Morphology, Behavior, and Interaction of Cultured Epithelial Cells after the Antibody-induced Disruption of Keratin Filament Organization

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ABSTRACT The organization of intermediate filaments in cultured epithelial cells was rapidly and radically affected by intracellularly injected monoclonal antikeratin filament antibodies. Different antibodies had different effects, ranging from an apparent splaying apart of keratin filament bundles to the complete disruption of the keratin filament network. Antibodies were detectable within cells for more than four days after injection. The antibody-induced disruption of keratin filament organization had no light-microscopically discernible effect on microfilament or microtubule organization, cellular morphology, mitosis, the integrity of epithelial sheets, mitotic rate, or cellular reintegration after mitosis. Cell-to-cell adhesion junctions survived keratin filament disruption. However, antibody injected into a keratinocyte-derived cell line, rich in desmosomes, brought on a superfasciculation of keratin filament bundles, which appeared to pull desmosomal junctions together, suggesting that desmosomes can move in the plane of the plasma membrane and may only be 'fixed' by their anchoring to the cytoplasmic filament network. Our observations suggest that keratin filaments are not involved in the establishment or maintenance of cell shape in cultured cells.

Intermediate filaments are a prominent component of the cytoskeleton in many eucaryotic cells (for reviews see references 2, 35, and 54). The intricate patterns they form have led some investigators to surmise that they are actively involved in determining cellular morphology and behavior. However, a number of observational and experimental results, most notably the reported absence of intermediate filaments in the early mouse embryo (9, 28, 42), their remarkably variable organization in cultured cells (33), and the absence of effect when the intermediate filaments of fibroblasts were collapsed by intracellularly injected antibodies (21, 30, 39), suggest that intermediate filaments are not directly involved in the determination of cellular morphology.

The keratin filaments of epithelial cells are by far the most complex class of intermediate filaments (for reviews see references 17 and 35). They are the first intermediate filaments to appear during development in the mouse (9, 28, 29, 42). Desmosomal (macula adherentes) (15) intercellular junctions form first and keratin filaments subsequently integrate into them (12, 28, 41). Linked through these junctions into a supracellular network, 8–12-nm thick, keratin filaments course throughout the cell, forming an intricate system of anastomosing filament bundles which also anchor the epithelial sheet, through hemi-desmosomes, to the underlying basal lamina. The keratin filament proteins are a complex and distinct group, although they do share ultrastructural features (50) and some antigenic determinants (11, 43) with other intermediate filament subunit types. The subunit composition of keratin filaments varies depending on the particular tissue in which they occur (17, and references therein), and such differences can be detected by monoclonal (32) and polyclonal (13) antibodies. Keratin filaments appear to be heteropolymers (37, 49). Genetic analysis has revealed that the keratins are coded for by at least two multigene families (19), but the functional significance of these families is unclear.

While much is known about the biochemical complexity of intermediate filaments, their functional role within cells is as yet unknown. We have used intracellularly injected monoclonal antibodies to specifically disrupt intermediate filament organization in living cells to examine the role of keratin

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filaments in the determination and maintenance of cell morphology and behavior in cultured epithelial cells. These experiments suggest that keratin filaments are not actively involved in many aspects of epithelial behavior in cultured cells. On the basis of these and similar observations (14, 21, 30, 31, 39), we conclude that although intermediate filaments probably act as a cellular skeleton, this is more important for the integrity of a three-dimensional tissue than for a monolayer of cells in tissue culture.

MATERIALS AND METHODS

Cells and Cell Culture: PtK_1 and PtK_2 kidney epithelial cell lines (52) and a simian virus 40-transformed, human keratinocyte line (SVK14) (51) were grown in bicarbonate-buffered, Dulbecco's modification of Eagle's minimal medium, supplemented with 10% fetal calf serum (FCS) and antibiotics. Cells for immunofluorescence and intracellular injection experiments were grown on glass coverslips or on specially prepared glass-bottomed petri dishes (8).

Intracellular Injection: Cells were injected according to Chambers (10) with minor modifications (30). Needles were pulled from internally threaded glass capillary tubes and back-filled with ~0.2 μl of antibody or buffer solution. Needle tips were <1 μm in diameter, and when filled with 3 M KCl had measured electrical resistances of from 20–40 megohms. Pressure was applied using a 50 ml air-filled syringe. The volume injected under these conditions has been estimated to be on the order of 10^{-13}/cell (24). Intracellular injection of IgG solution or of buffer alone usually caused a momentary but noticeable swelling of the injected cell. In many cases, juxtanuclear vacuoles rapidly appeared, which then shrank and disappeared over the course of an hour. In some cases, cells responded to injection by rapidly contracting and partially rounding up, but these cells became reextended within 10 to 30 min. The overall survival of cells after antibody injection was high, usually >90%. The antibody effects described here could easily be distinguished from trauma reponses to the injection process itself.

Antibodies Used for Injection: The monoclonal antibodies LE61, LE41 (32), LC46 and RT97 (53), all IgG_1, were purified from tissue culture supernatants using protein A-sepharose (Pharmacia, Uppsala, Sweden), binding at pH 8.5 and eluting at pH 6. The antibodies were concentrated by vacuum dialysis and dialyzed into a high potassium, low sodium buffer designed to mimic intracellular conditions (31, 47). They were injected into cells at concentrations between 1 and 20 mg/ml. Most experiments were done using antibodies at 7 mg/ml.

LE61 and LE41 both give clean, keratin-type filament patterns by immunofluorescence microscopy, and completely fail to react with any nonepithelial cells in tissue culture or frozen sections (32, 33; E. B. Lane, extensive unpublished observations). They are directed against different epitopes on the cytokeratin antigens, as seen by their individual "fingerprint" binding patterns on 2-D gels of the original immunogen (32, 33). The specific antigenic site for each antibody is found on one or more major polypeptides and several minor ones. In spite of extensive and ongoing assays, by cytochemical and whole-cell immunoblotting methods, we have so far found no evidence to suggest that LE61 or LE41 bind to anything other than cytokeratin polypeptides and their fragments. Probably only a fraction of all cytokeratin proteins have yet been definitely characterized (17) and the spectrum of polypeptides detected autoradiographically by monoclonal antibodies may represent proteolytic degradations and/or individual gene products. (It must be remembered that these are not the epidermal cytokeratins, but those of simple epithelia.)

LC46, which was used to localize the centriolar complex, and as a nonfilament binding control, is a monoclonal antibody which has a very distinct cellular distribution by indirect immunofluorescence microscopy: it binds to a centriole-associated structure in PtK cells, possibly to ciliary rootlets (E. B. Lane, unpublished data). It does not otherwise co-localize with tubulin antibodies. RT97, a monoclonal antineurofilament antibody (courtesy of J. Wood, Wellcome Laboratories, Beckenham, U. K. [53]), was also used as a control antibody. Neither LC46 or RT97 had any effect on keratin filament organization when injected into cells.

The different effects of LE61 and LE41 antikeratin antibodies and the lack of effects of LC46 and RT97 provide internal controls for the specificity of antibody effects. As an additional control, horseradish peroxidase-conjugated polyclonal rabbit anti-mouse immunoglobulin (anti-MIg-HRP) (Dako Immunoglobulins A/S, Denmark) was also injected (with no effect). This antibody was used at 2 mg/ml.

Immunofluorescence, Immunoperoxidase, and Electron Microscopy: Rabbit antitubulin antisera (antitubulin) (a gift from P. Sheterline [45], The University, Liverpool, U. K.), rabbit anti-calf lens vimentin antisera (antivimentin) (a gift from F. Ramaekers, Katholicke Universiteit, Nijmeigen, The Netherlands) and affinity-purified, rabbit antiactin (antiactin) (a gift from D. Lawson, University College, London, U. K.) were used, as well as the mouse monoclonal antibodies LE61, LE41 (32), and LC46. Fluorescein-conjugated goat anti-mouse immunoglobulin (anti-MIg-F1), tetramethylrhodamine-conjugated goat anti-mouse (anti-MIg-Rd) and anti-rabbit (anti-RIg-Rd) immunoglobulin were purchased from Cappel Laboratories Inc. (Cochranville, PA), and used diluted 1:100 for anti-MIg-F1 and 1:150 for anti-MIg-Rd and anti-RIg-Rd. All antibodies were diluted into HEPES-buffered minimal essential media (MEM) supplemented with 2.5% FCS and 0.02% sodium azide. Anti-RIg-Rd was diluted into this solution plus 10% normal mouse serum to block cross-reactivity (30, 31).

For immunofluorescence microscopy, cells were first washed with serum-free media and then fixed with 100% methanol for 10 min at −20°C. Injected mouse antibodies were recognized by labeling the cells with anti-MIg-F1. After incubating for 20 to 25 min at room temperature, the cells were washed with 80% basal salt solution: 20% HEPES-buffered media (wash buffer). The cells were then sequentially labeled with a second antibody and the appropriate rhodamine-conjugated antiserum. After the final labeling cells were washed with wash buffer and then with distilled water before being mounted in 14% Gelvatol (Monsanto Co., St. Louis, MO):30% glycerol in phosphate buffered saline (PBS). Cells were photographed on Tri-X film developed so as to give an effective speed of 800 to 1,600 ASA.

In some experiments, cells were labeled sequentially with LE41, anti-MIg-F1, rabbit second antibody, and anti-RIg-Rd. The injected cells were unambiguously recognizable. The specificity of anti-MIg-F1 and anti-MIg-Rd was demonstrated by their lack of labeling of neighboring, uninjected cells. The specificity of anti-RIg-Rd was also apparent.

Injected anti-MIg-HRP was visualized in control cells as follows: cells were washed in basal salt solution and then fixed for 10 to 15 min in 1.5% glutaraldehyde in PBS, then washed with 0.1 M Tris, pH 7.4 (Tris buffer). Cells were then soaked for 15 min in Tris buffer plus 0.5% $CoCl_2$. After washing in Tris buffer, the cells were incubated first for 5 min in 1% diaminobenzidine (DAB) in Tris buffer without H_2O_2 and then for 15 to 30 min in 1% DAB, 0.1% H_2O_2 in Tris buffer. DAB solutions were filtered before use. Cells were washed in distilled water, mounted in Gelvatol, and examined as soon as possible thereafter.

For electron microscopy, cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate, pH 7, for 30 to 60 min at room temperature and postfixed with 1% OsO_4 in phosphate buffer for 30 min in the dark at 0°C. The cells were then washed with distilled water and stained en bloc with 0.5% aqueous uranyl acetate. After this, the cells were washed, dehydrated, and embedded in Durcupan (Fluka AG., Switzerland). The location of the injected cells had been marked on the underlying coverslip using a diamond-scribing objective and could be followed throughout preparation. Markings were transferred to the overlying resin before removing the coverslip, before sectioning. Coverslips were removed with hydrofluoric acid or thermal shock. Thin sections of the cells were cut parallel to the substrate, stained with lead citrate, and examined on a Zeiss EM10.

RESULTS

LE61 (32, 33) is a monoclonal antibody directed against a determinant on the keratin filaments of simple epithelia (see Materials and Methods). When injected into PtK_2 cells at concentrations of 1 mg/ml and below, it had little or no effect on keratin filaments (not shown). Above 3 mg/ml, LE61 had a disruptive effect on keratin filament organization, and at 7 mg/ml the keratin filaments of all injected cells were effected (Figs. 1 and 2). LE61 was used at 7 mg/ml in most experiments. Increasing the antibody concentration from 7 to 20 mg/ml was only marginally more effective at completely disrupting keratin filaments (not shown). Irrelevant antibodies of the same class as LE61 (IgG_1) (LC46 and RT97) did not bind to or effect keratin filament organization when injected at any concentration (not shown). On decorating the keratin filaments, the injected LE61 antibody caused the keratin filament network to collapse away from the cell periphery (Fig. 1 *a–c*), sometimes leaving antibody-decorated fragments behind. As the antibody-decorated filaments collapsed they fragmented, and were ultimately reduced to scattered cytoplasmic granules (Figs. 1 *d*, *e*; and 2). In some cells keratin filaments, apparently unlabeled by the injected antibody could be seen upon secondary LE61 labeling, and were collapsed toward the nucleus (Figs. 1 *d*, *e*; and 2). After 1 h many cells contained no extended keratin filaments as determined by immunofluorescence microscopy using LE61 (Fig. 1 *d* and *e*) or any of a number of other monoclonal antikeratin filament antibodies (not shown). The

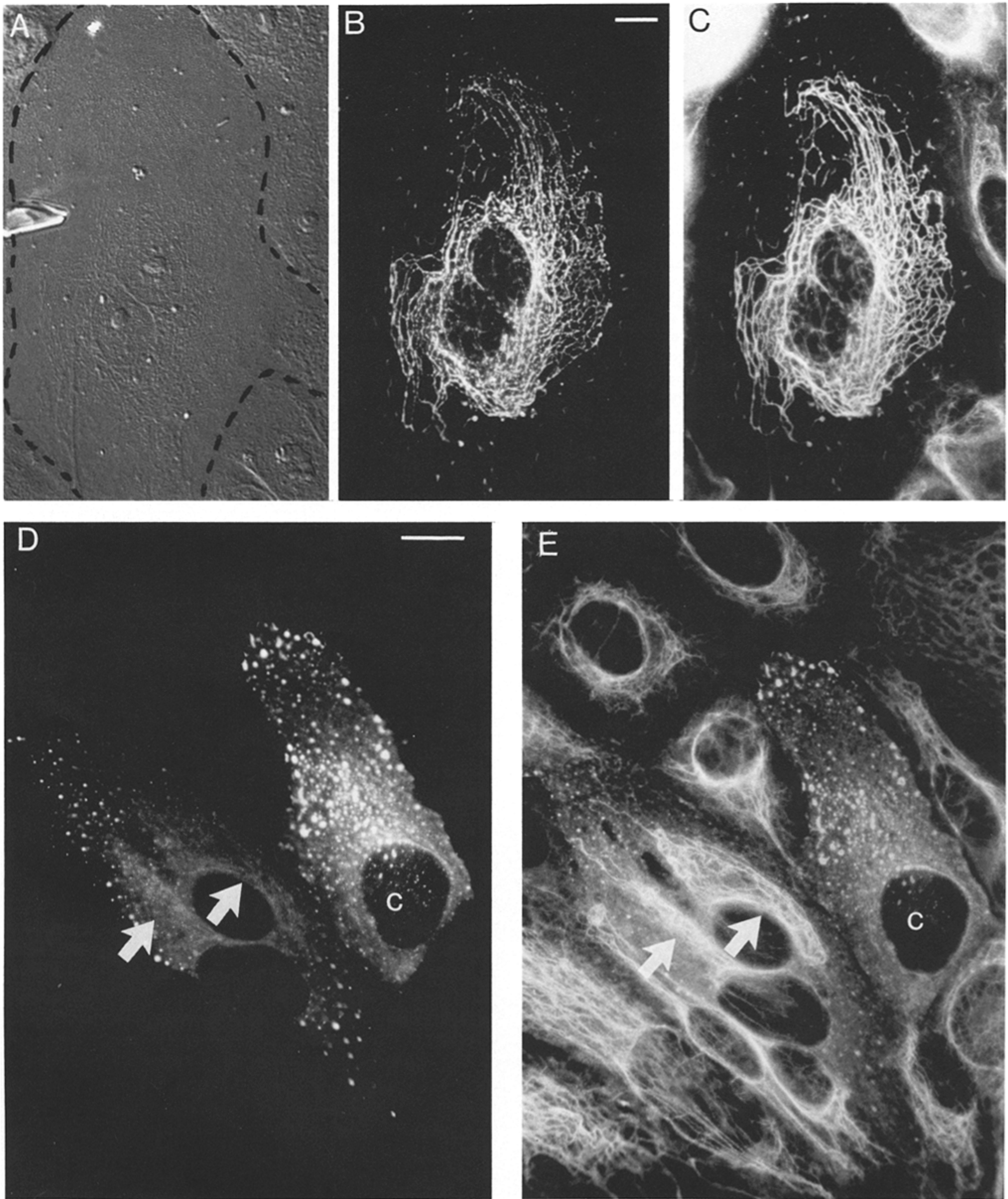
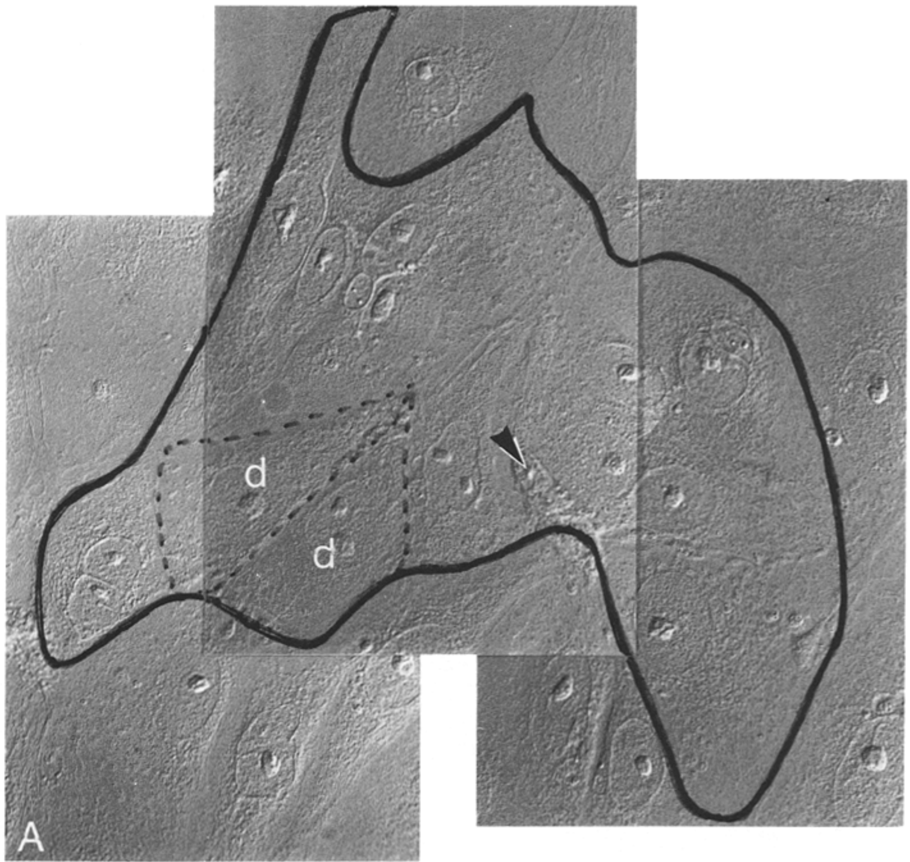


FIGURE 1 Two hours after LE61 injection (7 mg/ml) into PtK_2 cells, the keratin filament networks were found in the process of collapse and disruption (*A–C*) or completely disrupted (*c* in *D* and *E*). Some injected cells contained collapsed coils of keratin filaments (arrows, *D* and *E*) not labeled by the injected antibody. (*A*) Nomarski optics, boundary of injected cell outlined; (*B* and *D*) injected antibody visualized (after fixation) with anti-MIg-Fl; (*C* and *E*) all available antigen visualized by restaining with LE61 and anti-MIg-Rd. Bars, 10 μm. *A–C*: × 700; *D* and *E*: × 1,000.

full range of observed LE61 effects is illustrated in Fig. 2. A group of cells injected with LE61 were followed by time-lapse video microscopy for 23 h and then fixed and examined by immunofluorescence microscopy. The keratin filaments of all the injected cells were effected. During the course of the experiment an injected cell divided and its daughters (*d*) retained some sister-cell symmetry (1, 46) (Fig. 2).

Electron microscopy of LE61-injected cells revealed that,



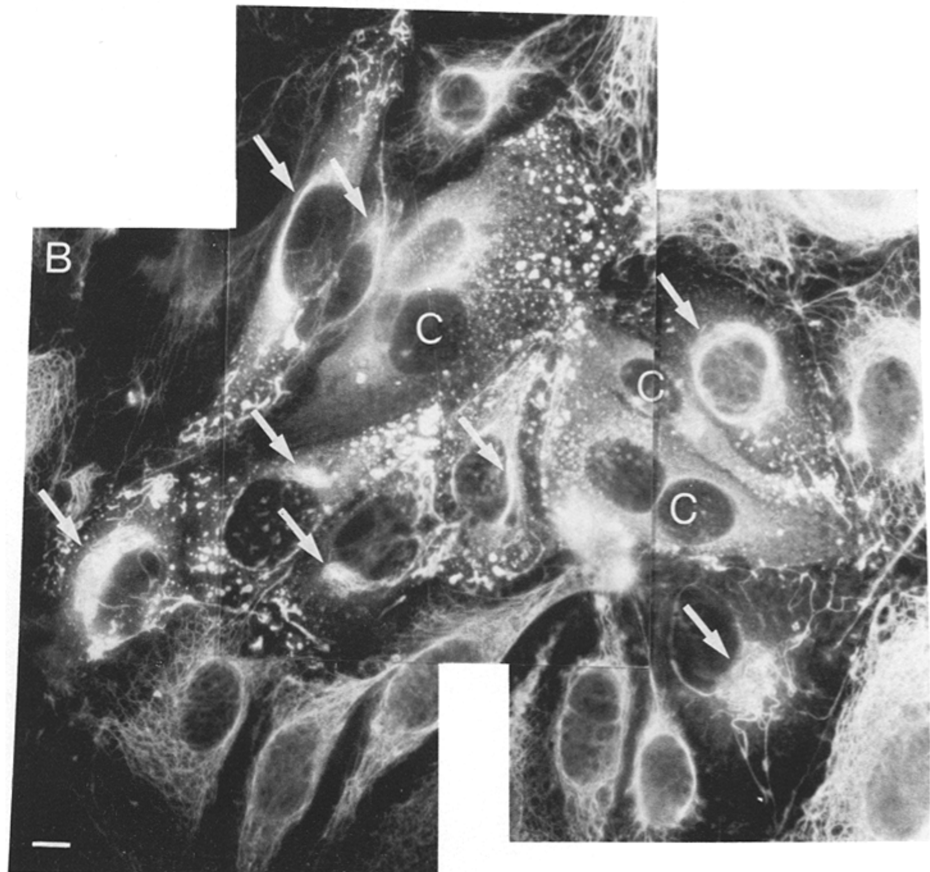
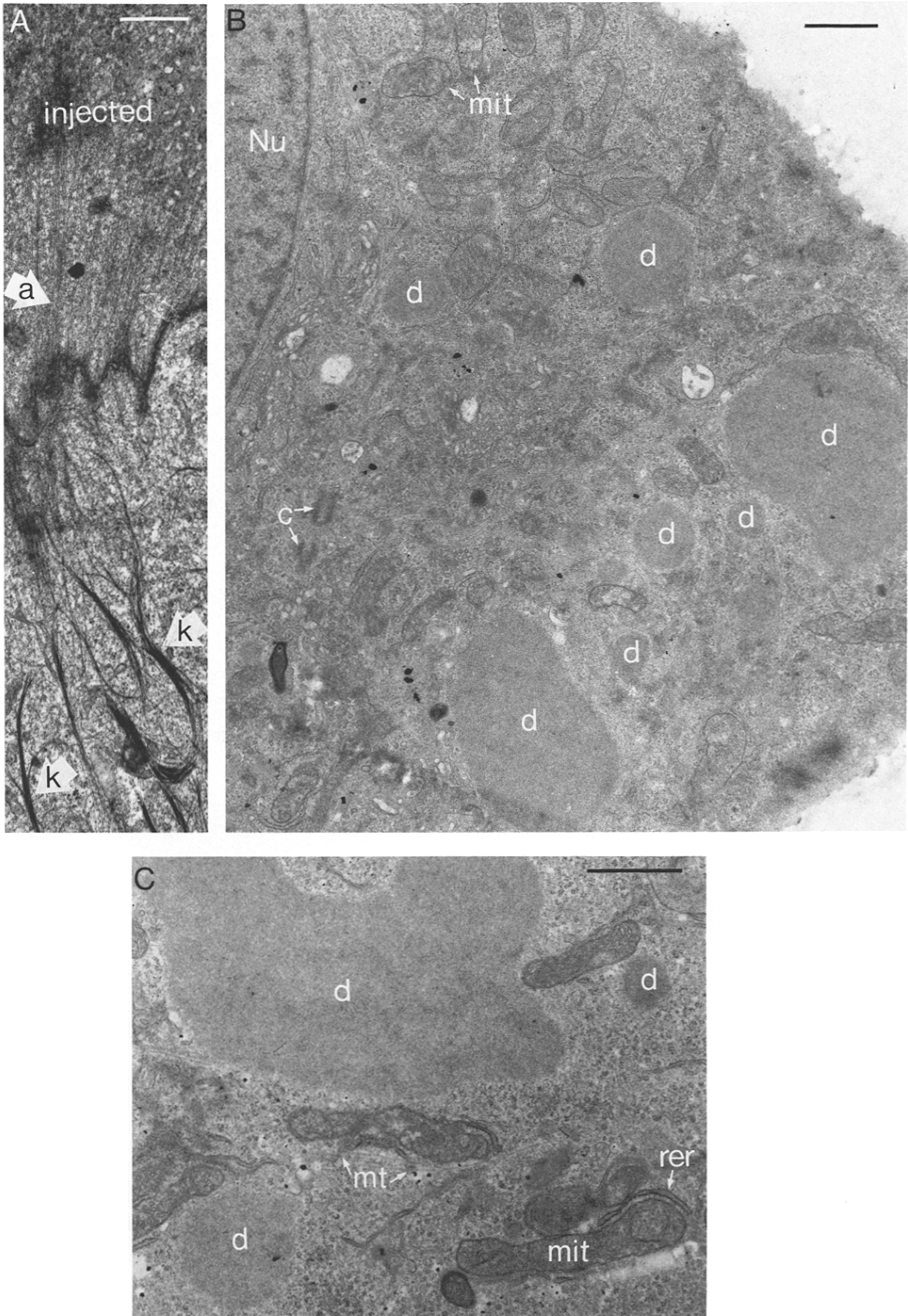


FIGURE 2 A group of PtK_2 cells injected with LE61 (7 mg/ml (*A*—Nomarski optics, injected cells within outlined region) were followed by time-lapse video microscopy for 23 h then fixed and labeled with LE41 and anti-MIg-Rd (*B*) to visualize keratin filaments. A cell which died after injection (black arrowhead in *A*) was expelled from the epithelial sheet. At ~8 h, an injected cell divided and its daughters (*d*, outlined by broken lines in *A*) still showed some sister cell symmetry 12 h later. All injected cells were effected by the injected antibody and were either without apparent keratin filaments (marked *C*) or contained perinuclear bundles of keratin filaments (white arrows) (*B*). Bar, 10 μm. × 500.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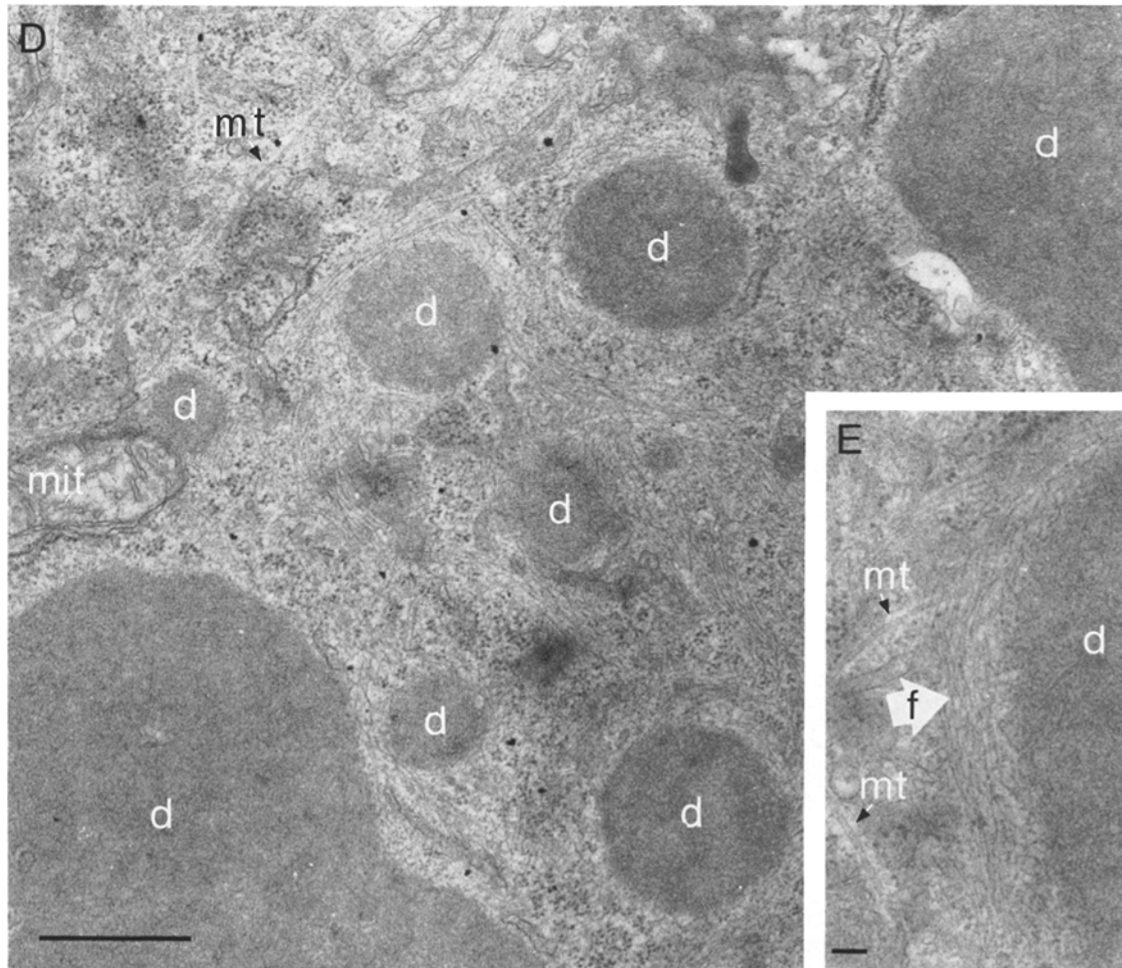

FIGURE 3 PtK₂ cells injected with LE61 (7 mg/ml) were fixed after 18 h and examined by thin-section electron microscopy. While normal cells contained prominent keratin filament bundles (arrows *k* in *A*), injected cells (*injected* in *A*, cells in *B* and *D*) contained none. Rather irregular granules with a fine, feltlike internal structure (*d*) were found scattered throughout the cytoplasm of injected cells (*B*). These granules were not bounded by membranes (*C* and *E*) and not obviously associated with other intracellular organelles or structures (*Nu*: nucleus, *c*: centrioles, *mit*: mitochondria, *rer*: rough endoplasmic reticulum, and *mt*: microtubules). In some cells however, coils of distinct, intermediate-sized filaments were found closely associated with the antibody-induced cytoplasmic granules (*D* and *E*), these resembled vimentin rather than keratin filaments. In *A*, actin filaments (arrow *a*) can be seen within the injected cell and inserted into the junction between cells. The plane of section passed close to the bottom of the injected cell. Bars (*A–D*), 1 μm. Bar (*E*), 0.1 μm. *A* and *B*: × 13,000; *C*: × 17,000; *D*: × 20,000; *E*: × 50,000.

unlike normal cells, many contained no dense keratin tonofilament bundles (Fig. 3 *a*) and few, if any, extended intermediate-sized filaments. The cytoplasm of the injected cells was littered with irregular clumps of homogeneous material (Fig. 3 *b*). These structures were not bounded by membranes (Fig. 3 *b* and *c*). Some fine fibrous substructure was seen within them, but the 8- to 12-nm keratin filaments did not remain intact (Fig. 3). The intermediate-sized filaments that were seen by electron microscopy in LE61-injected cells were single, looking more like vimentin than keratin filaments; residual filaments were usually found coiled around the nucleus or closely associated with the clumps of amorphous material (Fig. 3 *d* and *e*). Immunofluorescence microscopy revealed the same association between collapsed vimentin filaments and keratin filament remnants (31) (Fig. 5). LE61 itself has no affinity for vimentin, so this effect has to be an indirect one, a secondary result of keratin disruption (31).

After 3–4 d and one to two rounds of cell division, many of the injected cells and their descendants (see below) display few, if any, extended keratin filaments (Fig. 4). LE61-labeled keratin filament remnants were scattered throughout the cytoplasm (Fig. 4). Some cells had keratin filament bundles located near the nucleus. These bundles were not seen by indirect immunofluorescence of the injected LE61 (Fig. 4 *a–c*). Vimentin filaments, which had collapsed in response to injected antikeratin (31) (Fig. 5 *a* and *b*), had largely reemerged by 3 d after injection, although perinuclear bundles of vimentin filaments were still apparent in many cells (Fig. 5 *c* and *d*).

Aside from the transient injection trauma (see Materials and Methods), no obvious effects were seen on cell morphology or behavior throughout the course of antibody-induced intermediate filament disruption in PtK_2 cells. Membrane-ruffling and the saltatory motion of intracellular particles contained normally, as seen on time-lapse video films of injected cells (not shown). It should be noted that saltatory motions of particles could be temporarily stopped by excitatory illumination of cells containing fluorochrome-conjugated antibodies (Klymkowsky, unpublished observations).

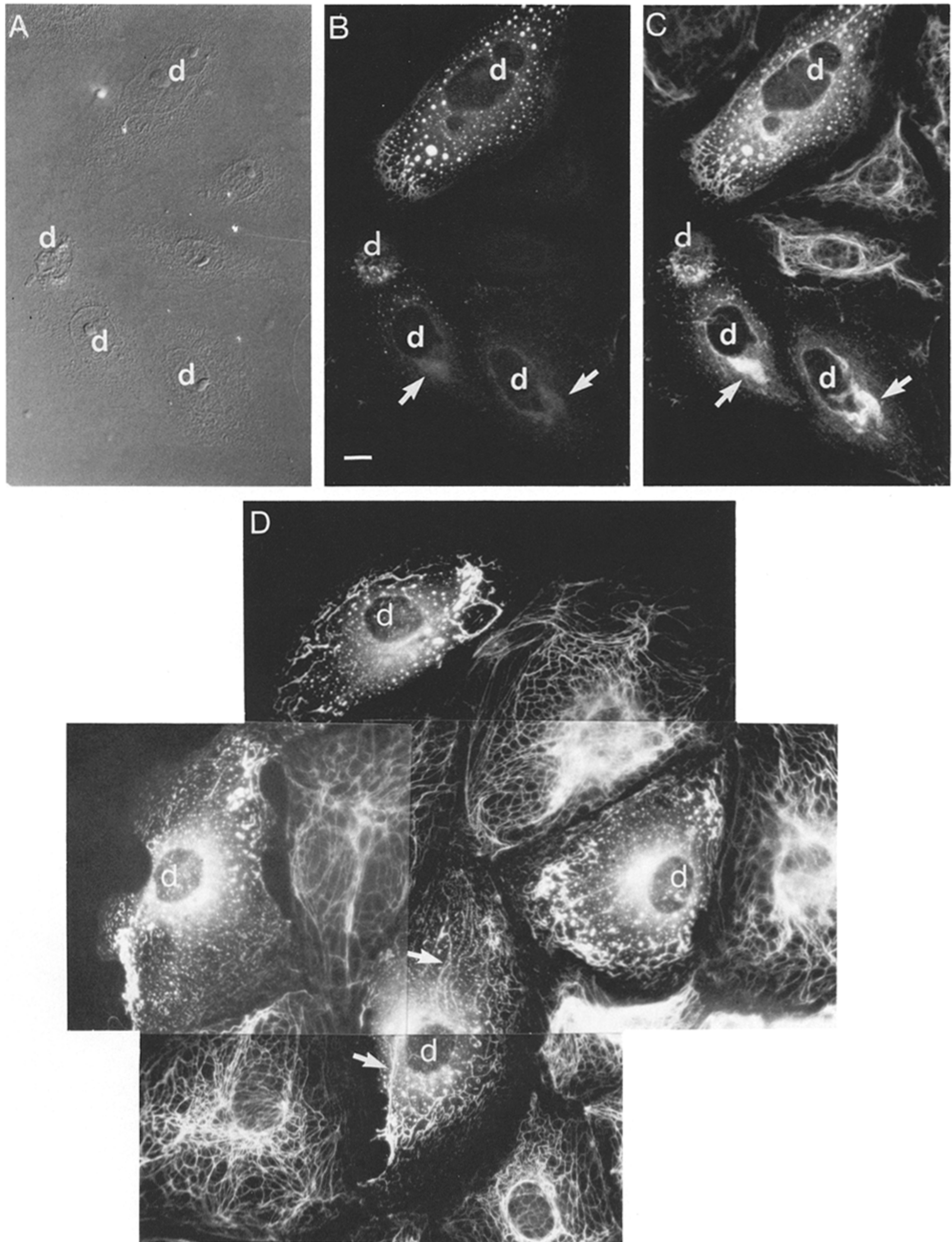

FIGURE 4 Two cases (*A–C*, and *D*) are shown of the effects of LE61 (7 mg/ml) on PtK_2 keratin filament organization 3 d after injection. A single cell within a cluster was injected and the cells fixed 3 d later (*A*; Nomarski optics), labeled with anti-MIg-Fl (*B*) to visualize the injected cell and its progeny, and then with LE41/anti-MIg-Rd (*C* and *D*) to reveal all keratin filaments. In both cases the original injected cell had divided twice in the course of the experiment (daughter cells, *d*). The keratin filament networks were still largely disrupted in all of the daughters (*C* and *D*), although clumps of keratin filaments near the nuclei (arrows in *A–C*) and some extended keratin filaments (arrows in *D*) were visible. Bar, 10 µm. × 500.

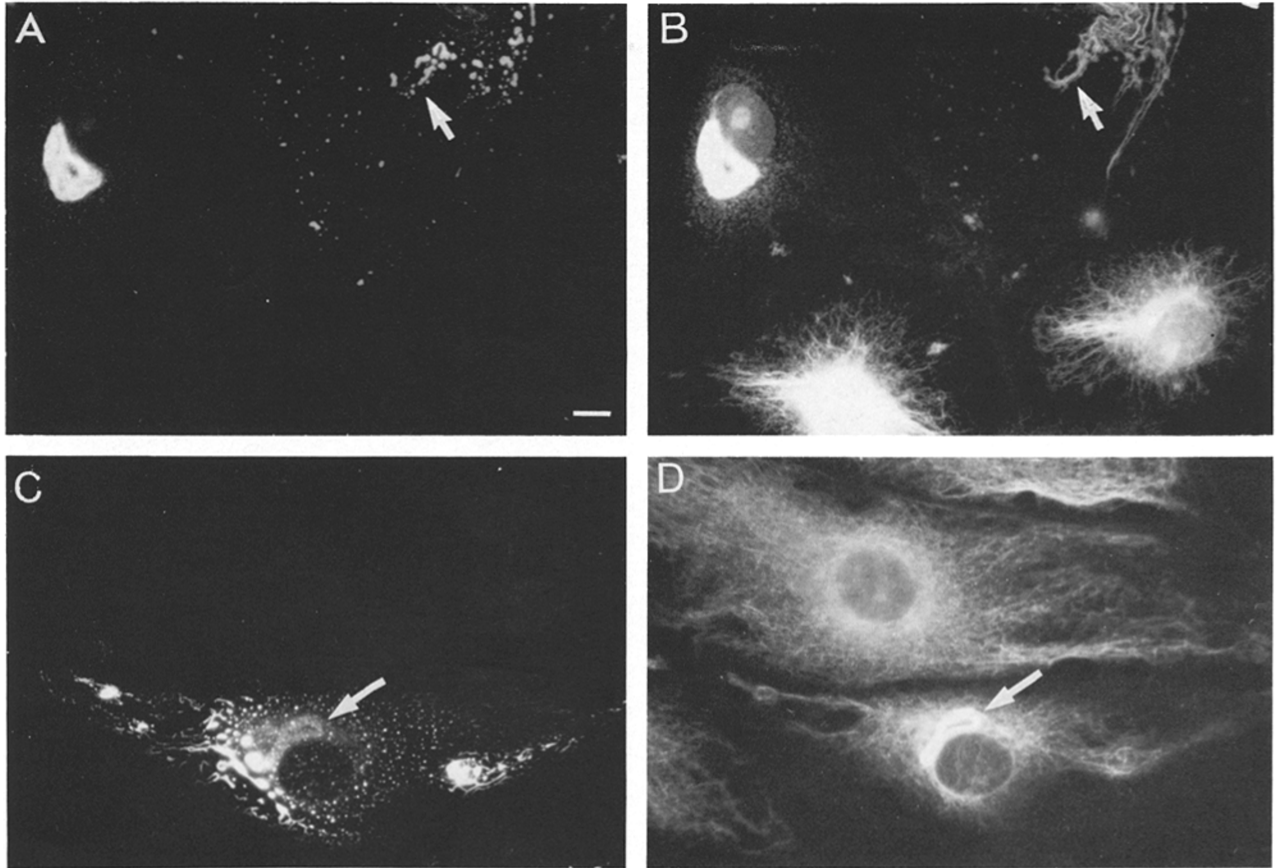

FIGURE 5 Four hours after LE61 injection (7 mg/ml) (*A*; revealed by anti-MIg-Fl) the vimentin filaments of injected PtK_2 cells (*B*; revealed by rabbit anti-vimentin/anti-RIg-Rd) were closely associated with keratin filament remnants and largely collapsed (arrows in *A* and *B*). By 3 d (*C*; injected antibody revealed by anti-MIg-Fl) a large portion of the injected cell's vimentin filaments (*D*; anti-vimentin/anti-RIg-Rd) were still collapsed in a coil near the cell nucleus (arrows *C* and *D*). The vimentin filament coil appeared to be associated with keratin filament remnants (arrows). The faint coil visible in *C* may be breakthrough fluorescence due to the intensely labeled vimentin filament coil in *D*. Bar, 10 μm. × 500.

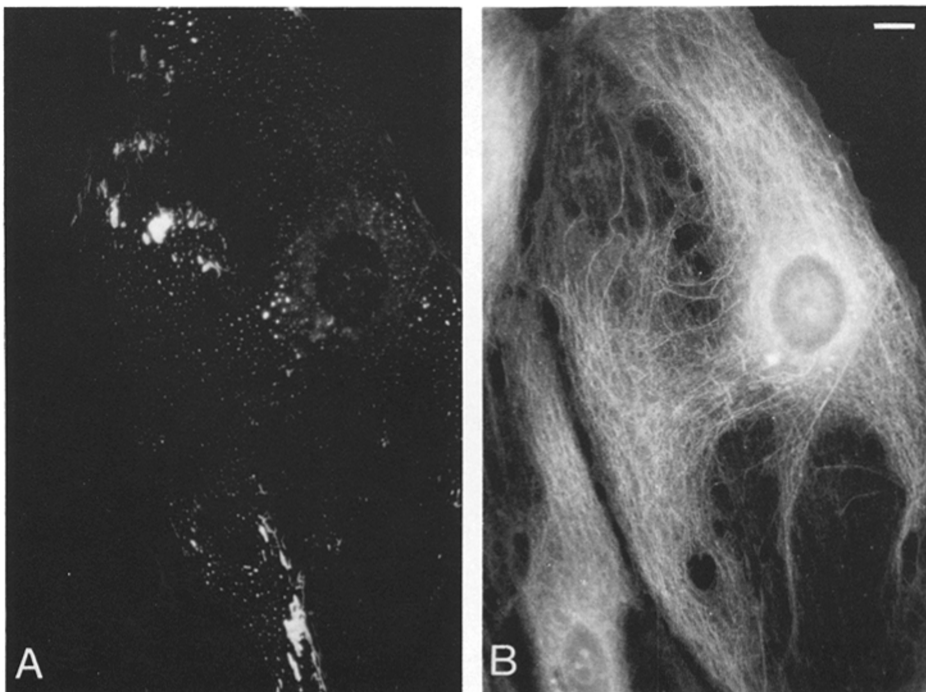

FIGURE 6 Three days after LE61 injection (7 mg/ml), PtK_2 cells were fixed and labeled by anti-MIg-Fl (*A*) to visualize the injected antibody and anti-tubulin/anti-RIg-Rd (*B*) to reveal the microtubular network. Bar, 10 μm. × 500.

At no time during the disruption of keratin and vimentin filament organization were there any obvious effects on either microtubule or microfilament organization (Figs. 6 and 8). The position of the nucleus appeared normal throughout keratin filament disruption (see Figs. 1, 2, 4–8). Likewise, the position of the centriolar complex was also unaffected (Figs. 7, 9, 10), as shown by the binding of LC46, a monoclonal antibody which recognizes a centriolar-associated antigen in PtK cells (E. B. Lane, unpublished data).[1]

During cell division, PtK cells, like other cell types, undergo substantial changes in morphology and a radical reorganization of their cytoskeletal systems (3, 4, 20), although they remain rather flattened by comparison with many other cultured epithelial cells. LE61-injected PtK_2 cells, with their intermediate filament systems disrupted, underwent all of the same gross morphological changes during mitosis as did normal, uninjected cells (see Fig. 11 *a–c*). They maintained their position within the epithelial sheet and showed a degree of postmitotic, sister-cell symmetry (1, 46) (Fig. 2 and unpublished time-lapse observations). This symmetry was progressively distorted in both normal and injected cells as they began to interact with other cells in clusters.

In experiments in which only interphase cells were injected with LE61, a number were later seen in prophase, metaphase, anaphase (Fig. 11 *a–c*), and teleophase. Cells were also followed by time-lapse video through mitosis (not shown). Since PtK cells grow in clusters, it was possible to record cell divisions by injecting a single cell per cluster. When examined at a later time, all of the cells found to contain injected antibody within one cluster were then known to be derived from a single injected cell. Examples of such an experiment are shown in Fig. 4, and clearly demonstrate that injected cells can go through at least two rounds of cell division with a disrupted keratin filament network.

LE41 is another monoclonal antibody against simple epithelium cytokeratins, but it recognizes a different determinant to that of LE61 (32, 33). We therefore injected similar amounts (7 mg/ml) of LE41 into PtK_2 cells for comparison with LE61. LE41 did not cause filament breakup. In many LE41-injected cells (Fig. 9), there was a partial, perinuclear coiling of keratin filaments, while the rest of the filaments appear as a fine, dense network. By contrast with the sharply defined filament cables in uninjected cells, it appears as though LE41 has induced separation and splaying out of the individual filaments within the bundles. Extended filaments labeled by injected LE41 could still be seen 4 d after injection (not shown). Neither LE61 nor LE41 had any obvious effect on the rate of cell division over the course of 3-day experiments when compared to anti-MIg-HRP injected cells (Table I, Fig. 10). The large number of cells that failed to divide at all during the 3 d of the experiment was not unexpected, given the low mitotic index of PtK_2 cells (4).

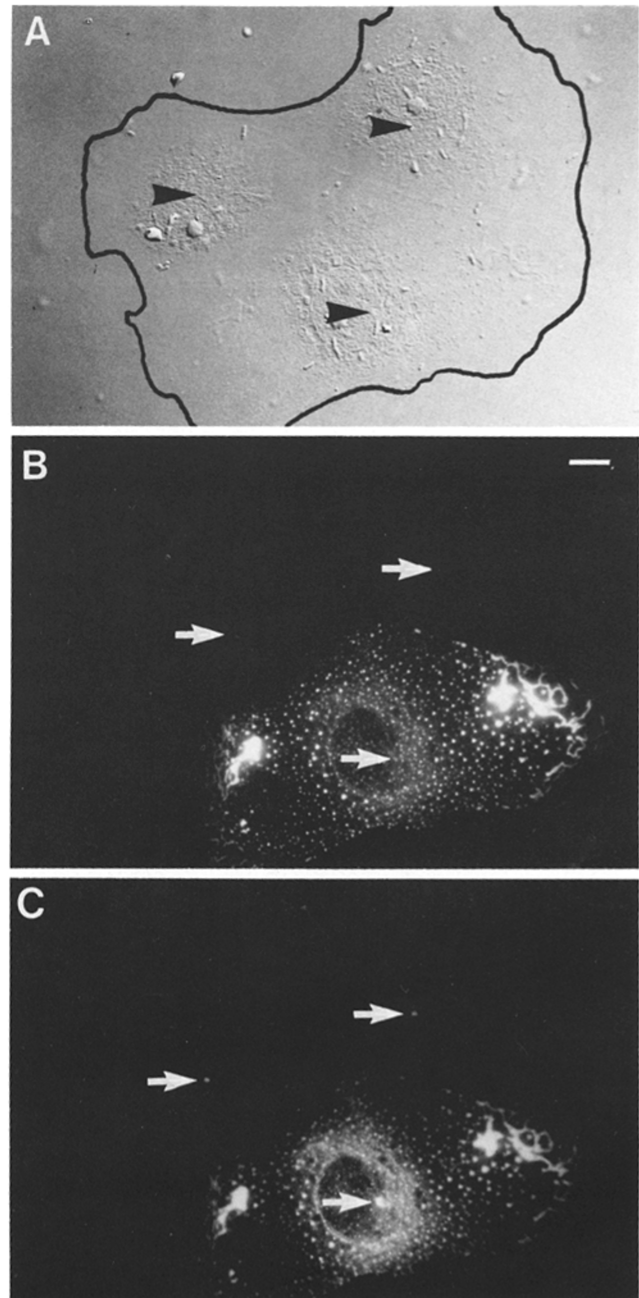

FIGURE 7 One day after LE61 injection (7 mg/ml), PtK_2 cells (*A*; Nomarski optics/cells outlined) were fixed and labeled with anti-MIg-Fl (*B*) to visualize the injected antibody and with the monoclonal antibody LC46/anti-MIg-Rd (*C*) to reveal the centriolar complex. Arrowheads and arrows mark the position of the LC46 labeled centriolar complex in both injected and uninjected cells, which in all cases was near the nucleus. Bar, 10 μm. × 500.

Effect of Injected Antikeratin Antibody on Other Cell Types

The injection of LE61 into PtK_1 cells caused the same kind of disruptive effect on keratin filament organization as seen in PtK_2 cells (Fig. 11), although less predictably. There were more filament remnants associated with the cell periphery (Fig. 11 *a–c*). Very small structures, labeled by the injected antibody, were often visible at sites where keratin bundles from neighboring, uninjected cells impinged on the boundary of the injected cell, presumably the sites of desmosomes (Fig. 11 *d* and *e*). The keratin filament networks of these uninjected neighboring cells were uneffected by antibody-induced disruption of keratin filaments in their neighbors (Fig. 11).

We examined the effect of injected LE61 on the keratin filaments of SVK14 cells, which are derived from human keratinocytes by SV-40 transformation, but, unlike normal

[1] When injected into PtK_2 cells, LC46 bound to the centriolar region but had no apparent effect on cell division, perinuclear centriolar localization, or keratin filament organization (unpublished observations).

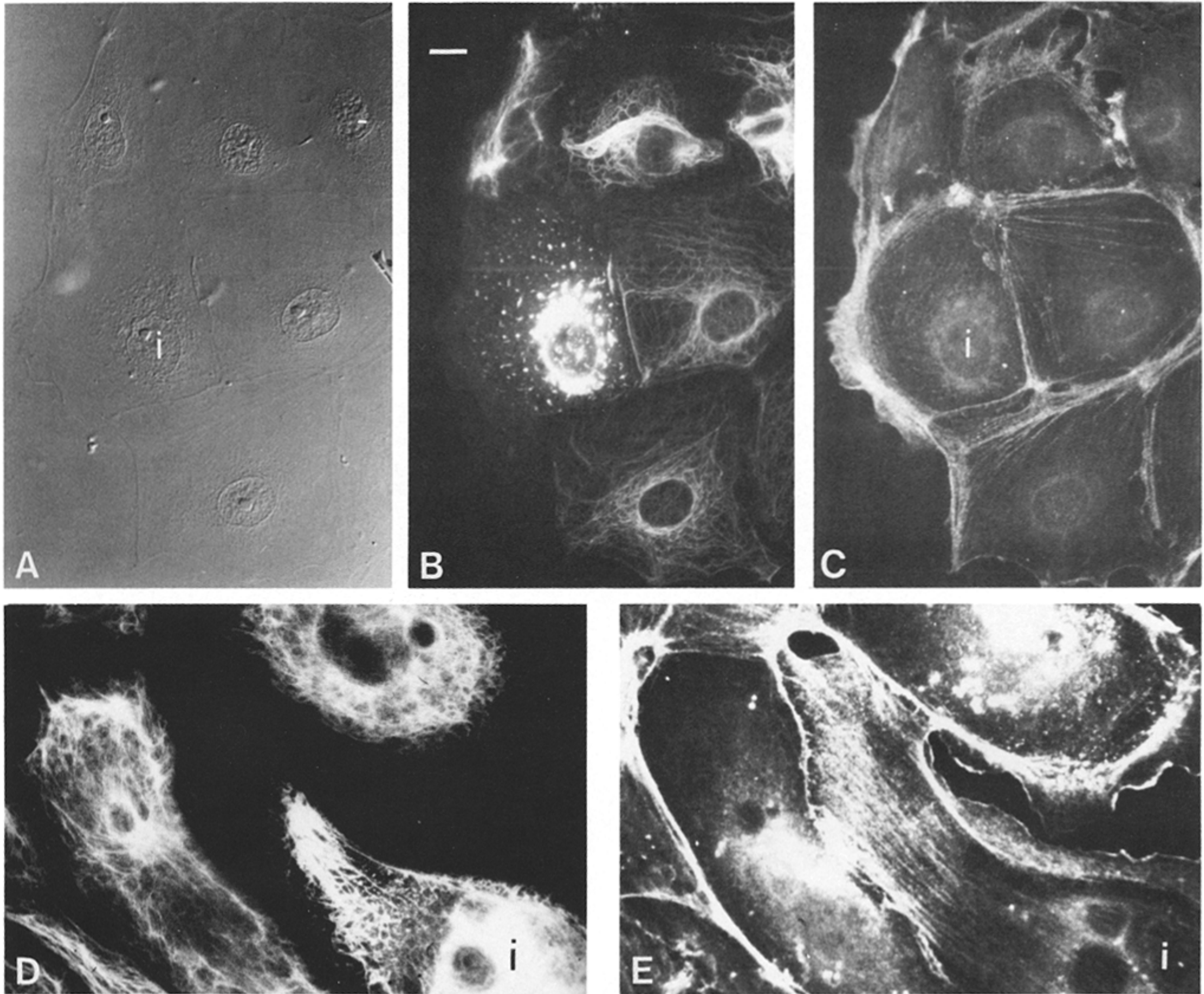

FIGURE 8 PtK_2 (*A–C*) and PtK_1 (*D* and *E*) cells were injected with LE61 (7 mg/ml) (*A*; Nomarski optics) and fixed after 4 (*A–C*) or 24 (*D* and *E*) h. The injected antibody disrupted keratin filament organization (*B* and *D*; visualized by LE41/anti-MIg-Fl) but had no obvious effect on actin organization (*C* and *E*; visualized by anti-actin/anti-RIg-Rd). Injected cells are marked by *i* in parts *A*, *C*, *D*, and *E*. Bar, 10 μm. × 500.

keratinocytes, display the LE61-recognized simple epithelial antigen (32, 33, 51) (Fig. 12*a* and *b*). LE61 was much less effective in SVK14 than in PtK_1 at disrupting filament organization, even when injected at concentrations of 20 mg/ml. Some fragmentation did occur, but the principal effect of the injected antibody was to retract and aggregate keratin filament bundles (Fig. 12*c–e*), as in the early stages of LE61-induced disruption in PtK cells. The distribution of desmosomal bridges, quite regular in uninjected cells (Fig. 12*a* and *b*), changed dramatically (Fig. 12*c–e*). In a rare case, nearly all of the injected antibody-labeled material was seen to have coalesced in one spot by the cell boundary, while in the adjacent, uninjected cell a symmetrical aggregation of keratin filaments had taken place (Fig. 12*f* and *g*). A couple of desmosomal bridges were still clearly visible between the two keratin-containing filaments bundles (Fig. 12*f–h*).

DISCUSSION

A number of functions have been ascribed to intermediate filaments, from being involved in positioning the cell nucleus and the determination of cell shape to being "mechanical integrators of cellular space" (35). We have used the intracellular injection of monoclonal antikeratin filament antibodies to examine the role of keratin filaments in cultured epithelial cells. These antibodies make it possible to manipulate keratin filament organization in different ways within living cells. When injected into cells, LE41 appeared to cause keratin filament bundles to splay apart (Fig. 9) (33) suggesting that the LE41-recognized antigenic determinant, located predominantly on polypeptides of 54 and 46 kilodaltons in PtK_1 cells, may be involved in the lateral organization of keratin filaments. On the other hand, LE61, a monoclonal antibody which recognizes a broadly distributed keratin filament determinant primarily on 41 and 43 kilodalton polypeptides (32, 33), can disrupt keratin filament organization in a more profound manner, such that the intermediate filament structure was lost (Fig. 3) (31, 33). LE65, monoclonal antibody directed against a determinant similar to LE61 (32, 33) had a similar effect on keratin filaments (not shown). The difference between the immunoblotting patterns of these monoclonal antibodies suggests that through intracellular injection it might be possible to

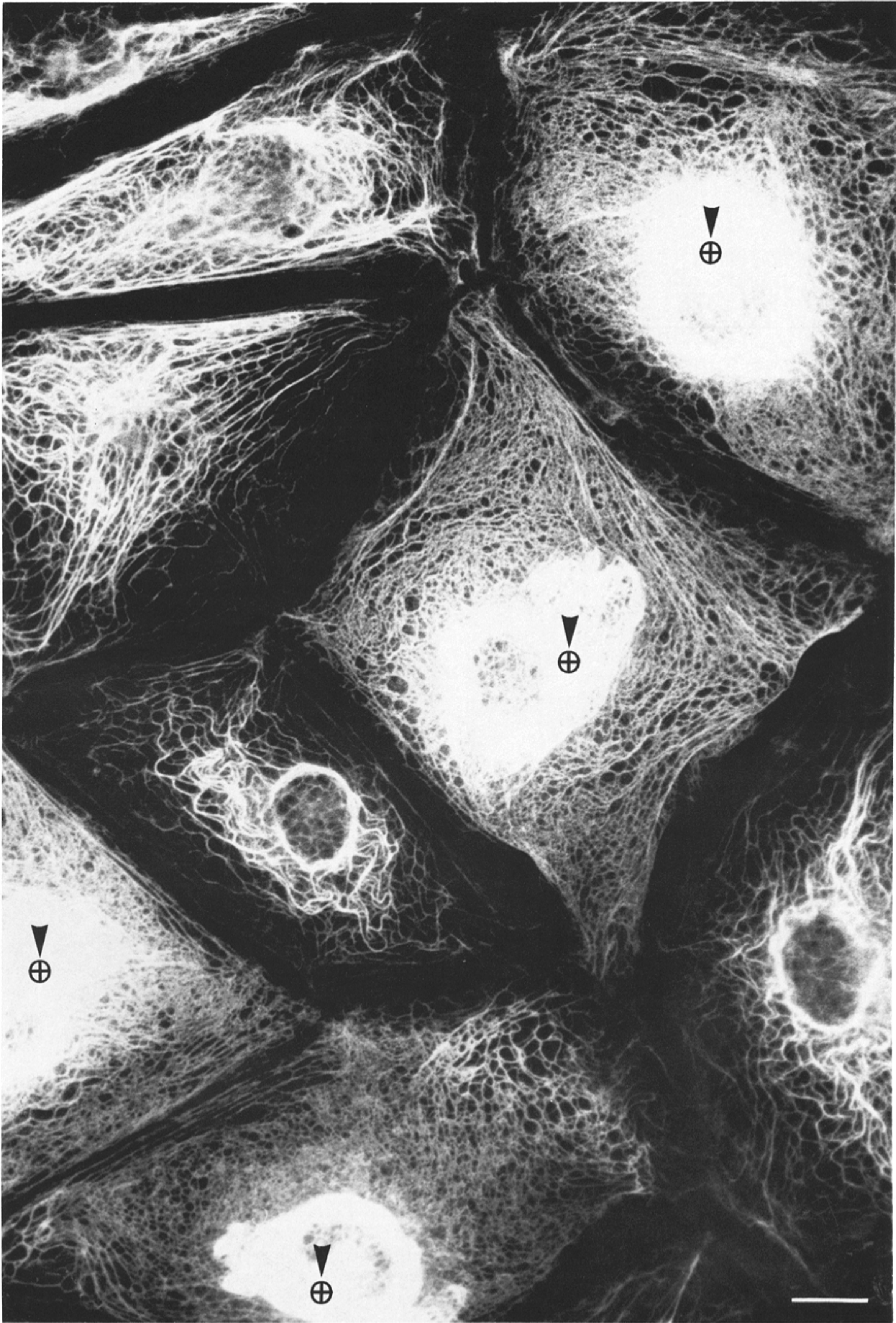

FIGURE 9 PtK_2 cells injected with LE41 (arrows and crossed circles) were fixed after 24 h (injected antibody recognized by anti-MIg-Fl (not shown) and cells were labeled by LE41/anti-MIg-Rd) revealed partial perinuclear collapse of the keratin filament network and a dense mesh of keratin filaments within the injected cells. Bar, 10 μm. $\times$ 1,350.

assign functions to different parts of the keratin com'plex (17), once more is known about keratin molecular structure.

When seen by electron microscopy (Fig. 3), the scattered cytoplasmic granules resulting from the injection of LE61 into PtK cells, are similar to the cytoplasmic bodies observed in two other cases of keratin filament disruption, that produced by cold shock in the skin of some fishes (44) and that seen during mitosis in some epithelial cell lines (18, 26, 34). In all these cases the 8- to 12-nm diameter filament structure is lost, but the finely fibrous, feltlike texture of the bodies suggests that some protofilamentous structure may persist. Such feltlike aggregates represent an alternative to the usual intermediate filament structure of the cytokeratins, perhaps analogous to the structure of the Z-disk associated intermediate filament proteins in muscle (35). This alteration in ultrastructure suggests that the binding of LE61 to sites on the keratin filament may interfere with the local forces which hold the protofilaments together.

LE61-induced keratin filament remnants appeared to be randomly scattered throughout the cytoplasm of injected cells (Figs. 1–9, and 11), even 3 (Fig. 4) to 5 d after injection. Unlike most foreign proteins, which seem to be quickly removed from the cytoplasm after intracellular injection and accumulated in lysosomes (48), we saw no signs of apparent autophagy of either LE61 or LE61-labeled keratin filament remnants (Figs. 3 and 4). On the other hand, antiintermediate filament antigen (43), a monoclonal IgG_1 antibody which caused the collapse of intact intermediate filaments in fibroblasts (30), seemed to induce the autophagy of keratin filaments when injected into PtK_2 cells (see Fig. 7 of reference 33; Klymkowsky, work in progress).

The disruption of keratin filament organization by LE61, when used at a concentration of 7 mg/ml, was complete by 2 to 4 h after injection in most cells (Figs. 1 and 2). PtK_1 and SVK14 cells were much less reproducibly effected by injected LE61. Increasing the concentration of injected antibody from 7 to 20 mg/ml had only a marginal effect on antibody-induced disruption, suggesting that most of the available antigenic sites had been saturated at the lower concentration. The observed differences on antibody susceptibility would appear to be due to variability among the cells. Both differences in keratin filament structure or the presence of mature desmosomes (see below) could be responsible.

Neither microtubule or microfilament organization was effected by antibody-induced keratin filament disruption (Figs. 3, 6, 8). PtK cells, like many cultured epithelial cells, contain a second, and distinct intermediate filament network of vimentin (4, 16, 23, 40) in addition to keratin filaments. Although LE61 had no effect on vimentin filaments when injected into cells without keratin filaments, it caused vimentin filament collapse in PtK_2 cells (31) (Fig. 5), indicating an interrelationship between the two filament systems (25, 31).

We could see no apparent gross effect on cell shape or behavior of the LE61-induced disruption of keratin filament organization in either PtK_2 or PtK_1 cells. Even though many injected cells contained no demonstrable keratin filaments (Figs. 1, 2, 3, and 4) and their vimentin filaments were completely collapsed (31) (Fig. 5), these cells were indistinguishable from uninjected, buffer-injected, or control antibody-injected cells (Figs. 1–11) (except for the occasional keratin filament debris seen in phase optics). Intermediate filaments often form cagelike structures around the cell nucleus (4, 7, 27), and it has been suggested that they may position the nucleus within the cell and provide "topological information" (7). In the absence of extended intermediate filaments there was no obvious change in the location of the nucleus or the centriolar complex (Figs. 1, 2, 4, and 7), or on postmitotic, sister-cell symmetry (1, 46) (Fig. 2 and unpublished time-lapse studies). It might be expected that the nucleus would be restricted to the thick central region of the cell in any case, so our observations do not rule out a role for intermediate filaments in nuclear positioning.

While small changes in the pattern of saltatory motions of intracellular particles may have escaped our notice, particles

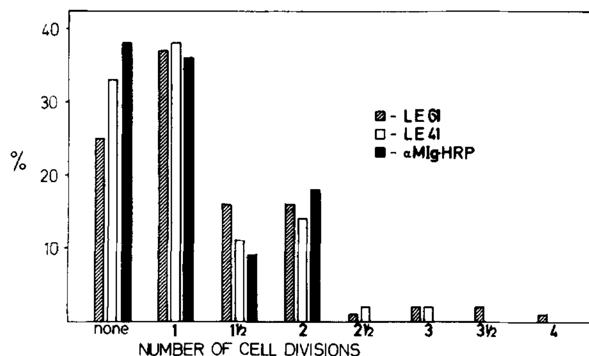

FIGURE 10 Plotting the percentage of injected PtK_2 (abscissa) cells which had given rise to no cell divisions (*none*), (*2*) one cell division (*1*), (*3*) marked (*1 ½*) etc. daughters by 3 d after injection shows that there was little difference in the number or pattern of cell divisions in PtK_2 cells injected with either LE61 (7 mg/ml), LE41 (7 mg/ml), or anti-MIg-HRP (2 mg/ml).

TABLE I

Fate of Cells at 3 Days after Antibody Injection

Injected antibody	Number of cell divisions*								Total no. of cells injected	Clusters with antibody‡	Cells with antibody§	Percent survival‖	Division index*
	None	1	1.5	2	2.5	3	3.5	4					
									A	*B*	*C*	*B/C* × 100	*C/B*
LE61	32	47	20	21	1	2	2	1	162	128	317	79	2.5
LE41	39	45	13	16	2	2	0	0	155	117	254	75	2.2
Anti-MIg-HRP	17	16	4	8	0	0	0	0	55	45	93	82	2.1

* The number of cell divisions was scored as none for a cluster containing only one cell with injected antibody, one for a cluster containing two cells with antibody, 1.5 for a cluster containing three cell, etc.

‡ The number of cell clusters found to contain injected cells or their progeny.

§ The number of individual cells found to contain injected antibody.

‖ Since only one cell per cluster was originally injected the percent survival is the number of clusters containing injected cells divided by the number of cells injected.

• The division index is the number of cells found to contain antibody divided by the number of cells which survived injection, i.e. the number of clusters with cells that contained injected antibody.

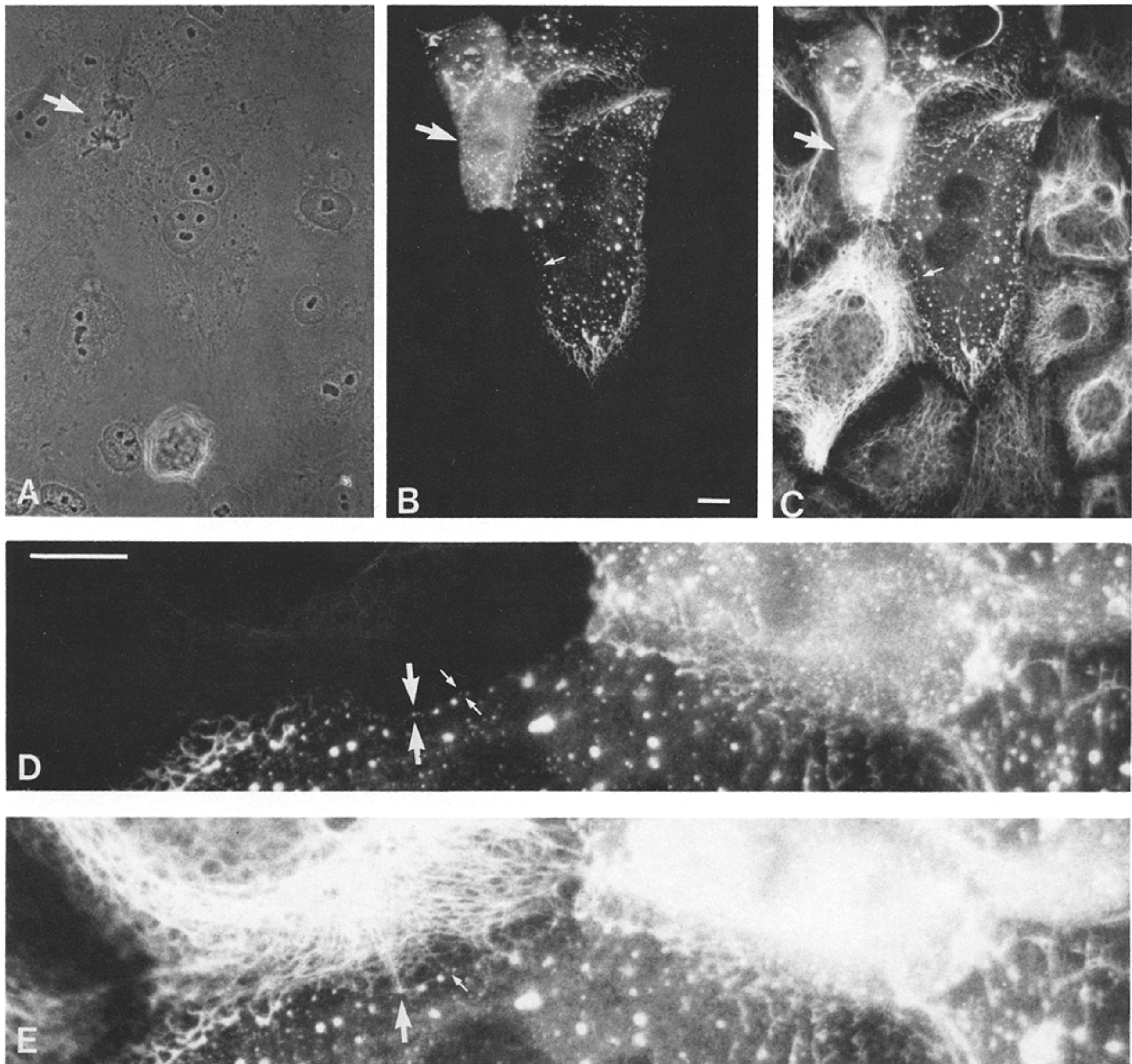

FIGURE 11 A group of PtK_1 cells within a sheet (*A*; Nomarski optics) was injected with LE61 (7 mg/ml) and fixed 24 h later. The injected cells (*B*; labeled by anti-MIg-Fl) remained integrated within the cell sheet even though the organization of their keratin filaments was disrupted (*C*; LE41/anti-MIg-Rd). An injected cell was caught in anaphase (large arrows, *A–C*). The small arrow in *B* and *C* points to a region of apparent desmosomal junctions. This region is shown at higher magnification in *D* (anti-MIg-Fl labeling of the injected antibody) and *E* (LE41/anti-MIg-Rd). The pairs of large and small arrows in *D* point out sites of impinging keratin filaments (*E*). Structures labeled by the injected antibody are visible at sites of cell-to-cell interaction of keratin filaments. Bars, 10 μm. *A–C*: × 500; *D* and *E*: × 1,500.

certainly continued to move in a saltatory manner after LE61-induced disruption of keratin and vimentin filament organization as seen on time-lapse video tapes (not shown). Likewise, vimentin filament collapse in fibroblasts had no effect on saltatory motion (21, 39), indicating that such intracellular movements do not require the presence of extended intermediate filaments. Membrane ruffling also continued in cells with no intact keratin filaments (not shown). A close association between intermediate filaments and microtubules has been reported (22). The collapse of intermediate filaments in mitotic cells (4, 27) and after drug-induced microtubule depolymerization (e.g., 27) suggests that intermediate filament organization may depend on microtubules. Microtubules, however, are clearly independent of intermediate filaments (Fig. 6) (5, 21, 30, 39). Likewise, the transformations in cell shape and microfilament organization during mitosis (3, 4) were not dependent on extended intermediate filaments (Figs. 8 and 11). Since it is now known that many epithelial cells also break up their keratin filaments during mitosis (SVK14 does, PtK do not) (18, 26, 34) it is perhaps not surprising that intact intermediate filaments are not necessary for normal cell division.

Some experiments have indicated that polyribosomes are associated with the detergent-insoluble cytoskeleton under suitable ionic conditions (36, 38). By autoradiography we could detect no difference in the cellular distribution of active polyribosomes in injected and uninjected cells (unpublished obser-

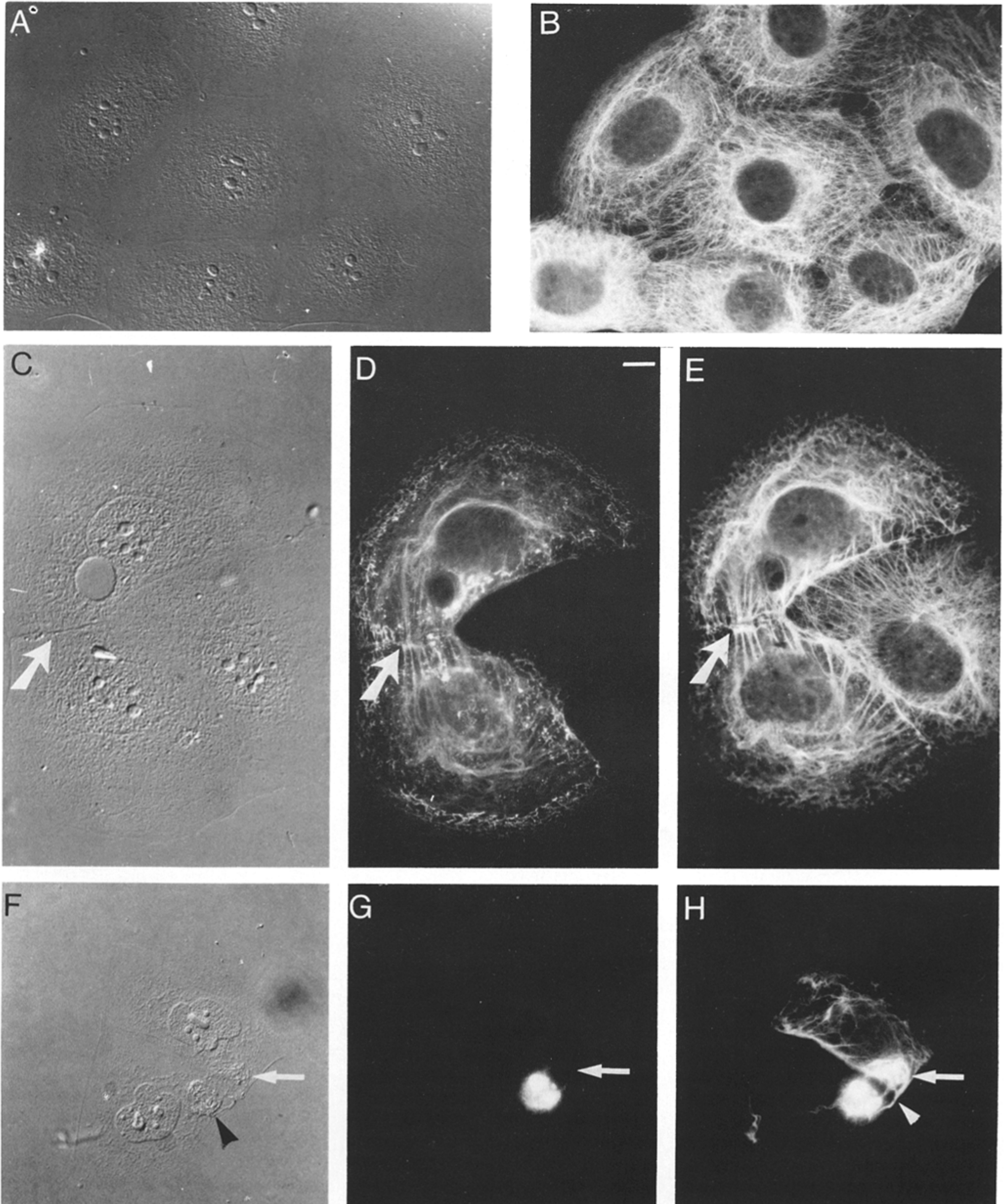

FIGURE 12 SVK14 cells, derived from keratinocytes, show cell-to-cell filament anchorage by way of desmosomal bridges, (*A*; Nomarski, *B*; = LE61-labeled uninjected cells). LE61 was injected at 20 mg/ml (*D* and *G*, visualized with anti-MIg-Fl) and usually caused partial filament disruption (*C–E*), with fragmented material collecting at the cell periphery; desmosomal bridges (arrows) are the most resistant part of the network. *E* and *H* show total antigen with secondary LE61/anti MIg-Rd. In the cell pair in *F–H*, all that remains of the network is a single phase-dense mass (*A*, arrowhead) at the cell boundary, but some desmosome bridges are still retained (*H*, arrowhead). This massive aggregate has furthermore induced symmetrical filament coalescence in its neighboring, uninjected cell (stemmed arrow), indicating desmosome mobility in the plane of the membrane. Bar, 10 μm. × 500.

vations). If bulk protein synthesis within PtK_2 cells depended on extended intermediate filaments, then the disruption of intermediate filament organization would have been expected to affect the rate of cell division, which depends at one level on protein synthesis. We observed no significant effect of antibody-induced perturbation or disruption of PtK_2 intermediate filament organization on mitotic rate (Fig. 10, Table I). This is not to say that the intact intermediate filament network or the injected antibodies themselves may not have had subtle effects on protein synthesis. The microtubule subunit protein tubulin, for example, can regulate its own synthesis (6).

Given that PtK_2 cells grow in clusters, although they are poor in desmosomes (55), it is not surprising that they remained in clusters in the absence of extended intermediate filaments (see Figs. 2 and 11). Intercellular actin filament alignment, through focal contact junctions analogous to zonulae adherentes (15) was unaffected by keratin filament disruption (Figs. 3 and 8), and it is presumably through actin coordination that the cells are maintained in integrated clusters.

The presence of desmosomes seems to confer some resistance to antibody-induced disruption. PtK_1 cells have more desmosomes than PtK_2 cells, and although the disruption of keratin filaments by injected LE61 was similar to that in PtK_2, it was less predictable and often not as extensive. The keratin filaments of SVK14 cells were even less susceptible to LE61-induced disruption than PtK_1 cells and SVK14 cells clearly have more desmosomes (Fig. 12*b*, *d*, and *e*).

Keratin filament remnants often remained attached to the cell edge at the sites of filament attachment in neighboring cells (Fig. 11), indicating that desmosomes persisted after keratin filament disruption. Disruption of filaments in one cell had no obvious effect on the keratin filament organization in neighboring, uninjected cells (Fig. 11). There was no suggestion that tension existed across desmosomal junctions. The effect of LE61, injected into SVK14, was most similar to the very early stages of filament collapse in PtK cells. The filament network of injected cells remained partly intact and held out at several anchorage sites, presumably desmosomes, where they bordered on other cells. Filaments only pulled away completely from free edges with no desmosomes. Frequently the filaments appeared to be prevented from total collapse by being looped around the nucleus. Even after the total collapse of the filament network seen in Fig. 12*f–h* the single remaining antibody-stained mass was still attached to desmosomes at the boundary with the single neighboring cell. By means of these desmosomes, the collapsed mass was in line with the keratin filaments of its neighbor, in which a symmetrical keratin aggregation had appeared. These observations suggest that desmosomal junctions can, like other membrane proteins, move in the plane of the plasma membrane.

CONCLUSION

Our observations on the keratin filaments of cultured epithelial cells, together with similar results obtained from the study of vimentin filaments in cultured fibroblasts (21, 30, 39), suggest that keratin filaments in particular and intermediate filaments in general are not actively involved in the maintenance of cell shape or movement in tissue culture, although the developmental complexity of the keratin protein argues for biochemical functions at another level. The insoluble intermediate filament network may indeed form the true "cellular skeleton," that is, a passive mechanical framework which strengthens cells and tissues against mechanical deformation. Intermediate filaments may not be required by cells cultured on a rigid substrate.

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