CONCERNING THE UNIVERSALITY OF A MICROTUBULE ANTIGEN IN ANIMAL CELLS

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INTRODUCTION

After the discovery that cytoplasmic microtubular elements can become aggregated into well-defined paracrystals (PC) under the influence of the Vinca alkaloid, vinblastine (1), it was possible for us to isolate and purify rapidly such PC from an established line of murine cells (2). This material was shown to possess an ATPase activity and a single major antigenic determinant (2). Antisera obtained from immunized rabbits contained an antibody that agglutinated readily the isolated PC and became attached specifically to both intracellular PC and the mitotic spindle (2). The finding that this antibody could bind to the spindle not only in

the cell of origin but also in avian and human cells (2) showed that all the higher vertebrates may possess either the same or a closely related antigen of the microtubules. The present survey was undertaken to extend the above observations and determine whether a common antigen could be demonstrated in microtubules of the lower animals.

MATERIALS AND METHODS

Cell Cultures

Established lines of strain HeLa, of human origin, CV-1, from African green monkey kidney, and L_2 murine fibroblasts were the same as those employed previously in this laboratory (2). A cloned subline of the murine neuroblastoma C-1300, originating from Jackson Laboratories, Bar Harbor, Maine, was provided by Dr. J. D. Broome, New York University Medical School. Primary and secondary cultures of chick embryo fibroblast (CEF) were prepared by the standard procedures. Continuous lines of heart cells TH-1 from the box turtle, *Terrapene carolina* (3), and IGH from the iguana, *Iguana iguana*, as well as GL gekko lung of *Gekko gecko*, VSW spleen from a tumor-bearing viper, *Vipera russelli*, were provided by Dr. H. F. Clark, Wistar Institute, Philadelphia, who originated these lines. Dr. Clark also supplied RTG-2 cells derived from the gonad of rainbow trout, *Salmo gairdneri* (4). An established line from the adult ovarian tissue of a mosquito, *Culex quinquefasciatus*, Say (5), was donated by Dr. D. W. Roberts, Boyce Thompson Institute, Yonkers, New York. The subline AeC8 or AeC10 of lepidopteran cells from *Antheraea eucalypti* orginated by Grace (6), was supplied by Drs. A. E. Green and W. W. Nichols, Institute for Medical Research, Camden, New Jersey. I am grateful to all the above mentioned for their generosity.

Culture Media and Conditions

With the exception of insect cells, all lines were propagated in Eagle's minimal essential medium (7) supplemented with 10% fetal bovine serum (FBS). The mosquito cells were grown in a mosquito medium with 20% FBS and moth cells in a modification (8) of Grace's insect tissue culture medium, both purchased from Grand Island Biological Co., Grand Island, New York. In preparation for cytological examination, cells were plated on pieces of cover slip glass lying in 50-mm Petri dishes and were kept in humidified incubators gassed continuously with a mixture of 5% CO_2 in air. Mammalian, avian, and iguana cells were maintained at 37°C, all other cells at 22°–25°C. Development of long "axonal" processes of neuroblastoma was induced by treatment with 10^{-4}–10^{-5} M bromodeoxyuridine (BUdR) for 72 hr, according to the report of Schubert and Jacob (9). Formation of microtubular PC was elicited by treating cultures for 18–24 hr with 10^{-5} M vinblastine sulphate (Eli Lilly and Co., Indianapolis, Ind.).

Antisera

Rabbit antisera were prepared after repeated immunization of albino rabbits with 50 μg portions of highly purified PC, as previously described (2). *Immunofluorescence staining* was carried out as described previously (2). Cover-glass cultures were washed in phosphate-buffered saline (PBS, 10), drained, and fixed in 100% acetone at −40°C for 5 min. After hydration in PBS the specificity of serum binding was tested by the indirect method (11). Briefly, preimmune or immune sera diluted 1:4 in PBS were layered on the cultures. After incubation at 37°C for 30–45 min unattached antibody was removed by repeated washing with PBS, then fluorescein conjugates of antirabbit globulin (Microbiological Associates, Inc., Bethesda, Md.) were applied in the same way as the rabbit sera. Finally, the preparations were mounted in a 1:1 mixture of glycerol and PBS and examined in the Zeiss Ultraphot microscope (Carl Zeiss, Inc., New York) under phase-contrast or UV optics. The cells of *A. eucalypti* do not attach firmly to the cover glass. They were therefore centrifuged into pellets, resuspended in PBS, spread on cover slips or slides, and air dried over ice before fixation and staining.

Electron Microscopy

Pellets of *A. eucalypti* cells were prepared, fixed, and processed for thin sectioning and electron microscopy as described previously (12).

RESULTS

Immunofluorescence Labeling

A survey of the various species of vertebrate, lepidopteran, and dipteran cells listed in Table I revealed that all of them may contain a common or closely related antigen in their microtubules. The antibody prepared against PC of mouse L cells was apparently bound with about the same affinity to all the vertebrate cells tested, ranging upward on the evolutionary tree from a teleostean fish to man (Table I, Figs. 1–6 and 9). Evidence was obtained indicating that moth cells of *A. eucalypti* and mosquito cells of *C. quinquefasciatus* also contained the same or related antigen, although the binding affinity in the invertebrates to microtubular elements was much weaker (Table I).

TABLE I

Intracellular Labeling with Specific Antiserum

Animal	Cell type or species	Vinblastine induced PC	Mitotic apparatus
Human	HeLa	++++	++++
Monkey	CV-1	++++	++++
Mouse	L_2	++++	++++
Mouse	neuroblastoma C-1300	++++	++++
Chicken	embryonic fibroblasts	++++	++++
Turtle	TH-1	++++	N.E.
Iguana	IGH	++++	N.E.
Gekko	GL	++++	N.E.
Viper	VSW	++++	N.E.
Teleost fish	RTG-2	++++	++
Mosquito	*Culex quinquefasciatus*, Say	++	+
Moth	*Antheraea eucalypti*	++	N.E.

+, ++, weak specific fluorescence; +++, moderate specific fluorescence; ++++, intense specific fluorescence; N.E., not examined

The neuroblastoma line C-1300 when proliferating rapidly in vitro possessed, as expected, the same microtubular antigen. When neuroblastoma cells were placed in a BUdR-containing medium so as to induce formation of "axons" or elongated processes (9), specific immunofluorescence could be observed in such processes, presumably because they contained micro- or neurotubules. Electron microscope examination of thinly sectioned "axons" revealed an abundance of such tubular elements in them (unpublished observation).

With the invertebrate species examined, immunofluorescence of the PC was apparently much lower than that of cells from the vertebrates. That such a low degree of antibody binding was not due to a paucity of microtubules was established by examining thinly sectioned cells from *A. eucalypti*. These cells possessed very numerous microtubules, especially in the centrosphere region and within prominent cell processes, as illustrated in Figs. 10 and 11. Abundance of microtubules in this cell line most probably accounts for the formation of rela-

FIGURES 1–9 Selected areas from cultures on cover glass, fixed and stained with fluorescein-conjugated anti-PC serum. When viewed under UV light, specific fluorescence of either PC or mitotic apparatus is demonstrable.

FIGURES 1–3 Selected examples of immunofluorescence of the mitotic apparatus (arrows) in CV-1 cells. Fig. 1, × 500; Fig. 2, × 720; Fig. 3, × 750.

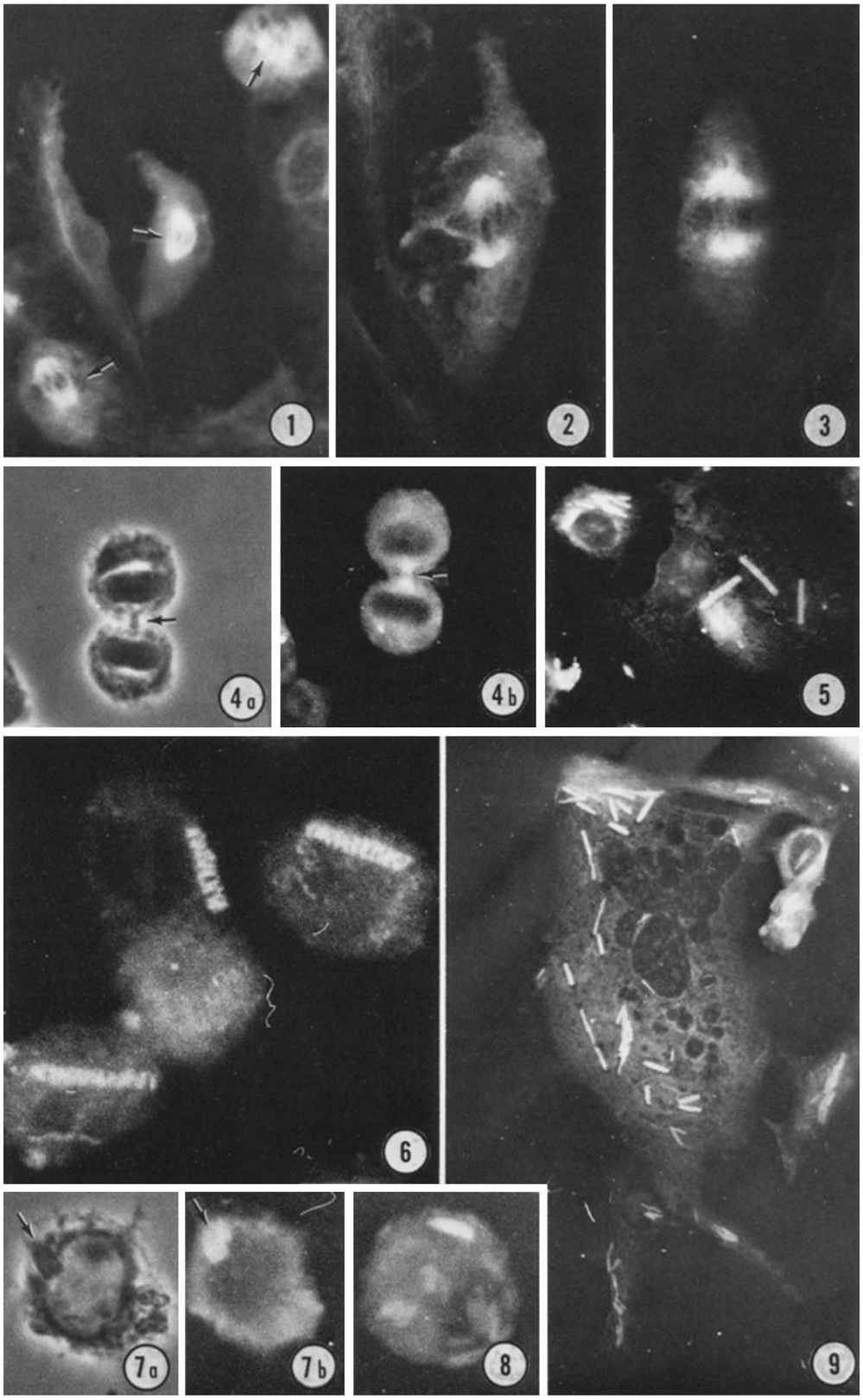
FIGURE 4 A neuroblastoma cell at telophase. In (*a*) note that the midbody or intercellular bridge (arrow) is clearly evident by phase contrast, and in (*b*) that it contains intensely fluorescing material (arrow). It is known that such bridges, which at the terminal stage of separation become the residual body of Flemming, are rich in microtubules (20). × 1100.

FIGURE 5 Neuroblastoma cells after treatment with vinblastine. The PC fluoresce intensely. × 375.

FIGURE 6 HeLa cells treated with vinblastine. The relatively large PC appear to be in the form of helices with prominent gyres. × 1600.

FIGURES 7 and 8 Cells of *A. eucalypti* after exposure to vinblastine. When observed by 7 *a* phase contrast the PC are dense, and 7 b under UV they possess low specific fluorescence. Fig. 8 is another example illustrating specific fluorescence. × 1400.

FIGURE 9 RTG-2 fish cells after treatment with vinblastine. Several brightly fluorescing PC are evident in the cytoplasm of the large cell occupying the center of the field. × 390.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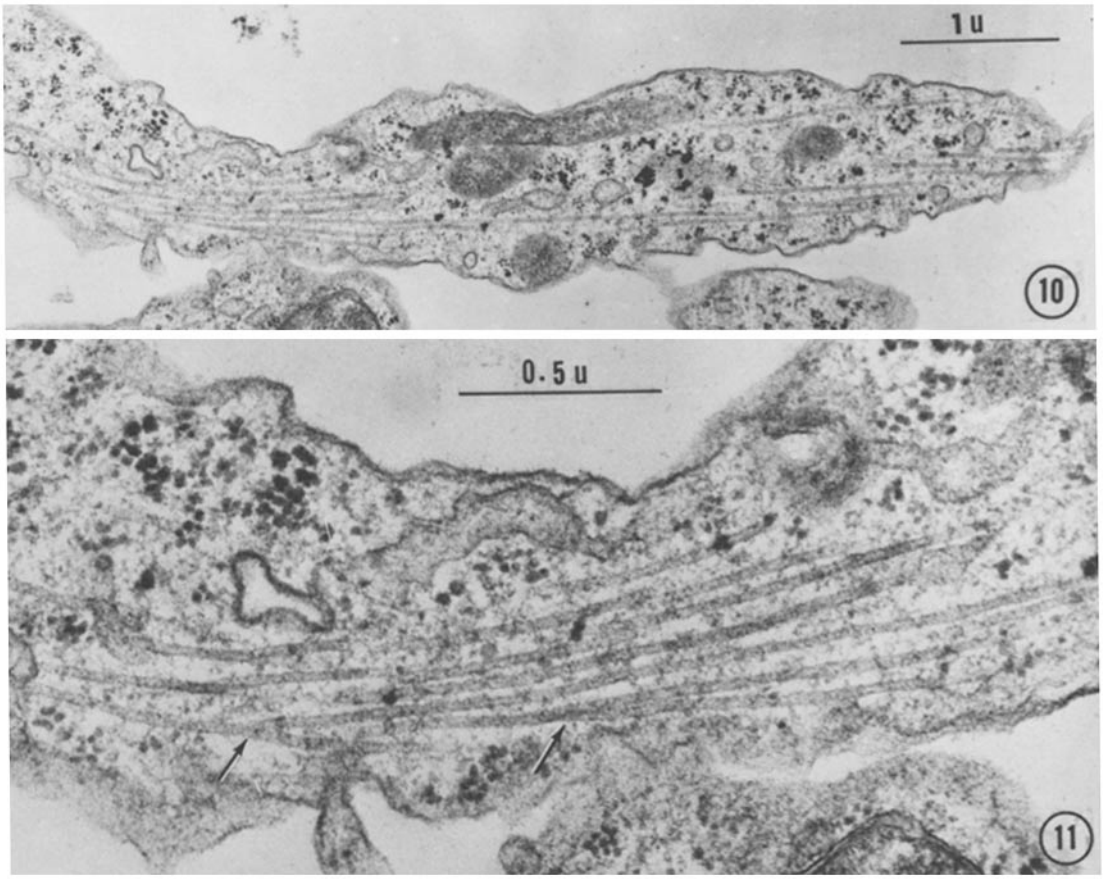

FIGURE 10 Thin section through an elongated process emanating from a cell of *A. eucalypti*. The microtubules, disposed in parallel with the long axis of the process, course through the cytoplasmic matrix which also contains the usual organelles including mitochondria, vacuoles, and ribosomes. × 22,000.

FIGURE 11 Selected area from the above example, illustrated at higher magnification to demonstrate the microtubules. Arrows point towards regions where branching of microtubules may have occurred. × 54,000.

tively massive PC under the influence of vinblastine (Figs. 7 and 8). The prominence of the mitotic spindle in these cells has been recognized previously by others (13).

Occasionally, the PC located within cells fixed and processed for examination by immunofluorescence were in the form of a tightly wound helix (Fig. 6). A correspondingly dense helical configuration of the PC was observed in the phase-contrast images. Such helical structures have been noted in HeLa and other mammalian cells. The tightness of coiling was, however, quite variable from culture to culture and from one region of the same culture to another. It appears most likely that some kind of uncoiling of the PC occurred during the fixation and hydration sequence. The significance, in molecular terms, of the change in configuration from a tightly packed paracrystalline into a looser helical form is not obvious at present but implies, as already noted (2), the existence of another order of organization in the PC, above the level of the microtubule.

Immunodiffusion and Agglutination Tests

Independent evidence concerning occurrence of a common or related antigen in various cells was sought by means of immunodiffusion in agar gels or by specific agglutination of purified PC in concentrated suspensions. The immunological tests on

isolated PC from HeLa, neuroblastoma, L_2, and *C. quinquefasciatus* cells conformed to the procedures described previously (2). Antisera at dilutions of 1/4 to 1/10 were used for testing agglutination. At a 1/4 dilution the immune serum caused the clumping of PC from all the four species, but at greater serum dilution only the PC from human and mouse cells became noticeably aggregated.

Immunodiffusion on a microscale by the Ouchterlony technique was conducted on glass slides with wells 1–1.5 mm in diameter, set about 2.5–3 mm apart. About 5–20 μg of each antigen was added to the peripheral wells and about 10 λ of undiluted antiserum to the center well. As clearly as could be judged within the limits of the microtechnique, single precipitin lines were formed between the center and each peripheral well loaded with PC from HeLa, L_2, and neuroblastoma cells. The precipitin lines became confluent, indicating the presence of a closely related antigenic determinant in the PC of the cell types tested. These observations support the results with immunofluorescence, summarized in Table I.

DISCUSSION

The present survey on the occurrence of an antigen in microtubules of animal cells extends our previously reported identification of a common or related antigen in cells of avian, murine, and human origin. It is yet unknown whether this antigen is a protein, glycoprotein, carbohydrate, or some other chemical species. It also remains to be determined whether this antigenic determinant occurs ubiquitously among the lower invertebrates and plants.

Ubiquitous occurrence of some proteins and antigens is known among eukaryotic cells. For example, histone IV, derived from the pea plant or bovine tissue, possesses very similar aminoacid sequences, implying unusual evolutionary stability of this protein (14). Purified trypsins originating from bovine, porcine, elasmobranch fish, and Echinoderm (starfish) sources possess immunological cross-reactivity, as determined by tests of immune precipitation and inhibition of enzymatic function (15). The degree of reactivity of the trypsins with heterologous antibodies is proportional to their relatedness according to the chronological evolutionary sequence.

Evidence against the universality of at least some microtubular antigens was provided recently by Fulton et al. (16). These authors showed that a common antigenic determinant occurs in a tubulin from the outer fibers of flagella derived from several species of sea urchins and a sand dollar but is conspicuously absent from a species of starfish, indicating that distribution of this antigen is restricted even within the phylum Echinodermata. It is, however, important to emphasize that tubulins from various classes of microtubules are a group of proteins each of which possesses a different chemical and antigenic composition (17–19). It is now assumed that various types of microtubules are organized from a composite of similar but not identical tubulins, each of which may contain a unique antigenic determinant. Future work may identify which of these tubulins contains the antigen identified in the mitotic apparatus of animal cells examined in the present study.

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