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Source: Zoological Science, 14(2) : 287-296

Published By: Zoological Society of Japan

URL: <https://doi.org/10.2108/zsj.14.287>

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Study on the Nature of Starfish Larval Muscle Cells *In Vitro*

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ABSTRACT—We describe a culture method in which larval muscle cells of the starfish *Asterias amurensis* develop from epithelial cells, probably deriving from the coelomic pouches. The nature of the muscle which appears in the culture is described morphologically, physiologically and ultrastructurally. Cells were dissociated from the late gastrula stage, treated with 0.6 M of glycine in half-strength sea water free of Ca^{2+} and Mg^{2+} for 12 hr, and cultured for various periods. Elongated cells appeared after about a week from small aggregates of epithelial cells which were found among the mesenchymal network on the 1st day of culture. The characteristics of the elongated cells are as follows: (1) they possess two or more arms; (2) they adhere to the cultural substratum, mesenchyme cells, and themselves at all parts of the cell body; (3) they contract in response to acetylcholine; (4) they contain an abundance of fibrous actin and myosin throughout their cytoplasm; (5) the cytoplasm contains bundles of thick (12–19 nm) and thin (5–8 nm) filaments without any dense material; and (6) no proliferative activity was observed while the cells were kept in culture for up to 14 days. These features were compared with those of the larval muscle cells *in vivo*.

INTRODUCTION

Echinoderm larvae possess a simple body structure of three germ layers and an extracellular matrix (ECM) which are easily discernible. Some tissues, such as muscles, the digestive tract and the ciliary band, are so simply arranged that the cells constituting these tissues can be directly correlated with larval functions.

In order to study the nature and behavior of these cells more closely, we recently devised a method for culturing the embryonic cells of starfish (Kaneko *et al.*, 1995). When cells were dissociated from the mid gastrula stage, treated for 12 hr in 0.6 M of glycine in half-strength of sea water free of Ca^{2+} and Mg^{2+} , and subsequently cultured for 24 hr in sea water supplemented with newborn bovine serum, aggregates of epithelial cells were found scattered in a continuous network of mesenchyme cells (Kaneko *et al.*, 1995). The aggregates consisted of a mixture of mesendodermal cells including esophageal, stomachic, intestinal and coelomic pouch cells (Kaneko *et al.*, 1995). However, when cells were dissociated from a slightly more advanced embryo, i.e., the late gastrula one, treated for 12 hr in the glycine solution and cultured for about a week, the cultural substratum was covered with

patches of mesenchymal syncytia and elongated cells. The elongated cells resembled in appearance the muscle cells of echinoderm larvae.

Muscle cells of pluteus larva of sea urchin and bipinnaria larva of starfish are elongated cells with one central bulge which contains the nucleus. These cells are located in close contact with the blastocoelic side of the basal lamina of three different body regions, i.e., encircling the esophagus and stretching parallel to the body axis in two separate clusters under the dorso-lateral ectoderm. The former functions in the swallowing of food material and the latter in the opening of the oral hood. Muscle cells arise from the coelomic pouch cells (Ishimoda-Takagi *et al.*, 1984; Cox *et al.*, 1986; Burke and Alvarez, 1988; Crawford, 1990; Cameron and Davidson, 1991; Ettensohn, 1992). The muscle cells of the pluteus larva are reported to contract in response to experimentally applied serotonin and acetylcholine (Gustafson *et al.*, 1972). In the cytoplasm of these cells are thick and thin filaments lacking the Z-band, as judged from the electromicrograph presented by Burke and Alvarez (1988).

In the present paper, we describe the morphological, physiological and ultrastructural characteristics of the elongated cells which appear in the culture of embryonic cells of the starfish *Asterias amurensis*. Its features are compared with those of the muscle cells *in vivo*. We concluded that the elongated cells found *in vitro* are indeed muscle cells.

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This is the first report, to our knowledge, which describes the *in vitro* nature of the muscle cells of an echinoderm larva.

MATERIALS AND METHODS

Embryos

Mature eggs of the starfish *Asterias amurensis* were obtained using 1-methyladenine (1-MA), inseminated and allowed to develop in artificial sea water (ASW) (Jamarin U; Jamarin Laboratory, Osaka) (Kanatani, 1969; Kaneko *et al.*, 1995). Embryos were collected either at the late gastrula stage (Fig. 1) or at the early bipinnaria stage (Fig. 8a, b).

Media

Dissociation medium (DM) (pH 6.6): 1.2 M glycine in distilled water supplemented with 1% (v/v) ASW and 6% newborn bovine serum (NBS; M.A. Bioproducts, Maryland).

Treatment medium (TM) (pH 6.7): 0.6 M glycine in a half strength Ca^{2+} , Mg^{2+} free Jamarin U (Jamarin Laboratory, Osaka).

Culture medium (CM) (pH 8.1): ASW containing 4% (v/v) NBS.

Mesendodermal cell culture

Primary culture of mesendodermal cells was prepared as described previously (Kaneko *et al.*, 1995), except that the embryos were of the late gastrula instead of the mid gastrula stage. In brief, embryos were dissociated into single cells with DM, transferred to TM and incubated at 15°C for 12 hr. Cells which survived this treatment were collected and inoculated in a glass Petri dish containing CM at 18°C. For fluorescence staining and scanning and transmission electron microscopy, glass coverslips (18 mm × 18 mm) were laid on the bottom of the dish. After 24 hr of cultivation, the CM was replaced to remove the cell debris. The culture was then continued for 5-15 days without further change of the CM. The cells were observed and photographed under a phase-contrast microscope (Nikon, TMD).

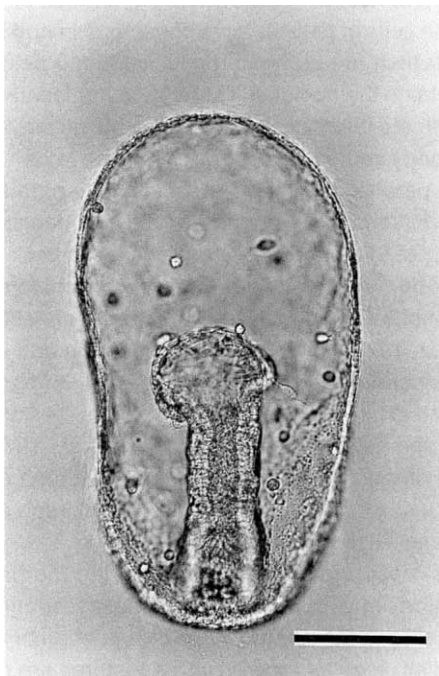


Fig. 1. Light micrograph of an embryo at the late gastrula stage (about 48 hr after insemination) used in this study. Bar, 100 μm .

Application of acetylcholine to cells in culture and to intact larvae

A drop of acetylcholine (1×10^{-5} M in ASW) was applied to the CM immediately above the target cell in the optic field of a phase-contrast microscope. Its reaction was monitored through a video system attached to the microscope. The video system was composed of a microscopic video camera (Nikon, DK-3001), a monitor television (Sony, KV21GR1) and a video cassette recorder (Victor, BR-9000) (Dan-Sohkawa *et al.*, 1995). The contraction-relaxation behavior of the cells was recorded at 30 frames/sec and analysed frame by frame. Intact larvae were directly transferred to 1×10^{-5} M acetylcholine in ASW.

Fluorescence staining of actin and myosin

N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) phalloidin (NBD-phalloidin; Wako Pure Chemical Industries, Ltd., Osaka) was used to detect intracellular F-actin (Burke and Alvarez, 1988). Cells cultured on a coverslip were rinsed with ASW to remove cell debris and were fixed with 4% paraformaldehyde in ASW at 20°C for 10 min. After washing the cells twice with phosphate-buffered saline (PBS, pH 7.4), they were permeabilized in acetone at 20°C for 3 to 5 min and air-dried. NBD-phalloidin in PBS was applied to the cells kept for 20 min at 20°C in a moist chamber, then the cells were quickly washed twice with PBS. The cells were mounted on a slide glass with PBS-nonfluorescent glycerin (1:1) mixture. They were observed under a fluorescence microscope (Olympus, BH-RFL) using a G beam (460-470 nm).

A polyclonal antiserum of rabbit against nematode (*C. elegans*) myosin was used as the test drug for myosin. This serum was kindly provided by H. Kagawa (Okayama University, Okayama, Japan). The reactivity of this antiserum to the molecular components of the cells in culture prepared by the present method was checked beforehand by western blotting, and it was confirmed to recognize a single band at 210 kDa (data not shown). The cells on a coverslip were washed and fixed as mentioned above. After washing twice with PBS, the cells were treated with the antiserum for 30 min at 20°C in a moist chamber. Excess antiserum was removed by 30-min washing with three changes of PBS. The cells were then treated with 1/30 diluted goat anti-rabbit fluorescein isothiocyanate-IgG (Sigma) for 30 min at 20°C in a moist chamber. After the final 30-min washing with three changes of PBS, the cells were mounted and observed as mentioned above.

Scanning electron microscopy (SEM)

Cells cultured on a cover slip were washed with ASW to remove the CM and cell debris. The cells were fixed with 2% OsO_4 in ASW for 15 min at 20°C and rinsed with double distilled water (D_2W) for 15 min. After dehydration by passage through a series of graded concentrations of ethanol, the cells were subjected to critical-point drying and sputter-coating with gold, as described previously (Kaneko *et al.*, 1990). The specimens were observed with a scanning electron microscope (JEOL, T-300).

Transmission electron microscopy (TEM)

Cells cultured on a coverslip were washed with ASW and fixed with 2% OsO_4 in ASW for 1 hr. After washing twice with ASW, the samples were treated with 0.5% triton X-100 in ASW for 10 min and washed three times with D_2W . They were subsequently stained with 1% uranyl acetate in D_2W for 1 hr and rinsed 3 times with D_2W . After dehydration by passage through a series of graded concentrations of ethanol, the cells were embedded in Epon (Taab, Berkshire). Semithin sections (1 μm) for light microscopy were stained with 0.1% toluidine blue to select target cells for electron microscopy. Ultrathin sections were cut with an ultramicrotome (Reichert Jung, OmU4), sequentially stained with uranylacetate and lead citrate, and examined with an electron microscope (JEOL, JEM100CX). All operations were carried out at 20°C. Whole embryos were processed similarly. The diameters of thick and thin filaments were measured on negative TEM film using

a standard micrometer under a stereoscopic microscope (Olympus, VE22).

Observation of muscle cells in vivo

In order to stop the larvae from swimming and also their muscle cells from contracting, 4-day larvae (bipinnariae) were mounted in a narrow space between the slide glass and the coverslip in 10 mM CoCl_2 in ASW. The muscle cells were observed with a differential interference microscope (Nikon, Nomarski HPD).

RESULTS

Appearance and morphology of the elongated cells

Figure 2a shows the cells after 1 day in culture. Epithelial cells form small aggregates of 10 to 50 cells arranged in one or a few cell layers. Mesenchyme cells are interconnected to one another to form a network.

Elongated cells make their appearance in the same culture after 5-7 days (Fig. 2b). At the time of their appearance, most of the epithelial cell aggregates have disappeared, with the exception of only a few. The mesenchyme cells are transformed into disc-like syncytia. The population of elongated cells reaches maximum between the 6th and 8th days. Some

of the elongated cells show spontaneous contraction-relaxation activity. The cells adhere stably either to the substratum or to the mesenchymal syncytia with all parts of their cell bodies. No proliferative activity was detected.

Detailed morphological features of the elongated cells are shown in Figs. 3 and 4. These cells have two or more arms and are about 100-200 μm in length. The arms are rod-like, often branched (Fig. 3b, c) and develop a small lamellipodium at their distal ends (Fig. 4a). The nucleus is located in the central, bulged portion (Figs. 3 and 4b). In a dense population of cells, they become interconnected (Figs. 3c and 4b). These morphological characteristics are maintained, at least, until the 14th day, after which the cells gradually disappear.

Contraction of the elongated cells in response to acetylcholine

In order to find whether the elongated cells have the ability to respond to neurotransmitter, we tried to stimulate the cells with acetylcholine. When $1 \times 10^{-5}\text{M}$ of acetylcholine was dropped into the medium, all cells in the vicinity contracted within a few seconds. The mode of contraction is similar to the spontaneous one. These cells do not contract on application of D_2W , ASW or 1-MA.

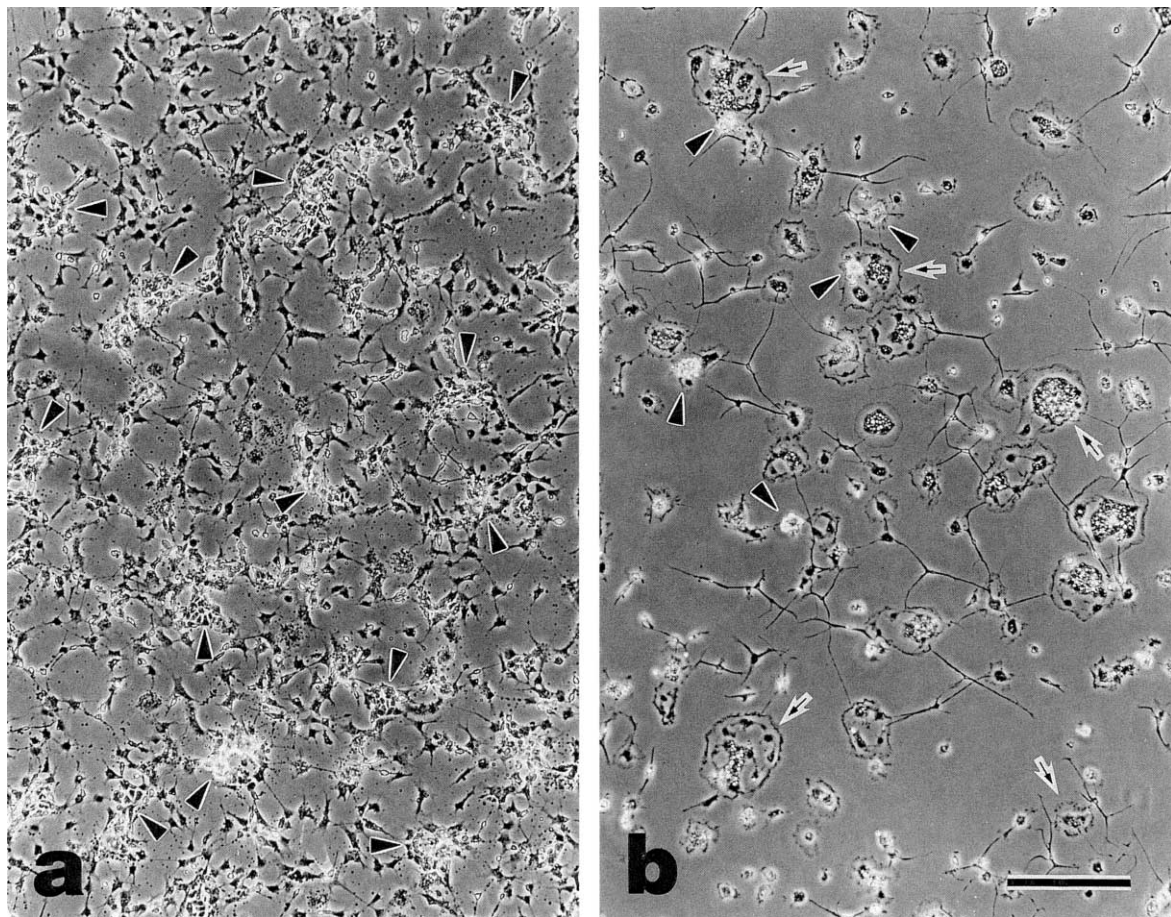


Fig. 2. Phase-contrast micrographs of cells in culture derived from embryos at the late gastrula stage. (a) One day in culture. Aggregates of epithelial cells (arrowheads) are scattered among the network of the mesenchyme cells. (b) Seven days in culture. Elongated cells have made their appearance. Epithelial cells and mesenchyme cells are distinguishable as small refractile aggregates (arrowheads) and disc-like syncytia (arrows), respectively. Bar, 200 μm .

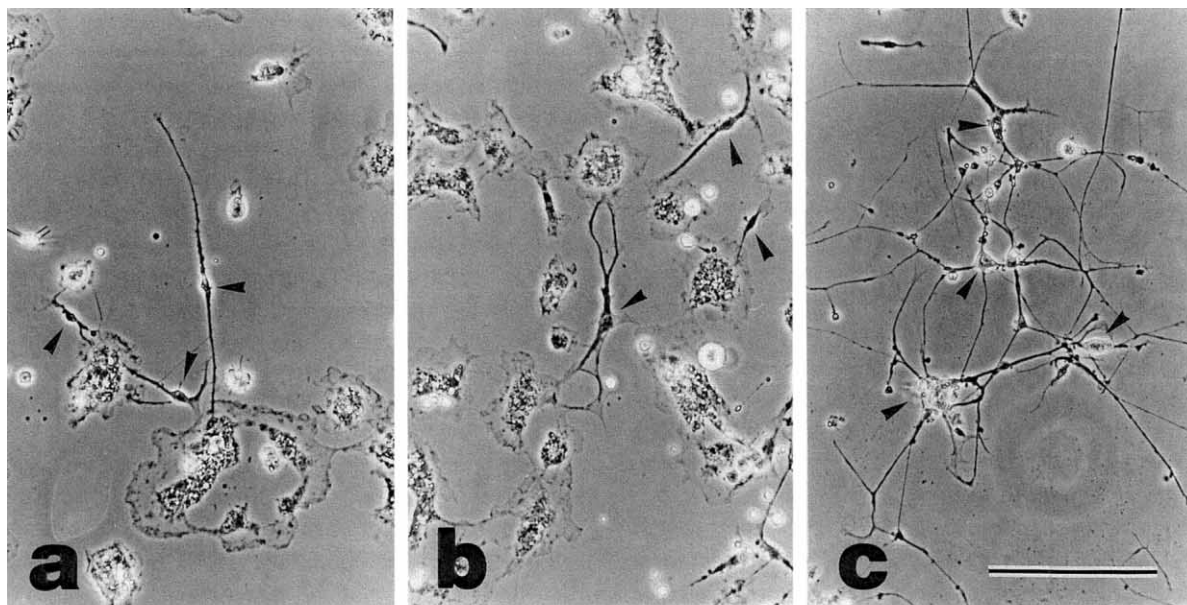


Fig. 3. Morphology of the elongated cells (7 days in culture). They possess two (a) or more arms (b), (c). A single nucleus is located in the central, slightly bulged portion of the cell (arrowheads). The cells are frequently observed in contact with the mesenchyme cells, as shown in (a) and (b). Interconnection of the elongated cells is also found (c). Bar, 100 μ m.

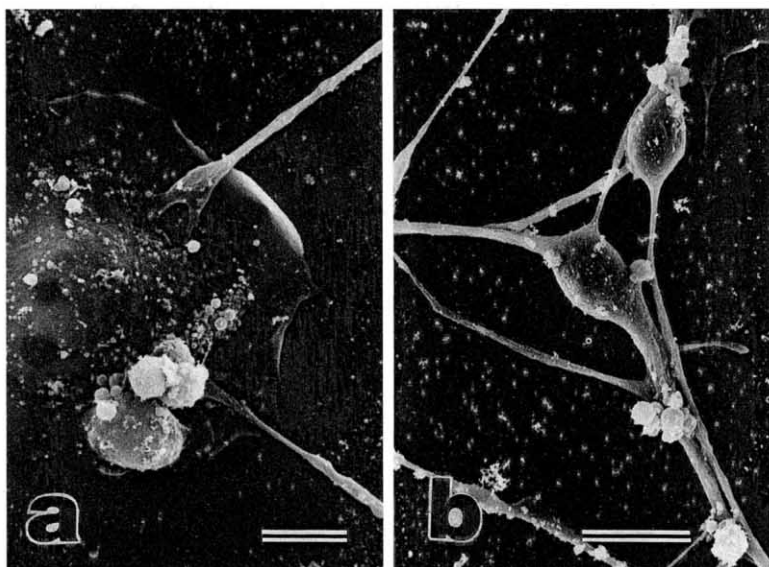


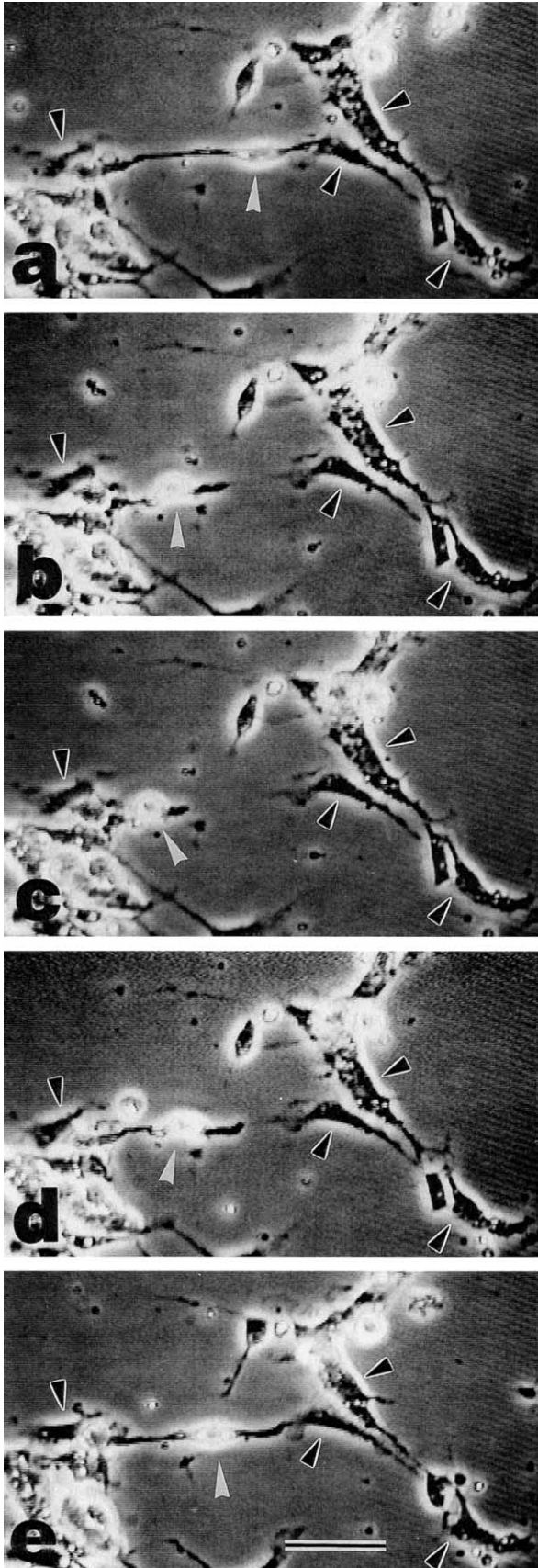
Fig. 4. Scanning electron micrographs of the elongated cells (7 days in culture). (a) Distal ends of two arms adhere close with small lamellipodia to a mesenchyme cell. (b) Central, bulged portion of two cells, in which the nucleus is contained. Bars, 10 μ m.

Figure 5 shows the detailed analysis of one cycle of the contraction-relaxation process as captured on video. The elongated cell finishes contracting within 2 sec after application of 1×10^{-5} M acetylcholine (Fig. 5a-c). The speed of contraction was the same for all cells examined. The process of relaxation, on the other hand, was more gradual and variable among the cells. The cell shown in Fig. 5 recovered its original length after 6 min and 13 sec (Fig. 5c-e). The duration of recovery ranged from a few seconds to a few hours depending on the cell. The degree of contraction also varied from cell to cell,

ranging between 80% and 20% of the original length. The mesenchyme cells did not react to acetylcholine (Fig. 5).

Contractile apparatus of the elongated cells

In order to elucidate the contractile apparatus of the elongated cells, we examined the distribution of actin and myosin by staining the cells with NBD-phalloidin and polyclonal antiserum against nematode myosin, respectively. Panels a and b of Fig. 6 show that both of these proteins were abundant throughout the cell body along its long axes and



that of the branches.

Ultrastructural examination revealed that bundles of filaments run longitudinally along the arms and through the edges of the bulged portions (Fig. 7a). At a higher magnification, thick and thin filaments are clearly discernible (Fig. 7b). Although no dense body or Z-band was found to conjugate them, the arrangement was quite orderly when seen in cross section (Fig. 7c). The diameter of the thick filament varies from 10-25 nm (average 16.3 ± 3.3 nm, $n = 53$), while that of the thin filament is about 5-8 nm.

Location and fine structure of the larval muscle cells in vivo

In order to find evidence that the elongated cells correspond to the larval muscle cells, the muscle cells were examined *in vivo* in a manner similar to that used for the elongated cells. When observed using Nomarski optics, muscle cells both encircling the esophagus (Fig. 8c) and lining

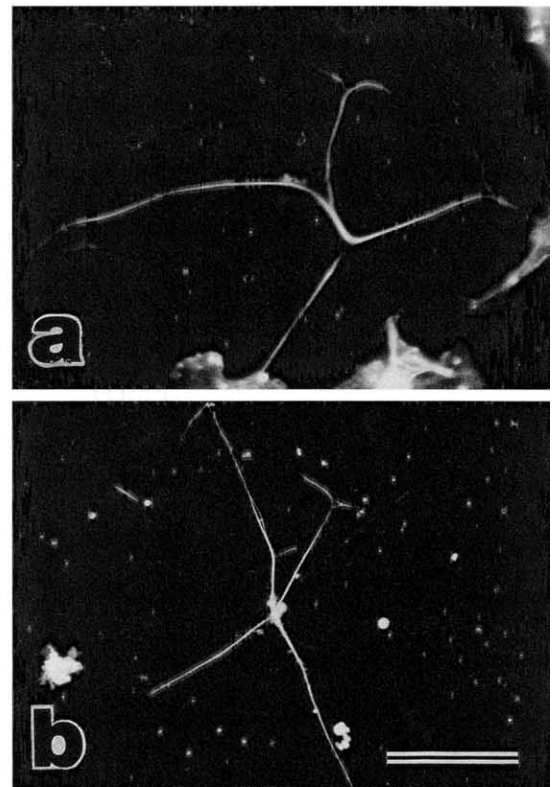


Fig. 6. Distribution of actin and myosin in the elongated cells. Fluorescence micrographs of the staining pattern after treating the cells with (a) NBD-phalloidin and (b) anti-myosin polyclonal antiserum. The cells are shown to contain large amounts of F-actin and myosin. Bar, 100 μ m.

Fig. 5. Reaction of an elongated cell to acetylcholine. Micrographs were taken at (a) 0 sec, (b) 1 sec, (c) 2 sec, (d) 23 sec, and (e) 6 min 13 sec after application of acetylcholine (1×10^{-5} M). This cell (white arrowhead) contracted to a maximum degree within 2 sec, and recovered its full length by 6 min 13 sec. Mesenchyme cells showed no reaction (black arrowheads). Bar, 30 μ m.

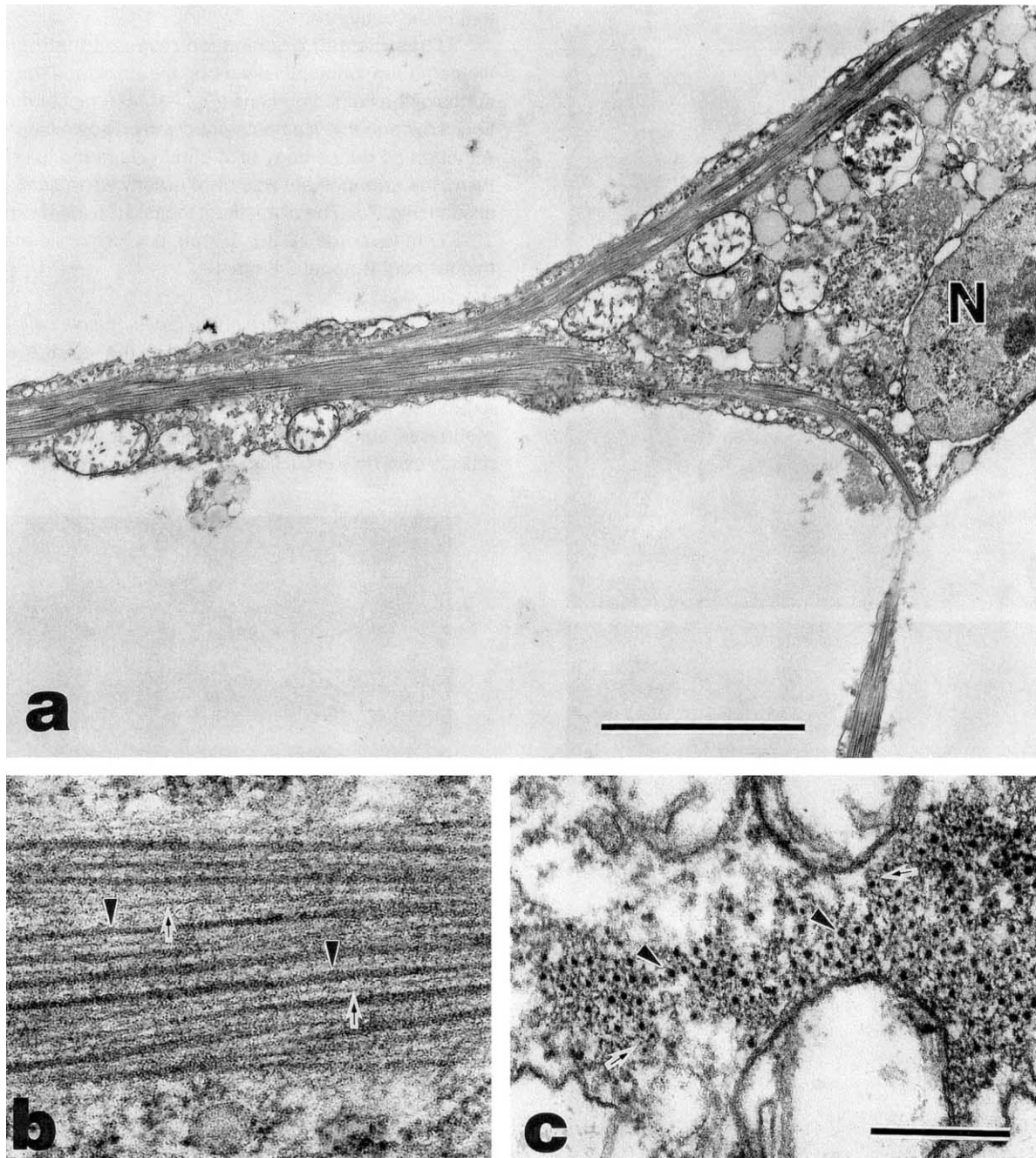


Fig. 7. Transmission electron micrographs of the contractile apparatus of the elongated cells (7 days in culture). (a) Bundles of fine filaments can be seen running along the arms and through the edges of the bulged region containing the nucleus (N). Bar, 3 μ m. (b) Longitudinal section of the filaments. Thick (arrowheads) and thin (arrows) filaments are distinguishable. There is no periodic structure. (c) Cross section of the filaments. Symbols are same as for (b). Bar for (b) and (c), 0.3 μ m.

the dorso-lateral ectoderm (Fig. 8d) of these groups show the characteristic rod-like, two- or multi-armed forms with a bulge in the central region. These cells contract, although asynchronously, when intact embryos are treated with 1×10^{-5} M acetylcholine in ASW (Fig. 8b).

Figure 9 shows the ultrastructure of myofilaments of the two groups of larval muscle cells. Myofilament bundles of the esophageal muscles lie adjacent to the plasma membrane

apposing the basal lamina of the esophageal epithelium (Fig. 9a), while those of the dorsal muscles lie along the plasma membrane while closely adhering to one another (Fig. 9b). The thick filaments are conspicuous in longitudinal sections at a higher magnification (Fig. 9e, f). Spaces between the thick myofilaments are so narrow that it is difficult to locate the thin filaments. Neither the dense bodies nor the Z-bands are found. In cross sections, however, both thick and thin filaments are

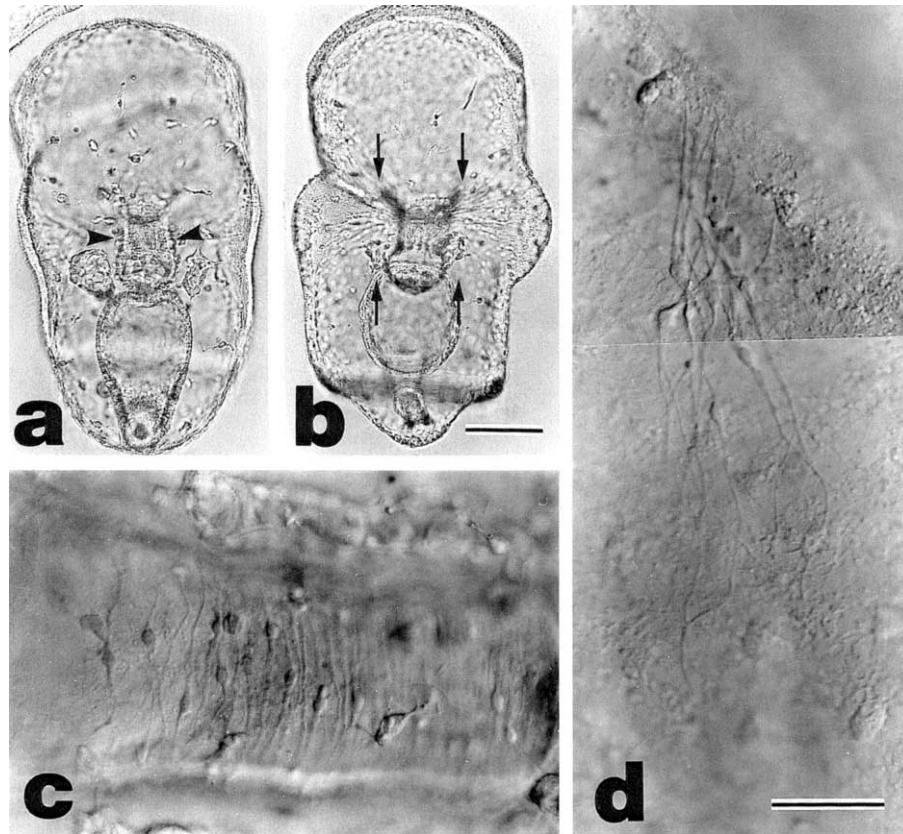


Fig. 8. Localization of larval muscle cells *in vivo*. Light micrographs of two bipinnaria larvae (4 days after insemination) in relaxed state (a) and in fully contracted state in response to applied acetylcholine (b). Arrowheads in (a) and arrows in (b) show the sites of esophageal and dorsal muscle cells, respectively. Bar for (a) and (b), 100 μ m. Differential interference micrographs of the esophageal (c) and the dorsal (d) muscle cells. Their shape agrees well with that of the elongated cells *in vitro* (Fig. 3). Bar for (c) and (d), 50 μ m.

obvious (Fig. 9c, d). The diameter of the thick filaments of esophageal muscle cells varies from 13-20 nm (average 15.7 ± 4.0 nm, $n = 20$), while that of the dorsal muscle cells varies from 12-28 nm (average 19.4 ± 5.0 nm, $n = 20$). The diameter of the thin filaments, on the other hand, is 5-8 nm for both groups.

DISCUSSION

We have shown, in this paper, the appearance of elongated cells from embryonic cells of starfish, dissociated at the late gastrula stage, treated with glycine and cultured for about a week (Figs. 2b and 3). We conclude that these elongated cells are genuine larval muscle cells for the following reasons: (1) their elongated shape with multiple arms (Fig. 3) resembles that of the larval muscle cells (Fig. 8); (2) these cells contain an abundance of F-actin and myosin which are distributed throughout the entire cytoplasm (Fig. 6); (3) bundles of thick and thin filaments (Fig. 7) are present in their cytoplasm resembling those found in larval muscle cells (Fig. 9); and (4) these cells contract in response to acetylcholine (Fig. 5) as seen with larval muscle cells (Fig. 8).

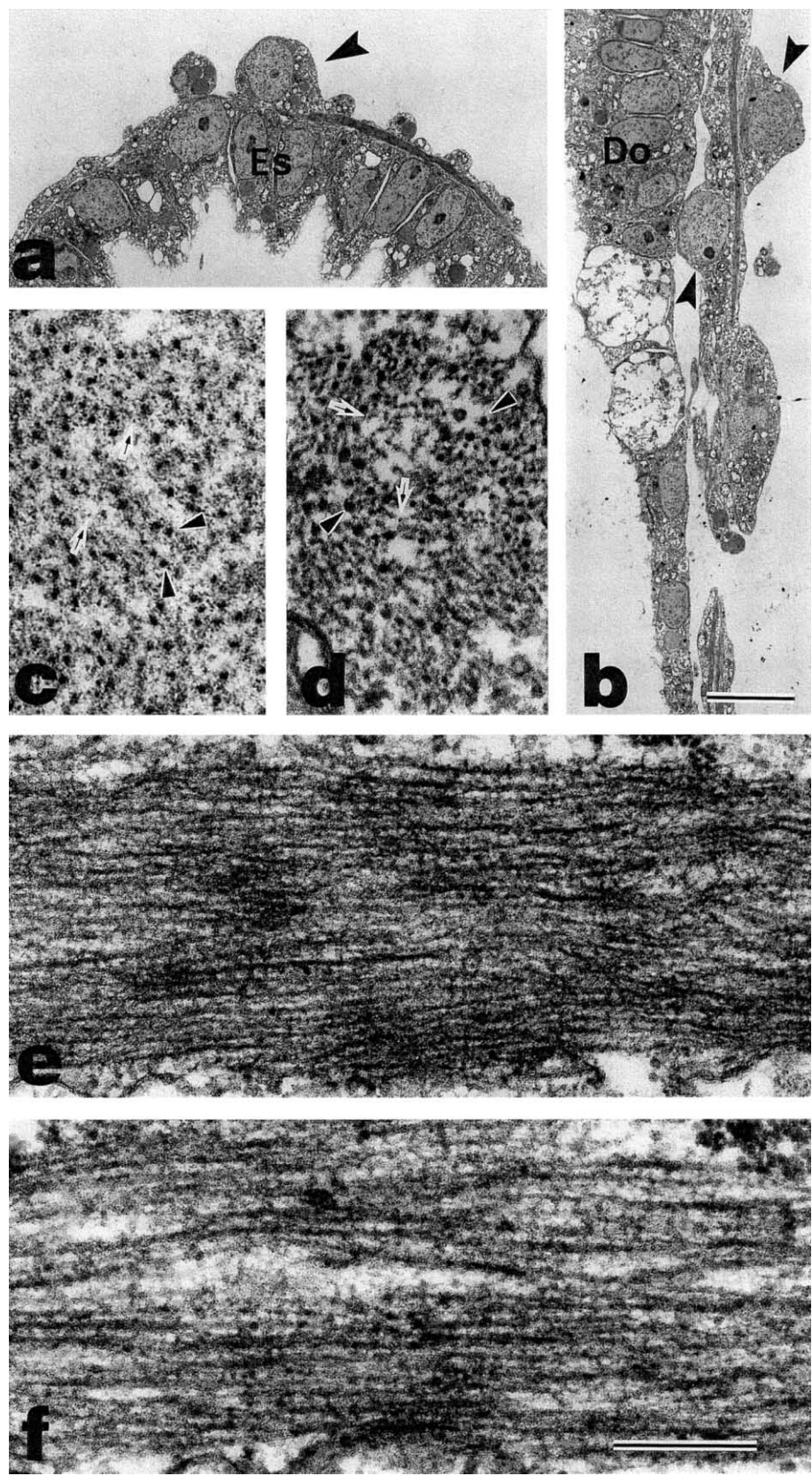
We have reported earlier that there is an order of resistibility to glycine treatment among the embryonic cells,

i.e., in descending order, mesenchyme cells, cells constituting the coelomic pouches, endodermal cells and ectodermal cells (Kaneko *et al.*, 1995). The only change in the experimental conditions made in the present study from the earlier one was using embryos from the late gastrula stage rather than the mid gastrula one. The fact that the size of the epithelial aggregates were much smaller in the present case (compare Fig. 2a with Fig. 3 in our previous paper (Kaneko *et al.*, 1995)) indicates that the susceptibility of the epithelial cells to the glycine treatment increases somewhat with development. This, in turn, suggests that the majority of the cells constituting the aggregates found in the present culture may be coelomic pouch cells, which are more closely related to the mesenchyme cells that display the strongest resistance to the treatment. Further decrease in the size of the aggregates paralleling the appearance of elongated muscle cells (Fig. 2) and the fact that muscle cells originate from the coelomic pouches *in vivo* (Ishimoda-Takagi *et al.*, 1984; Cox *et al.*, 1986; Burke and Alvarez, 1988; Crawford, 1990; Cameron and Davidson, 1991; Ettensohn, 1992) support this possibility.

Our present findings offer some insights into the nature of the larval muscle cells of the starfish. One of the prominent characteristics of the muscle cells *in vitro* is their adhesiveness. In spite of the apparent lack of adhesive devices, except at

the every tips of their arms (Figs. 3 and 4), the muscle cells are either tightly fixed to the substratum throughout the length of their cell bodies or partly to the adjacent mesenchyme cells

or to the arms of other muscle cells. This adhesiveness is maintained at the time of contraction in response to acetylcholine even though the length of the cell body is reduced



to 20% of the original length (Fig. 5). The cells seem to regain their original length after the contraction by tracing back their original positions (Fig. 5). This adhesiveness *in vitro* agrees with that observed for the muscle cells *in vivo*; that is, while esophageal muscle cells are found in tight association with adjacent basal lamina (Fig. 9a), dorsal muscle cells adhere along their body length to fellow muscle cells (Fig. 9b). These small bundles seem to become focally attached to the basal lamina (Fig. 9b), fixed both with ends probably by small lamellipodia, such as those shown in Fig. 4a. Focal attachment of the muscle bundles is considered to be effective for generating strong contraction of the dorsal epithelium (Fig. 8b).

The larval muscle cells of the starfish reacted to acetylcholine (Fig. 5). In contrast to this finding, cholinergic neurons have not been detected, to our knowledge, in either starfish or sea urchin embryos, although acetylcholine itself has been detected in the sea urchin embryo (Buznikov *et al.*, 1968). On the other hand, Nakajima reported finding catecholaminergic neurons in starfish larva, distributed along the dorsal ciliary band (Nakajima, 1988) and serotonergic ones forming two ganglia in the same ciliary band (Nakajima, 1987). Moss *et al.* (1994) have also reported that in starfish larva, cells bearing native echinoderm neuropeptide (GFNSALMF amide) are localized in the vicinity of the serotonergic neurons. They suggest that these nervous systems control the ciliary movement instead of the muscular movement. Similar observations have been made for sea urchin larva (Gustafson *et al.*, 1972; Bisgrove and Burke, 1987). Our present finding on the reaction of larval muscle cells to acetylcholine predicts the presence of a cholinergic neuron system which controls muscular movement.

Larval muscle cells of the starfish are of the smooth muscle type, which lacks a periodic structure (Figs. 7 and 9). They share characteristics with the most primitive type of smooth muscle cells according to Matsuno's classification (Matsuno, 1987), i.e., containing thick filaments 14 nm in diameter and relatively few dense bodies. This type of smooth muscle cells is also characteristic of long-term contraction-relaxation periodicity (Matsuno, 1987). The contraction (1~2 sec) and relaxation (few seconds~) features of the larval muscle cells of the starfish (Fig. 5) also agree with this characteristic.

Smooth muscle cells obtained from several sources of mammal vascular tissues exhibit proliferative activity in culture (Chamley *et al.*, 1977). This activity is coupled with loss of intrinsic features, such as cell shape, contraction activity and smooth muscle specific contractile proteins (Chamley *et al.*,

1977). In our culture system, starfish larval muscle cells show neither proliferative activity nor dedifferentiation. We do not know whether starfish larval muscle cells are incapable of such activity or our culture system failed to support them.

The culture method presented in this paper provides a model system for studying the development of primitive muscle cells. By using the difference in susceptibility of the embryonic cell types to the glycine treatment (see above), this method preferentially leaves coelomic pouch cells on the culture substratum, from which the muscle cells develop. The difference between the results of our previous study (Kaneko *et al.*, 1995) and this one as to whether or not the muscle cells appear indicates that commitment of the muscle cells has not yet occurred in the mid gastrula stage. This notion is strengthened by the fact that muscle cells did not make their appearance even after coelomic pouch cells of the mid gastrula stage were maintained in culture for up to 12 days (data not shown). In contrast, the commitment and/or determination and differentiation of muscle cells is observed in the culture of late gastrula cells. A study is in progress to provide further information on the developmental process of the larval muscle cells.

ACKNOWLEDGMENTS

We gratefully acknowledge the guidance of Dr. M. Yamamoto in taking the scanning electron micrographs and the the generous gift of antimyosin polyclonal antiserum from Dr. H. Kagawa, both of Okayama University. We are grateful to Mr. M. Isozaki and Mr. W. Godo for kindly providing the starfish and all other members of the Ushimado Marine Laboratory of Okayama University for allowing us to use their facilities throughout the course of this investigation.

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Fig. 9. Transmission electron micrographs of the esophageal and dorsal muscle cells *in vivo*. (a) An esophageal muscle cell (arrowhead) adheres to the blastocoelic side of the basal lamina of the esophageal epithelium (Es). (b) Dorsal muscle cells (arrowheads) adhere closely to one another in close vicinity to the ectodermal epithelium (Do). Bar for (a) and (b), 6 μ m. (c), (d) Cross sections of myofilaments of esophageal and dorsal muscle cells, respectively. In both sections, thick (arrowheads) and thin (arrows) myofilaments are distinguishable. (e), (f) Longitudinal sections of myofilaments of esophageal and dorsal muscle cells, respectively. In both sections, thick myofilaments are conspicuous, while thin myofilaments are difficult to find. This may be due to artificial shrinkage which occurred during fixation. Note that both filaments are discernible in the cultured elongated cells (Fig. 7b). No periodic structure can be seen. These ultrastructural characteristics are in good accord with those of elongated cells *in vitro* (Fig. 7). Bar for (c), (d), (e) and (f), 0.3 μ m.

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(Received August 23, 1996 / Accepted October 8, 1996)