Distribution and activity of endogenous lectin during myogenesis as measured with antilectin antibody

(CELLULAR DIFFERENTIATION/SURFACE PROTEIN/IMMUNOFLOURESCENCE)

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ABSTRACT Antibodies to electrolactin, a lectin endogenous to embryonic skeletal muscle, have been used to study the distribution of electrolactin during myogenesis in L6 cells and rat primary muscle cultures. Antibody binding is highest to mononucleated cells and is low to myotubes in both systems. Binding is much lower to fibroblasts in the primary cultures. Binding appears to be on the surface of these cells, although evidence is presented for there being binding on the inside of cells as well. When observed on myotubes, binding is generally associated with highly stained patches and in some instances is near regions where fusion may be occurring. In L6 cells, binding sites can be exposed by treating mononucleated cells with trypsin. These results are discussed in terms of their possible role in myogenesis and synaptogenesis.

Teichberg et al. (1) discovered and partially characterized a lectin from electric organs and other excitable tissues. This lectin, called electrolactin, caused the agglutination of rabbit erythrocytes and this agglutination was inhibited by galactose-containing sugars. Subsequently, a protein with these properties has been isolated and has been found in several different tissues (2–4).

Because this lectin was found in high activities in embryonic skeletal muscle, several laboratories investigated the possibility that electrolactin was involved in the differentiation of skeletal muscle. Although all these studies showed that the activity of electrolactin changed as the muscle cells differentiated, no definitive function for electrolactin has been proven (5–8).

In our studies, using the L6 line of myogenic cells, we have found that the agglutination due to electrolactin is blocked by another protein, and evidence for the surface location of this protein has been provided (9). We have suggested that this blocking protein may be identical to another agglutinating protein present in these cells, which we have called myonecit. Because the blocking protein is soluble under our homogenization conditions, its presence in the supernatant fraction can prevent the accurate measurement of the soluble form of electrolactin (s-electrolactin). There is another form of the electrolactin which is bound to particolar organelles but can be solubilized by sugars containing β-galactose; we have called this particulate form of electrolactin p-electrolactin. All three proteins—i.e., s-electrolactin, p-electrolactin, and myonecit—undergo characteristic changes in activity as the cells differentiate. Myonecit and p-electrolactin activities are very low after the cells are plated into fresh medium, but s-electrolactin activity is high. As the cells grow to confluency and begin to fuse, both myonecit and p-electrolactin activities increase, but s-electrolactin activity decreases. These changes in activities can represent changes in specific activities of >10-fold (9).

The studies reported here used antibodies directed against electrolactin to determine the localization of electrolactin in L6 cells and rat primary skeletal muscle cells as they underwent differentiation.

MATERIALS AND METHODS

Electrolactin was prepared from homogenates of L6 cells. Cells were homogenized in phosphate-buffered saline containing 2 mM EDTA, 2 mM 2-mercaptoethanol, and 1 mM phenylmethanesulfonyl fluoride. The homogenate was centrifuged at 100,000 × g; the electrolactin in the supernatant fraction is called crude s-electrolactin and that in the pellet is p-electrolactin. p-Electrolactin can be completely solubilized with 10–50 mM lactose. After this sample is recentrifuged and the supernatant fraction is dialyzed, it is called crude p-electrolactin. The method for purifying electrolactin from L6 cells will be described in detail elsewhere, but it is substantially similar to that reported by de Waard et al. (2) and Den and Malinzak (3). Electrolactin was eluted with a continuous lactose gradient from an affinity column made by attaching fetuin to Sepharose 4B. The peak protein eluted from this column showed one band on 15% sodium dodecyl sulfate/polyacrylamide gel electrophoresis with an approximate molecular weight of 13,000.

Rabbits were immunized with three injections, 1 week apart. The first was 200 μg of purified electrolactin emulsified in complete Freund’s adjuvant, injected intradermally into multiple sites between the shoulder blades; the second was 200 μg near the original sites; the third was 100 μg intravenously. The animals were sacrificed 4–6 weeks after the first injection and the serum was stored frozen.

The cells were labeled with antilectin antibodies by using the second-antibody technique (10). The cells were washed several times with cold 0.1 M phosphate pH 7.1 buffer (P, buffer) containing 0.1 mg of bovine serum albumin per ml. All the remaining steps were done at 4°C with constant shaking. The buffer was removed and the cells were fixed for 60 min in 0.01 M sodium periodate/0.075 M lysine/2% paraformaldehyde/0.037 M phosphate, pH 6.9, with one change of fixative after 30 min. This method of fixation has been shown by others to be compatible with preservation of cellular ultrastructure and surface antigens (11, 12). After fixation, the cells were washed with P, buffer several times and then washed for 30–60 min, with changes every 10 min, in P, buffer/albumin. The cells were rinsed in 0.1 M sodium borate pH 8.4 buffer for 5 min and then incubated in 0.05 M sodium borohydride in the borate buffer for 10 min with one change. This was followed by another borate buffer rinse and 30-min washing with P, buffer/albumin.

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Abbreviations: s- and p-, soluble and particulate forms, respectively; P, 0.1 M phosphate buffer, pH 7.1.
After the above treatment the cells were incubated with either immune or preimmune serum diluted 1:20 in Pi buffer/albumin. Prior to dilution the serum was heated to 56°C for 30 min. Preimmune sera (from rabbits before they were injected with electrolectin) were used to determine the level of non-specific binding. After the cells were washed for 60 min with Pi buffer/albumin with changes every 10 min, the cells were treated with either 125I- or fluorescein isothiocyanate-labeled goat anti-rabbit IgG. The fluorescent labeled and purified goat anti-rabbit IgGs were purchased from Miles and used without further purification. 125I labeling was carried out by the chloramine-T method (13). The fluorescent IgG used was diluted 1:20 in Pi buffer/albumin; the 125I-labeled IgG was diluted 1:50. After the cells were labeled, they were washed extensively in Pi buffer/albumin; the labeled cells were then dissolved in 0.5 M NaOH at 37°C for 12 hr and assayed for radioactivity in a gamma counter.

After they were fixed, cells were made permeable by treatment for 2 min with acetone diluted 1:1 with distilled water, with undiluted acetone for 2 min, and with acetone diluted 1:1 with distilled water for 2 min, all at −20°C. Cells were then labeled by using the scheme given above.

Intact cells were labeled with 125I (Amersham/Searle), by using lactoperoxidase and glucose oxidase, before they were removed from the dishes (14). Control cells, treated with the same reagents, remained impermeable to trypan blue.

The growth conditions for the L6 cells (15) and primary rat skeletal muscle cultures have been described (9, 10), as have the agglutination assays (5, 6, 9).

Characterization of Antielectrolectin Antibodies. A reaction of identity in the Ochterlony double-diffusion test was noted between antielectrolectin serum and s- and p-electrolectins, indicating that these proteins are identical (Fig. 1). The same results were observed with crude and affinity-purified preparations of p-electrolectin. There were no reactions with preimmune sera. Immunoelectrophoresis of these preparations resulted in a single band (data not shown). These results indicate that the antibodies react with a single antigen in the L6 cells.

Antisera have also been produced in rabbits to affinity-pu-

Table 1. Antielectrolectin antibodies inactivate agglutination of erythrocytes by electrolectin

<table>
<thead>
<tr>
<th>Addition</th>
<th>Electrolectin activity/50 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>7.5; 7.5</td>
</tr>
<tr>
<td>Preimmune serum</td>
<td>5.5; 6.0</td>
</tr>
<tr>
<td>Immune serum</td>
<td>0; 0</td>
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</tbody>
</table>

Preimmune and immune sera were diluted 1:20 with buffer before 125 µl was added to the 125-µl sample of electrolectin. After the samples were mixed, they were incubated at room temperature for 60 min. Then, 8.7 µl of goat anti-rabbit IgG was added to all samples and they were incubated at room temperature for another 30 min before they were centrifuged at 12,000 × g for 10 min. The supernatant fractions were assayed for lectin activity (5).

rified electrolectin from fetal rat and fetal calf skeletal muscle. Antibodies against L6 or fetal rat muscle cells crossreacted with lectin from these two sources but not with fetal calf lectin.

![Image](https://example.com/image1)

**Fig. 1.** Immunological characterization of s- and p-electrolectins by Ochterlony immunodiffusion tests using antisemur produced against affinity-purified p-electrolectin. (A) Wells a, antielectrolectin antiserum diluted 1:1 in phosphate-buffered saline; b, crude p-electrolectin; c, affinity-purified p-electrolectin; d, crude s-electrolectin. (B) Wells: a, antielectrolectin antiserum diluted 1:2 in phosphate-buffered saline; b, affinity-purified s-electrolectin; c, affinity-purified p-electrolectin. A is unstained; B is stained with Coomassie blue.

![Image](https://example.com/image2)

**Fig. 2.** (B and D) Indirect immunofluorescent staining of L6 cells, showing distribution of antielectrolectin antibody binding. (B) Trypsinized cells were plated into growth medium 60 min before being fixed and stained. (D) A myotube located in a culture that was largely comprised of myoblasts. In D the cells were plated 2 days before they were labeled, which is 2–3 days before fusion became prominent and therefore the myotube illustrated must be one that had formed in less than 2 days. (A and C) Phase-contrast photographs of the same cells shown in B and D, respectively. (Bar: A, 20 µm; C, 50 µm.)
Antibodies against L6 or fetal rat crossreacted with lectin from adult rat and mouse skeletal muscle. Table 1 shows that the antiserum is able to inactivate the agglutination due to electrolecin, and we have determined by sodium dodecyl sulfate/polyacrylamide gel electrophoresis that the antiserum precipitates a protein that comigrates with purified electrolecin.

**Binding of Antieleclectin Antibodies to L6 Cells.** As characterized by the antibody binding, the distribution of electrolecin (i) is not uniform, (ii) decreases as the cells grow to confluency, and, (iii) is largely absent from myotubes except for an occasional patch.

Mononucleated cells treated with trypsin (100 µg/ml) and then with immune sera are illustrated in Fig. 2 A and B. Cells treated in the same way but with preimmune sera showed a diffuse labeling at a much lower intensity. Fixed or unfixed cells in suspension or cells freshly plated into fresh growth medium, as those illustrated in Fig. 2 A and B, all showed a similar binding pattern. The cells in suspension were surrounded by a halo of fluorescence on their circumference such as seen on the middle cell in Fig. 2B. After the cells became attached to the substratum, however, the ruffled region of the cell attaching to the substratum was labeled (as seen in the bottom cell in Fig. 2B), as was the thicker region surrounding the cell body. It is difficult to determine the precise region of the cells that is stained from these micrographs, but the pattern of labeling such as seen on the middle cell in Fig. 2B is commonly attributed to surface labeling.

One day after the cells were plated into fresh growth medium, the label was uniformly distributed over the surface. As the cells grew toward confluency, the labeling became much less intense and patches of fluorescence were more common. The fact that labeling decreased before fusion is shown in Fig. 3 (fusion started on day 5 and continued to about day 10). For the observations illustrated in Fig. 3, we used 125I-labeled goat anti-rabbit IgG as the second antibody in order to quantify the binding. The observations made with fluorescent microscopy, however, are consistent with those made by using 125I-labeled IgG. The cells used in Fig. 3 were trypsinized and transferred into fresh medium from a pooled source of cells on day 0. The source of cells was from a plating done 4 days before day 0. Day 0 cells were labeled 2 hr after plating, and day 1 cells were labeled 24 hr after, etc. Therefore, the only difference between day 0 and day 4 cells is the treatment with trypsin and the plating into fresh medium. Control experiments, done simply by labeling after trypsinization without plating into fresh medium, showed an effect similar to that illustrated in Fig. 3—namely, day 0 cells showed the highest binding of antieleclectin antibodies. It is concluded that trypsinization exposes surface electrolecin.

Staining of myotubes was characterized as being extremely
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Table 2. Immunoprecipitation of 125I-labeled p-electrolectin from intact cells labeled with 125I

<table>
<thead>
<tr>
<th>Crude</th>
<th>With preimmune sera, cpm</th>
<th>With immune sera, cpm</th>
<th>Difference, cpm</th>
</tr>
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<tr>
<td>0</td>
<td>29</td>
<td>29</td>
<td>0</td>
</tr>
<tr>
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<td>223</td>
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</tr>
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<td>40</td>
<td>207</td>
<td>407</td>
<td>200</td>
</tr>
<tr>
<td>80</td>
<td>361</td>
<td>893</td>
<td>532</td>
</tr>
</tbody>
</table>

Crude p-electrolectin was prepared from cells that had been iodinated by the lactoperoxidase procedure. The lectin was prepared as described in Methods. To the samples was added 200 μl of preimmune or immune serum diluted 1:5 with buffer. The final volume was brought to 0.25 ml with 0.2 M lactose in phosphate-buffered saline, and the mixture was incubated at 4°C for 120 min. Goat anti-rabbit IgG (10 μl) was then added and the samples were incubated at 4°C for 90 min. The samples were then centrifuged at ~12,000 x g for 10 min. The supernatant fractions were discarded. The pellets were dissolved in 10 μl of 10% sodium dodecyl sulfate, added to 10 ml of Aquasol II (New England Nuclear), and assayed in a scintillation counter. The supernatant fraction from the dialyzed lactose-extracted pellet was applied to an affinity column, and labeled protein with electrolectin activity eluted from the column at the same volume as did unlabeled electrolectin.

low with an occasional patch of fluorescence. These patches seemed to be more prevalent in young myotubes (Fig. 2C and D) compared with older myotubes, which rarely show binding of antibodies. When found on myotubes in the oldest cultures studied (i.e., 5 days after fusion started) patches had a granular appearance in contrast to the uniformly stained patches in Fig. 2D.

Binding of Antielectrolectin Antibodies to Rat Primary Muscle Cultures. Fig. 4 shows the binding pattern of antielectrolectin antibodies to primary rat muscle cultures. As in the L6 cultures, labeling was most intense on the mononucleated cells. The most intensely stained cells were generally the spindle-shaped ones, a shape characteristic of myoblasts. The other cells, with a triangular shape, probably are fibroblasts and stained lightly if at all. Arrays of myoblasts were lined up end to end in a chain (Fig. 4A) which, as described by others (18), probably represents fusing myoblasts. When the cells were aligned in this way, the staining became more localized toward the side of the two cells making contact with one another. For example, the cell on the far left in Fig. 4 shows more labeling on its right side than on its left (i.e., labeling is more intense on the side making contact with the chain of cells).

Primary rat myotubes generally were stained lightly compared to myoblasts. Exceptions to this generalization have been encountered, as noted for the L6 cells. Intensely stained patches were observed which resembled those seen with L6 cells. In the primary muscle cultures, however, we also found regions in myotubes that appeared to be in the process of fusing with myoblasts; these regions often stained intensely with antielectrolectin antibodies (Fig. 4E–H). Label was oriented toward the contact between cells and may have been coalescing in these contact regions.

Specificity and Location of Antielectrolectin Binding Sites. The labeling of the cells described above appears to be specific for antielectrolectin antibodies. (i) Labeling of cells under identical conditions with preimmune sera showed low levels of labeling, between 1/10th and 1/2 that when 125I-labeled goat anti-rabbit IgG was used. (ii) The pattern of labeling in fluorescence microscopy with preimmune sera was different from that observed with immune sera; with preimmune sera the labeling was generally diffuse and very faint. (iii) Pretreatment of the cells with preimmune sera did not block the binding of the immune sera. (iv) The antisera binding clearly was much more intense for some classes of cells than for others—e.g., fibroblasts were stained much less than myoblasts.

From these experiments it is probable that at least some of the electrolectin is on the surface of cells for the following reasons: (i) Cells were fixed in paraformaldehyde for 1 hr before being treated with antisera. This fixation procedure has been used in electron microscope studies of the localization of antibody binding in similar cells and no disruption of cells was noted (12). (ii) Occasionally, groups of cells were encountered that were labeled uniformly and their nuclei stood out as unstained areas in a background of intense fluorescence. Often these cells were in regions that had been damaged in the preparation of the cells, but in other instances no damage could be identified. These cells probably represent cytoplasmic labeling. (iii) The labeling of intact cells with 125I by the lactoperoxidase procedure produced a labeled protein that was precipitated by the antielectrolectin antisera (Table 2). (iv) Additional antielectrolectin binding sites could be exposed by making the cells permeable with acetone (Table 3). All permeabilized cells prepared for fluorescent microscopy were heavily and uniformly labeled, whereas nonpermeabilized cells were not all stained and the staining pattern was more likely to be nonuniform. (v) Considerable variation was observed in the binding of goat antirabbit 125I-IgG to live cells treated with antisera or preimmune serum. Unfixed cells, on day 4, 5, or 10 after plating, were labeled 40% higher than 50% less than fixed cells (see Note Added in Proof). Additional work on this point will be required. (vi) This finding is a result of fixation does not expose binding sites but rather that permeabilization of the cells exposes sites that are presumably in the cytoplasm of the cell.

DISCUSSION

The antibody-binding studies show that electrolectin decreases as the L6 cells grow to confluence and that fusion is not required for electrolectin to become inaccessible to antibody binding. This observation is surprising because a number of biochemical studies have shown that total cellular electrolectin activity increases until just before fusion begins, and then it decreases (5–9). We believe that there are two possible explanations for the difference between these results. (i) The antibody labeling technique only measures a portion of the total cellular electrolectin, as the studies on acetone-permeabilized cells showed. Presumably, largely surface lectin is measured in antibody-binding studies whereas biochemical studies measure total cellular lectin. (ii) We have shown elsewhere that there is a protein that blocks the agglutination due to electrolectin (9).

We have suggested that this blocking protein is likely to be another agglutinating protein which we have called myoneclectin. Because both myoneclectin and the blocking protein are inacti-
vated by the trypsinization of intact cells and, consequently, higher levels of antielectrolectin activity are measured after trypsinization, we suggest that the removal of these proteins may be responsible for the increased antielectrolectin antibody binding observed following trypsinization. There are several alternative interpretations of the results obtained with trypsin, but we believe that this interpretation is the one most consistent with all the data. If this interpretation proves to be correct, local proteolysis may expose electrolectin and play a role in differentiation.

The general pattern of binding of antibodies to primary muscle cultures is similar to that observed with L6 cells—i.e., high on myoblasts and low on myotubes. However, there are striking morphological differences between the two cultures. Myoblasts are characteristically spindle-shaped in primary muscle cultures but not in L6 cultures. These spindle-shaped cells are commonly the most heavily stained cells in the cultures. Also, the alignment of cells believed to be in the process of fusing (18), seen in primary muscle cultures, is not seen with L6 cells. Such alignment is illustrated in Fig. 4A, and the accompanying fluorescence micrograph shows that electrolectin is not distributed uniformly in these cells but is oriented more toward the side of the cell making contact with another myoblast or myotube. In some cases, which may represent myoblast-to-myotube fusion, the degree of electrolectin localization is even more concentrated in regions where fusion is likely to be occurring. We have observed, in time-lapse studies (unpublished observation), cells in the process of fusing which look very much like those illustrated in Fig. 4.

We have preliminary evidence that antielectrolectin antibodies bind to neurons present in spinal cord explants, and we have found that PC-12 cells bind antielectrolectin antibodies. Others have presented evidence for the binding of similar antibodies to neurons in the optic tectum and spinal cord (19-21). Our results suggest, therefore, that, when the innervating arbor reaches the muscle fiber during embryogenesis, no electrolectin is present on the muscle membrane, but it may be present in the neuron. If these observations are substantiated, it would be interesting to determine if the disappearance of electrolectin from the surface of the myotube is accompanied by the loss of the membrane binding site of the lectin.

Note Added in Proof. We have recently found that fixing cells with 3% paraformaldehyde in phosphate-buffered saline (pH 7.2) for 30 min at 21°C has a marked effect on the binding of antielectrolectin antibodies compared to unfixed cells. Unfixed cells have none of the patches observed with fixed cells, and there is a reduction in the overall magnitude of binding which may be dependent upon the developmental stage of the cells. The patches seen after fixation, however, are very near the surface of the cell as determined by the plane of focus at magnification × 1200.

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