+	+	−	+	+	Clubbing
2	71	M	+	+	+	+	?	Lung cancer, clubbing
3	37	M	+	+	+	Unmarried	−	Heart failure, clubbing
4	17	M	+	+	+	Unmarried	+	Bronchiolitis
5	36	M	+	+	Dextrocardia	+	−	?

maceutical Company, Tokyo, Japan), and 20 normal human volunteers (33–72-years-old, mostly taken by bronchoscopy) were evaluated. A previous electron microscopic examination of WIC-Hyd male rats showed a complete lack of outer and inner dynein arms of the cilia in respiratory tracts and ependymal cells [16]. Each WIC-Hyd male rat had situs inversus and immotility of the tracheal cilia was confirmed under light microscopy immediately after vivisection. Rat and human respiratory epithelial samples were freshly frozen in OCT compound and smeared on poly-L-lysine-coated glass slides at a thickness of 6 μm.

Epithelial specimens from the 20 normal volunteers and five PCD patients (17–71-years-old, Table 1) were taken by bronchoscopy, open lung biopsy, or from resected tissue of patients with other disease (such as lung cancer) and fixed in 10% formalin for 24 h. They were then embedded in paraffin, and 3 μm-thick sections were mounted on glass slides. Four of the five patients were diagnosed with PCD with both inner and outer dynein arms determined to be defective on previous electron microscopic examination [6, 9, 19]. The electron microscopic appearance of the specimen from patient number 5 was unknown.

AD2 was used as primary antibody for each immunostaining procedure and Western blotting. This antibody was raised in a rabbit against sea urchin *Anthocidaris crassispina* sperm flagellar dynein (dynein 1) heavy chain antigens, which were obtained by electrophoresis of the ATPase-active fraction of 21S proteins extracted from a high salt extracting solution [20].

For immunofluorescence specimens were treated with 4 mM magnesium chloride ($MgCl_2$) and 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 20 min on glass slides, then fixed with 4% paraformaldehyde in PBS for 40 min. (Paraffin-embedded specimens were pretreated with 0.4% pepsin in 0.01 N hydrochloric acid (HCl) for 20 min at 37° C after deparaffinization). They were then equilibrated in PBS, blocked with 5% normal goat serum for 20 min and incubated with AD2 (50 μg/ml) overnight at 4° C. After PBS washes, they were incubated with fluorescein isothiocyanate-labelled goat anti-rabbit IgG antibody (100 μg/ml, MBL, Nagoya, Japan).We examined the specimens with a laser scanning confocal imaging system (MRC-600, Bio-Rad Laboratories, Tokyo, Japan).

The peroxidase-antiperoxidase (PAP) technique was used as follows. Specimens were pretreated with 4 mM $MgCl_2$ and 0.1% Triton X-100 in PBS for 20 min on glass slides, then fixed with 4% paraformaldehyde in PBS for 40 min. They were then washed in PBS, blocked with 5% normal goat serum for 30 min and incubated with AD2 (5 μg/ml) for 1 h at room temperature. After PBS washes, they were incubated with peroxidase-conjugated goat Fab to rabbit IgG (165 μg/ml, Cappel, Organon Teknika Corporation, Durham, N.C., USA) and stained with 0.02% diaminobenzidine, 0.065% sodium azide and 0.003% hydrogen peroxide in 0.05 M TRIS/HCl buffer (pH 7.6) for 5–8 min. The nuclei were stained with methyl green.

For immunoelectron microscopy fresh-frozen normal human specimens were prepared exactly the same as in the PAP technique until the washing step after incubation with AD2. They were then incubated with 15 nm of gold-conjugated goat anti-rabbit IgG (diluted to 1/100, Zymed Laboratories, San Francisco, Calif., USA) for 1 h at room temperature, washed in PBS, refixed with 1% glutaraldehyde for 1 h, washed again in phosphate buffer, treated with 1% osmium tetroxide for 1 h, and dehydrated with alcohol and acetone. After embedding in Spurr's resin (Polysciences, Warrington, Pa., USA) on glass slides, they were removed from the slides, cut into ultrathin sections, and examined with an electron microscope (JEM-1200EX, JEOL, Tokyo, Japan).

Western blotting was performed by a slightly modified method reported previously [20]. Briefly, the tracheobronchial wash from normal Wistar Imamichi rats was centrifuged at 500 *g* for 5 min. The supernatant containing the cilia was subsequently centrifuged at 12000 *g* for 10 min. The pellet was resolved in a sample buffer for the Laemmli system [8]. Slab gel electrophoresis was performed using a 5–10% gradient gel. Proteins were transferred electrophoretically from the gel to nitrocellulose membranes and the blotted proteins were incubated with AD2 (1 μg/ml) overnight at 4° C. Detection was by peroxidase-catalyzed chemiluminescence (ECL, Amersham Japan, Tokyo, Japan).

Results

Rat study

All (n=10) fresh-frozen respiratory cilia from normal Wistar Imamichi rats stained clearly by the immunofluorescence and PAP techniques (Fig. 1). Staining with non-specific rabbit γ-globulin (Cappel, Organon Teknika Corporation) instead of AD2 was used as a control (data not shown). Triton X-100 was used in the fixation step in order to allow the antibodies to penetrate into the tight structure of cilia. The 4% paraformaldehyde fixation immediately after treatment with 0.1% Triton X-100 was essential in the PAP technique. Although this procedure is considered to be optimal for immunofluorescence, fixation with 0.1% Triton X-100 in 10% formalin solution was also adequate to obtain clearly stained images.

None of the fresh-frozen respiratory cilia from WIC-Hyd male rats (n=10) reacted positively when stained by either the immunofluorescence or the PAP technique (Fig. 2). In one case, some pathological cilia appeared to stain very faintly by both methods, but similar background staining was seen in the non-specific rabbit γ-globulin-stained controls (data not shown).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and Western blotting of normal rat tracheobronchial washings yielded a single band of about 450 kDa stained with AD2. The 500 *g* supernatant of crude tracheobronchial washings was examined under a phase-contrast microscope, and abundant respiratory cilia were found without significant contamination (data not shown).

Human study

All (n=20) fresh-frozen normal human respiratory cilia stained clearly by both the immunofluorescence and PAP

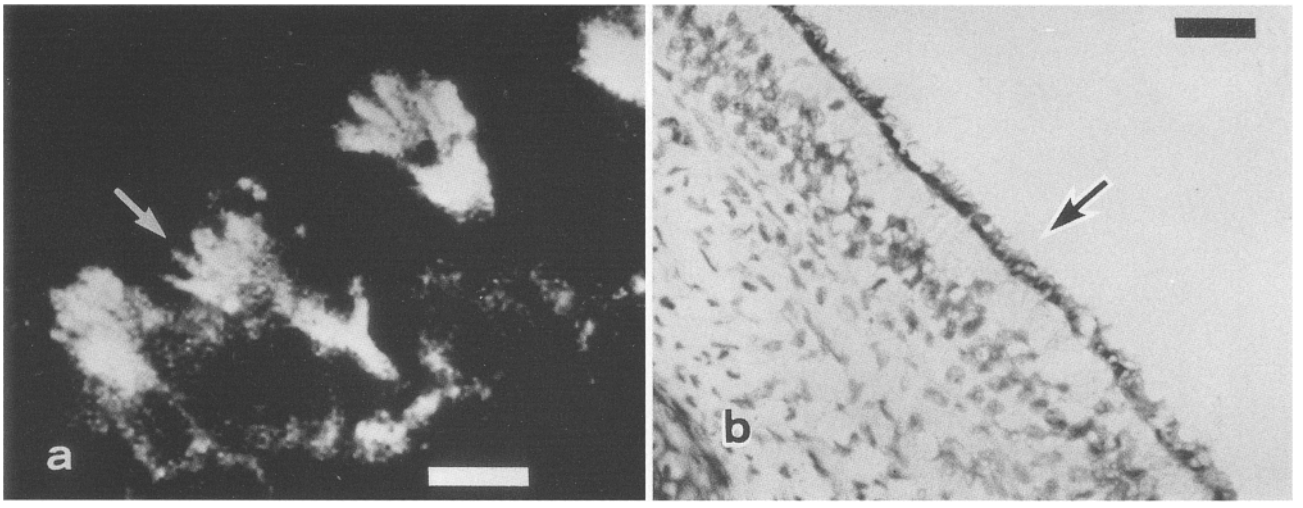


Fig. 1a, b Immunofluorescence and peroxidase-antiperoxidase (PAP) staining of fresh-frozen normal Wistar Imamichi rat respiratory cilia using AD2. **a** Immunofluorescence, *bar*=20 μm. **b** PAP, *bar*=40 μm. The respiratory cilia (*arrows*) are clearly stained by both techniques

techniques (Fig. 3). Staining with non-specific rabbit γ-globulin instead of AD2 was used as a control (data not shown). Not only respiratory cilia but also the cytoplasm of basal cells were clearly stained by immunofluorescence (Fig. 3a).

Figure 4 shows the electron microscopic appearance of normal human respiratory cilia by the ordinary method using tannic acid and glutaraldehyde in the fixation step [15]. Both inner and outer dyneim arms are clearly seen. Immunoelectron microscopy with AD2 labelled the outer dynein arms of normal human respiratory cilia (Fig. 5). Although not every outer dynein arm was stained with colloidal gold, almost no gold was observed in other locations.

Some normal human cilia embedded in paraffin (n=12) stained clearly on immunofluorescence, but not as strongly as the fresh-frozen specimens (Fig. 6a, *right panel*). Other paraffin-embedded normal human cilia (n=8) failed to react by this technique (data not shown). None of the paraffin-embedded human cilia from PCD patients (n=5) reacted positively on immunofluorescence (Fig. 6b, *right panel*). The orientation of respiratory cilia was easily determined by comparing negative immunofluorescence images with the transmission images (Fig. 6b, left panel).

Discussion

Kartagener's syndrome is reported to have an incidence of 1:18000–229000 [11]. The incidence of immotile cilia syndrome is thought to be about twice that of Kartagener's. PCD may occur even more frequently. The genetic type of PCD is thought to be mainly autosomal recessive [11], but in Wistar Imamichi rats it seems to be X-linked [16]. In Japan, 65 cases of PCD have been examined by electron microscopy, and in 94% of these cases dynein arms were abnormal, including defective inner arms

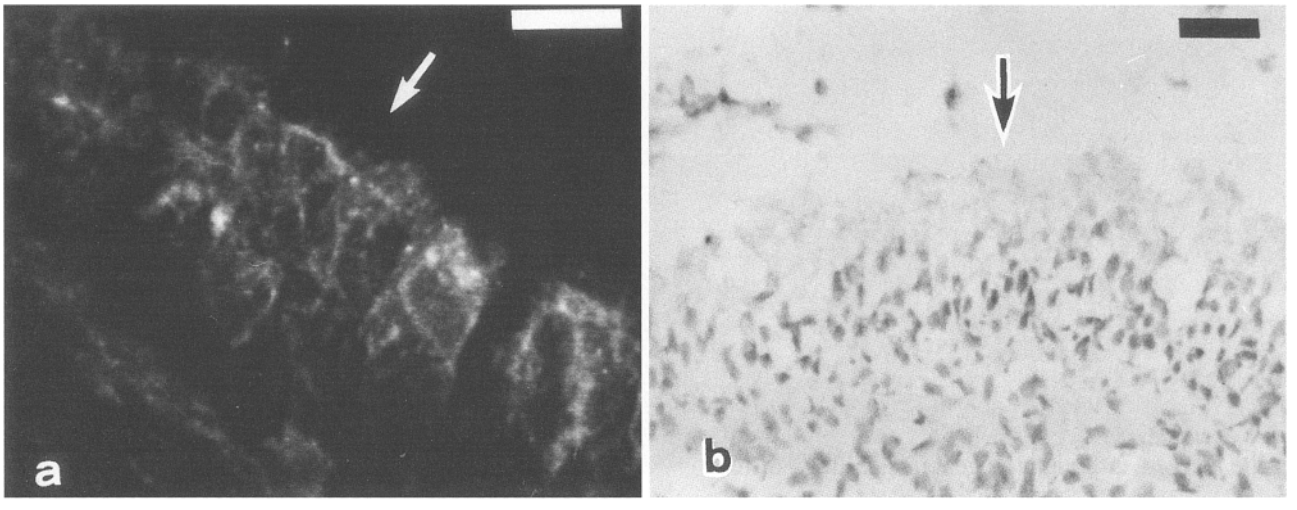


Fig. 2a, b Immunofluorescence and PAP staining of fresh-frozen pathological respiratory cilia of WIC-Hyd male rats using AD2. **a** Immunofluoescence, *bar*=20 μm. **b** PAP, *bar*=40 μm. No respiratory cilia (*arrows*) have been stained by either technique

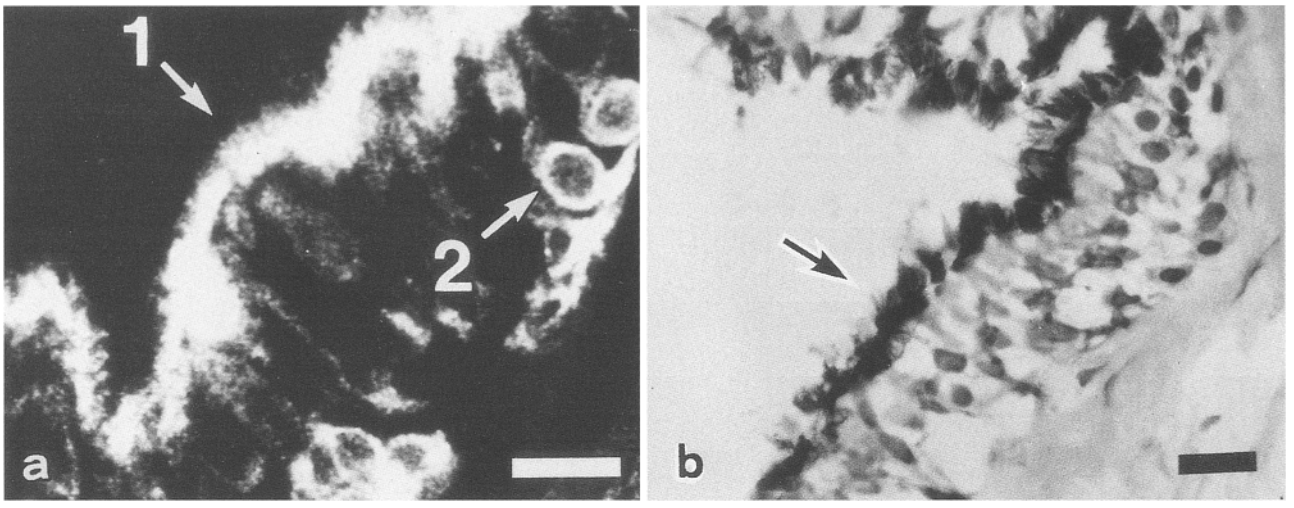


Fig. 3a, b Immunofluorescence and PAP staining of fresh-frozen normal human respiratory epithelia with AD2. **a** Immunofluoescence, *bar*=20 μm. Not only respiratory cilia (*arrow 1*) but also the cytoplasm of basal cells (*arrow 2*) are clearly stained. **b** PAP, *bar*=20 μm. The respiratory cilia (*arrow*) are clearly stained

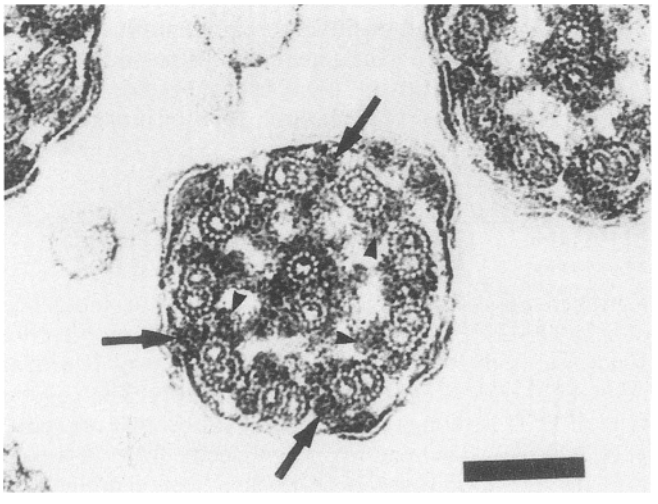


Fig. 4 Electron microscopic appearance of normal human respiratory cilia by the ordinary method using tannic acid-containing fixation. Both inner (*arrowheads*) and outer (*arrows*) dynein arms are clearly seen. *Bar*=0.1 μm

(47%), outer arms (6%), or both arms (47%) [6, 9, 19]. When dynein arms are absent or shortened, the axonemal dynein heavy chains, with their ATPase activity, may be lacking.

In this study, AD2 recognized both rat and human normal respiratory cilia, but not the cilia of WIC-Hyd male rats or patients with PCD. This finding suggests that there is homology between the heavy chains of sea urchin sperm flagellar dynein and those of rat and human respiratory ciliary dynein. Furthermore, PCD patients and WIC-Hyd male rats are similar in that they are both lacking the antigen, although the genetic aetiology differs.

It remains questionable whether there is any difference between the dynein of the inner and outer arms of cilia. The axonemal outer dynein arms have been reported to be composed of two or three heavy chains (two in mammals) whose molecular weight is about 450 kDa, three intermediate chains of 70–125 kDa and several light chains of approximately 15–25 kDa [17]. However, the inner dynein arms of Chlamydomonas flagella have been reported to have six heavy chains [18]. Functionally, the inner dynein arms appear to be essential for ciliary motility, while the outer arms appear to regulate the beating velocity of cilia [3]. In this study, AD2 labelled only the outer dynein arms (Fig. 5). Thus, there may be a difference in antigenicity between the inner and outer dynein arms. Because it is possible that the antibodies could not penetrate fully into the cilia, any difference in antigenicity must be confirmed by post-embedding immunoelectron microscopy.

The molecular weight of 450 kDa on Western blots is about the same as that previously reported for both Chlamydomonas flagellar and sea urchin sperm flagellar dynein heavy chains [17]. It seems likely therefore, that the blotted protein represents at least one of the two heavy chains in the outer dynein arms of rat respiratory cilia.

In order to distinguish the cilia of PCD patients from normal cilia using paraffin-embedded specimens, they should be stained simultaneously by immunofluorescence, since normal human specimens stain more clearly by immunofluorescence than by the PAP technique. In addition, it is very helpful to compare the staining of pathological cilia with the transmission image in order to conclude that staining is negative, since the orientation of cilia is unclear when they do not stain by immunofluorescence (Fig. 6). Some normal control human paraffin-embedded cilia, especially old specimens, fail to stain by this method, perhaps because high molecular weight protein is easily degraded during the paraffin-embedding procedure. Thus, immunostaining of paraffin-embedded specimens can yield false negative results, but if the respiratory cilia stain clearly, they inevitably contain the dy-

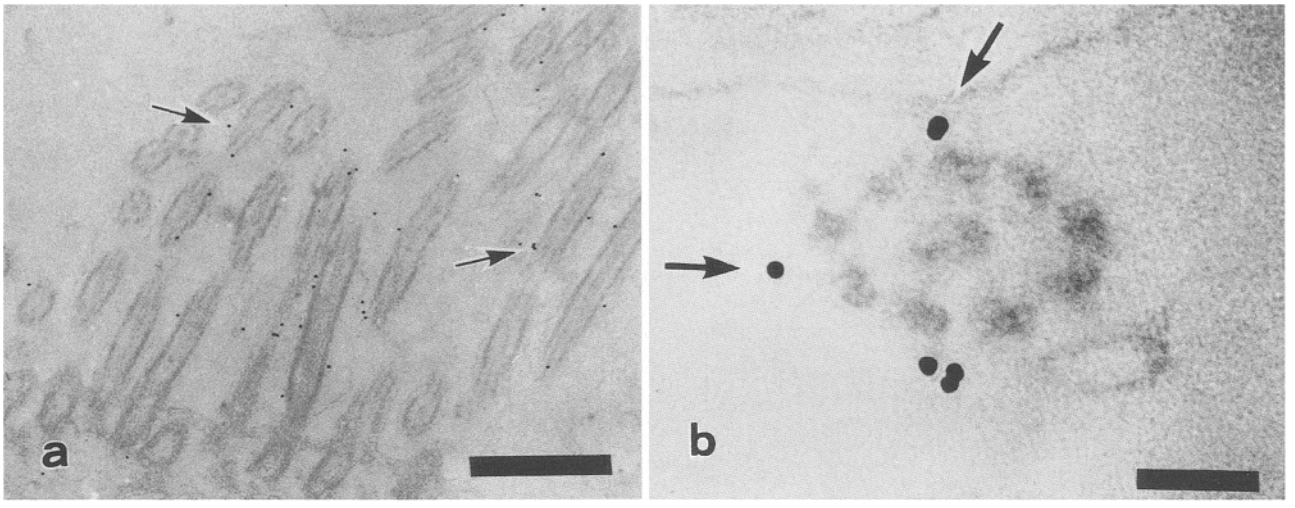


Fig. 5a, b Pre-embedding immunoelectron microscopy of fresh-frozen normal human respiratory cilia with AD2. *Arrows* indicate colloidal gold with a diameter of 15 nm. Almost every gold particle was located at the position of the ciliary outer dynein arms. **a** *Bar*=1 μm, **b** *bar*=0.1 μm

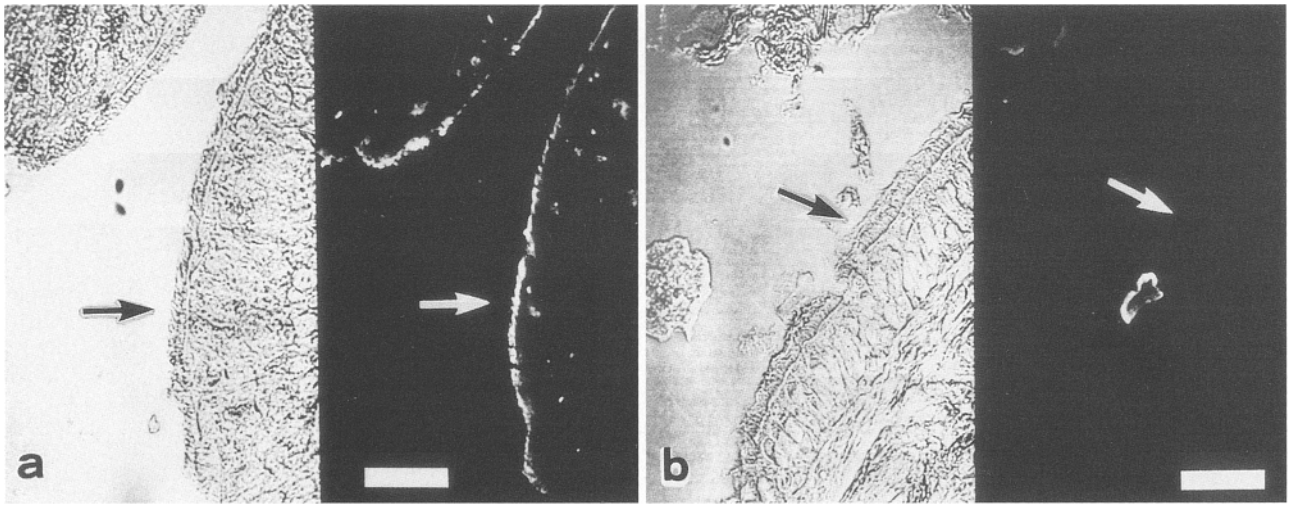
nein fragments. In order to improve retention of antigen, other methods of fixation (fixing in formol calcium or the AMeX method) before embedding in paraffin, or embedding in low melting point wax may be effective [2, 10, 13]. Specimens suspected of PCD may need to be embedded in paraffin in this manner for future immunohistochemical analysis.

Because axonemal dynein heavy chains are assumed to be produced in the cytoplasm of basal cells and then move into the cilia during the differentiation from basal cells to ciliated cells, intense staining of fresh-frozen normal human cytoplasm from basal cells should localize dynein heavy chains along this route (Fig. 3a). Further investigation is needed to confirm this finding.

The direct roles of dyskinetic cilia on the pathogeneses of many congenital abnormalities such as situs inversus, congenital heart diseases (atrial septal defect, ventricular septal defect, transposition of great vessels and Fallot's tetralogy) and skeletal malformations are still not well known. A rotation abnormality during embryogenesis due to ciliary immotility has been suggested though not yet studied. Further study on dynein may yield important information on these disorders.

In conclusion, AD2 recognizes the outer arm dynein heavy chains of normal respiratory cilia, and all the diseased cilia in PCD examined here were found to lack the antigen. When paraffin-embedded respiratory cilia clearly stained by AD2 immunostaining, they were found to express the dynein fragments. Thus, AD2 may be useful in diagnosing and classifying PCD via light microscopy especially when only paraffin-embedded specimens are available. This approach may be of potential use for better defining and classifying PCD.

Fig. 6a, b Immunofluorescence image of paraffin-embedded human respiratory cilia using AD2. **a** Normal control, *bar*=40 μm. **b** Primary ciliary dyskinesia, *bar*=40 μm. The left panel represents the transmission image. The respiratory cilia (*arrows*) of (**a**) stained clearly, but those of (**b**) did not react positively by this method



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References

1. Afzelius BA (1976) A human syndrome caused by immotile cilia. Science 193:317–319
2. Brandtzaeg P (1982) Tissue preparation methods for immunocytochemistry. In: Bullock GR, Petrusz P (eds) Techniques in Immunocytochemistry, vol 1. Academic Press, London, pp 1–75
3. Brokaw CJ, Kamiya R (1987) Bending patterns of Chlamydomonas flagella: IV. Mutants with defects in inner and outer dynein arms indicate differences in dynein arm function. Cell Motil Cytoskeleton 8:68–75
4. Camner P, Mossberg B, Afzelius BA (1975) Evidence for congenitally nonfunctioning cilia in the tracheobronchial tract in two subjects. Am Rev Respir Dis 112:807–809
5. Gibbons IR, Rowe AJ (1965) Dynein: a protein with adenosine triphosphatase activity from cilia. Science 149:424–426
6. Hachiya T, Koizumi T, Hayasaka M, Honda T, Kubo K, Sekiguchi M, Mochizuki I (1993) A case of Kartagener's syndrome with lung cancer. J Jpn Soc Bronchol 15:179–184
7. Koto M, Miwa M, Shimizu A, Tsuji K, Okamoto M, Adachi J (1987) Inherited hydrocephalus in Csk: Wistar-Imamichi rats; Hyd strain: a new disease model for hydrocephalus. Exp Animal 36:157–162
8. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
9. Nogawa S, Toyoda T, Kamo T, Onaka A, Kawai T, Torikata C (1989) A case of immotile cilia syndrome and a review of Japanese cases. Jpn J Thorac Dis 27:505–512
10. Pollard K, Lunny D, Holgate CS, Jackson P, Bird CC (1987) Fixation, processing, and immunochemical reagent effects on preservation of T-lymphocyte surface membrane antigens in paraffin-embedded tissue. J Histochem Cytochem 35:1329–1338
11. Rott H-D (1979) Kartagener's syndrome and the syndrome of immotile cilia. Hum Genet 46:249–261
12. Rutland J, Cole PJ (1980) Non-invasive sampling of nasal cilia for measurement of beat frequency and study of ultrastructure. Lancet Sept. 13:564–565
13. Sato Y, Mukai K, Watanabe S, Goto M, Shimosato Y (1986) The AMeX method: A simplified technique of tissue processing and paraffin embedding with improved preservation of antigens for immunostaining. Am J Pathol 125:431–435
14. Sleigh MA (1981) Primary ciliary dyskinesia. Lancet Aug. 29:476
15. Torikata C, Kawai T, Nogawa S, Ikeda K, Shimizu K, Kijimoto C (1991) Nine Japanese patients with immotile-dyskinetic cilia syndrome: an ultrastructural study using tannic acid-containing fixation. Hum Pathol 22:830–836
16. Torikata C, Kjimoto C, Koto M (1991) Ultrastructure of respiratory cilia of WIC-Hyd male rats. Am J Pathol 138:341–347
17. Witman GB (1989) Perspective: Composition and molecular organization of the dynein. In: Warner FD, Satir P, Gibbons IR (eds) The dynein ATPases. (Cell movement, vol 1). Alan R Liss, New York, pp 25–35
18. Witman GB (1992) Axonemal dyneins. Curr Opin Cell Biol 4: 74–79
19. Yokota T, Ohno N, Tamura K, Seita M, Toshimori K (1993) Ultrastructure and function of cilia and spermatozoa flagella in a patient with Kartagener's syndrome. Intern Med 32:593–597
20. Yoshida T, Katsuta K, Takanari H, Izutsu K (1989) Analysis of mammalian dynein using antibodies against A polypeptides of sea urchin sperm flagellar dynein. Exp Cell Res 184:440–448