

# RAPID COMMUNICATIONS

## MYOSIN TYPES IN CULTURED MUSCLE CELLS

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### ABSTRACT

Fluorescent antibodies against fast skeletal, slow skeletal, and ventricular myosins were applied to muscle cultures from embryonic pectoralis and ventricular myocardium of the chicken. A number of spindle-shaped mononucleated cells, presumably myoblasts, and all myotubes present in skeletal muscle cultures were labeled by all three antimyosin antisera. In contrast, in cultures from ventricular myocardium all muscle cells were labeled by anti-ventricular myosin, whereas only part of them were stained by anti-slow skeletal myosin and rare cells reacted with anti-fast skeletal myosin. The findings indicate that myosin(s) present in cultured embryonic skeletal muscle cells contains antigenic determinants similar to those present in adult fast skeletal, slow skeletal, and ventricular myosins.

The nature of myosin(s) synthesized by striated muscle cells in the course of differentiation is controversial. Myosin in differentiating muscle cells differs from that present in nonmuscle cells (1) but it is not clear whether (a) it is identical to adult fast skeletal myosin (10, 11, 12), (b) it is a distinct embryonic type (4, 5, 16, 17, 18), or (c) it is a mixture of fast skeletal and slow skeletal myosins (3) or even a mixture of skeletal and cardiac myosins (6, 7).

The discrepancy between different results may be attributable to the material examined and the techniques used. However, even when comparable systems were analyzed by the same procedure, conflicting results were reported. For instance, in one immunofluorescence study (6), cultured muscle cells from chick embryo pectoralis were found to react with antibodies against both skeletal and cardiac myosins, whereas, in a more recent study (10), muscle cells were found to react with anti-fast skeletal but not with anti-slow skeletal myosin.

Using antimyosin antibodies specific for fast skeletal, slow skeletal, and ventricular myosins, we have therefore reinvestigated the antigenic properties of myosin(s) present in cultured muscle cells from embryonic chicken pectoralis muscle. Cultures of ventricular myocardium and co-cultures

of pectoralis and ventricular myocardium were also examined for comparison. A preliminary report on antimyosin staining of cardiac muscle cells in culture has been previously presented (14).

### MATERIALS AND METHODS

#### Cultures

Primary muscle cultures were prepared from pectoralis muscle and ventricular myocardium of 11-d chick embryos, by use of previously described methods (15), except that cultures were grown on gelatin-coated glass coverslips placed in 100-mm plastic petri dishes. Co-cultures of skeletal and cardiac muscle were prepared by first plating pectoralis cells, at a density of  $4 \times 10^6$  cells per dish, and 2 d later ventricular cells, at a density of  $1 \times 10^6$  cells per dish. Formation of cardiac-skeletal heterokaryons should be negligible under these conditions (9). After various time intervals from 3 up to 7 d after plating, coverslips were removed and processed for immunofluorescence.

#### Antibodies

Antibodies against column-purified myosins from chicken pectoralis (fast skeletal), ALD<sup>1</sup> (slow skeletal), and ventricular myocardium were raised in rabbits, as previously described (13). Six rabbits were immunized with fast skeletal myosin, six with slow skeletal myosin, and five with ventricular myosin. Antisera

<sup>1</sup> Abbreviations used in this paper: ALD, anterior latissimus dorsi; PBS, phosphate-buffered saline; CNBr, cyanogen bromide.

giving a single strong precipitin line with the corresponding immunogen in double immunodiffusion and immunoelectrophoresis were selected for immunofluorescence studies and affinity purified on the corresponding insolubilized immunogens (8). Anti-fast skeletal and anti-slow skeletal myosin antisera did not cross-react with heterologous myosins in immunodiffusion and immunoelectrophoresis tests and did selectively stain fast and slow skeletal muscle fibers, respectively, in immunofluorescence tests performed on cryostat sections of ALD and pectoralis muscle (8). To rule out any possible cross-reaction undetected by the above procedures, anti-fast skeletal myosin was absorbed with insolubilized slow skeletal myosin and anti-slow skeletal myosin with fast skeletal myosin. In a typical experiment, 10 ml of anti-ALD antiserum were passed through an immunoabsorbent column prepared by coupling ~27 mg of pectoralis myosin to 5 g of CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N.J.). This immunoabsorbent, which had the capacity to bind ~12 mg of antibodies from an anti-pectoralis myosin antiserum, retained about 0.5 mg of anti-ALD myosin antibodies. The unretained fraction was applied to an immunoabsorbent prepared by coupling ~23 mg of ALD myosin to Sepharose; bound antibody (~4 mg) was eluted by lowering the pH to 2.8 and used for immunofluorescence. A similar procedure was used for cross-absorption of anti-fast skeletal myosin. Anti-ventricular myosin antisera gave a cross-reaction with slow skeletal myosin detectable by immunodiffusion and immunofluorescence, which was abolished by two sequential passages through an ALD myosin affinity column. Immunodiffusion tests with purified myosin heavy and light chains showed that anti-slow skeletal and anti-ventricular myosin antibodies used for immunofluorescence studies were specific for the heavy chains of slow skeletal and ventricular myosin, respectively (S. Sartore, manuscript in preparation).

### Immunofluorescence

Coverslips with muscle cultures were washed in PBS, fixed in acetone at  $-20^{\circ}\text{C}$  for 5 min and air-dried (2). This treatment did not affect the intensity of antimyosin staining of muscle fibers in fresh-frozen cryostat sections of adult muscle. In contrast, fixation in 1% paraformaldehyde in PBS or in an acetone-paraformaldehyde mixture for 5 min was found to inhibit markedly the intensity of immunofluorescence reactions in both cryostat sections and muscle cultures.

For direct immunofluorescence tests, fluorescein- or tetramethylrhodamine-labeled antimyosins were used. Fluorescein-isothiocyanate was coupled to antimyosin antibodies as described elsewhere (8); tetramethylrhodamine-isothiocyanate-labeled antimyosins were obtained as described by Fujiwara and Pollard (2). Conjugates with a fluorochrome/protein ratio of ~2 were separated by ion-exchange chromatography (8) and tested on cryostat sections of adult chicken muscles to establish the appropriate dilution that was generally used also for muscle cultures. Cultured cells were incubated with the selected conjugate at  $37^{\circ}\text{C}$  for 30 min, washed in PBS, fixed in 2% paraformaldehyde in PBS for 5 min, and mounted in glycerol or Elvanol.

For indirect immunofluorescence tests, cells were first incubated ( $37^{\circ}\text{C}$ , 30 min) with unlabeled antimyosin, washed in PBS, and treated with fluorescein- or tetramethylrhodamine-labeled goat anti-rabbit IgG (purchased from Miles Laboratories Inc., Elkhart, Ind., and from N. L. Cappel Laboratories, Inc., Cochranville, Pa. respectively). The specificity of direct and indirect immunofluorescence reactions was assessed by performing different controls, as described elsewhere (8).

For double immunofluorescence tests, two procedures were used. In the first method, cells were allowed to react simultaneously with two antimyosins, one labeled with fluorescein and the other with rhodamine. In the second method, cells were first stained by indirect immunofluorescence with one antimyosin, and subsequently by direct immunofluorescence with another antimyosin conjugated with a different fluorochrome. The latter method should give a strong advantage to the first antibody in case of competition for binding to myosin. To rule out binding of the second antimyosin to free anti-IgG combining sites that may exist after the reaction with the first antibody and fluorescent anti-IgG, control cultures were treated with preimmune serum before application of the second antibody.

The microscope used was a Leitz Dialux with epifluorescence optics and with a Ploemopak containing specific filter combinations for fluorescein and rhodamine. The rhodamine filter combination completely abolished fluorescein fluorescence, whereas an additional filter (S-525) was used with the fluorescein filter set to eliminate rhodamine fluorescence.

### RESULTS

In muscle cultures from embryonic pectoralis, two cell types were stained by our antimyosin antisera: a number of mononucleated spindle-shaped cells (Fig. 1), presumably differentiating myoblasts, and multinucleated myotubes (Fig. 2). In contrast, as shown in Fig. 1, the majority of mononucleated cells, most of which showed a flattened fibroblast-like morphology, did not react with any of the three antisera. Staining was diffuse throughout the cytoplasm in myoblasts and young myotubes, and restricted to the A-bands of the myofibrils in more mature myotubes (Fig. 2*e* and *f*). Cultures treated with a preimmune, fluorescein-labeled antiserum showed no reaction; specific reactions were completely blocked by prior absorption of antibodies with the corresponding immunogens but not with heterologous myosins. In cultures processed for sequential double immunofluorescence, the staining reaction was essentially unchanged by treatment with unlabeled preimmune serum before addition of the second fluorescent antibody.

All identifiable myotubes in muscle cultures processed for immunofluorescence with anti-fast skeletal myosin appeared labeled, and the same was true for cultures stained with anti-slow skeletal or anti-ventricular myosin. Double-labeling tests confirmed that the same myoblast or myotube was labeled by two distinct antimyosins whether applied simultaneously or in sequence (Fig. 2). The following combinations were used in double-labeling tests and gave essentially similar results: simultaneous tests: anti-fast skeletal plus anti-slow skeletal myosin, anti-fast skeletal plus anti-ventricular myosin, anti-ventricular plus anti-slow skeletal myosin; sequential tests: anti-fast

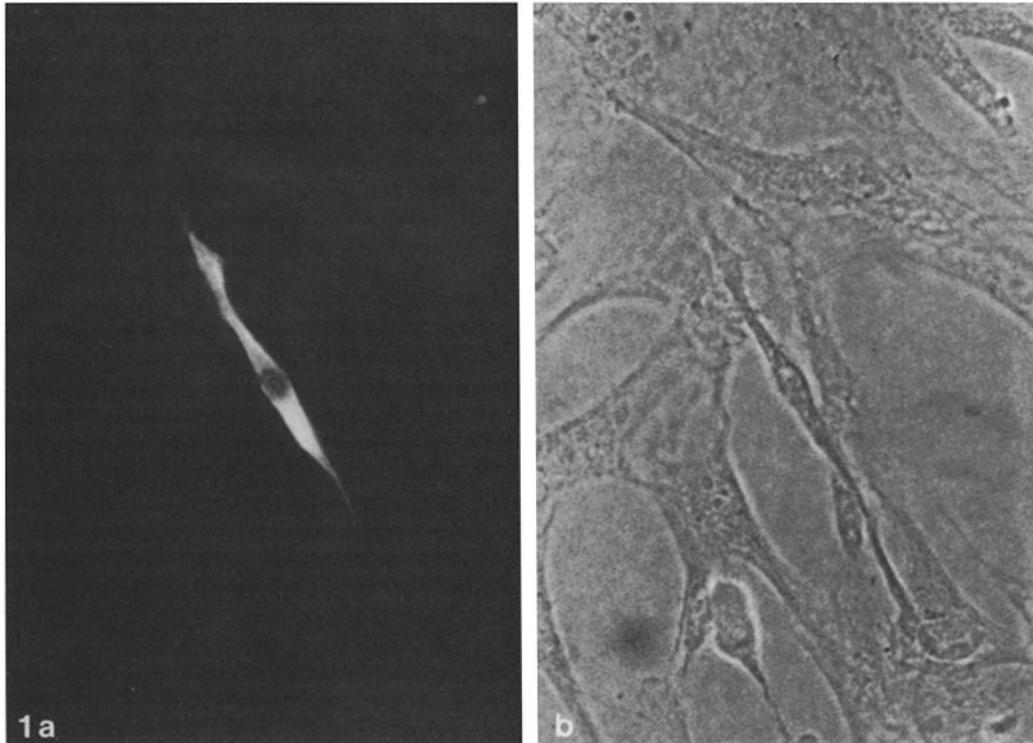


FIGURE 1 Muscle culture from chicken embryonic pectoralis after 3 d in vitro, stained with fluorescein anti-ventricular myosin (direct immunofluorescence). (a) Fluorescence micrograph; (b) phase-contrast micrograph. Only a spindle-shaped mononucleated cell is labeled; no myotube is present in this field.

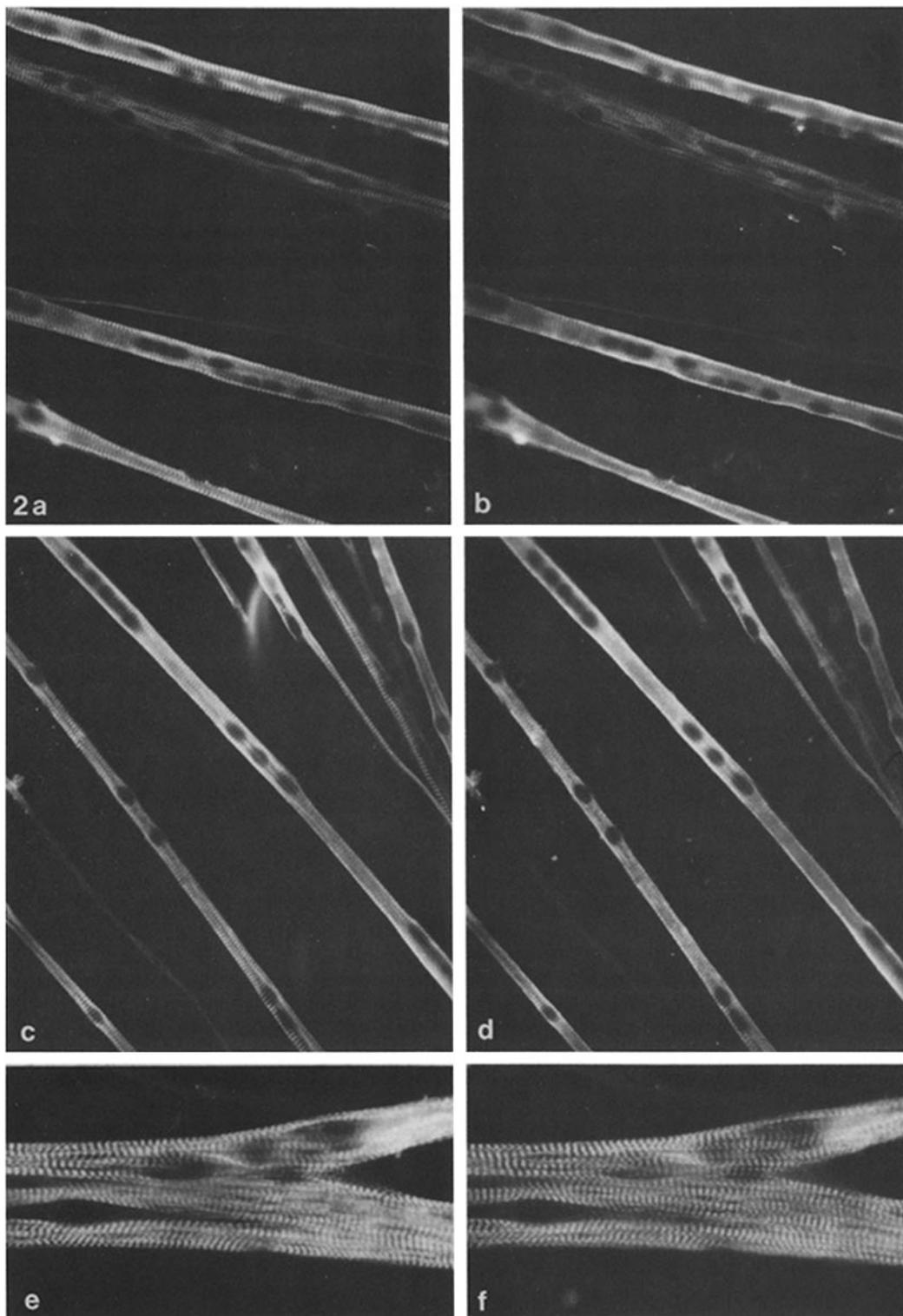
skeletal followed by anti-slow skeletal myosin, anti-fast skeletal followed by anti-ventricular myosin. The finding that myotubes reacted with the second antiserum even in sequential tests indicates that there is no significant competition for binding to myosin; the determinants recognized by the two antimyosins must therefore be located on different molecules or on different portions of the same molecule. Myofibrils reacting only with one or another antimyosin were never identified within double-labeled myotubes: the same myofibrils thus appear to react with both antimyosins. Though the level of response of muscle cells to the three antimyosin antisera was not significantly different, there was some variation in the intensity of staining among different myoblasts or myotubes with any antimyosin. In double-labeling experiments, myotubes showing less intense staining with one antimyosin stained also less intensely with the other antimyosin.

The reactivity of cardiac muscle cells to antimyosin antibodies differed strikingly from that of

skeletal muscle cells. In muscle cultures from ventricular myocardium, all muscle cells stained brightly with anti-ventricular myosin, whereas only part of them were stained, with variable intensity, by anti-slow skeletal myosin; rare muscle cells reacted also with anti-fast skeletal myosin (Fig. 3). The morphology of labeled cardiac cells was variable in these cultures and there was no obvious correlation between cell shape and reactivity with antimyosin antisera. The contrasting response of ventricular and skeletal muscle cells was clearly demonstrated in co-cultures stained simultaneously with anti-ventricular and anti-fast skeletal myosin. Multinucleated skeletal myotubes, which were easily identified in the co-cultures, reacted with both antimyosins, whereas mononucleated muscle cells, mostly cardiac muscle cells in 5- or 7-d cultures, reacted only with anti-ventricular myosin (Fig. 4).

#### DISCUSSION

This study shows that skeletal muscle cells grown



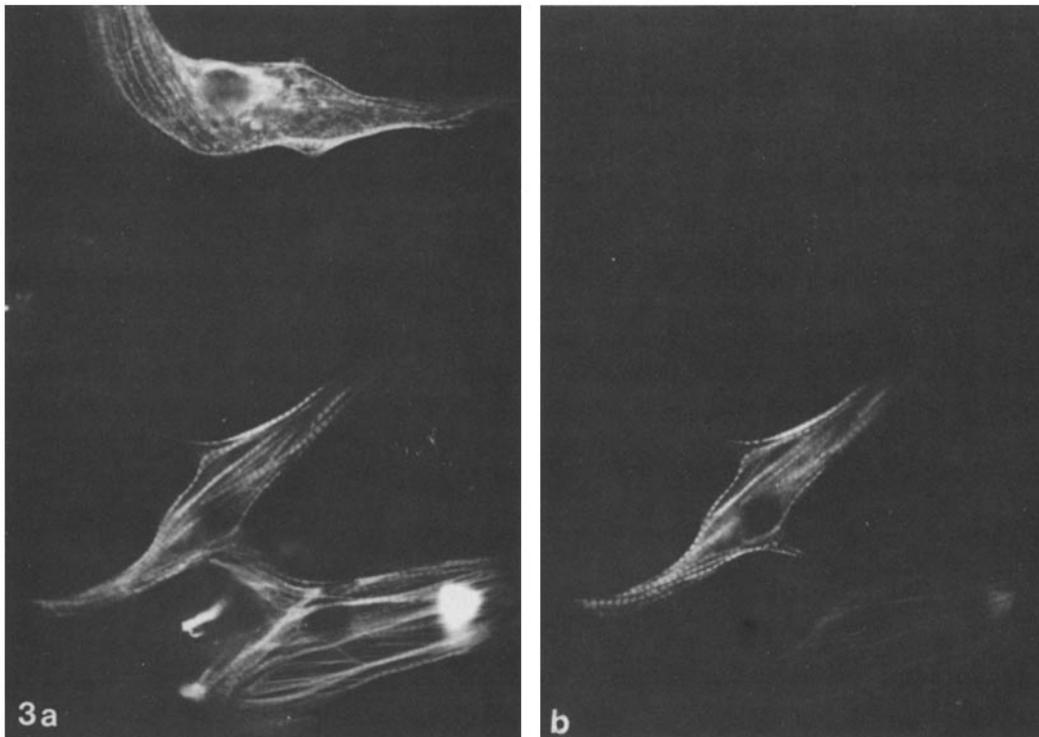


FIGURE 3 Muscle culture from chicken embryonic ventricular myocardium after 3 d in vitro, stained simultaneously with fluorescein anti-ventricular myosin (*a*) and rhodamine anti-fast skeletal myosin (*b*). Only one out of the three muscle cells present in this field and labeled by anti-ventricular myosin is also stained by anti-fast skeletal myosin.

in vitro from chicken embryonic pectoralis display multiple reactivity to antimyosin antisera raised against three different myosins from adult striated muscle, including ventricular myosin. Cultured myotubes show no evidence for selective compartmentalization of “fast” or “slow” antigenic types of myosin, such as seen in muscle fibers from adult chicken muscles. In contrast, cultures of embryonic ventricular myocardium reveal muscle cell heterogeneity when stained with the same antisera. The significance of this heterogeneity is presently unknown: it might be the expression of an early differentiation of muscle cells of working myocar-

dium and conduction tissue, which are known to differ in their reactivity to antimyosin antisera in the adult chicken heart (13). Correlated electrophysiological and antimyosin immunofluorescence studies of cultured cardiac cells would be needed in this respect.

As regards the response of cultured skeletal muscle cells to antimyosin antisera, our results are in agreement with those previously obtained by Masaki and Yoshizaki (6), whereas they are in conflict with those of Rubinstein and Holtzer (10) showing reactivity of cultured myotubes with anti-fast skeletal but not with anti-slow skeletal

FIGURE 2 Muscle cultures from chicken embryonic pectoralis after 6 d in vitro, stained simultaneously with two different antimyosin antisera. (*a* and *b*) Myotubes stained with fluorescein anti-slow skeletal myosin (*a*) and rhodamine anti-fast skeletal myosin (*b*). (*c* and *d*) Myotubes stained with fluorescein anti-ventricular myosin (*c*) and rhodamine anti-fast skeletal myosin (*d*). (*e* and *f*) Higher magnification of the same myotube stained with fluorescein anti-ventricular myosin (*e*) and rhodamine anti-fast skeletal myosin (*f*).

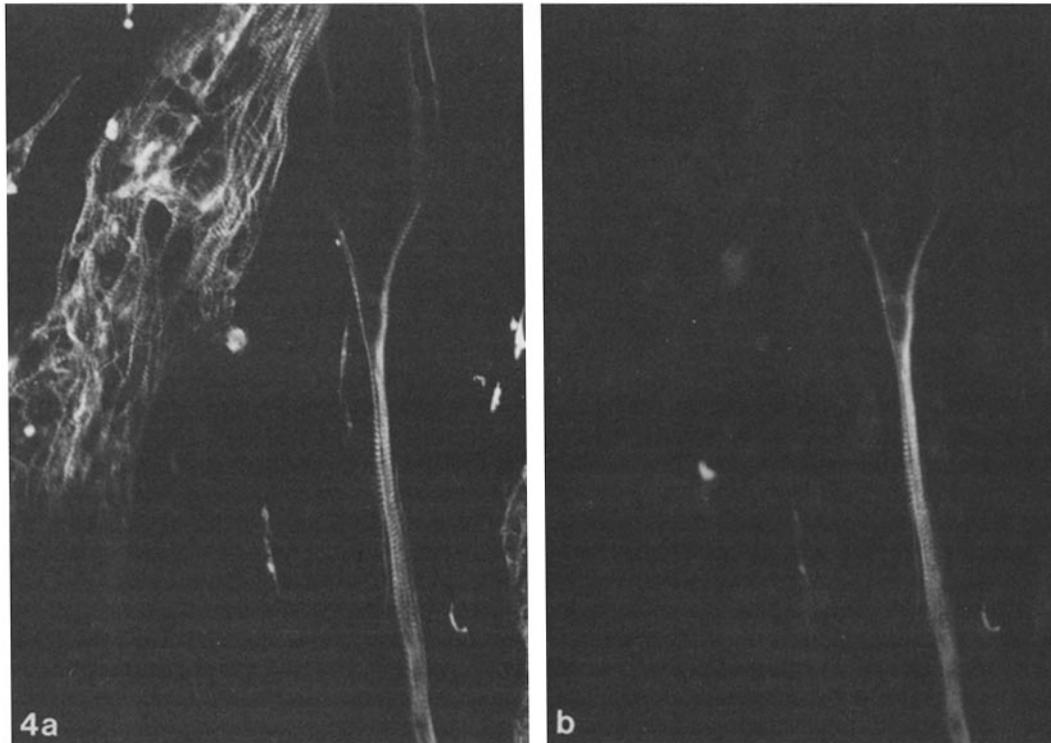


FIGURE 4 Coculture of chicken embryonic pectoralis and ventricular myocardium after 5 d in vitro, stained sequentially with fluorescein anti-ventricular myosin (a) and rhodamine anti-fast skeletal myosin (b). A small aggregate of cardiac muscle cells (upper left) is stained only by anti-ventricular myosin, whereas a skeletal myotube (lower right) is labeled by both antimyosins.

myosin. The discrepancy between these findings may be a result of the different properties of antibodies raised in different animals, or of the fixation procedure used to permit fluorescent antibodies access to the cytoplasm of cultured cells. We have found that acetone-paraformaldehyde mixtures, such as used by Rubinstein and Holtzer (10), markedly depress the intensity of immunofluorescence reactions with different antimyosin antisera. Negative results should therefore be regarded with caution under these conditions.

It appears from this study that cultured muscle cells from chicken embryonic pectoralis synthesize myosin(s) with antigenic determinants similar to those present in adult fast skeletal, slow skeletal, and ventricular myosin. This finding, though not sufficient in itself to define the nature of myosin in skeletal muscle cultures, helps in restraining the number of possible interpretations. Immunofluorescence results are thus not compatible with the presence in differentiating muscle cells of one

exclusive myosin type identical to adult fast skeletal myosin (10); they may be compatible with the presence of several distinct myosin types corresponding to those found in adult skeletal and cardiac muscles, or with the presence of one or more unique embryonic myosins cross-reacting with antibodies to adult muscle myosins. Embryonic myosin has been shown to be present in cultured muscle cells from mammalian skeletal muscle (17, 18), but so far there is no direct biochemical evidence for the existence of similar myosin in cultured chicken muscle cells.

There is an analogy between the findings reported here and those of Gauthier et al. (3) concerning staining of skeletal muscle fibers from the newborn rat with both anti-fast skeletal and anti-slow skeletal myosin. The multiple reactivity of neonatal muscle fibers was interpreted as a result of the coexistence of slow and fast isoenzymes of myosin within the same fiber and was related to the polyneuronal innervation of developing mus-

cle. Our results seem to suggest that the multiple reactivity of differentiating skeletal muscle cells to different antimyosins may be an intrinsic property of developing muscle, even independent of innervation.

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