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Visualization of a system of filaments 7–10 nm thick in cultured cells of an epithelioid line (Pt K2) by immunofluorescence microscopy

(intermediate filaments/tonofilaments/keratin/autoantibodies/mitotic drugs)

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ABSTRACT **During our studies with antibodies against structural proteins of the cytoskeleton of eukaryotic cells we have observed that sera from many normal rabbits decorate a fiber system in cells of the established rat kangaroo cell line Pt K2. The display and organization of these fibers are different from those of microfilament bundles (decorated by antibody to actin) and microtubules (decorated by antibody to tubulin). This new fiber system can be further distinguished by its resistance to reorganization when cells are treated with Colcemid or cytochalasin B. The decoration of this fiber system is not detected if Pt K2 cells are fixed with formaldehyde. Such sera also appear to decorate swirls of perinuclear fibers in mouse Neuro 2a cells, and in mouse 3T3 cells treated with mitotic drugs. Comparison of the immunofluorescence pictures with electron microscopic data suggests that the sera are visualizing bundles of intermediate 7- to 10-nm filaments.**

Electron microscopical analysis of a variety of tissues and cells grown in culture has documented three major fiber systems: microfilaments (5–6 nm), intermediate filaments (7–10 nm), and microtubules (20–25 nm) (see, for instance, ref. 1). Microfilament bundles contain nonmuscle actin in an F-actin-like form since they can be decorated by heavy meromyosin (2) or by actin-specific antibodies in immunofluorescence microscopy (3). Microtubules contain tubulin as the major structural protein (for a review see ref. 4) and can be decorated *in situ* by monospecific antibody against tubulin (5–7). Much less is known about intermediate filaments although they have been identified by electron microscopy in a variety of cell types, including fibroblasts, muscle cells, and glial cells (see, e.g., refs. 1, 2, and 8 and other references in these papers). Other examples of such filaments include the neurofilaments of nerve cells (9, 10), the tonofilaments of epithelial cells, and the prekeratin fibrils of keratinizing cells (11, 12). All these filaments have diameters of 7–10 nm but in most cases their biochemical nature and possible biochemical relation remain to be demonstrated.

Antibodies against specific cellular proteins, when used in immunofluorescence microscopy, can be useful probes of the *in situ* localization of the respective antigens (e.g., refs. 3, 5–7). In the case of intermediate filaments, an antibody raised against the major neurofilament polypeptide [molecular weight 55,000 (9)] decorates, in mouse Neuro 2a cells, fibrous swirls of material around the nucleus, identified by electron microscopy as bundles of intermediate filaments (10). In addition, an antibody to a 50,000 molecular weight protein from gizzard, thought to be derived from 10-nm filaments, has been reported (13) but it is not known what structures are decorated by this antibody in nonmuscle cells.

Here we document that most normal rabbit sera contain autoantibodies that decorate a fiber system in rat kangaroo Pt K2 cells and in other cell types. Comparison of the immunofluorescent data with electron micrographs of Pt K2 cells suggests that in this cell line these sera visualize bundles of tonofilament-like intermediate filaments.

MATERIALS AND METHODS

Pt K2 cells, originally derived from rat kangaroo kidney epithelium and Neuro 2a cells, a neuroblastoma line from mouse, were from the American Type Culture Collection. Pt K2 cells were maintained in Eagle's minimal essential medium supplemented with nonessential amino acids, glutamine, sodium pyruvate, and 10% (vol/vol) fetal calf serum. Neuro 2a cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Growth conditions for mouse 3T3 cells have been described (6).

Cells on glass cover slips were fixed in methanol for 5 min at −20°, rinsed in acetone at −20°, and air-dried. Formaldehyde fixation, when used, was for 10 min at room temperature in 3.7% formaldehyde in phosphate-buffered saline (14) prior to the methanol step. The procedures used for indirect immunofluorescence microscopy have been described in detail (3, 14).

A total of 30 normal rabbit sera was tested at a 1:6 dilution on Pt K2 cells, omitting the formaldehyde fixation step. The sera were obtained from different sources: 21 from Germany (12 from Göttingen, 3 from Hamburg, and 6 from Sulzfeld; the last 6 rabbits were supposedly "pathogen free"), 8 from England (Cambridge), and 1 from Israel. With the exception of two sera, all sera were positive on Pt K2 cells although at different intensities. Sera H17 and H23, the sera used in the detailed studies, were from rabbits from Göttingen and gave strong reactions. The actin antibody was raised against chicken gizzard actin denatured by dodecyl sulfate (15) and was used as the IgG fraction at 1 mg/ml. Its properties are similar to those of our previous antibody (3). The monospecific rabbit antibody against tubulin from pig brain has been described in detail (6, 16). The fluorescein-coupled goat antibodies against rabbit gamma globulins were from Miles Co. or from Hyland Co.

For electron microscopy, cells were fixed as monolayers and processed for ultrathin sectioning as described (17). Initial fixation was in 2.5% glutaraldehyde, buffered with cacodylate and supplemented with ions (described under I in ref. 17) either in the cold or at room temperature. Micrographs were taken with a Siemens Elmiskop 101.

RESULTS

Sera from a variety of normal, uninoculated rabbits were tested in indirect immunofluorescence microscopy on rat kangaroo Pt K2 cells. When the formaldehyde fixation step was omitted and the cells were fixed only with organic solvents, most sera decorated a fibrous system extending through the cytoplasm of the cells. Results with two of these sera (H17 and H23) are shown in Fig. 1 *a* and *b*. A system of wavy, intermingled fibers, some of them running to points at the cell boundary, is clearly seen. These bundles are not of uniform thickness and seem to be composed of subfibers. Differential focussing shows that

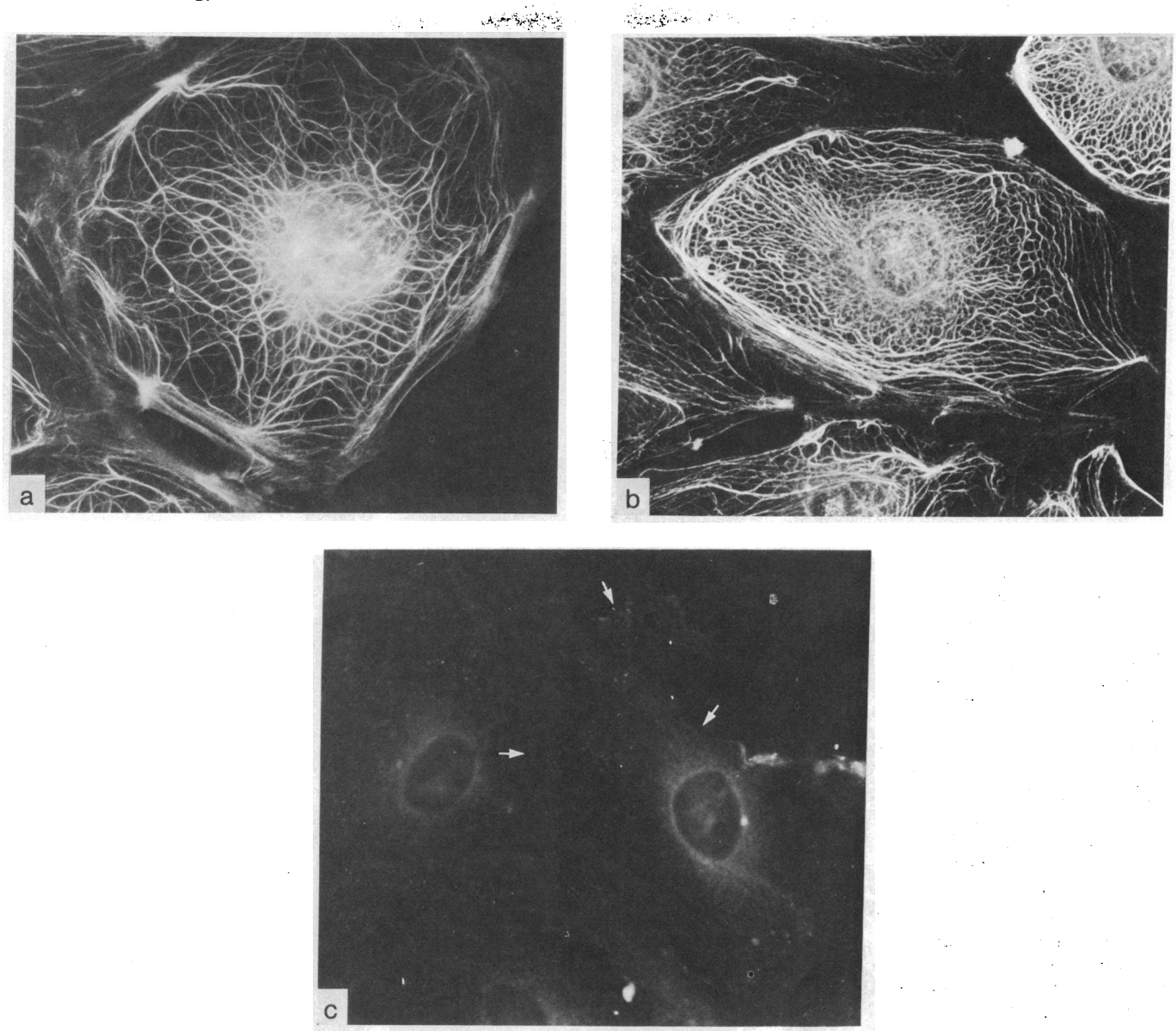

FIG. 1. Pt K2 cells decorated with rabbit sera from H17 (*a*) and H23 (*b*). Note the wavy system of fibers running throughout the cells. (*c*) As *a* except that the cells were treated with formaldehyde. Arrows indicate cell boundary. (×900.)

these fibers are also found in the perinuclear area and sometimes curl around the nucleus. Similar results were obtained with Pt K1 cells. Decoration of the fiber system in Pt K2 cells was abolished if the cells were fixed in formaldehyde (Fig. 1*c*).

The pattern of the fiber system decorated by "normal" rabbit antisera is different from that of both the microfilament system and the microtubule system, as judged by the following experiments. (*i*) Fig. 2 *a–c* compares the structures seen by indirect immunofluorescence microscopy on Pt K2 cells after the formalin fixation is omitted, using antibodies either to actin or to tubulin or the H17 serum. The actin (*a*) and tubulin (*b*) antibodies decorate fiber systems that are similar to those reported by us (3, 5, 6) and by others (7) for other cell lines after formalin fixation and that are clearly different from the fibrous system decorated by H17 (*c*). (*ii*) The three fiber systems can be distinguished in Pt K2 cells treated either with the "microfilament-rearranging" drug cytochalasin B or with the tubulin-specific drug Colcemid. Fig. 2 *d–f* shows Pt K2 cells treated with cytochalasin B (10 μg/ml, 30 min). As previously shown for mouse 3T3 cells (15), cytochalasin B induces a disorganization of microfilamentous actin and gives rise to "star-like heaps" visualized by the antibody to actin (*d*). The microtubule pattern is unchanged by cytochalasin (compare *e* and *b*), as is the pattern obtained with H17 serum (compare *f* and *c*). Fig. 2 *g–i* shows Pt K2 cells treated with Colcemid (5 μg/ml, 4 hr). The actin pattern (*g*) is unchanged whereas, as expected (1, 5–7), the microtubule pattern is abolished (compare *h* and *b*). The distribution of the fibers visualized by the H17 serum is only slightly affected by Colcemid, and under these conditions the fibers are still present over a large area of the cytoplasm (compare *i* and *c*).

Serum from H17 also decorates fibers in certain other cell lines, although we have not made an extensive study. Primary skin cells from mouse epidermis (for characteristics see ref. 18) show fibers that are distinct from the microfilament and microtubule systems in these cells (data not shown). In Neuro 2a cells, H17 serum decorates swirls of fibers, often in the perinuclear cytoplasm (Fig. 3*a*). These swirls are similar in organization and display to those recently described by Jorgensen *et al.* (10) using the same cell line and an antiserum against the major neurofilament polypeptide. In mouse 3T3 cells, which

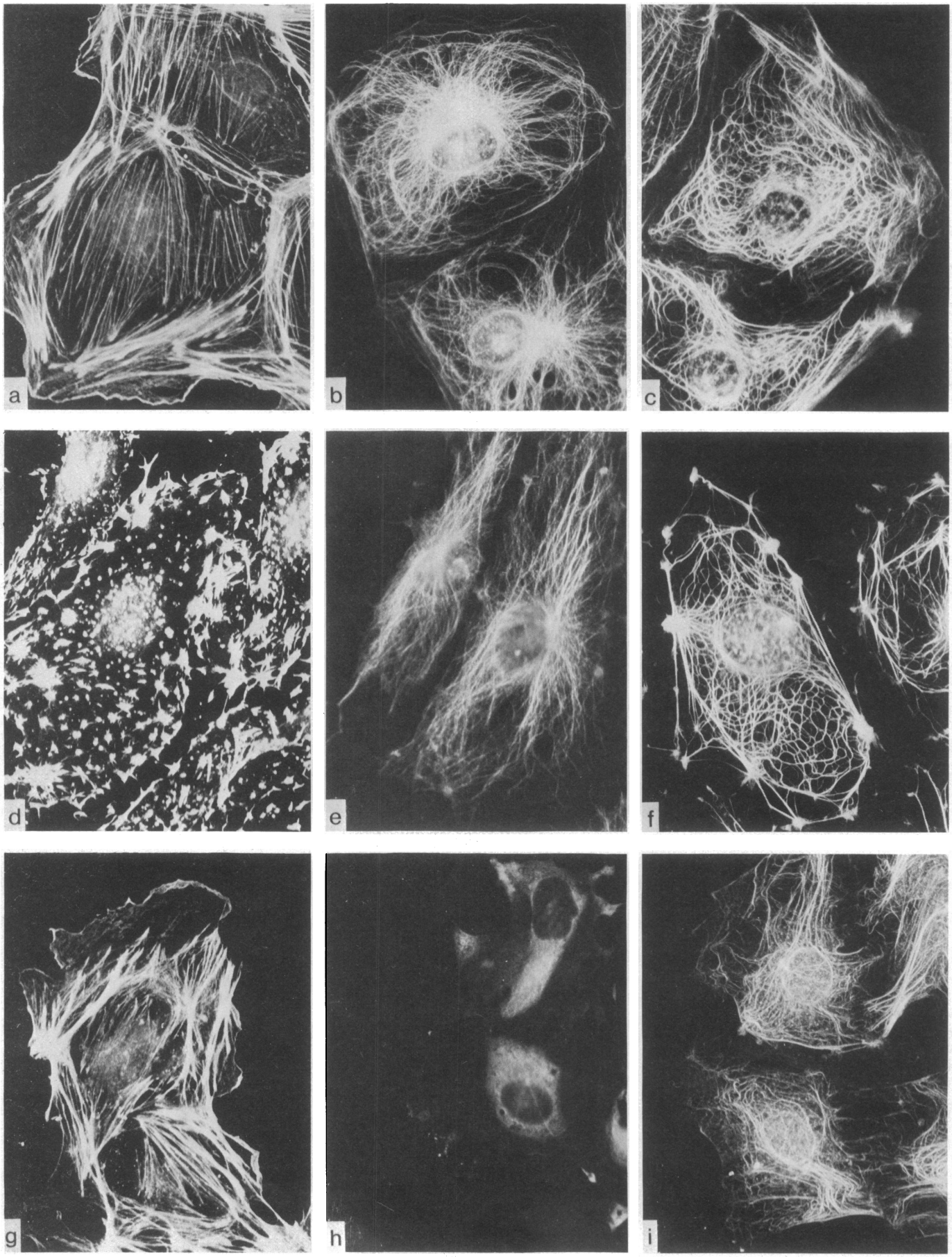

FIG. 2. (*Legend appears at bottom of the following page.*)

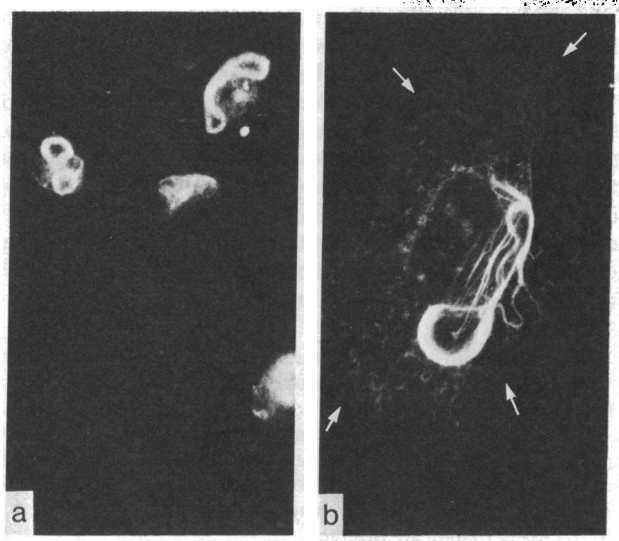

FIG. 3. (*a*) Mouse Neuro 2a cells stained with H17 serum. Note the swirls of fibers around the nucleus. (×1200.) (*b*) Mouse 3T3 cells treated for 4 days with Colcemid (0.5 μg/ml) and then stained with H17 serum. (×400.) Arrows indicate the cell boundary.

have been used extensively in previous studies in indirect immunofluorescence microscopy, (3, 5–7, 15, 16), H17 serum gives weak decoration of a few thin fibers in the cytoplasm and around the nucleus. If, however, the H17 serum is used on 3T3 cells treated with mitotic drugs like Colcemid, colchicine, or Janssen 17934, thick swirls of fibers around the nucleus are visualized (Fig. 3*b*). The thickness of these bundles increases with longer times of treatment and with higher concentrations of the mitotic drug. Similar drug treatments are known to induce a juxtanuclear cap of 10-nm filaments in other cells (19).

The results with the H17 and H23 sera are not an isolated case. Indeed, in screening 30 rabbits from three locations in Germany and from England and Israel, we found in almost all the sera a noticeable titer for the decoration of the fiber system described in Pt K2 cells.

Electron microscopy of Pt K2 cells shows not only aggregates of microfilaments in the form of extended straight "cables" (see ref. 20), but also aggregated bundles of filaments 7–10 nm thick. These bundles of intermediate sized filaments frequently appear flexible and wavy (Fig. 4*a*) and show a variety of bizarre branchings and interconnections. They resemble the arrays described by Brecher in Pt K1 cells (8) and also the tonofilaments and prekeratin fibrils present in various epithelia, especially epidermis (11, 12, 18, 21). The filaments in Pt K2 cells differ from microfilaments not only by their greater diameter, but also in other properties. In cross sections they often show an unstained core which represents an internal hollow channel, as demonstrable by the penetration of phosphotungstic acid into isolated fibers (data not shown), similar to the hollow core described in tonofilaments (21). Frequently, single 7- to 10-nm filaments reveal small, blunt lateral projections which are sometimes rather regularly spaced (e.g., Fig. 4*b*). They further exhibit a conspicuous tendency to aggregate laterally and form heavily stained spicule- or rod-like bundles in which the individual fibrous constituents are no longer distinguishable (Fig. 4), a situation which closely resembles the fibril–keratohyalin granule associations during early steps of keratinization processes (11, 12, 18, 21). Like the fibrous bundles of keratinizing epithelia, the 7- to 10-nm filaments of Pt K2 cells also show frequent associations with clusters of ribosomes, probably polyribosomes (Fig. 4*b*). These fiber bundles sometimes abut on, but do not terminate at, the extended intercellular junctions

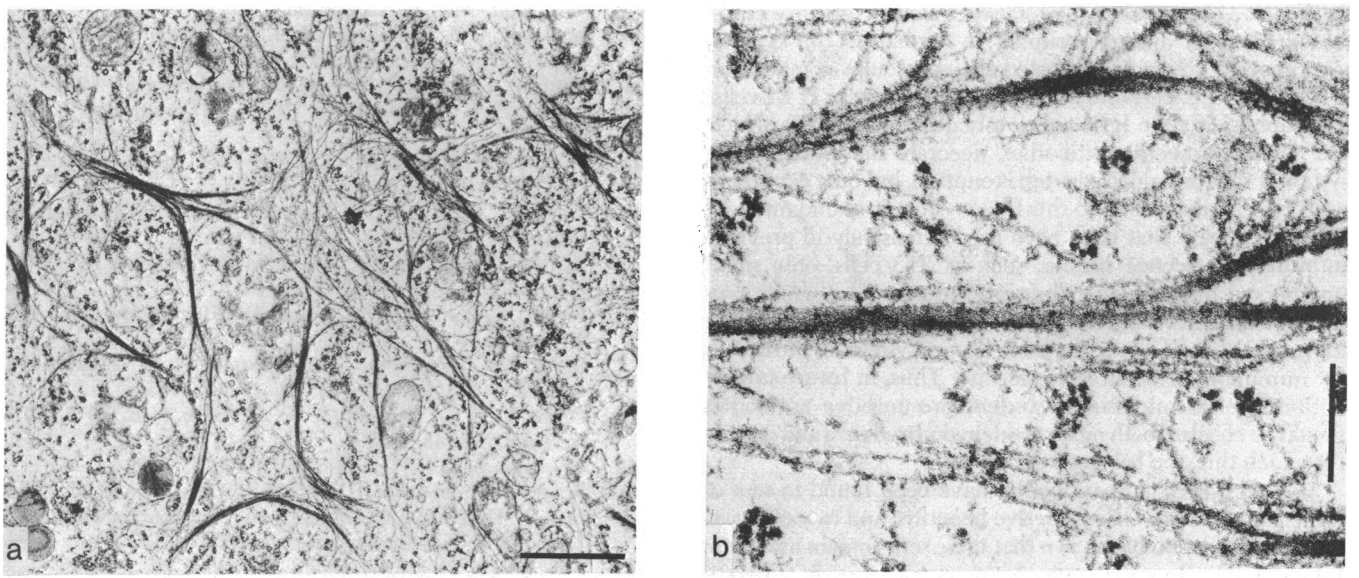

FIG. 4. (*a*) Survey electron micrograph showing typical arrangements of "thick" filaments in Pt K2 cells. Bar denotes 1 μm. (*b*) Electron micrograph of aggregated and single "thick" filaments of Pt K2 cells at higher magnification. Note the "keratin-like" densely stained lateral aggregations as well as the lateral side projections on the individual filaments. Note also their frequent and close associations with ribosomes. Bar denotes 0.2 μm. (×78,000.)

FIG. 2 (*on preceding page*). Pt K2 cells stained directly (*a–c*), after treatment of the cells with 10 μg/ml of cytochalasin B for 30 min (*d–f*), or after treatment with 5 μg/ml of Colcemid for 4 hr (*g–i*). Cells were stained with (*a, d,* and *g*) antibody to actin, (*b, e,* and *h*) antibody to tubulin, and (*c, f,* and *i*) H17 serum. Cells were fixed only with organic solvents (except for *e*, where formaldehyde fixation was used). Note that cytochalasin B destroys the microfilament system, leaving the microtubules and H17 system intact, and that Colcemid destroys the microtubules, leaving the microfilaments unaffected and the H17 system only slightly disturbed. (×600.)

between adjacent Pt K2 cells, which are desmosomes of a fascia adherens-like organization.

DISCUSSION

Our results show that many "normal" rabbit sera can be used in immunofluorescence microscopy to demonstrate in Pt K2 cells a system of fibers with characteristics similar to those expected for intermediate filaments. This fiber system is different in display from bundles of microfilaments visualized with antibody to actin (3) and microtubules visualized with antibody to tubulin (5, 6) in the same cell line (Fig. 2). Furthermore, it is unaffected by treatment with cytochalasin B at concentrations that rearrange the microfilament system and only slightly affected by Colcemid at concentrations that result in the disappearance of the microtubular system. Electron microscopic studies of these (Fig. 4) or similar cells (8) also show the presence of fibers that can be distinguished from microfilaments and microtubules both by their diameter (7–10 nm) and by their ultrastructure (see *Results*). A comparison of the immunofluorescence data (Fig. 1 *a* and *b*) with the electron microscopy data (Fig. 4*a*) suggests that the same fiber system is being visualized by the two different methods. The function of these intermediate filaments is unknown. However, Pt K2 cells are very flat and stick extremely tightly to the substratum. Thus, it may be that the intermediate filament system of Pt K2 cells is involved in some manner with anchorage and/or the maintenance of the epithelioid cell shape.

The presence in most rabbit sera of antibodies decorating intermediate filaments in Pt K2 cells is unlikely to have interfered with previous studies using immunofluorescence microscopy to visualize microfilaments and microtubules. These studies all used formaldehyde-fixed cells, and the formaldehyde fixation step abolishes the decoration of intermediate filaments in Pt K2 cells by spontaneous rabbit antibodies (Fig. 1*c*). Furthermore, some of the previous studies used only monospecific antibodies isolated from the total IgG pool by affinity chromatography on antigen coupled to Sepharose (6, 7). Our monospecific antibodies, directed against tubulin or myosin or α-actinin, do not decorate intermediate filaments in Pt K2 cells. The rabbit antiactin IgGs previously described (3, 15), which are not monospecific antibodies, decorate the tonofilament system if the formaldehyde step is omitted, but only extremely weakly. Finally, although this fiber system is abundant in Pt K2 cells, in cells that have been used extensively in previous immunofluorescence studies, such as 3T3 cells, only small numbers of intermediate fibers are present. The results presented here show that formaldehyde fixation is not obligatory for preservation of any of the three fibrous systems in Pt K2 cells for immunofluorescence microscopy. Thus, in future immunofluorescence microscopy studies, presumptive antibodies should be checked both on formaldehyde-fixed cells and on cells for which this step has been omitted.

Smooth muscle autoantibodies have been found in sera of some patients with chronic active hepatitis, and in some cases antigen absorption has shown that these sera contain anti-actin IgGs (22, 23). Recently spontaneous smooth muscle autoantibodies have been reported also in some normal rabbit sera, although the corresponding antigens were not identified (24). Since smooth muscle contains not only actin filaments but also 7- to 10-nm filaments (for references see ref. 25), our results suggest that at least some of these anti-smooth muscle antibodies might in part be directed against intermediate filaments. Currently we do not know if our rabbit sera recognize a major or a minor protein of the 7- to 10-nm filament or whether they recognize another structural component. Nor do we know if the decoration of tonofilament-like structures in Pt K2 cells and of 10-nm neurofilaments in Neuro 2a cells and in Colcemid-treated 3T3 cells is due to the presence of the same antigen in these fibers or to the presence of a mixture of spontaneous antibodies in our "normal" rabbit sera. Only a detailed protein chemical and immunological characterization of the proteins of tonofilaments, neurofilaments, keratin fibers, and other types of intermediate filaments will permit determination of whether these structures contain common elements.

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