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Relationship between movement and aggregation of centrioles in syncytia and formation of microtubule bundles

(cell fusion/cytoskeleton/10-nm filaments/nuclear movement/simian virus 5)

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ABSTRACT **Previous reports from this laboratory have provided evidence suggesting that microtubules and 10-nm filaments serve both cytoskeletal and force-generating functions in the intracellular movement and positioning of nuclei in syncytia. It has been found that, during the process of cell fusion and nuclear migration in syncytia induced by the paramyxovirus simian virus 5, centrioles are transported in the cytoplasm and form large aggregates. These aggregates are usually found in regions adjacent to rows of aligned nuclei and large bundles of microtubules and 10-nm filaments. Colchicine prevents the translocation and aggregation of centrioles, but cytochalasin B has little effect on this process. These results suggest that the same cytoskeletal elements that are involved in nuclear migration and positioning—i.e., microtubules and 10-nm filaments—are also involved in the transport of centrioles. The possibility that aggregates of centrioles may serve as centers for the organization of microtubules and 10-nm filaments into the large bundles observed in the syncytia is discussed.**

The paramyxovirus simian virus 5 (SV5) causes massive fusion of infected baby hamster kidney cells (BHK21-F) (1–3). The large syncytia that result may contain hundreds or thousands of nuclei. Studies with time-lapse cinematography showed that nuclei migrate in a rapid and orderly fashion and become aligned into tightly packed parallel rows in the syncytia (1, 2). On the basis of these results it was suggested that microtubules demarcated the direction of nuclear movement, and that they might also be involved in the production of force for this motion (2). More recent studies (4) have indicated that, in addition to microtubules, 10-nm filaments also play a role in nuclear motility, but no evidence was found for the involvement of organized actin-containing microfilaments. It thus appears that microtubules and 10-nm filaments are two components of a system that mediates the migration and positioning of nuclei in the cytoplasm (4).

In single fibroblastic cells, microtubules and 10-nm filaments usually appear as a network of filaments radiating from the perinuclear region to the cell periphery (5–7), whereas in fused multinucleated syncytia they are packed into large bundles that may exceed the length of many cells (1, 2, 4). The size of these bundles suggests that an efficient mechanism must exist for the assembly and organization of these cytoplasmic fibers in the syncytia. Because the centriole-containing centrosphere region has been implicated as an organizing center that could control microtubule assembly and distribution in the cell (6, 8), and because this centrosphere region (9) or centrioles (10) can nucleate microtubule assembly *in vitro*, it seemed appropriate to investigate the relationship between the centrioles and the organization and distribution of the large bundles of microtubules present in the syncytial cytoplasm.

In electron microscopic studies of virus-induced syncytia, aggregates containing as many as 16 or more centrioles have been observed (2, 3). However, because only a limited area can be examined by thin-sectioning and electron microscopy, it is difficult to follow the fate of centrioles during the process of cell fusion without the aid of a specific marker for centrioles. Recently, sera collected from several nonimmunized rabbits were shown to give specific fluorescence staining of centrioles in a wide variety of cell types (11). These sera interact specifically with material associated with the centrioles, but not with tubulin, the major component of the microtubules in the walls of the centrioles. The availability of such sera thus provides a powerful tool with which to investigate the distribution of centrioles and their possible function in syncytia.

MATERIALS AND METHODS

Cells and Viruses. Baby hamster kidney cells (BHK21-F) were grown as described (1). Briefly, the cells were grown in monolayer cultures in 100-mm petri dishes or on glass coverslips (no. 1) in reinforced Eagle's medium (REM) supplemented with 10% calf serum and 10% tryptose phosphate broth. Confluent monolayers were washed with phosphate-buffered saline (P_i/NaCl, pH 7.2) and inoculated with the W3 strain of SV5 (12) at a multiplicity of 1–5 plaque-forming units per cell. After 2 hr at 37°C, the inoculum was removed and fresh REM was added to the cultures.

Antisera to Centrioles and Microtubules. The nonimmunized-rabbit sera that react specifically with centrioles (11) and the preparation of rabbit antiserum to purified tubulin (4) have been described.

Immunofluorescence Microscopy. For visualization of centrioles, cells were fixed and treated with centriole-staining sera as described (11). For immunofluorescence staining with anti-tubulin antiserum (4), cells grown on glass coverslips were fixed in 3.7% (wt/wt) formaldehyde in P_i/NaCl for 30 min at room temperature, washed three times in P_i/NaCl, and extracted with acetone for 2 min at −20°C. Cells were then allowed to react with antiserum to tubulin (1:20 dilution) at 37°C for 30 min. After washing three times in P_i/NaCl, cells were incubated with fluorescein-conjugated goat anti-rabbit IgG (0.5 mg/ml). After washing three times with P_i/NaCl, cells were mounted in Elvanol and examined with a Zeiss Photomicroscope III equipped with epifluorescence optics.

Preparation of Media Containing Colchicine and Cytochalasin B. A stock solution of colchicine, 200 μg/ml (Sigma), was made in REM. One milligram of cytochalasin B (Sigma) was dissolved in 0.1 ml of dimethyl sulfoxide and 9.9 ml of REM was added to give a stock solution concentration of 100 μg/ml. Stock solutions were kept frozen and were diluted with fresh REM to the appropriate final concentrations for each experiment. In most experiments, colchicine or cytochalasin B was added to the end of the 2-hr adsorption period to eliminate any possible effects of the drug on virus adsorption or penetration.

Abbreviations: SV5, simian virus 5; REM, reinforced Eagle's medium; P_i/NaCl, phosphate-buffered saline.

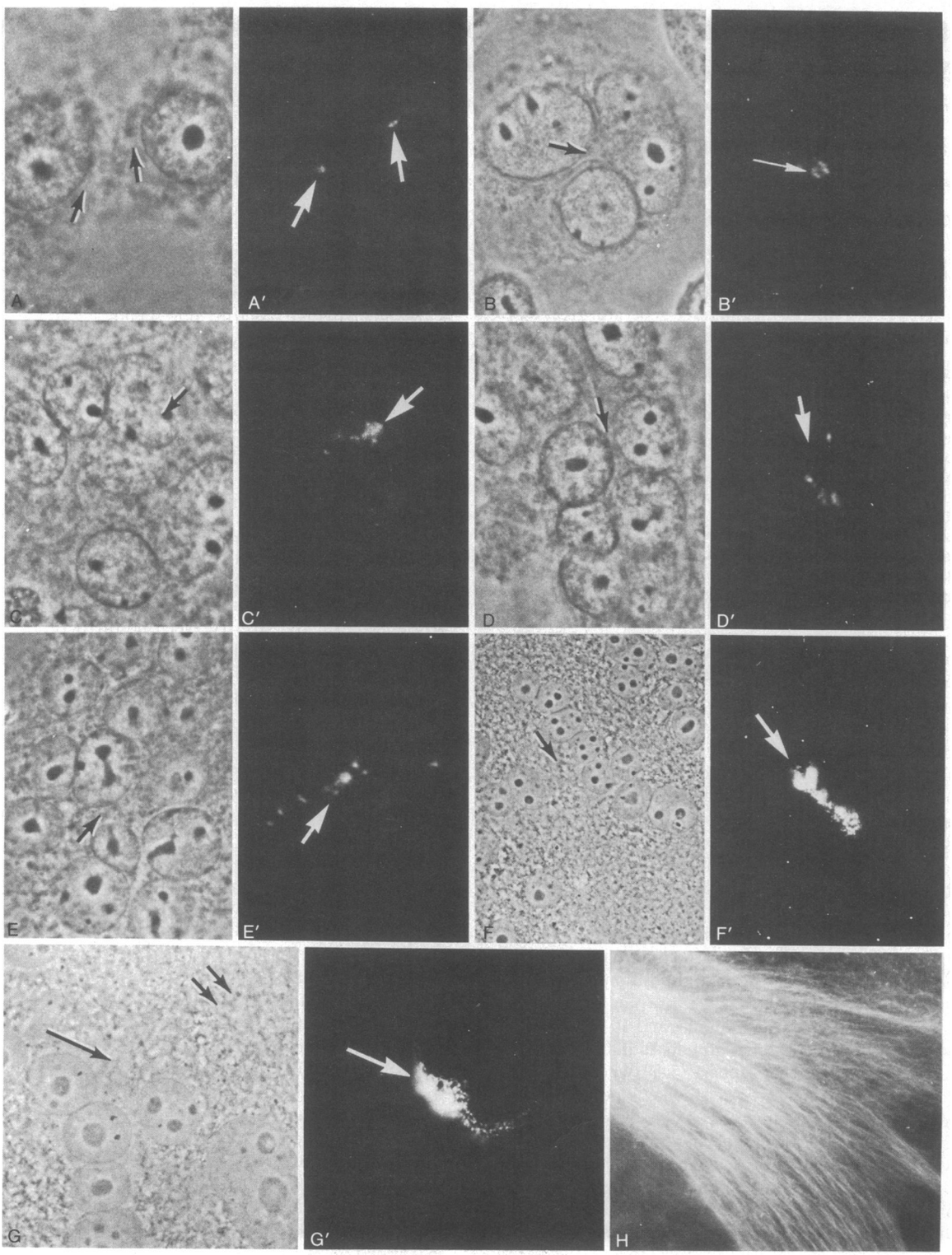

FIG. 1. (*A–F′*) Phase contrast and fluorescence micrographs of syncytia formed by fusion of BHK21-F cells infected with SV5 and stained by the indirect immunofluorescence technique using rabbit serum that specifically labels centrioles. *A–F* are phase-contrast micrographs and the corresponding A′–F′ are the identical areas with fluorescence optics. The arrows in the phase-contrast micrographs indicate the specific locations of the fluorescent granules indicated by arrows in the corresponding fluorescence micrographs. (*A*) Uninfected cells. (×1700.) (*B–E*) Syncytia derived from fusion of 3, 5, 8, and 13 cells, respectively. (×1500.) (*F*) Area of syncytia containing many (>100) nuclei. (×1000.) (*G*, *G′*, and *H*) Correlation of the locations of aligned nuclei, centrioles, and large microtubule bundles in syncytia. (×1200.) (*G*) Phase-contrast micrograph showing aligned nuclei; (*G′*) immunofluorescenee microscopy of centriole aggregate located in the area indicated by single arrow in *G*; (*H*) immunofluorescence microscopy using antiserum to tubulin, showing the presence of a large microtubule bundle located in a region similar to that indicated by double arrows in *G*.

Electron Microscopy. Monolayer cultures of SV5-infected BHK21-F cells were fixed with 1% glutaraldehyde in P_i/NaCl and postfixed with osmium tetroxide at room temperature. After fixation, cells were dehydrated in a graded series of alcohol (50–100%) and embedded in Epon 812 (13). Thin sections were stained with hot uranyl acetate (14), followed by lead citrate (15), and examined in a Philips 300 electron microscope.

RESULTS

Observation of uninfected BHK21-F cells by indirect immunofluorescence microscopy with centriole-staining sera indicated that, in most cells, a pair of fluorescent granules could be seen near the edge of the nucleus (arrows Fig. 1 *A* and *A'*). A previous report (11) has demonstrated that these fluorescent granules represent a pair of centrioles. Their perinuclear location also coincides with the typical position of centrioles revealed by electron microscopy. Sometimes the two centrioles did not appear in the same focal plane, thus giving an overlapping fluorescence image that appeared as a single granule (left arrow, Fig. 1*A'*).

To study the behavior of centrioles during cell fusion and nuclear migration, we examined syncytia derived from the fusion of differing numbers of cells. As described previously (4), relatively synchronized cell fusion can be obtained by leaving infected cultures overnight at 33°C and then shifting them to 39.5°C. Almost immediately after the temperature is raised, fusion begins and proceeds rapidly. By 4 hr after the shift to 39.5°C, most of the cells in the monolayer had fused into one very large syncytium. During this 4-hr period the nuclei became aligned in closely associated parallel rows, and microtubules and 10-nm filament bundles formed parallel arrays between the aligned nuclei. Cultures at different stages of cell fusion were monitored at various times after the temperature shift. Giant cells containing 3–8 nuclei were usually observed in the first half hour after the shift to 39.5°C, whereas cells with 10–12 nuclei were usually observed about 1 hr after the temperature shift. Between 2 and 4 hr after the temperature shift, large syncytia containing many nuclei were observed.

Fluorescence staining revealed that, as nuclei moved toward each other in the syncytia, pairs of centrioles were also translocated from their original positions to form aggregates, which were usually located in the middle of the nuclear clusters. Fig. 1 *B* and *B'* shows an aggregate of three pairs of centrioles surrounded by three nuclei in a syncytium. Fig. 1 *C* and *D* illustrates the nuclear regions of two syncytia formed by the fusion of five and eight single cells, respectively. Groups of centrioles were recognizable as groups of fluorescent granules among the nuclei in the central part of these syncytia (Fig. 1 *C'* and *D'*). No fluorescent staining was noticed in other areas of the syncytial cytoplasm. As the cell fusion progressed, the size of syncytia became larger, as did the number of fluorescent granules in centriole aggregations. Fig. 1 *E* and *E'* shows another syncytium derived from the fusion of 13 cells. Again, centrioles were grouped together in the internuclear region. By 4 hr after the temperature shift, almost all the cells had fused into a single large syncytium (Fig. 1*F*), and an area of intense fluorescence, composed of large numbers of centrioles, was present in the region containing large clusters of nuclei (Fig. 1*F'*). These fluorescent aggregates were usually at the edges of large microtubule bundles and rows of nuclei. The spatial relationships among centrioles, nuclei, and microtubule bundles are illustrated further in Fig. 1 *G*, *G'*, and *H*. Examination of the same area by both phase-contrast and fluorescence optics showed an aggregate of many centrioles present at the edge of a row of aligned nuclei (Fig. 1 *G* and *G'*). Immunofluorescence microscopy using antiserum to tubulin (Fig. 1*H*) showed that such areas contain large bundles of microtubules. Previous studies (2, 4) showed that these areas also contain large numbers of 10-nm filaments. The series of photographs shown in Fig. 1 *A–F'* demonstrates that during cell fusion, centrioles moved to form a cluster in the internuclear region of the syncytium. Their location usually coincided with the position of microtubule bundles in the cytoplasm.

Treatment of cells with colchicine, which causes the disruption of microtubules, prevents nuclear movement and alignment (2, 4). Nuclei in colchicine-treated syncytia are not found in the parallel rows seen in untreated cells but are scattered in a random pattern in the syncytial cytoplasm. In the present studies we have found that colchicine also prevents the movement and aggregation of centrioles in these syncytia. Pairs of centrioles, seen as two fluorescent granules (arrows, Fig. 2*A'*), remained scattered throughout the syncytial cytoplasm after colchicine treatment and were found in the perinuclear region of each individual nucleus (Fig. 2*A*). Thus, colchicine not only depolymerized microtubules but also inhibited the translocation of centrioles in the cytoplasm.

Cytochalasin B treatment of cells at 20–50 μg/ml causes marked morphological changes in the syncytia (4). As shown in Fig. 2*B*, a large central body of syncytial cytoplasm was formed with numerous long filamentous processes radiating from it. Although at this high concentration cytochalasin B inhibits microfilament function, it does not inhibit cell fusion, nuclear migration, or the formation of microtubule bundles and 10-nm filament bundles (4). Fluorescent staining of cytochalasin B-treated syncytia with centriole-staining sera revealed that centrioles had moved to the central region of the syncytia in the presence of the drug (Fig. 2*B'*), although they did not form as compact an aggregate as that found in untreated cultures (Fig. 1 *F'* and *G'*).

Thin-section electron microscopy confirmed the results obtained by indirect immunofluorescence microscopy using the centriole-staining sera. Groups of centrioles were frequently observed in the syncytial cytoplasm. Fig. 2*C* shows two pairs of centrioles among the helical SV5 virus nucleocapsids. Often a large number of microtubules and 10-nm filaments radiated out from the centriolar region (Fig. 2*D*). Groups of 8, 16, or more centrioles in the SV5-induced syncytia have been reported (3).

DISCUSSION

By indirect immunofluorescence using sera that specifically label centrioles, we have shown that pairs of centrioles were translocated in the cytoplasm of syncytia and aggregated into groups adjacent to the rows of nuclei and bundles of microtubules and 10-nm filaments that form in the syncytial cytoplasm. The number of centrioles in the aggregates was proportional to the number of nuclei in the clusters. Colchicine prevented the translocation and aggregation of centrioles, but cytochalasin B did not, although the aggregates formed in the presence of cytochalasin B were not as tightly packed in the absence of the drug. Previous studies showed that microtubules and 10-nm filaments are involved in the movement and alignment of nuclei in syncytia, apparently providing both direction and motive force (2, 4). The present results indicate that the movement and positioning of centrioles involves the same cytoplasmic fibers.

Centrioles have long been associated with phenomena such as spindle fiber formation, ciliogenesis, and distribution of the microtubule network in cells (16, 17). In these processes, centrioles and the materials in their immediate environment have been implicated as organization centers for microtubule polymerization. Osborn and Weber (6), Brinkley and coworkers (7,

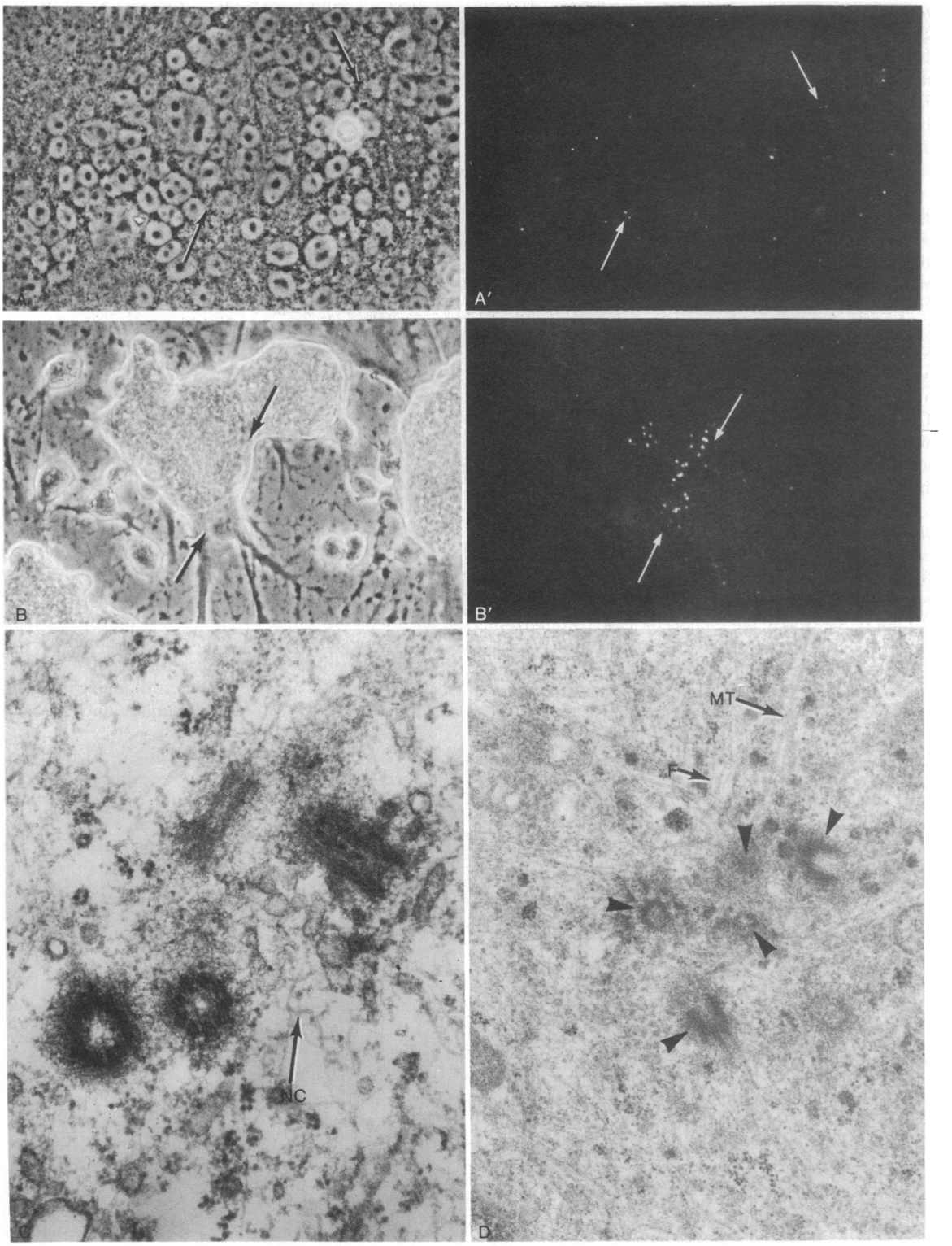

FIG. 2. (*A*) Phase-contrast and (*A'*) fluorescence micrographs of the same area of an SV5-infected BHK21-F syncytium treated with colchicine (10 μg/ml) for 18 hr. Note the random, nonaligned distribution of nuclei (*A*) and the centrioles (arrows) near the individual scattered nuclei. (×830.) (*B*) Phase-contrast and (*B'*) fluorescence micrographs of SV5-infected BHK21-F syncytium treated with cytochalasin B (20 μg/ml) for 18 hr. The centrioles have been translocated to the center of the syncytia (arrows) and form an aggregate, but this is not as tightly packed as the aggregates seen in untreated syncytia. (×1050.) (*C*) Electron micrograph of a syncytium, showing four centrioles surrounded by nucleocapsids (NC) of SV5 virus. (×51,900.) (*D*) Electron micrograph of a syncytium showing a large number of microtubules (MT) and 10-nm filaments (F) radiating out from a region containing five centrioles (arrows). (×29,900.)

18) and Frankel (8) independently found that, after breakdown with Colcemid or cold, microtubules repolymerize from the centrosomal region—i.e., from the region containing the centriole-associated material, towards the peripheral cytoplasm. These investigators suggested that centrioles may play a key role in determining microtubule distribution in interphase cells.

It is interesting to note that, in our studies of virus-induced syncytia, aggregates of centrioles were usually located near a large microtubule bundle. The size and distribution of these microtubule bundles suggest that the aggregated centrioles could be functioning in the organization of the microtubule bundles. It is possible that these centriole aggregates could serve as "stations" connecting microtubule bundles, which form the long track-like structure along which the nuclei move in the syncytial cytoplasm (2, 4).

Because 10-nm filaments usually depend on microtubule distribution for their organization in the cytoplasm (4, 19, 20), centrioles and the pericentriolar material may also govern indirectly the formation and distribution of the large bundles of 10-nm filaments that have been observed in association with the microtubule bundles (4). Centrioles could thus act as the center coordinating the organization of both microtubules and 10-nm filament bundles.

Translocation of centrioles also takes place in mitosis. Normally during prophase, two centriole pairs separate and move to opposite ends of the cell to establish the poles of the mitotic spindle. Treatment with colchicine prevents not only spindle fiber formation but also the migration of centrioles from the cell center to the polar region. Our results with colchicine treatment of syncytia are consistent with these observations. Stubblefield and Brinkley (21) suggested that inability of centriole translocation is the main reason for the failure of spindle fiber formation. However, it is still not clear whether in the syncytia described here the centriole translocation precedes the microtubule bundle formation—i.e., centrioles first migrate and form the aggregates and then the microtubules organize into bundles—or, alternatively, that, during the process of elongation and organization of microtubules into bundles, centrioles are moved with them. However, the fact that many centrioles are gathered in a small area suggests that they are specifically aggregated. Experiments examining microtubule growth in syncytia after recovery from colchicine treatment may shed light on microtubule organization and assembly *in vivo* in relation to centriolar function.

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