Detection of Desmin-containing Intermediate Filaments in Cultured Muscle and Nonmuscle Cells by Immunoelectron Microscopy

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ABSTRACT Antibodies raised against chicken gizzard smooth muscle desmin were shown to be specific by immunofluorescence cytochemistry and immunoautoradiography after two-dimensional polyacrylamide gel electrophoresis. Embryonic chick heart cell cultures (permeabilized with Triton X-100) and enucleated adult chicken erythrocyte ghosts (Granger, B. L., E. A. Rapasky, and E. Lazarides, 1982, *J. Cell Biol.* 92:299–312) were then used for immunoelectron-microscopic localization of desmin. As expected, all intermediate filaments (IF) of the cardiac myocytes were labeled heavily and uniformly with the desmin antibodies. No periodicity or helicity was detectable along the labeled IF. Of interest was the intermittent but clear labeling of the IF of the nonmuscle, fibroblastic cells in the identical cultures. These antibodies did not bind vimentin from embryonic chick heart homogenates; furthermore, they did not label IF of avian erythrocytes known to contain vimentin but not desmin. We conclude that IF of cardiac fibroblastic cells contain low, but significant, concentrations of desmin and that this protein probably forms a copolymer with vimentin in these cells.

Intermediate filaments (IF) are cytoplasmic fibrous elements of 8–12 nm diameter ubiquitous among eukaryotic cells. In spite of their apparent morphological similarities, IF in different cell types can be distinguished on the basis of their constituent subunit proteins (IFP). Biochemical and immunological differences divide the IFP into five classes (1): vimentin, in fibroblasts and many other cells of mesenchymal origin, as well as in many cells grown in tissue culture; desmin, in muscle; cytokeratin, in various epithelia; neurofilament triplet proteins, in neurons; and glial fibrillary acidic protein, in astroglia. Although it had been assumed earlier that a given cell type had only one characteristic IFP, it is now becoming clear that this histological specificity is not absolute. Vimentin, which has the broadest tissue distribution, has also been shown to be coexpressed with cytokeratin in cultured epithelial cells (2, 3), with glial fibrillary acidic protein in cultured astrocytes and glioma cells (4, 5), with desmin in skeletal muscle (6, 7) and in vascular smooth muscle (8–10).

The occurrence of IFP other than vimentin in "noncharacteristic" cell types has been the subject of some controversy. This is especially true with respect to desmin. Early studies employing indirect immunofluorescence claimed that desmin was present only in muscle cells (11–13). Subsequently, desmin was shown to coexist with vimentin in baby hamster kidney (BHK-21) cells (14), reputedly a fibroblastic cell line. It has been suggested recently, however, that these cells are in fact derived from vascular smooth muscle (9). Although Gard, Bell, and Lazarides (15) reported the presence of desmin in chick embryo fibroblasts derived from myogenic cultures, it may be argued that the desmin seen in the gels was derived from contaminating myoblasts. Furthermore, the immunofluorescence, which was weak and required Colcemid-induced aggregation of the IF to be detectable, might have been due to slight cross-reactivity of the antisera with vimentin. Hence, to obtain a definitive answer as to whether or not desmin is found in nonmuscle cells, two requirements must be met: a method of sufficient sensitivity, and a way of proving the absolute specificity of the probe. We have used immunocytochemical tech-

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niques at the electron microscopic (EM) level to study the distribution of desmin in embryonic chick heart cells grown in monolayer culture. Using the biochemically pure vimentin filaments in chicken erythrocytes as a negative control, we have shown that antibodies, which are completely unreactive with the erythrocyte IF, label not only the IF of cardiac myocytes heavily and uniformly but also produce intermittent decoration of the IF in the nonmuscle cells. From these data, we conclude that: (*a*) desmin is the predominant IFP in cardiac myocyte IF and is found uniformly distributed throughout all IF, and (*b*) the nonmuscle cells in these cultures also contain desmin, but in much lower amounts, interspersed along IF which may be heteropolymers with vimentin.

MATERIALS AND METHODS

Preparation and Characterization of Antibodies: Antibodies against chicken gizzard smooth muscle desmin were raised in rabbits essentially according to Lazarides and Hubbard (16). Desmin (skeletin) was obtained by extraction with either 8 M urea (16) or 1 M acetic acid (17) and purified by preparative polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS PAGE) (18) before use as an immunogen. All animals used were screened to ensure that they did not have auto-antibodies to IF proteins before immunization.

Immunoglobulins were purified from immune sera by ammonium sulfate fractionation and chromatography on DEAE-cellulose (Whatman DE52, Whatman Inc., Clifton, NJ). For affinity purification of the antibodies, an acetic acid extract containing desmin and actin (17) was brought to neutral pH, and the resulting precipitate was cross-linked with 0.3% glutaraldehyde.[1] After extensive washing in 0.5 M Tris-HCl, pH 8, this material was used as an affinity adsorbent. Since this adsorbent contains a substantial amount of a 43,000-dalton protein presumed to be actin, the antibodies eluted from it were additionally passed through a column of F-actin covalently linked to 6-aminohexanoic acid-activated Sepharose 4B (Sigma Chemical Co., St. Louis, MO) to ensure that no actin antibodies were present. The actin-Sepharose column was judged to be active by its reversible binding of myosin subfragment 1 and actin antibodies kindly provided by Dr. R. Schwartz (Baylor College of Medicine, Houston, TX; reference 20). No difference in antibody labeling was observed when passage through the actin column was omitted. In some experiments, the antibodies were absorbed with an acetone powder of chicken erythrocyte ghosts before use.

Two-dimensional isoelectric focusing/SDS polyacrylamide gel electrophoresis (IEF/SDS PAGE) was performed as described for a miniature gel system by Mikawa et al. (21) except that the IEF gel contained 0.2% pH 3.5–10, 1.6% pH 5–7 and 0.8% pH 4–6.5 ampholines. The sample was prepared by homogenizing two hearts from 7-d-old embryonic chicks into 1 ml of a buffer containing 8.5 M urea, 2% Nonidet P-40, 1% 2-mercaptoethanol. The extracts were clarified in a Beckman microfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) for 2 min before application to the IEF gel. After focusing, two equivalent IEF gels were subjected to electrophoresis on the same SDS polyacrylamide slab gel, which consisted of a 4.5% acrylamide stacking gel over a 10% separating gel. The gel was then sliced vertically to give two two-dimensional gels, one of which was stained directly with Coomassie Brilliant Blue (R-250) for protein; the other was transferred electrophoretically to nitrocellulose (S&S BA45) by the method of Towbin et al. (22) except that the electrophoresis was carried out for 3 h at 100 mA.

Immunoautoradiography (immunoblot; "Western" transfer) was carried out as follows: after drying the nitrocellulose, excess protein binding sites were blocked by preincubation with 1% globulin-free bovine serum albumin in phosphate-buffered saline (1% BSA-PBS) for 15 min at room temperature, after which the nitrocellulose paper was reacted with 50 μg/ml DEAE-cellulose purified antidesmin IgG in 0.1% BSA-PBS for 1 h at room temperature; the paper was then washed extensively with several changes of PBS, again preincubated with 1% BSA-PBS, and incubated with 1×10^6 cpm/ml ^{125}I-protein A (prepared by iodination of protein A by the chloramine T method (23) for 1 h at room temperature; following this incubation, the paper was washed for 1 h with several changes of PBS and then overnight in 0.15 M NaCl, 0.05 M Tris HCl, 1% sodium desoxycholate, 1% Triton X-100, 0.1% SDS, pH 7.5. After washing, the paper was air-dried, exposed for 8 h at −70°C to Kodak X-OMAT AR-5 X-ray films with intensifying screen (Cronex Lightning Plus), and the autoradiogram developed in D-19.

[1] Only after this antibody was prepared did a method for preparing actin-free desmin appear in the literature (19).

The vimentin antibodies were a kind gift from Dr. Stephen Blose, Cold Spring Harbor Laboratory, and were used at a dilution of 1:40.

Unlabeled goat-anti-rabbit IgG was obtained from Cappel Laboratories Inc. (Cochranville, PA) and further purified on an affinity column of rabbit IgG covalently linked to CNBr-activated Sepharose 4B (24). Ferritin-labeled goat-anti-rabbit IgG was obtained from Miles-Yeda (Rehovot, Israel). All antibody solutions were used at a concentration of ~50 μg/ml.

Frozen Sections: Pieces of breast muscle, cardiac muscle, and gizzard (5 mm^3), from a freshly killed chicken, were frozen in liquid nitrogen-cooled isopentane and mounted in O.C.T. compound (Tissue Tek, Lab-Tek Div., Miles Laboratories Inc., Naperville, IL). Frozen sections 4-μm thick were cut on a Slee cryostat (Slee International Inc., New York, NY), air dried, and fixed in acetone at −20°C before using for indirect immunofluorescence experiments.

Cell Cultures: Ventricles of seven- or eight-d-old chick embryos were dissociated by incubation with trypsin (25). For light microscopy, dispersed cells were plated onto glass coverslips previously coated with Formvar. For electron microscopy, cells were plated onto stainless-steel electron microscope grids sandwiched between a Formvar film and a 12-mm round glass coverslip. The cultures were maintained for at least 48 h at 37°C in Ham's F-12 medium supplemented with 10% fetal bovine serum and antibiotics, in an atmosphere of 95% air, 5% CO_2, before being used.

Preparation of Cardiac Cytoskeletons for Microscopy: Cytoskeletons were prepared essentially by the method of Webster et al. (26). The coverslips bearing cells were rinsed briefly in buffer P (0.1 M PIPES, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM EDTA, 10 μM taxol), extracted for 6 min in buffer P containing 0.5% Triton X-100, and again thoroughly rinsed. Indirect immunolabeling of filaments in the cytoskeletons was performed in a humid environment, employing either unlabeled or ferritin-conjugated goat-anti-rabbit IgG as second antibodies. Unlabeled goat-anti-rabbit-IgG was equally effective in localizing the intermediate filaments because the accumulation of antibody molecules thickened the filaments sufficiently for identification in the electron microscope. After labeling and rinsing, the cytoskeletons were fixed in buffered 2% glutaraldehyde, rinsed in distilled water, stained in 1% uranyl acetate, dehydrated in ethanol, and critical-point-dried from CO_2 in a Tousimis Samdri 790 instrument. Some samples were rotary shadowed with a thin layer of platinum and carbon to stabilize them against melting in the electron beam during electron microscopy (see Figs 4*a*, *b*, and *c*; Fig. 5); others were not (see Fig. 4*d*).

For fluorescence microscopy, cytoskeletons were similarly prepared except that affinity-purified, fluorescein- or rhodamine-conjugated second antibodies were used. Coverslips were mounted either in 90% glycerol, 20 mM Tris, pH 8.7, or in Gelvatol (Monsanto Co., St. Louis, MO) and photographed on Kodak Tri-X film.

Preparation of Avian Erythrocytes for Microscopy: Enucleated erythrocyte ghosts were prepared from chickens exactly as described by Granger et al. (27). For light microscopy, ghosts were allowed to adhere to Alcian Blue-coated glass coverslips before being processed for indirect immunofluorescence (not shown). For electron microscopy, ghosts were allowed to adhere to glow-discharge Formvar-carbon-coated grids and then floated successively on drops of buffer P, first antibodies, buffer P twice, ferritin-conjugated goat-anti-rabbit-IgG, and finally buffer P, for 30 min each. They were then fixed in buffered 2% glutaraldehyde and negatively stained with 1% uranyl acetate.

Electron Microscopy: Grids were examined in a JEOL 100CX electron microscope equipped with a side-entry goniometer, at 80 or 100 KV. Negatives of critical-point-dried samples were contact-reversed and the resulting "positives" were used for printing.

RESULTS

Antibody Specificity

The specificity of the antibodies for desmin was first assessed by indirect immunofluorescence and immunoautoradiography. Immunofluorescence microscopy of glycerinated adult chicken skeletal myofibrils, cultured embryonic chick heart cells, and frozen sections of adult muscle tissues showed characteristic staining patterns for desmin (Fig. 1). In agreement with published reports (7, 16), the antibodies stained the Z-lines of adult myofibrils, as well as a lacy scaffold that appeared to link adjacent myofibrils into register (Fig. 1 *a* and *b*).

Cultures of embryonic chick cardiac cells contain two predominant cell types: glycogen-rich, contractile myocytes and

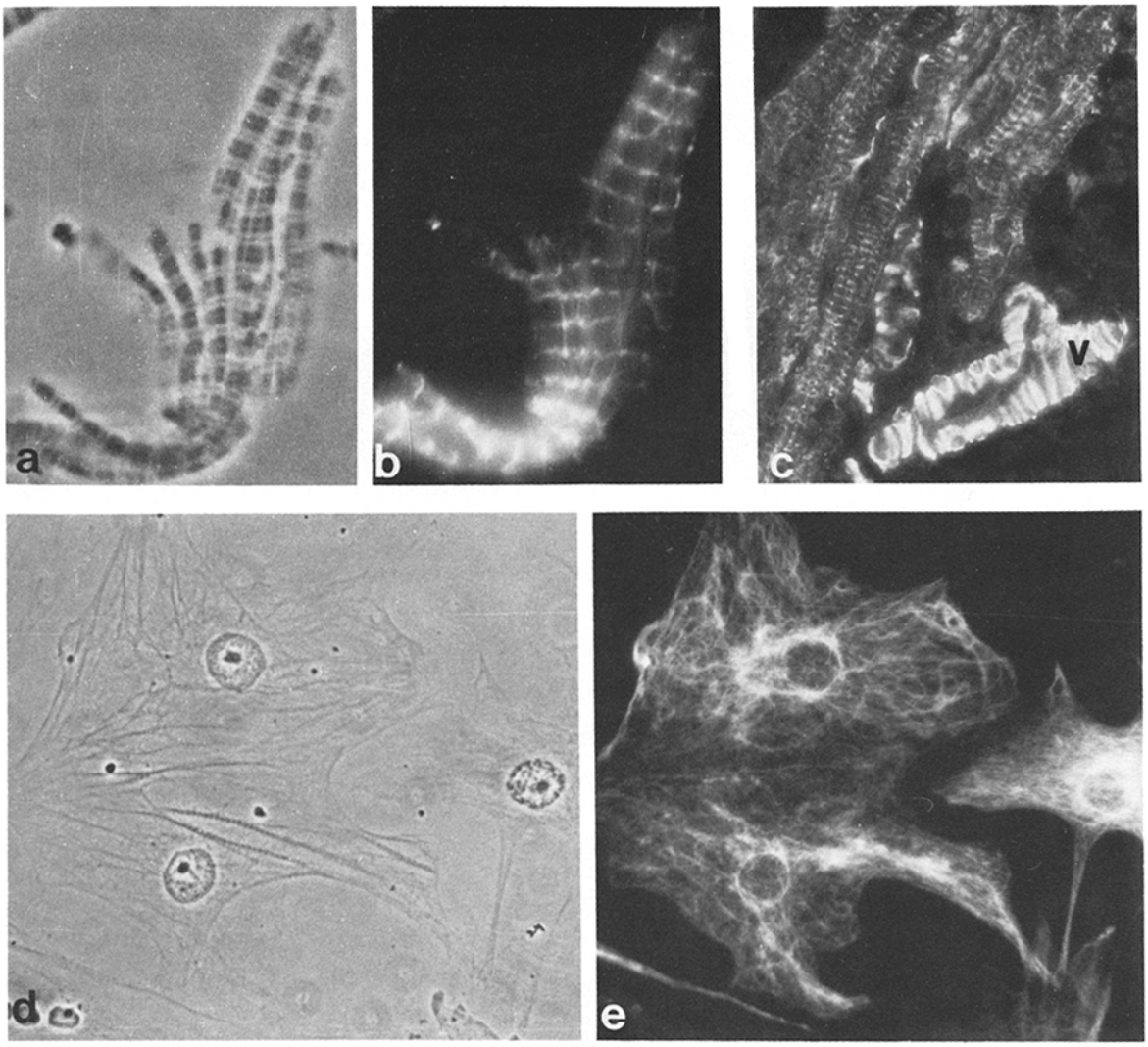

FIGURE 1 Characterization of the desmin antibodies by immunofluorescence. (*a* and *b*) Phase contrast and fluorescent images of adult chicken skeletal myofibrils labeled with the desmin antibodies. × 1,600. (*c*) Frozen section of adult chicken cardiac muscle. *v*, blood vessel. × 700. (*d* and *e*) Phase contrast and fluorescent images of embryonic cardiac myocyte cytoskeletons labeled with desmin antibodies. × 700.

more flattened, pleomorphic, noncontractile cells, presumably fibroblasts (28). Myocytes stained intensely with desmin antibodies, primarily as a fibrous network (Fig 1 *d* and *e* and references 29, 30, 31) although a diffused background staining was always seen in the cytoplasm. A weak but significant level of staining was consistently observed in fibroblasts during visual examination although this level of fluorescence was invariably too low to be captured on film.

In frozen sections of adult skeletal (not shown) and cardiac muscle (Fig. 1 *c*), desmin was also localized at Z-lines and longitudinally between myofibrils. It was prominent in the tunica media of blood vessels, presumably within smooth muscle cells. Invariably, the fluorescent staining was more intense in the smooth muscle of the blood vessels than at the Z-lines of the striated muscle.

When homogenates of whole, 7-d embryonic chick hearts were displayed on gels by IEF/SDS PAGE, desmin was resolved from other proteins, notably vimentin and synemin (Fig. 2 *a*). Immunoautoradiography of such gels using the desmin antibodies (Fig. 2 *b*) produced only one spot which was identified as desmin by its R_f in the IEF and SDS PAGE dimensions. The isoelectric variants of desmin were not resolved in these miniature IEF gels.

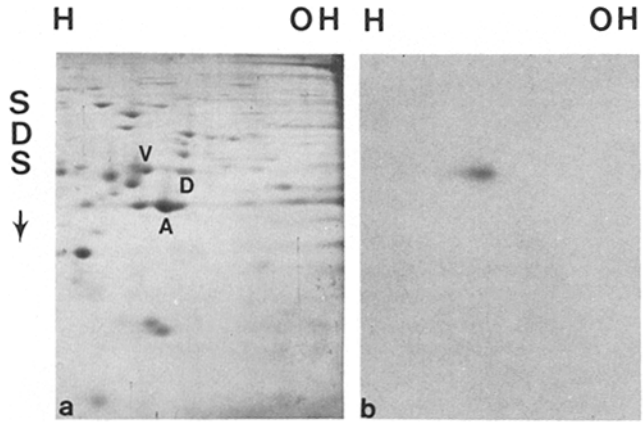

FIGURE 2 Immunoautoradiographic analysis of desmin antibody reactivity. ~5 μl of an 8.5 M urea-2% NP40 extract of 7-d embryonic chick heart were displayed by two-dimensional IEF/SDS PAGE. (*a*) Electrophoretic pattern after staining for protein with Coomassie Brilliant Blue R-250; *V*, vimentin, *D*, desmin (α + β), *A*, actin (α, β, γ). (*b*) Autoradiogram of an identical gel that had been transferred electrophoretically to nitrocellulose paper and stained indirectly with the desmin antibodies followed by ^{125}I-protein A. 8-h exposure. The spot was identified by its R_f in the IEF and SDS PAGE dimensions.

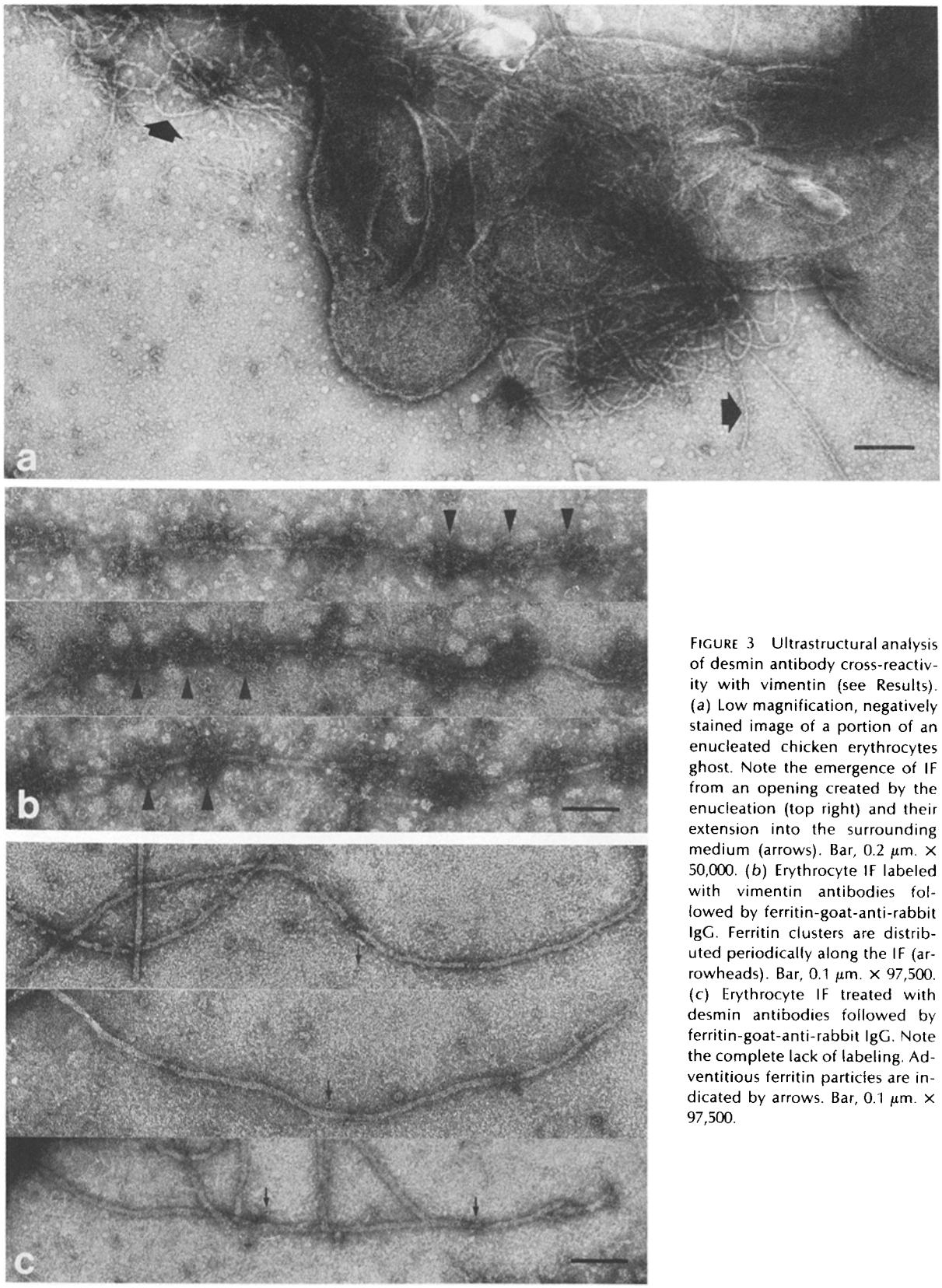

FIGURE 3 Ultrastructural analysis of desmin antibody cross-reactivity with vimentin (see Results). (*a*) Low magnification, negatively stained image of a portion of an enucleated chicken erythrocytes ghost. Note the emergence of IF from an opening created by the enucleation (top right) and their extension into the surrounding medium (arrows). Bar, 0.2 μm. × 50,000. (*b*) Erythrocyte IF labeled with vimentin antibodies followed by ferritin-goat-anti-rabbit IgG. Ferritin clusters are distributed periodically along the IF (arrowheads). Bar, 0.1 μm. × 97,500. (*c*) Erythrocyte IF treated with desmin antibodies followed by ferritin-goat-anti-rabbit IgG. Note the complete lack of labeling. Adventitious ferritin particles are indicated by arrows. Bar, 0.1 μm. × 97,500.

Experiments with Chicken Erythrocytes

It may be argued that desmin antibodies with a low but significant affinity for vimentin would produce false-positive labeling of native IF, detectable by electron microscopy, but escape detection during immunoautoradiography of the denatured protein. To eliminate this possibility, we performed indirect labeling on chicken erythrocyte IF, recently shown by Granger et al. (27) to be composed of vimentin and synemin but to contain no detectable amount of desmin (Fig. 3). Expulsion of hypotonically lysed erythrocytes through a fine-gauge needle resulted in the extrusion of their nuclei. This left

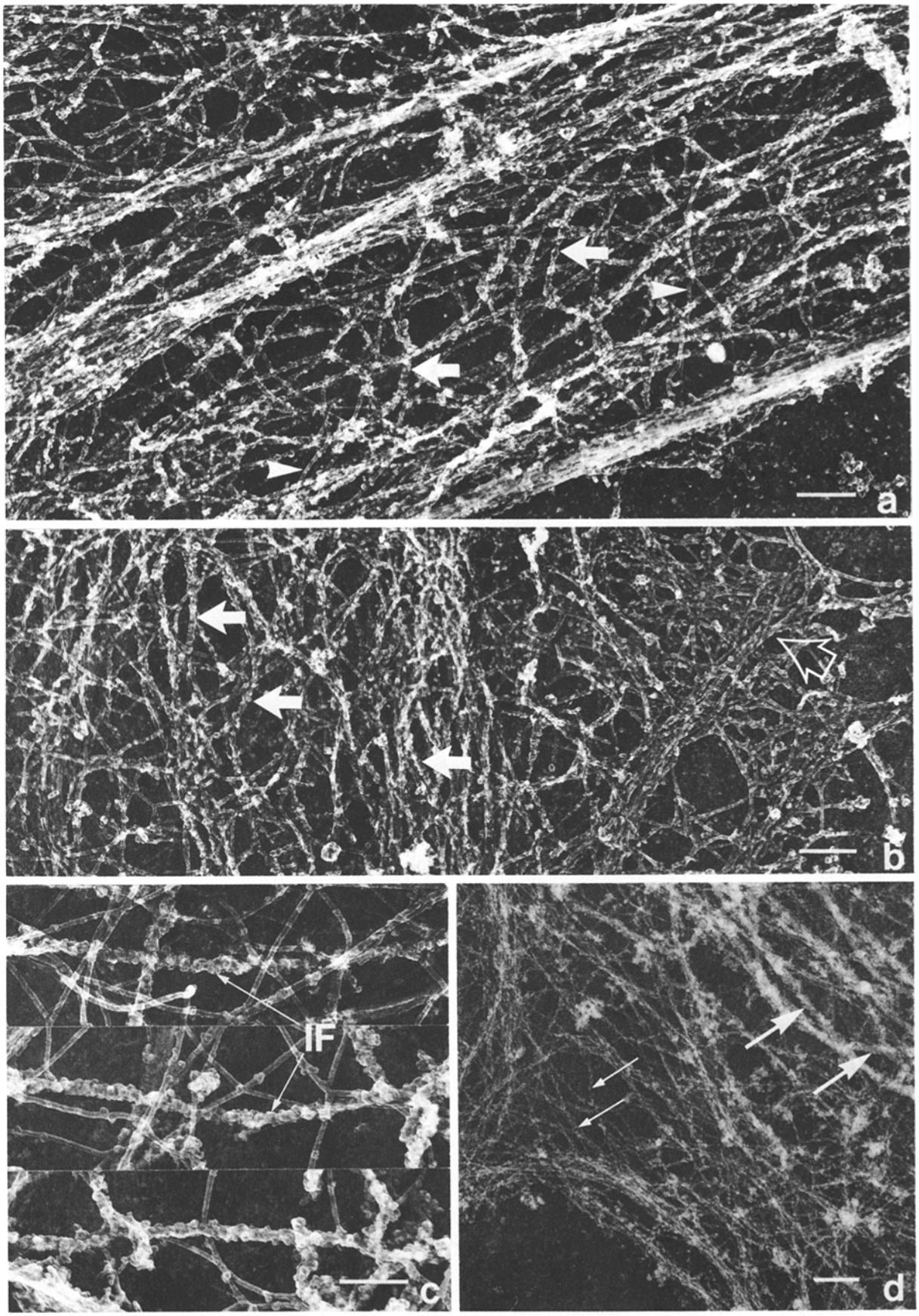

FIGURE 4 Desmin antibody labeling of cardiac myocyte IF. (*a*) Cortical region of a cardiac myocyte cytoskeleton labeled with desmin antibodies followed by ferritin-goat-anti-rabbit IgG. A population of wavy filaments is now greatly thickened (~30 nm) and studded along their length with ferritin particles (arrows). Thinner, unlabeled filaments remain smooth-contoured (arrowheads). Bar, 0.2 μm. × 50,000. (*b*) The antibody-labeled IF (solid arrows) are absent from thinly spread regions of the cell, which are populated by actin filaments (open arrow). Bar, 0.2 μm. × 50,000. (*c*) High magnification views of antibody-decorated IF. Note that the entire filament is labeled without apparent periodicity. Bar, 0.1 μm. × 120,000. (*d*) Myocyte cytoskeleton simultaneously labeled with myosin subfragment 1 (thin arrows) and desmin antibodies followed by unlabeled goat-anti-rabbit IgG (thick arrows) to contrast the locations of actin and intermediate filaments respectively (see text). Bar, 0.2 μm. × 40,000.

an opening in the ghost, which was presumably too large to be resealed. Since in the intact cell the nucleus was probably positioned by IF (32), the enucleation usually left a tuft of IF at the opening (Fig. 3 *a*). Very often, the IF extended well beyond the margin of the cell into the surrounding medium. These completely exposed IF proved to be ideal subjects on which we could perform antibody labeling. Enucleated erythrocyte ghosts adhering to Formvar-coated grids were incubated in a manner identical to that for the muscle cell cultures with either vimentin antibodies (kindly provided by Dr. Stephen Blose) or desmin antibodies, followed by ferritin-conjugated goat-anti-rabbit IgG. The antibody binding was then visualized by negative-stain electron microscopy. As shown in Fig. 3 *b*, the IF of chicken erythrocytes were labeled intensely by antibodies to vimentin, but not at all by the desmin antibodies (Fig. 3 *c*). It should be pointed out that, although the IF in Fig. 3 *b* show intermittent labeling by the vimentin antibodies, full decoration could be achieved by increasing the antibody concentration or incubation time. This, however, completely obscured the IF themselves.

Immuno-EM Analysis of Cytoskeletons Derived from Primary Cardiac Cell Cultures

Cardiac cytoskeletons produced by detergent permeabilization and critical point drying closely resembled intact cells in gross morphology. Most membranous material was solubilized, leaving behind a filament network and an electron-dense nuclear residue. Myocytes were defined by the presence of striated myofibrils. Most of the nonmuscle cells contained long bundles of filaments that are probably identical to the stress fibers observed by light microscopy. These fiber bundles often ter-

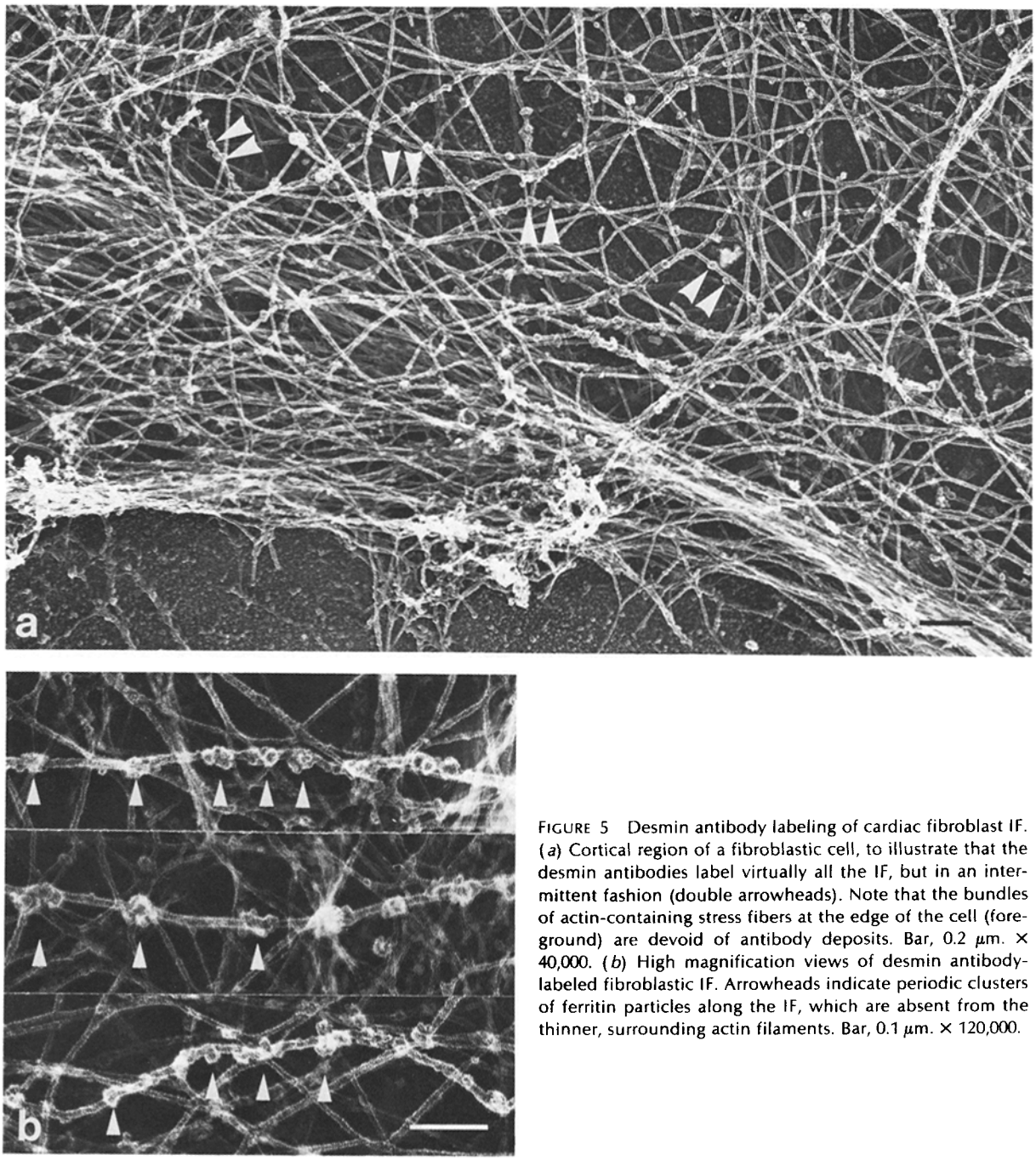

FIGURE 5 Desmin antibody labeling of cardiac fibroblast IF. (*a*) Cortical region of a fibroblastic cell, to illustrate that the desmin antibodies label virtually all the IF, but in an intermittent fashion (double arrowheads). Note that the bundles of actin-containing stress fibers at the edge of the cell (foreground) are devoid of antibody deposits. Bar, 0.2 μm. × 40,000. (*b*) High magnification views of desmin antibody-labeled fibroblastic IF. Arrowheads indicate periodic clusters of ferritin particles along the IF, which are absent from the thinner, surrounding actin filaments. Bar, 0.1 μm. × 120,000.

minated at ruffled borders in which were located complex, anastomotic networks of actin-containing filaments.

The cytoskeleton contained, in addition to microtubules, a dense network of filaments, the diameters of which ranged from 3–12 nm. In contrast to embedded and thin-sectioned material, filaments in whole-mount, critical-point-dried cytoskeletons could not be categorized as thin or intermediate filaments with any degree of certainty by morphological criteria alone. However, they could be easily distinguished from one another by specific labeling procedures (Fig. 4). All of the IF within myocyte cytoskeletons were uniformly labeled with antibodies to desmin (Fig. 4*a*). These filaments exhibited a gently curvilinear and undulating pattern in most regions of the cell (29, 30, 31) and formed an intricate woof around all myofibrils although specific associations with them were not apparent. They occupied a different intracellular domain than microfilaments and were completely absent in the actin-rich ruffled border regions (Fig. 4*b*). That desmin and actin are found in mutually exclusive filament populations could be demonstrated by simultaneous labeling of the IF with desmin antibodies and of actin with myosin subfragment 1 (Fig. 4*d*). At this level of resolution, the antibody labeling of desmin-containing filaments did not exhibit any obvious periodicity (Fig. 4*c*).

In fibroblastic cells found in the same cultures, indirect labeling with desmin antibodies resulted in the intermittent decoration of the IF (Fig. 5). Although the extent of labeling was considerably less than that of myocyte IF, virtually all IF in the fibroblasts assumed a knobby appearance periodically along their length (Fig. 5*a*). This appearance was brought about by the deposition of clusters of ferritin particles (Fig. 5*b*). Furthermore, this represents the maximal degree of labeling achievable with the desmin antibodies; doubling the incubation time or increasing the antibody concentration by 10- and 50-fold did not bring about appreciably more complete labeling of the fibroblastic IF. Since the same antibodies did not decorate the vimentin-containing IF in chicken erythrocytes under identical conditions (Fig. 3), we conclude that their labeling of fibroblastic IF reflects the presence of desmin.

DISCUSSION

The first major finding of the present studies is that antibodies to desmin labeled the IF of cardiac myocytes uniformly and completely. This strongly suggests that desmin is an integral, structural protein of cardiac IF, probably forming the core of these filaments. Recent evidence (33, 34) indicates that muscle IF have a number of accessory proteins, viz., synemin (mol wt 230,000 daltons) and paranemin (mol wt 280,000 daltons). Since these proteins are found in much smaller quantities than desmin (34) and have not been shown to polymerize into IF by themselves, they are probably distributed periodically along a filamentous core composed of desmin. Further studies are necessary to substantiate this possibility.

Simultaneous labeling with desmin antibodies and myosin subfragment 1 demonstrated that desmin and actin form mutually exclusive populations of filaments in myocytes and nonmuscle cells in primary cardiac cell cultures. This result, together with the finding that purified desmin is polymerization-competent (19, 35), argues against the copolymerization of desmin and actin into IF in vivo (36). The significance of the persistent copurification of actin with desmin through several cycles of polymerization-depolymerization remains to be established.

The second major finding is that desmin antibodies that do not cross-react with vimentin under either native (in enucleated erythrocyte ghosts) or denaturing (in immunoautoradiography) conditions do label the IF in cardiac fibroblasts. This provides strong evidence that desmin is present in these filaments.

In immunocytochemical studies at the EM level, it is often difficult to distinguish low levels of labeling, due to the small amount of the antigen present, from binding due to slight cross-reactivity of the antisera, even if no such cross-reactivity is observable by immunofluorescence or immunoblotting. This complication is even more acute in the case of the IF that have been shown to share antigenic determinants (37, 38), and in the case of desmin and vimentin in particular, which show extensive amino acid homologies (39, 40). Thus, a negative control is critical. Luckily, recent studies of the IF network of chicken erythrocytes (27) have demonstrated that these filaments contain vimentin but no desmin. Since these structures proved completely unreactive with the desmin antibodies at both the immunofluorescent and EM levels, but the IF of cultured cardiac myocytes and fibroblastic cells were decorated with them, we conclude that desmin is present in both of these cardiac cell types. Comparing the extent of labeling in the two cell types suggests that fibroblastic IF contain relatively little desmin, which might explain the weak to absent immunofluorescence in our hands and others' (11–13, 15).

The low desmin content in the IF of chick cardiac fibroblasts agrees closely with the low molar ratio of desmin to vimentin established biochemically for chick embryo fibroblasts (15), but does not appear commensurate with the molar ratio of approximately unity seen in BHK-21 cells. Although the high desmin content in BHK-21 cells might be due to species differences, it more likely reflects their origin from embryonic vascular smooth muscle (9).

Since all filamentous elements present in the embryonic myocyte could be identified morphologically (microtubules, thick and thin filaments within myofibrils) or by specific labeling with S_1 (cytoplasmic actin filaments) or antibodies (intermediate filaments), it may be concluded that desmin is present in all intermediate filaments. Therefore, the fibroblastic IFP, vimentin, present in these cells must occur as a copolymer with desmin. Copolymers of IFP have been shown to be the preferred conformation when mixtures of these proteins are allowed to polymerize in vitro (41) and have been demonstrated in BHK-21 cells by biochemical cross-linking in situ (42). Insofar as the fibroblastic cells are concerned, the sparse labeling of their IF again points to copolymers of desmin and vimentin, with vimentin being the predominant species.

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