Immunocytochemical Analysis of Intermediate Filaments in Embryonic Heart Cells with Monoclonal Antibodies to Desmin

SPENCER I. DANTO and DONALD A. FISCHMAN
Department of Anatomy and Cell Biology, State University of New York/Downstate Medical Center, Brooklyn, New York; Department of Cell Biology and Anatomy, Cornell University Medical College, New York 10021

ABSTRACT Monoclonal antibodies (McAbs) have been generated against a preparation of intermediate filament proteins (IFP) from adult chicken gizzard. Two antibodies, D3 and D76, have been characterized in detail. They bind specifically to desmin but recognize different epitopes. In the adult chicken, both McAbs produced equivalent immunofluorescent staining patterns, reacting in frozen sections with all forms of muscle tissue, including vascular smooth muscle, but with no other tissue types. In isolated skeletal myofibrils and in longitudinal frozen sections of cardiac and skeletal muscle, desmin was detected with both McAbs at the Z-band and in longitudinally-oriented filament bundles between myofibrils. In contrast to these results in the adult, the intermediate filaments (IF) of embryonic cardiac myocytes in primary cultures were decorated only with McAb D3, whereas McAb D76 was completely unreactive with these cells. Similarly, frozen sections through the heart at early stages of embryonic chick development (Hamburger-Hamilton stages 17–18) revealed regions of myocytes, identified by double immunofluorescence with myosin-specific McAbs, that were unstained with McAb D76 even though similar regions were stained by McAb D3. That McAb D76 reacted with desmin in all adult cardiac myocytes but not with all embryonic heart cells indicates that embryonic and adult cardiac IF are immunologically distinct and implies a conversion in IF immunoreactivity during cardiac development.

Intermediate filaments (IF) constitute a heterogeneous class of cellular organelles present in most, if not all, eucaryotic cells. Although IF exhibit many morphological, biochemical, and biophysical similarities (8, 17–19, 28, 34, 38, 39, 44, 47–49, 53–55), differences in the antigenicity, molecular weights, and isoelectric points of their subunit proteins (IFP) have allowed their classification into five categories, more or less characteristic of the cell type of origin (see 34). Since no biochemically assessable activity of IF has yet been demonstrated, the identification and localization of these proteins in cells has relied heavily upon immunologic and electrophoretic techniques. While the conventional antisera, which have been used in these studies to date, were adequate for the gross detection of, or discrimination among, the various IFP in cells, there are problems in using them for more detailed investigations of the function of IF in cellular organization and development. These problems stem from (a) the polyclonal nature of antisera which precludes an absolute assignment of their specificities, thus making it difficult to exclude the possibility that weak immunological cross-reactions result from contaminating antibodies; in addition, antisera to a given antigen vary from animal to animal, and even from bleed to bleed; (b) the difficulty in distinguishing between specific (antigen-antibody) and nonspecific interactions (see e.g., 1, 26); (c) the presence of idiopathic auto-antibodies to IFP in many preimmune sera (20, 31, 41); (d) the existence of regions of amino acid sequence homology between different IFP and between IFP and supposedly unrelated molecules (17, 19) increasing the probability of obtaining cross-reactive antisera; (e) the occurrence, as demonstrated with monoclonal

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antibodies, of epitopes common to all IFP (9, 42), and another shared between an IFP, vimentin, and a nonrelated molecule, tropomyosin (5); (f) the fact that conventional antisera, by virtue of their polyclonality, produce a modal response in which minor antigenic differences are masked.

One way to circumvent these problems is to use monospecific probes to unambiguously identify IFP. Thus, as a first step in our study of the IF of muscle, a series of monoclonal antibodies (McAbs) have been generated to IF purified from adult chicken gizzard. Two of these, D3 and D76, have been shown, by several criteria, to react exclusively with desmin, the predominant IFP in muscle, and have been used to validate the tissue and cellular distribution of desmin as determined with polyclonal antisera (3, 6, 14, 22, 23, 33, 34). In addition, they highlight the presence of desmin in the walls of all nonelastic blood vessels, and in longitudinally disposed filaments running between adjacent myofibrils in adult skeletal muscle which have recently been visualized by immunoelectron microscopy (50). Although all muscular IF appear immunologically identical with conventional antibodies to desmin (2, 15) in spite of morphological differences between smooth and striated muscle (6, 22, 35, 45), and between developing and mature muscle (3, 14, 16), McAb D3 and McAb D76 are shown to display divergent reactivities towards embryonic chick myocytes. Although McAb D3 is reminiscent of polyclonal antisera in recognizing desmin in all cells that contain it, irrespective of the age of the heart, McAb D76 uniquely fails to react with the filaments in embryonic chick cardiac myocytes in culture or, in specific cases, in vivo, suggesting the existence of antigenic differences between embryonic and adult cardiac IF.

MATERIALS AND METHODS

Production of Hybridoma Secreting Antidesmin Antibodies: C3H mice were immunized to desmin as follows: acetic acid solubilized desmin (tide infra) was neutralized to pH 7 with NaOH and emulsified with an equal volume of complete or incomplete Freund's adjuvant (CFA, IFA, respectively); on day 1, the mice were primed with 1.5 mg desmin in CFA equally divided among two subcutaneous and one intraperitoneal (i.p.) sites; subsequent injections of 1.0 mg desmin in IFA were administered i.p. on days 8 and 15. Fusion of immunized splenocytes with SP-2 myeloma cells (a nonsecreting, K-chain producing, HAT-sensitive myeloma line, kindly provided by Dr. C. Bianco) was performed 4 d after the final immunization according to the procedure of Claffin and Williams (7). Hybrid cells were selected by growth in HAT-medium (Dulbecco's modified Eagle's medium containing 4.5 g/l glucose (DME), 20% (vol/vol) fetal bovine serum, 10% (vol/vol) NCTC 109, 0.1 mM hypoxanthine, 0.01 mM aminopterin, 0.03 mM thymidine, and penicillin and streptomycin. All tissue culture reagents were purchased from Gibco Laboratories (Grand Island, NY). After 2 wk they were gradually weaned into medium lacking HAT. Cells were fed as necessary by replacing half the medium in the well with fresh medium. During this period, wells containing growing cells were periodically screen for antisera activity by a solid-phase radioimmunoassay (RIA, see below). Positive wells were gradually expanded, cloned in soft agar, and/or frozen in 10% (vol/vol) dimethyl sulfoxide-90% fetal bovine serum in liquid N2. Antibody-secreting colonies, selected by overlaying the plates with goat anti-mouse antisera (GAM), were picked, recultured, and the positive colonies recloned by limiting dilution. Clonality was proven by two dimensional isoelectric focusing/SDS-PAGE whose chromatographically-purified mouse IgG (Miles-Yeda, Rehovot, Israel) had been coupled. Specifically bound antibody was eluted at acid pH (0.2 M glycine-HCl, pH 2.3) and immediately neutralized with equimolar Tris(hydroxymethyl)aminomethane methanol hydrochloride (Tris-HCl), pH 8.6. 125I-labeled goat anti-mouse IgG (125I-GAM) was prepared by a modification of the chloramine-T method in which the iodination was performed while the goat antibodies were bound to a mouse IgG affinity column (24). Labeled specific antibody fractions were eluted above and further purified by gel filtration through a Sephadex G-200 or Sepharose 6-300 column. The pooled 125I-GAM peak was diluted to about 1 x 10⁶ cpm/ml in PBS (10 mM sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl) containing 0.1% BSA before use.

Rabbit antisera to desmin were as prepared and characterized by W. Ip in our laboratory (29). MF-20, a McAb, and then frozen in 10% nonsecreting, K-chain producing, HAT-sensitive myeloma line, kindly provided by D. Herzlinger and G. Ojakian, (25) for the antidesmin McAbs. All gave comparable background levels, usually <0.03% of input radioactivity.

Sample Preparation: Myofibrils were prepared as described (10) by Dounce homogenization of small fascicles of stretched, glycinated chicken pectoralis major muscle into PBS (100 mM EGTA). For immunofluorescence, the myofibrils were allowed to adhere to glass slides or coverslips for 1 min, rinsed in PBS, and then rinsed in PBS containing 0.1% BSA to reduce non-specific protein interactions.

Frozen sections of 4-10 μm thicknesses were cut from tissue blocks rapidly frozen in isopentane cooled to its fusion point by liquid N2 and embedded in O.C.T. compound (Tissue-Tek II, Lab-Tek Div., Miles Laboratories, Inc., Naperville, IL). The sections were then air-dried and stored desiccated at -20°C until use. For immunofluorescence, they were pretreated for 5 min with PBS at room temperature.

Desmin was isolated from adult chicken gizzard either by the procedure of Small and Sobieszek (45) or by that of Hubbard and Lazarides (27) with equivalent results. The acetic acid solubilized proteins were further enriched in desmin by at least two cycles of isoelectric precipitation at pH 4.1. One-dimensional SDS PAGE (not shown) revealed the presence of desmin (50 kD), an almost equal amount of actin, small amounts of myosin, a 230-kD protein, presumably synemin, and traces of other, unidentified, contaminants.

Myosin was prepared from adult chicken breast muscle as described by Reinach et al. (43) and alpha-actin from the acetone powder of the residue by the procedure of Spudich and Watt (46). Smooth muscle beta, gamma-actin was similarly purified from adult gizzard.

Primary cardiac muscle cultures were established from hearts of 7-10 d-old embryonic chicks by gentle trypsinization and trituration (13). Cells (0.5 x 10⁶) were plated onto gelatinized 60-mm tissue culture dishes containing formvar-coated or polystyrene coverslips in Ham's F-12 nutrient medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. They were allowed to grow for 1-2 d at which time the majority of the myocytes were beating. Immunofluorescence was performed on Triton X-100 extracted cells (cytoskeletons) prepared by incubating cultures in PEM buffer (0.1 M PIPES-HCl, 1.0 mM EGTA, 0.5 mM MgCl₂, pH 6.8) containing 0.5% Triton X-100 (PFTMx) for 5 min at room temperature. The cytoskeletons were then immersed in PFTMx containing 0.1-0.2% BSA (PFTMB) for 5 min before treatment with antibodies.

Immunofluorescence: All immunofluorescence experiments were performed on the specimens prepared as described above without postfixation as it was discovered that the use of fixatives such as formalin, at concentrations as low as 0.38% in PBS, ethanol, methanol, or acetone, at -20°C, abolished reactivity. The preparations only received a preincubation in a BSA-containing monthly lots and stored at 4°C in the presence of 0.1% (wt/vol) NaN₃. The same lot of each antibody was used for all experiments reported here.

Other Antibodies: Fluorescein- and rhodamine-conjugated goat and rabbit anti-mouse IgG antibodies were purchased from Cappel Laboratories (West Cochranville, PA) and affinity purified on a Sepharose-4B (Sigma Chemical Co., Louis, MO) column to which chromato graphically-purified mouse IgG (Miles-Yeda, Rehovot, Israel) had been coupled. Specifically bound antibody was eluted at acid pH (0.2 M glycine-HCl, pH 2.3) and immediately neutralized with equimolar Tris(hydroxymethyl)aminomethane methanol hydrochloride (Tris-HCl), pH 8.6. 125I-labeled goat anti-mouse IgG (125I-GAM) was prepared by a modification of the chloramine-T method in which the iodination was performed while the goat antibodies were bound to a mouse IgG affinity column (24). Labeled specific antibody fractions were eluted above and further purified by gel filtration through a Sephadex G-200 or Sepharose S-300 column. The pooled 125I-GAM peak was diluted to about 1 x 10⁶ cpm/ml in PBS (10 mM sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl) containing 0.1% BSA before use.

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of clones were screened by RIA against the immunizing preparation in selectivemedium, screening by RIA against the immunizing preparation, rather than desmin purified by SDS PAGE. This antigen proved to be highly immunogenic. Of the 98 original wells, all of which contained at least one hybridoma as indicated by cell growth in selective medium, screening by RIA against the immunogen revealed 38% to be positive at greater than twofold background levels with nine wells (9.2%) reacting at greater than five times background values. On the basis of preliminary immunofluorescence and RIA results, two McAbs, D3 and D76, were selected for further study. In an indirect RIA in which the concentration of radiiodinated second antibody was such as to provide maximum sensitivity in the range of low antigen concentrations (37), McAbs D3, and D76 produced the dilution curves shown in Fig. 1. Since by Ouchterlony immunodiffusion (not shown) McAb D76 is an IgG1 and McAb D3 is an IgG2a, the discrepancy between the saturation binding values might reflect differences in the affinity of the second antibody for these two IgG subtypes. In contrast, use of 125I-labeled protein A produced higher binding values with D3 than with D76 (Table I), as predicted by the relative affinities of protein A for these IgG subclasses (30).

Of the proteins present in the immunizing preparation, desmin was proven to be the antigen recognized by the McAbs through a variety of complementary techniques. By immunofluorescence (vide infra), the cellular disposition of the antigens was that expected for muscle intermediate filament proteins (33, 34). This pattern was distinct from that of actin (the major contaminant in the desmin preparation), detected with fluorescently labeled myosin S-1 and from that of myosin, localized with either polyclonal or monoclonal antibodies (not shown). Essentially no reactivity was seen with either McAb in RIA against skeletal muscle myosin, skeletal or smooth muscle actin at up to 10 times the standard amount of antigen, although they reacted strongly with the acetic acid solubilized filament proteins (Fig. 2). The slight reaction seen with gizzard actin was probably due to contamination of this sample with desmin (SDS gel results, not shown).

Selected adult chicken tissues (brain, enucleated erythrocyte ghosts [prepared according to 22], skin, heart, and gizzard) were solubilized directly in SDS and beta-mercaptoethanol. After electrophoresis and electrophoretic transfer to nitrocellulose, the samples were reacted with McAb D3 or D76 followed by 121I-GAM. The autoradiogram (Fig. 3) demonstrated antibody binding to a 50-kd protein in gizzard, heart, and skin but not in the erythrocyte ghosts or in brain tissue. Frozen sections of skin (results not shown) indicated that both McAbs bound exclusively to smooth muscle of the dermis; no reactivity with the stratified squamous epithelium was observed. Since the erythrocyte ghosts contain vimentin but not desmin (22), these results demonstrate no binding of either McAb to vimentin. Taken together, this experiment proves that McAbs D3 and D76 bind exclusively to desmin, and to no other IFP of chickens.

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**RESULTS**

**Production and Characterization of Monoclonal Antibodies Against Desmin**

To avoid problems associated with SDS denaturation and to potentiate the production of antibodies that would react with desmin in its native state, repolymerized intermediate filaments that were prepared in vitro by the neutralization of the desmin preparation were used as the immunogen, rather than desmin purified by SDS PAGE. This antigen proved to be highly immunogenic. Of the 98 original wells, all of which contained at least one hybridoma as indicated by cell growth in selective medium, screening by RIA against the immunogen revealed 38% to be positive at greater than twofold background levels with nine wells (9.2%) reacting at greater than five times background values. On the basis of preliminary immunofluorescence and RIA results, two McAbs, D3 and D76, were selected for further study. In an indirect RIA in which the concentration of radiiodinated second antibody was such as to provide maximum sensitivity in the range of low antigen concentrations (37), McAbs D3, and D76 produced the dilution curves shown in Fig. 1. Since by Ouchterlony immunodiffusion (not shown) McAb D76 is an IgG1 and McAb D3 is an IgG2a, the discrepancy between the saturation binding values might reflect differences in the affinity of the second antibody for these two IgG subtypes. In contrast, use of 125I-labeled protein A produced higher binding values with D3 than with D76 (Table I), as predicted by the relative affinities of protein A for these IgG subclasses (30).

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**TABLE I**

| Protein A added (μg) | Specific cpm bound | McAb D3 | McAb D76 |
|----------------------|--------------------|---------|---------|
| 50                   | 19,365             | 2,631   |
| 10                   | 4,052              | 1,369   |
| 1                    | 2,319              | 439     |

To each well 5 μg acetic acid solubilized desmin (in 5 μl) were added and the protein immobilized by air-drying. The McAbs (50 μl) were added as 1:1 dilutions in PBS. The assay was performed as described for the standard RIA in the Materials and Methods except that the appropriate dilution of 125I-protein A in PBS containing 0.1% globulin-free BSA was substituted for the 121I-GAM.
FIGURE 2 RIA analysis of McAb specificity for various purified muscle proteins. Undiluted McAb D3 and McAb D76 (50 μl) were assayed for reactivity with the indicated proteins purified as described in Materials and Methods at concentrations up to 100 times those used in the standard assay. Counts were not corrected for background binding to BSA which is also given. □, desmin; X, myosin; ○, α-actin; ●, β-γ-actin; ▲, BSA.

FIGURE 3 Reactivity of McAbs D3 and D76 with adult chicken tissues. Pieces of brain (B), enucleated erythrocyte ghosts (E) (22), skin (S) (including dermis), heart (H) (ventricle), and gizzard (G) were solubilized in boiling 2% SDS-5% beta-mercaptoethanol and then displayed by SDS PAGE. Proteins were transferred to nitrocellulose and either stained with amido black (A) or reacted with McAb D3 (B) or D76 (C), followed by 125I-GAM. Bound antibody was detected by autoradiography. The total protein loaded in each lane was approximately normalized for the actin concentrations seen by Coomassie Blue staining.

manifested different binding activities in the RIA, and since Ouchterlony immunodiffusion showed that McAb D76 was an IgG, while McAb D3 was an IgG2a, it seemed likely that their epitopes were also distinct. To prove this, a competitive binding experiment was performed. As shown in Table II, irrespective of the amount of desmin used as antigen, the combined RIA values of the two McAbs was approximately equal to the sum of their individual values. Assuming no polymorphism of the desmin molecule, this result, which was conducted in both McAb and 125I-GAM excess, indicates independent binding sites for each antibody.

Immunofluorescent Localization of Desmin in Adult Tissues

The monoclonal antibodies to desmin were used in indirect immunofluorescent studies of frozen-sectioned adult tissues to re-evaluate the data obtained with polyclonal antisera (21, 23, 33, 35). Desmin-specific immunoreactivity was restricted exclusively to muscle irrespective of its histological type: in longitudinal sections of skeletal (Fig. 4) and cardiac (Fig. 5) muscle fibers, a transverse staining pattern was evident. In addition, longitudinal staining was observed beneath the sarcolemma and between adjacent myofibrils. Considering the

Table II

|              | Desmin bound/ | Monoclonal antibody | cpm - background |
|--------------|---------------|---------------------|-----------------|
|             | μg            | D3                  | D76             |
| 0.039        | 592           | 2,231               |                   |
| 0.020        | 296           | 1,149               | 1,445            |
| D3 + D76     | 1,325         |                     |                 |

All values are averages of triplicate measurements. The 50 μl of antibody added to each well contained either 25 microliters of each McAb supernatant or 50 μl of a 1:1 dilution of the McAb with PBSB.

RIA data (Fig. 1) it was interesting that the intensity of immunofluorescent staining with McAb D3 was as bright or brighter than that with McAb D76. In smooth muscle, such as in the intestinal muscularis externa (Fig. 6) or gizzard, the entire myocyte was stained. The smooth muscle in the tunica media of all muscular arteries and veins was also intensely stained by both McAbs. Nonmuscle cell types, such as intestinal epithelia (Fig. 6), liver parenchyma (Fig. 7), or connective tissue elements, which contain IFP other than desmin, were unreactive.

Further immunofluorescent analysis of striated muscle using myofibrils prepared from glycerinated skeletal muscle localized desmin at the Z-band (Fig. 8). This staining was resistant to treatment of the myofibrils with 0.6 M KI, with 1% Triton X-100, and 0.1% SDS, or with PBS at pH 9.5. Furthermore, no reactivity was ever observed in controls that included the omission of the McAbs and their substitution with McAbs of similar IgG type but of different specificities. As in the frozen sections, longitudinally disposed elements were often observed running along the periphery of the myofibril. However, it was impossible with light optics to define precisely the relationship of these filaments to the myofibrillar apparatus. Longitudinally aligned filaments that react with antibodies to desmin have recently been visualized with ultrathin cryosections by transmission electron microscopy (50).

Immunofluorescent Analysis of Cardiac Cell Cultures

Screening the panel of McAbs to desmin against Triton X-100 extracted cardiac cell cultures (cytoskeletons) by immu-
FIGURE 4 Immunofluorescence of adult striated muscle. Phase (A and C) and FITC-fluorescent images of 4-μm thick frozen sections of longitudinally oriented adult pectoralis muscle reacted with McAb D3 (A and B) and McAb D76 (C and D). Note the longitudinal fluorescent elements (arrowheads). Bar, 10 μm.

FIGURE 5 Immunofluorescence of adult cardiac muscle. Phase (A and C) and FITC-fluorescent pairs of 10-μm thick, longitudinal cryostat sections of adult cardiac muscle. (A and B) reacted with McAb D3; (C and D) reacted with McAb D76. Arrowheads indicate regions of inter-Z-band fluorescence. Bar, 10 μm.
MacAbs D3 and D76 towards them. McAb D3 produced an immunofluorescent image identical to that of rabbit antiserum to desmin, namely, an intensely stained perinuclear whorl from which emanated a network of finer filaments (Fig. 9); in distinct contrast, no reactivity was ever observed with McAb D76. Classification of the immunoreactive cells as myocytes was based upon the following criteria: (a) the presence of myofibrils that could be stained with antibodies to striated muscle myosin; (b) an extended, oblate shape that was generally more refractile than adjacent nonmuscle cells; and (c) the presence of phase-dense glycogen granules stainable with the periodic acid-Schiff reagent. Although the majority of these cells were contractile before permeabilization and contained well-developed myofibrils demonstrable by phase-contrast optics (Fig. 9) or antimonyosin immunofluorescence (not shown), no Z-band, myofibril-associated, nor intercalated disc fluorescence was observed as has been described in adult muscle (this work, 29, 33) and in cultured mammalian embryonic cardiac myocytes (6, 14). The nonmuscle cell types, also present in these cultures were not labeled by either McAb.

Immunofluorescent Analysis of Embryonic Chick Heart

Frozen sections of hearts from 3 and 7 d-old embryonic chicks were processed for double immunofluorescence with McAbs D3 and D76 and a fluorescein-conjugated monoclonal antibody to myosin, MF-20 (36), to identify the myocytes. The results in Fig. 10 clearly demonstrate that all myocytes in 7 d-old hearts contained IF identically decorable with both McAbs. Hearts at 3 d of development in ovo (Fig. 11), although uniformly reactive with McAb D3, were found to contain regions of myosin-positive cells which were unreactive with D76. In the ventricular myocardium of 3 d-old embryos, all myocytes identified with MF-20 (myosin positive cells) were also reactive with D3 (desmin positive). However, only a subpopulation of these cells were stained by D76. Interestingly, the myotome present in these sections seemed to show no difference in the binding of the two desmin McAbs.

DISCUSSION

Monoclonal antibodies have been generated to an intermediate filament preparation from adult chicken gizzard. Two of these, D3 and D76, have been shown by immunofluorescent and immunoblot criteria to be specific for desmin and not to cross-react with any other intermediate filament protein. Additive binding of the antibodies in a solid-phase radioimmunoassay indicated that their epitopes are probably different. Immunofluorescent analysis of the McAb binding to frozen tissue sections revealed exclusive staining of the muscular elements, including vascular smooth muscle. While confirming the antigenic specificity of the antibodies, the
FIGURE 7  Immunofluorescent staining of adult liver. Cryostat sections through adult chicken liver (4 μm thick) were reacted with McAb D3 (A and B) and McAb D76 (C and D). A and C are the phase, and B and D the corresponding fluorescent images. Note the lack of staining of the liver parenchyma (p) but the intense reaction with the walls of a small vein (v) and artery (a). The vimentin-containing erythrocytes (arrowheads in C) were also completely unreactive. Bar, 16 μm.

FIGURE 8  Reaction of McAb D3 and McAb D76 with myofibrils isolated from adult skeletal muscle. Myofibrils prepared from stretched, glycerinated pectoralis muscle were treated sequentially with McAb D3 (A and B) or McAb D76 (C–E) and FITC-GAM. Comparison of the fluorescent (B and D) with the corresponding phase contrast (A and C) images revealed Z-band staining. Also evident were areas of longitudinal fluorescence (arrowheads), which were especially prominent in thicker myofibrils (E). Bars, 10 μm.

tissue distribution of desmin obtained with McAbs reciprocally validated previous results with polyclonal antisera (2, 21, 23, 33, 35). More interestingly, these two monoclonal antibodies displayed divergent reactivities towards embryonic cardiac myocytes, properties that may be useful in exploring the role of desmin in myofibrillar assembly.

Characterization of the Monoclonal Antibodies

Before an immunological reagent can be used as a molecular probe, its epitopic specificity must be precisely defined. This is especially critical when one is dealing with a family of biochemically related molecules such as the IFP which share both antigenic (9, 42) and amino acid sequence (17, 19) homologies. That the two McAbs used in these studies react selectively and exclusively with desmin is proven independently by immunofluorescence, RIA, and gel electrophoresis. Of particular importance is the failure of the McAbs to bind to vimentin, either in immunoblots or in tissues that contain it, since vimentin appears highly similar to desmin in peptide mapping (15) and in sites of phosphorylation (15, 38, 39, 47) in addition to amino acid sequence (17, 19). From this antigenic specificity as well as from the ability of the McAbs to recognize desmin both in its native state and denatured by acetic acid, SDS, or urea, certain conclusions about their epitopes may be inferred. First, they are more likely to reflect the primary structure of the molecule than higher order
FIGURE 9 Reactivity of the McAbs with cytoskeletal preparations of primary cardiac cell cultures. (A and C) phase contrast; (B and D) the corresponding FITC-fluorescent images after treatment with McAb D3 (A and B) and McAb D76 (C and D). Note the diffuse staining of myocytes with McAb D3 and its lack of reactivity with the Z-bands of myofibrils (arrowheads) and nonmuscle cells (f). McAb D76 did not react with any cells in these cultures. Bar, 10 μm.

conformations. Second, since they consist of amino acid sequences unique to desmin, current models of desmin’s secondary structure (17, 19) would place the D3 and D76 epitopes outside of the highly conserved, alpha-helical, rod domains and in the head or tail regions of the molecule.

It is also apparent that the antigenic determinants recognized by McAb D3 and McAb D76 cannot be identical. First, since the two McAbs are different IgG subclasses, they must be different molecules and it is improbable that two antibodies would have identical idiotypes unless the antigen contains many redundancies or the epitope is exceptionally immunogenic. Second, the McAbs are additive in the RIA. Third, they produced distinct immunofluorescent staining patterns.

Immunofluorescent Analysis of Adult Tissues

The use of heterologous antisera to study intermediate filaments is complicated by the immunologic (9, 42) similarity of the IFP and the frequent occurrence of contaminating auto-antibodies (20, 31, 41) to them. A monoclonal antibody known to react uniquely with a single IFP permits the unambiguous identification of that protein in tissues and cells. Monoclonal antibodies also often produce cleaner, and hence more easily interpretable, immunofluorescence. The McAbs to desmin thus facilitated the confirmation in frozen tissue sections results previously obtained with polyclonal antisera utilizing tissue culture cells (2, 3, 6, 15, 16, 26, 33) or other subcellular preparations. Although it was not surprising that desmin was found to be localized exclusively within muscle cells, direct confirmation in situ of the results obtained with polyclonal antisera is reassuring. They also extend these results in several ways. First, no binding of the McAbs was ever observed to nonmuscle cells either in frozen sections or in tissue culture in spite of reports that desmin is present in chick embryo fibroblasts isolated from myogenic cell cultures (15). Second, we observed intense staining with both antibodies of all the muscular arteries, veins, and arterioles within all the organs examined. In other species, the aorta, and possibly other elastic vessels, seem to be heterogeneous in their expression of desmin and vimentin with myocytes variously expressing only desmin, only vimentin, or some combination of both (4, 12, 40). The work presented here extends the localization of desmin in vascular smooth muscle to the chicken thereby implying that the vessels of the peripheral vasculature must, at least, express both desmin and vimentin. Third, in longitudinal sections of skeletal or cardiac muscle, and in myofibrils prepared from the former muscle, antibodies fluorescence was frequently observed subjacent to the sarcolemma and coursing along the periphery of the myofibril between sequential Z-bands. A similar finding was described by Lazarides and Hubbard (35) in skeletal muscle, but they ascribed the fluorescence to desmin filaments that had been involved in interconnecting Z-discs and had retracted against the myofibrils during sample preparation. Longitudinal filaments have been noted during skeletal myogenesis in vitro and viewed as intermediates in the assembly of IF at the Z-disc (3, 16). The detection of such elements in frozen sections of...
adult tissue documents their existence as true components of the muscle fiber in vivo. Although their detailed relationship to the myofibrillar and membranous systems could not be established with fluorescent optics, it seems plausible to suggest that they might correspond to the KI-resistant filament seen interconnecting Z-bands and membranes by Granger and Lazarides (21). As noted above, these filaments are probably identical to those described by Tokuyasu (50) with cryosections of cardiac muscle.

**Developmental Divergence of McAb D3 and McAb D76 Immunoreactivity**

Although both McAbs bound identically to adult tissues, they varied in their reactivity with immature cardiac cells. McAb D3 labeled IF in myocytes in culture as well as in sections of 3 and 7 d-old embryonic heart. McAb D76, on the other hand, did not bind to the cultured cardiac cells at all and failed to recognize desmin in specific region of 3 d-old...
cardiac anlage, although interacting normally with other areas of these, and all regions of older, embryonic hearts. This lack of immunofluorescence cannot be an artifact of antibody titer nor of antigen concentration since the solid phase RIA (Fig. 1) and estimates of the desmin content of the specimens suggest that saturating levels of each McAb have been used. Furthermore, the experiments have an internal control in that certain regions of the heart stained equivalently with polyclonal antisera to desmin but differently with the two McAbs. In addition there are regions of comparable IF density stained equivalently with both McAbs. Selective proteolysis of desmin is unlikely to be responsible for the lack of reactivity with McAb D76 since desmin can be shown to be present in these areas using polyclonal antisera or McAb D3; furthermore, only parts of the sectioned 3-d-old hearts were nonreactive and the 7-d-old hearts were always completely reactive. Thus, the implication of these results is that there is a developmental change in IF immunoreactivity, to which McAb D76 is sensitive, and that the cardiac cells in culture apparently mimic this earlier, nonreactive state although derived from older embryos. Presently, the significance of the change in D76 reactivity remains obscure. Possible mechanistic explanations include altered binding of IF accessory proteins to expose the McAb D76 binding site, conformational changes of the molecule, or de novo synthesis of a new desmin isotype. To date, we have been unable to discriminate among these alternatives. Attempts to disrupt the filament structure and unmask the McAb D76 epitope using acetone or ethanol, which have such effects on myosin filament structure (36) have thus far been unsuccessful (data not shown). An investigation of the possible relationship between the state of reactivity to McAb D76 and the morphological rearrangement of IF that occurs during skeletal myogenesis is currently in progress and the results will be reported separately (Danto, S., Fischman, D., and Lazarides, E. in preparation).

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DANTO AND FISCHMAN
Monoclonal Antibodies to Desmin

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