IMMUNOLOGICAL STUDIES OF THE 
EMBRYONIC MUSCLE CELL SURFACE 

Antiserum to the Prefusion Myoblast 

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ABSTRACT 

Xenogeneic antisera raised in rabbits have been used to detect compositional changes at the cell surfaces of differentiating embryonic chick skeletal muscle. In this report, we present the serological characterization of antiserum (Anti-M-24) against muscle tissue and developmental stage-specific cell surface antigens of the prefusion myoblast. Cells from primary cultures of 12-d-old embryonic chick hindlimb muscle were injected into rabbits, and the resulting antisera were selectively absorbed to obtain immunological specificity. Cytotoxicity and immunohistochemical assays were used to test this antiserum. Absorption with embryonic or adult chick heart, brain, retina, liver, erythrocytes, or skeletal muscle fibroblasts failed to remove all reactivity of Anti-M-24 for myogenic cells at all stages of development. After absorption with embryonic myotubes, however, Anti-M-24 no longer reacted with differentiated myofibers, but did react with prefusion myoblasts. The myoblast surface antigens detected with Anti-M-24 are components of the muscle cell membrane: (a) these macromolecules are free to diffuse laterally within the myoblast membrane; (b) Anti-M-24, in the presence of complement, induced lysis of the muscle cell membrane; and (c) intact monolayers of viable myoblasts completely absorbed reactivity of Anti-M-24 for myoblasts. These antigens are not loosely adsorbed culture medium components or an artifact of tissue culture because: (a) absorption of Anti-M-24 with homogenized embryonic muscle removed all antibodies to cultured myoblasts; (b) Anti-M-24 reacted with myoblast surfaces in vivo; and (c) absorption of Anti-M-24 with culture media did not affect the titer of this antiserum for myoblasts. We conclude that myogenic cells at all stages of development possess externally exposed antigens which are undetected on other embryonic and adult chick tissues. In addition, myoblasts exhibit surface antigenic determinants that are either masked, absent, or present in very low concentrations on skeletal muscle fibroblasts, embryonic myotubes, or adult myofibers. These antigens are free to diffuse laterally within the myoblast membrane and may be modulated in response to appropriate environmental cues during myodifferentiation.
The molecular processes underlying cellular recognition, growth regulation, and morphogenesis are largely unknown although each represents a major problem in developmental biology (36). Because many developmental events are preceded by specific cell surface interactions, there has been increasing interest in exploring the relationships between environmental cues, alterations of the cell surface, and genomic expression. Surface macromolecules that can be serologically identified probably play a role in establishing specific intercellular contact relationships and subsequent morphogenesis in a number of developing invertebrate systems including slime molds (17) and sea urchins (31). Similar molecules may serve to modulate cell interactions during the development of vertebrates; surface receptors for various lectins change during embryonic development (7, 25) and transformation (47), but whether these glycoproteins play a direct role in embryogenesis or carcinogenesis remains uncertain. Alterations in cell surface glycoproteins are associated with various developmental, growth, or metabolic states (52, 62), but there has been no demonstration of a unique series of morphogenetic and developmental events that can be correlated with the appearance or disappearance of specific cell surface antigens. Cell culture systems for synchronizing the differentiation of embryonic chick skeletal muscle (41) provide an opportunity for correlating a known set of temporally defined events with the appearance or disappearance of specific surface macromolecules.

Cell interactions before myotube formation exhibit an exquisite degree of tissue and temporal specificity (4, 66), and it is not unreasonable to assume that unique arrays of surface markers may play a role in this recognition process. The search for myoblast-specific surface molecules is not unprecedented; others have used sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to analyze radiolabeled, solubilized muscle cell membranes (22, 24, 37), but have been unable to define unique surface components associated with specific developmental stages of myogenesis. Lectins have been isolated from embryonic muscle cells (39), but the developmental role of these molecules is uncertain (26).

We have chosen an immunological approach because of its potential selectivity and sensitivity in discriminating subtle changes of membrane proteins that may accompany myogenesis. Serological and immunohistochemical studies in other systems (2, 17, 18, 23, 31, 33, 50, 64) and preliminary studies of our own (15, 16) indicated that this approach has considerable promise, and may eventually permit the isolation of those membrane antigens observed to accompany differentiation. This communication describes the serological and immunohistochemical properties of antisera we have raised against prefusion embryonic chick muscle cells (Anti-M-24). The biological effects of Anti-M-24, and an immunohistochemical characterization of the antigens it recognizes, will be described in a future report.

MATERIALS AND METHODS

Cell Culture

Muscle cells were obtained from the hindlimbs of 12-d-old embryonic chicks as previously reported (12). After trypsin (15,000 U/ml, Tryptar, Armour Pharmaceutical Co., Phoenix, Ariz.) dissociation of the muscle tissue, cell suspensions were washed extensively with E-HS medium, consisting of 83% Eagle's Basal Medium, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.0 mM glutamine, 10% heat-inactivated horse serum (Grand Island Biological Co., Grand Island, N. Y.), and 1.0 μg/ml soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, Mo.). These cell suspensions consisted of 85–95% myoblasts as assessed by morphological scoring of 12-h-old monolayers established from such suspensions. While we recognize that the use of morphological criteria for distinguishing myoblasts from fibroblasts is not}

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1 Abbreviations used in this paper: Anti-F, antisera produced in rabbits against embryonic chick skeletal muscle fibroblasts; Anti-M-24, antisera produced in rabbits against 24-h-old cultures of cells dissociated from the hindlimb muscle of 12-d-old embryonic chicks; Anti-M-96X, antisera produced in rabbits against 96-h-old cultures of x-irradiated cells dissociated from the hindlimb muscle of 12-d-old embryonic chicks; Anti-F, antiserum produced in rabbits against 96-h-old cultures of cells dissociated from the hindlimb muscle of 12-d-old embryonic chicks; Anti-M-24, antiserum produced in rabbits against embryonic chick skeletal muscle (41) to analyze radiolabeled, solubilized muscle cell membranes (22, 24, 37), but have been unable to define unique surface components associated with specific developmental stages of myogenesis. Lectins have been isolated from embryonic muscle cells (39), but the developmental role of these molecules is uncertain (26).

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Preparation of Antisera

Myogenesis in vitro has been described extensively in the literature and may be viewed as consisting of three phases of cellular activity: proliferation, fusion, and muscle cytodifferentiation. While there is no obligatory prerequisite for the first two for myodifferentiation to proceed, under the culture conditions described above, the cells undergo a brief (12-24 h) period of mitotic division followed by extensive, and relatively synchronous, cell fusion and, finally, extensive myodifferentiation. While the differential adhesion procedure referred to above enhances the relative proportion of muscle to nonmuscle cells in these cultures, we have found that exposure of primary muscle cell suspensions to x-irradiation is an effective means of eliminating fibroblasts from later stage (72-96 h) cultures. This procedure and a detailed description of myogenesis in this culture system have been reported previously (14). In this report, “myoblasts” or “prefusion myoblasts” refer to mononucleated myogenic cells found in primary dissociated tissue suspensions or in monolayer for the first 24 h of culture. “Myotubes” are multinucleated syncytia that arise from the fusion of myoblasts and represent differentiated muscle.

Preparation of Antisera

Suspensions of freshly dissociated, single muscle cells were placed in 25-ml Erlenmeyer flasks with E-HS (5-8 x 10^6 cells/3.0 ml) and rotated at 120 rpm at 37°C for 3-4 h in a New Brunswick Gyroratory Shaker (New Brunswick Scientific Co., Inc., Edison, N. J.). Others have suggested that such a period of culture may permit the renewal of surface components denuded by trypsin treatment (18). In support of this, it has been observed that after such a period of suspension culture, cells will reacquire ruthenium red-stainable surface materials (6) and by 2-4 h will exhibit tissue-specific cell sorting and aggregation (46). Embryonic chick muscle cells will also exhibit such aggregative phenomena in suspension culture after a short (2-4 h) recovery period. These cells fuse and differentiate into well developed myofibers. The sake of simplicity, we refer to all flattened, pleomorphic nonspindle-shaped cells as fibroblasts. Further immunological analysis of the nonmyogenic cell population may be necessary in the future. (13). While freshly trypsinized muscle cells require a 2-to 4-h recovery period before such aggregative phenomena may be observed, even freshly trypsinized cells apparently express an extensive complement of cell surface antigens as evidenced by their ability to elicit a high titer antibody response in rabbits (16), to effectively inhibit cytotoxicity of Anti-M-24 for myoblast target cells (16), and to react with Anti-M-24 as detected by ultrastructural observations of immunoperoxidase-stained, trypsin-treated myoblasts.²

After the 3- to 4-h suspension culture period, cells were collected, washed four times in cold Tyrode’s solution, and prepared for immunization as illustrated in Fig. 1. For each rabbit, 5 x 10^6 cells were resuspended in 1.0 ml of Tyrode’s solution and emulsified with an equal volume of complete Freund’s adjuvant (CFA) in a VirTis blade homogenizer (VirTis Co., Inc., Gardiner, N. Y.). The primary injection was administered via multiple portal routes and included intramuscular, subcutaneous, intradermal, and intraperitoneal sites. 2 wk later, 10^7 viable cells in 2.0 ml of Tyrode’s solution were injected intravenously via the marginal ear vein. The rabbits received two additional weekly boosts of 10^6 cells and were bled via the central artery of the ear on alternate days, 7-15 d after the final immunization. Serum was separated from whole blood after permitting clot retraction overnight at 4°C. All serum was heat inactivated for 30 min at 56°C, sterilized by Millipore filtration (Millipore Corp., Bedford, Mass.), and stored at 4°C or -20°C.

Three rabbits were immunized with myoblasts prepared as described above, and two additional animals were injected with myoblasts that had been x-irradiated (5,000 rad, General Electric Maxitron unit [General Electric Co., Wilmington, Mass.]) before immunization (14). These cells were irradiated before immunization, for two reasons: (a) it had been reported that x-irradiation rendered embryonic cells more immunogenic than identical, but nonirradiated, cells (1); and (b) x-irradiation was used to eliminate nonmyogenic cells from late stage cultures that were used to raise antisera to myotubes. Because we intended to compare antiserum against late stage, irradiated cultures (Anti-M-96X) to that against earlier ones (Anti-M-24), we decided to use irradiated myoblasts as immunogens in each case. The sera from all but one of the rabbits were very similar with regard to serological and immunohistochemical properties and were pooled for the study to be described in this report. For earlier work from our laboratory we used antisera obtained from four rabbits immunized by a slightly different schedule (12, 15). We observed that collection of blood over a longer period of time (5-19 d instead 5, 7, and 9 d) resulted in antisera of higher titer (Fig. 2). The cytotoxicity curves of all unabsorbed

² Deutsch, A., and D. A. Fischman. Unpublished observations. See also Deutsch, A., and D. A. Fischman. 1978. J. Cell Biol. 79(2):49a (Abstr.).
FIGURE 1 Schematic representation of protocol used to obtain antisera to embryonic chick skeletal muscle cells at prefusion (Antiprefusion Myoblast, Anti-M-24) or postfusion (Antimyotube, Anti-M-96X) developmental stages. Hindlimb muscles of 12-d-old embryonic chicks were carefully dissected and dissociated with trypsin as described in the text. After differential adhesion (65) to enrich for myoblasts, the cells were used to prepare one of three antisera. Anti-M-24 was prepared by immunizing four rabbits with mononucleated myoblasts that were harvested immediately after a 3- to 4-h suspension culture period on a gyratory shaker water bath to permit renewal of cell surface components denuded by trypsin used to remove the cells from monolayer cultures. For antiserum to x-irradiated prefusion myoblasts, the cells were x-irradiated before suspension culture. Antiserum to well differentiated myotubes (Anti-M-96X) was generated by collecting monolayers of 96-h-old muscle cultures that had been x-irradiated after differential adhesion. X-Irradiation substantially reduced the number of nonmyogenic cells in monolayer cultures of embryonic chick muscle (14). PBS, phosphate-buffered saline.

Antisera presented in Fig. 2 exhibited prozone phenomena at concentrations from 1/1 to 1/20 with maximal cytotoxicity between 1/20 and 1/40. To avoid working within the prozone, we chose to use antisera diluted to its Pa level for absorptions (see below). We found no significant differences in either the titer or specificity of antisera to control or irradiated myoblasts when tested on either type of target cell (Fig. 2 inset). The titer of pooled Anti-M-24 was higher than that of the sera from individual rabbits.

In addition to Anti-M-24, two additional antisera were prepared by the immunization schedule described for myoblasts. Skeletal muscle fibroblasts were obtained, as described above, and injected into two rabbits. This
FIGURE 2 Cytotoxicity of serially diluted antiserum to myoblasts (Anti-M-24) tested on 24-h-old muscle cell monolayers. Antiserum used in previous experiments (13, 15) was obtained from two rabbits by pooling bleedings from 5, 7, and 9 d after the third boost (▲). The antiserum used in the current set of experiments was generated in four rabbits against nonirradiated or x-irradiated myoblasts and represents sera pooled from bleedings on alternate days, 1-3 wk after the second and third boosts. (●). The inset lists the P0 values (see text) for each of these antisera tested on monolayers of skeletal muscle myoblasts that had ("X-ray") or had not ("control") been x-irradiated. Anti-M-24-7, Anti-M-24-19, and Anti-M-24-22 were prepared by immunizing rabbits with nonirradiated prefusion myoblasts; Anti-M-24-21X was prepared by immunizing rabbits with prefusion myoblasts that had been x-irradiated before immunization. Anti-serum will be referred to as "Anti-F". Anti-myotube serum (Anti-M-96X) was obtained by immunizing three rabbits with cells scraped from 96-h-old cultures of well differentiated muscle established from cell suspensions that had been x-irradiated.

$^{51}$Cr-Release Cytotoxicity Assay

Our modification of the method described by Wigzell (63) has been described in detail elsewhere (12, 16). Briefly, cells were plated in Falcon microtiter plates (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) at a density of 1-6 $\times$ 10$^6$ cells/well in 0.1 ml of media. 6-10 h after plating, 1.0 $\mu$Ci of $^{51}$Cr (New England Nuclear, Boston, Mass.) was added in 0.1 ml of E-HS, and the cultures were returned to the incubator. After 12 h, excess $^{51}$Cr was removed by washing the wells two times with E-HS, reincubating them for 2-4 h, and washing them two more times. For the cytotoxicity assay, 0.05 ml of antiserum and 0.05 ml of agar-washed guinea pig complement (final dilution of 1/10, Beckman Instruments Inc., Fullerton, Calif.) were added and the cells were incubated at 37°C for 45 min. Cold Tyrode's solution (0.1 ml/well) was added to the cultures, and the entire microtiter plate was centrifuged for 10 min at 3,000 g at 4°C. 1/10-ml samples were removed from the supernate of each well and measured for $^{51}$Cr radioactivity in a Searle Autogamma Counter (Searle Radiographics Inc., Des Plaines, Ill.). Percent cytotoxicity was calculated by the following formula:

Percent cytotoxicity

\[
\frac{(\text{Cr released in antisera} + \text{complement})-\text{background}}{(\text{Cr released in Triton X-100})-\text{background}}
\]

Background counts were those released by complement alone or by preimmune serum and complement. All samples were tested on triplicate wells. As a measure of cytotoxicity, we have used "P" values to compare the serological titer of antiserum with different target tissues. The "P" value is the dilution of antiserum necessary to induce release of a specified percentage of the $^{51}$Cr contained within a cell population upon the addition of complement (e.g., $P_{50}$ is the dilution of antiserum that induced 50% release of $^{51}$Cr, as described above, in the presence of complement). A single lot of complement was used whenever possible, and if nonspecific release of $^{51}$Cr was >10%, the complement was absorbed with 40 $\mu$g of agar/ml of guinea pig serum for 10 min to remove nonspecific cytotoxicity.

Absorption of Antisers

Antisera were absorbed with acetone powders, homogenized tissues, or intact cells grown in monolayer or suspension culture. Acetone powders were prepared by the method of Chou and Lipman as described by Tabor (59), washed with E-HS immediately before use, and gently mixed with antiserum in 2-3 absorptions. 10-150 mg of dry powders were used to absorb 1.0 ml of undiluted sera by repeated mixing. Absorbed antiserum was removed from the acetone powders by centrifugation at 10,000 g for 30 min and then filtered through a Millipore membrane (0.45 $\mu$m). Homogenized tissues were obtained by dissecting various embryonic organs from 10- to 14-d-old embryonic chicks, mincing them with scissors, and then by using five strokes each of the A and B pestles of a Dounce homogenizer (Kontes Co., Vineland, N. J.). Before further use, the homogenate was washed four times with cold saline and packed into a small volume by centrifugation (1,500 g $\times$ 5 min). Antiserum was absorbed with 2 equal vol of tissue homogenate diluted 1/10 with E-HS and assessed for residual cytotoxicity on myoblast monolayers for 1 h in the presence of complement. Antiserum to be quantitatively absorbed with cultured cells was first diluted with E-HS, absorbed with increasing numbers of cells, and tested on various target cell populations for residual cytotoxicity. These absorptions were carried out over-
night at 4°C, and the serum was used immediately after absorption in cytotoxicity or immunohistochemical assays.

**Immunoperoxidase Staining of Cells**

Live cultures were washed several times with cold Dulbecco's phosphate-buffered saline (DPBS), exposed for 30 min to rabbit antiserum diluted 1/10-1/20 with 20% heat-inactivated normal goat serum (NGS) in DPBS, and washed again with DPBS. Next, a solution of goat anti-rabbit IgG (GAR) conjugated to peroxidase (PO) (N. L. Cappel Laboratories Inc., Cochranville, Pa.), NGS, and DPBS (in the ratio of 1:1:6, respectively) was added to each dish for 45 min. All these steps were carried out at 4°C on a table-top platform shaker. At this point, the cultures were washed free of excess GAR-PO, fixed for 10 min in 0.5% glutaraldehyde, and again washed with DPBS. Cultures were reacted for peroxidase activity by incubation in the dark at saturated humidity and 37°C in a reaction mixture containing 1.0 mg/ml diaminobenzidine (Sigma Chemical, Co., St. Louis, Mo.), 3.0% hydrogen peroxide, and 0.05 M Tris-HCl (pH 7.4). Stained plates were prepared for observation by two additional washes in Tris-HCl and then overlaid with glycerol and a glass coverslip. Photomicrographs were taken on a Zeiss microscope (Carl Zeiss, Inc., New York, N. Y.) by using a 40 x Zeiss planapo oil immersion lens.

**Immunofluorescence**

Live cells or cells that had been fixed for 10 min in 2.0% paraformaldehyde (at room temperature or 50°C) were washed thoroughly with DPBS and exposed for 30 min to rabbit antiserum diluted 1/10 in 0.5% glutaraldehyde, and again washed with DPBS. Cultures were reacted for peroxidase activity by incubation in the dark at saturated humidity and 37°C in a reaction mixture containing 1.0 mg/ml diaminobenzidine (Sigma Chemical, Co., St. Louis, Mo.), 3.0% hydrogen peroxide, and 0.05 M Tris-HCl (pH 7.4). Stained plates were prepared for observation by two additional washes in Tris-HCl and then overlaid with glycerol and a glass coverslip. Photomicrographs were taken on a Zeiss microscope (Carl Zeiss, Inc., New York, N. Y.) by using a 40 x Zeiss planapo oil immersion lens.

**RESULTS**

**Kinetics of ³¹Cr Uptake and Release**

Complement-dependent ³¹Cr release cytotoxicity essays have been used to examine the reactivity of complement-fixing immunoglobulins with a variety of cell types (8, 58). Most of these studies used target cell populations which were homogeneous in cell type and geometry. Because primary embryonic cell cultures, such as those used in this study, contained a heterogeneous population of cells, it was necessary to compare the kinetics of isotope uptake and its antibody-complement-dependent release with several embryonic cell types.

Uptake of ³¹Cr by muscle cell cultures was linear with increasing cell number, and the level of incorporation over a 12-h period was proportional to the amount of isotope added to the cultures. In all experiments, we have used a 12-h incubation period in 1.0 μCi of ³¹Cr/microtiter well containing 3 x 10^4 cells. While different tissue monolayers took up different amounts of ³¹Cr (Table I) as assessed by Triton X-100-released counts, the kinetics of release in the presence of antibody (unabsorbed Anti-M-24) and complement were very similar for all cell types. Immune sera, in the presence of complement, released 90-95% of the Triton X-100-released counts; this value was reached 10-25 min after the addition of complement to antibody-treated cells and, in the case of the antisera diluted to induce <95% cytotoxicity, remained constant for at least 90 min (Fig. 3).

We have observed cells undergoing antibody-complement-induced lysis at the light microscopic and ultrastructural levels. Any of the nucleated cell types we have observed reacted to antibody and complement within minutes of its application by forming slowly growing membranous vesicles.

**TABLE I**

| Tissue and time postplating | Cell number initially plated per well (x 10⁴) | cpm of ³¹Cr |
|-----------------------------|---------------------------------------------|-------------|
| Muscle, 24                  | 3.0                                         | 870 ± 90    |
| Muscle, 96                  | 1.0                                         | 1,190 ± 200 |
| Fibroblast, 24              | 3.0                                         | 2,720 ± 450 |
| Heart, 24                   | 3.0                                         | 1,200 ± 100 |
| Liver, 24                   | 3.0                                         | 1,180 ± 200 |
| Retina, 24                  | 6.0                                         | 470 ± 70    |
| Brain, 24                   | 3.0                                         | 500 ± 60    |

Cell monolayers were labeled with ³¹Cr as described in the text, lysed with Triton X-100, and the cpm of ³¹Cr released were determined. Values represent the mean of at least three independent experiments, each done in triplicate, ± 1 SD of the mean. Radiochromate of comparable activity was used for all experiments. However, each of the separate experiments included all of the tissues listed above. Thus, the relative differences in uptake of ³¹Cr for the different cell types could be compared.
over the entire cell surface. Damaged cells exhibited, in addition to surface herniations, nuclear swelling followed by pyknosis, aggregation of organelles to a perinuclear position, and rapid rounding up and lifting off from the substratum of the entire cell.

If cultures treated with complement and antibody diluted to the \( P_{50} \) value were examined in the phase-contrast microscope, nearly all cells exhibited some degree of the surface herniation characteristic of antibody-complement-induced membrane lesions. When cultures were treated with specifically absorbed antiserum and complement, these lesions were observed on only a specific subpopulation(s) of the entire cell culture as will be discussed below.

Skeletal muscle cultures at 24-h post-plating consisted of two predominant cell types: the spindle-shaped myoblast, and the flattened, more pleiomorphic fibroblast (28, 30). Because the geometry of these cells differs, we considered that each may differ in the amount of \( {^{51}}\text{Cr} \) taken up and bound intracellularly. It is clear from the data presented in Table I that, on a cell-to-cell basis, fibroblasts take up 3-4 times more \( {^{51}}\text{Cr} \) than do myoblasts. To obtain a more quantitative estimate of the amount of \( {^{51}}\text{Cr} \) taken up by fibroblasts within a heterologous 24-h-old muscle culture, we mixed varying proportions of pure fibroblasts with freshly trypsinized muscle cell suspensions before plating. After 24 h of culture, which included a 12-h incubation in \( {^{51}}\text{Cr} \), the monolayers were treated either with Triton X-100 to obtain maximum release values, or with Anti-M-24 or Anti-F diluted to the \( P_{50} \) values for the cells against which the sera were prepared. We observed that there was a linear relationship between the number of fibroblasts present and the amount of \( {^{51}}\text{Cr} \) released by heterologous cultures in response to detergent- or antibody-complement mediated cytotoxicity (Fig. 4). From these data, we calculated that while fibroblasts represent only 10-20% of the cells present in a 24-h-old muscle monolayer, they account for 40-60% of the \( {^{51}}\text{Cr} \) taken up in these cultures.

**Absorption of Anti-M-24 with Tissue Homogenates or Acetone Powders**

Xenogeneic antisera to whole cells cross-reacted with many cell types of embryonic chick tissues. To remove antibodies to species-related antigens,
we extensively absorbed Anti-M-24 with various embryonic chick tissues. Presumably, nonmuscle-specific antibodies could be removed by such absorption. By absorbing antisera with tissues prepared by a variety of procedures (intact cells, homogenized tissues, and acetone powders), we hoped to demonstrate residual serological activity specifically related to muscle and/or myoblast cell surface antigens.

We have previously reported the demonstration of muscle tissue specificity in Anti-M-24 after absorption with homogenates of various embryonic chick tissues (12, 15). By using the more recently generated Anti-M-24, we have reproduced and extended these results to include acetone powders as tissue absorbants (Table II). Unabsorbed Anti-M-24 diluted 1/10 was highly cytotoxic for embryonic chick erythrocytes, hepatocytes, cardiac myocytes, and retina and brain cells (Table II). Absorption with acetone powders prepared from each of these tissues removed all cytotoxicity for the tissue with which it was absorbed while leaving high titers of antibody directed against the muscle cell surface (Table II). Visual observation of 24-h-old muscle cultures treated with Anti-M-24 absorbed with embryonic heart tissue did not reveal any alterations in cell morphology or membrane integrity (Fig. 5a). Within 10 min after the addition of complement, however, the spindle-shaped myoblasts exhibited characteristic antibody-complement-induced lesions (Fig. 5b). If these cultures were scored for the percent of cells that exhibited cytotoxic lesions, 65–85% of the cells were positive; nearly all of these cells were spindle shaped. A small number of spindle-shaped myoblasts remained unaffected by the specifically absorbed antiserum, and an occasional flattened fibroblast-like cell exhibited membrane blebbing. Thus, absorption with heart cells or tissue homogenates removed nearly all cytotoxicity of Anti-M-24 and complement for skeletal muscle fibroblasts while leaving a substantial cytotoxic titer against myoblasts. If preimmune serum was used in dilutions as high as 1/5, <10% cytotoxicity was always observed. Absorption of Anti-M-24 with myoblast monolayers or live cell suspensions removed all reactivity of this antiserum with muscle cell cultures. Absorption of Anti-M-24 with brain or retina removed all cytotoxicity for these tissues, but the antiserum was still highly cytotoxic for nearly all the cells in cardiac and skeletal muscle monolayers of any age. Apparently, striated muscle cells possess surface antigens not shared by ectodermally derived neural tissues. This is consistent with the earlier observations of others (18).

Indirect immunoperoxidase staining of 24-h-old muscle cultures treated with Anti-M-24 confirmed the serological and morphological data described above. After exposure of 24-h-old cell monolayers to unabsorbed Anti-M-24, both fibroblasts and myoblasts possessed extensive dark-brown granular reaction product along the cell periphery. Antiserum absorbed with embryonic heart or myotube monolayers, however, bound to nearly all myoblasts (Fig. 6a) but only to an occasional fibroblast. Most fibroblasts, however, were negative (Fig. 6b). If cells were fixed with low concentrations of glutaraldehyde before immunohistochemical treatment, a different pattern of staining was observed. While immunological specificity was retained, the reaction product was distributed throughout the entire cell and not restricted to the

### Table II

| Target cells | Absorbing tissue | Homologous cells used for absorption | Unabsorbed Anti-M-24 on absorbing tissue |
|--------------|------------------|-------------------------------------|-----------------------------------------|
| Embryonic muscle | A* | 1 ± 1 | 3 ± 2 | 92 ± 6 |
|               | H  | 0     |          |          |
| Adult muscle  | A  | 38 ± 2 | 58 ± 4 |          |
|               | H  | 57 ± 6 | 46 ± 3 |          |
| Embryonic liver | A  | 43 ± 6 | 52 ± 6 | 2 ± 1 | 87 ± 6 |
|                | H  | 60 ± 8 | 45 ± 9 | 4 ± 2 |          |
| Adult liver   | A  | 70 ± 8 | 80 ± 9 | 25 ± 2§ |          |
|                | H  | 62 ± 4 | 57 ± 7 | 33 ± 8§ |          |
| Embryonic heart | A  | 64 ± 5 | 42 ± 6 | 10 ± 4 | 90 ± 6 |
| Embryonic brain | A  | 70 ± 9 | 64 ± 4 | 2 ± 2 | 86 ± 8 |

Mean ± SD.

* A, acetone powders.

‡ H, homogenized tissues.

§ Tested on embryonic liver target cells.
Phase-contrast photomicrograph of a 24-h-old skeletal muscle culture exposed for 20 min to Anti-M-24 absorbed with 10-d-old embryonic heart in the (a) absence, or (b) presence, of guinea pig complement. Note the presence of characteristic complement-induced lesions on the spindle-shaped myoblasts while the more flattened, pleiomorphic fibroblasts retained their normal morphology. Unabsorbed Anti-M-24 lysed both cell types within 10 min of the addition of complement. Bar, 10 μm.

Fig. 7 illustrates such fixed monolayers treated with either preimmune serum, Anti-M-24 absorbed with embryonic heart cell homogenate, Anti-M-24 absorbed with 24-h-old muscle monolayers, or Anti-M-24 absorbed with myotube monolayer cultures. After absorption with embryonic heart or myotubes, mononucleated myoblasts still possessed dark granular reaction product at the cell surface while the fibroblasts did not. Homologous tissue used for absorption stained strongly with unabsorbed antiserum but demonstrated no reactivity with Anti-M-24 after absorption. Control experiments using preimmune serum, antiserum, and either GAR-PO without diaminobenzidine or GAR-PO without antiserum produced no reaction product on cultured cells.

Fig. 6 Bright field photomicrographs of representative (a) myoblast or (b) fibroblast in the same 24-h-old skeletal muscle monolayer after treatment with Anti-M-24 absorbed with well differentiated muscle cultures. For the immunoperoxidase reaction, live cultures were treated with absorbed Anti-M-24 and GAR-PO at 4°C, fixed with glutaraldehyde, and processed for peroxidase activity as described in the text. After exposure to unabsorbed Anti-M-24, both fibroblasts and myoblasts possessed extensive dark-brown granular reaction product along their periphery. Specifically absorbed antiserum, however, bound to nearly all myoblasts but only to an occasional fibroblast. Bar, 10 μm.
Extensive cross-reactivity was observed between Anti-M-24 and well-differentiated myotube cultures. If Anti-M-24 was absorbed with embryonic chick heart, retina, brain, erythrocytes, or with adult liver or skeletal muscle, the serum no longer reacted with homologous tissue used for absorption, but did bind to embryonic myotubes as assessed by $^{3}^{1}$Cr-release assays (Table II) and immunohistochemical visualization (Fig. 8). Absorption with 7-d-old myotube cultures, or homogenized neonatal muscle, removed all reactivity with myotubes while leaving antibodies reactive with myoblasts (Table II and Fig. 6).

Quantitative Absorption of Anti-M-24 with Isolated Cells

Although we were able to demonstrate serological specificity of Anti-M-24 for myoblasts after bulk absorption with various embryonic chick tissues, it was not certain whether the antigenic specificities of the prefusion myoblast were qualitatively or quantitatively unique. To approach this question, we absorbed Anti-M-24 with increasing numbers of viable embryonic chick cells. We reasoned that if the antigens detected on myoblasts and other cell types were quantitative (i.e.,
the same antigens distributed in differing amounts on different cell types), the cytotoxicity for myoblasts should decrease to background, although at an absorption level well beyond that for the homologous tissue used for absorption. If the differences detected were qualitative, then the cytotoxicity for myoblasts should plateau at some significant level of cytotoxicity, even after extensive absorption.

By using increasing numbers of various embryonic chick cell types, we examined the effects of quantitative absorption on the inhibition of cytotoxicity of Anti-M-24 for 24-h-old muscle monolayers. Absorption of a 1/100 dilution of antiserum with up to $10^7$ fibroblasts (Fig. 9A), hepatocytes (Fig. 9B), cardiac myocytes (Fig. 9C), or brain cells (Fig. 9D), never lowered cytotoxicity for myoblasts by >60%, whereas all cytotoxicity was removed for the monolayered cells of the tissue used for absorption. Absorption with $2 \times 10^6$ freshly trypsinized myoblasts, or $10^7$ myoblasts cultured in suspension, removed all cytotoxicity of this antiserum for myoblasts (Fig. 9E). If well differentiated myotubes were used as absorb-
ants, 50% cytotoxicity of Anti-M-24 for myoblast monolayers was retained at a point well beyond that at which all cytotoxicity was lost for myotube monolayers (Fig. 9F). After absorption with as many as 40 million erythrocytes, Anti-M-24 was still 80% cytotoxic for myoblast monolayer targets. Unabsorbed antiserum was highly cytotoxic for all embryonic chick tissues examined; the P50 ranged from 1/40 for liver and brain to 1/100 for skeletal muscle fibroblasts.

To determine whether absorbed Anti-M-24 could be used to specifically lyse skeletal myoblasts in a monolayer of cells from different embryonic tissues, the following experiment was performed. Varying percentages of skeletal myoblasts and cardiac myocytes were plated such that the final cell number was always 3 x 10^6/well. Triplicate wells were then treated with guinea pig complement and either unabsorbed Anti-M-24, Anti-M-24 absorbed with embryonic and adult chick liver acetone powders, or Anti-M-24 absorbed with embryonic brain and heart acetone powders. The percent cytotoxicity was nearly the same for each well (90% in wells treated with unabsorbed Anti-M-24; 65% in those treated with Anti-M-24 absorbed with liver), regardless of the proportion of cardiac-to-skeletal myocytes. However, after absorption of Anti-M-24 with heart and brain, the cytotoxicity of the serum was directly proportional to the percentage of skeletal myoblasts present in the heterologous cardiac-skeletal monolayer; nearly 50% of the 51Cr was released in pure skeletal muscle cultures while <15% cytotoxicity was observed in pure cardiac tissue cultures (Fig. 10). Visual observation of such cultures revealed that those cells lysed by antibody and complement were predominantly spindle shaped. An occasional flattened, pleiomorphic fibroblast or cardiac myocyte was observed to lyse.

**Lateral Mobility of Myoblast Surface Antigens**

Complement-dependent cytotoxicity results from lesions initiated at the site of surface antigen, antibody, and complement interaction. While it is generally accepted that such sites are associated with cell membrane components (58), it is conceivable that cytolysis may ensue from complement fixation by antibody bound to extracellular antigens loosely absorbed to the cell surface, such as components of culture media that may have been present in the immunizing cell suspension. If the myoblast-specific antigens detected with Anti-M-24 were cell surface-associated proteins, one would predict that they might move within the plane of the cell membrane under appropriate conditions.

To test whether the antigens detected with Anti-M-24 were free to move within the myoblast surface, live cells were treated with absorbed antiserum and GAR-FITC at varying temperatures and examined by fluorescence optics. Between 80 and 90% of the cells in single-cell suspensions of embryonic chick hindlimb muscle exhibited uniform fluorescence around their periphery immediately after treatment at 4°C with Anti-M-24 absorbed with embryonic brain acetone powder (Fig. 11a). If the cells were warmed to room temperature, fluorescent patches (Fig. 11b) were observed within 5-10 min, followed by the aggregation of these patches into a cap at one pole of the cell (Fig. 11c and d). Patches and caps were not observed if the cells were maintained at 4°C or pretreated with 50 mM sodium azide. When small aggregates of 4-10 cells formed by gyratory shaking for 4 h were incubated in Anti-M-24 and GAR-FITC, patches of fluorescence were observed. However, cap formation was
FIGURE 11 Immunofluorescent staining of primary skeletal muscle cell suspensions with Anti-M-24 absorbed with embryonic brain acetone powder. Live, stained cells were brought to room temperature and observed (a) immediately, (b) 5 min, and (c and d) 10 min later. When similar single-cell suspensions were permitted to form multicellular aggregates by gyratory rotation at 90 rpm for 4–24 h, treated in the cold with Anti-M-24 and GAR-FITC, and then warmed to room temperature, patches were observed on aggregates of 3–5 cells (e and f). Caps did not form in such small groups of cells, and neither caps nor patches were observed on larger multicellular aggregates (g). Trypan blue dye exclusion experiments indicated that >95% of these cells are viable in the presence of heat-inactivated immune serum. Bar, 50 μm.
rarely observed in these small aggregates, even when they were left at room temperature for 3-4 h after addition of antibody (Fig. 11 e and f). If the cells were allowed to form larger multicellular aggregates for 24 h before immunofluorescence treatment, neither patch nor cap formation was observed, even after exposure to immune serum for several hours at room temperature (Fig. 11 g). Cells in such suspensions were judged viable by the observation that 98% excluded trypan blue. If a suspension comparable to that used for immunofluorescence was plated onto plastic culture dishes, 85-95% of the cells attached and spread onto the substratum. Suspensions of the cells used for immunofluorescence were capable of forming full differentiated muscle as assessed by electron microscope examination of suspension cultures at varying times after initiation of the cultures (13).

When 18- to 24-h-old monolayers of mononucleated myogenic cells were treated with unabsorbed Anti-M-24 or serum absorbed with embryonic brain and liver, the cells rounded up and lifted off from the substratum. When these cells were rinsed with DPBS and treated with GAR-FITC immediately after lifting off from the substratum, uniform fluorescence was observed around the entire cell surface. If floating cells were removed at varying times after antibody treatment, and prepared for observation in the fluorescence microscope, extensive patching and capping was seen. After removal of excess antibody with a saline rinse, cells reattached to the substratum within 24 h and differentiated normally. Supernatant medium from such cultures contained many small vesicles that stained positively when treated with GAR-FITC.

Older monolayers of multinucleated myotubes (72-96 h post-plating) responded differently to treatment with this antisera; the cells remained firmly attached to the substratum. If Anti-M-24 and GAR-FITC were added to 96-h-old myotube cultures, a uniform surface fluorescence was observed in nearly all myotubes even up to 24 h after the addition of antisera and fluorescein conjugate (Fig. 12 a-d). No caps were observed, but a small number (15-25%) of the smaller myotubes did exhibit some fluorescent patches at the sarcolemma. Between 24 and 72 h after the addition of Anti-M-24 and GAR-FITC, large intracellular aggregates of fluorescent vesicles were present throughout the sarcoplasm of most cells (Fig. 12 e and f). If 96-h-old muscle cultures were treated with antiserum prepared against well differentiated embryonic chick myotubes (Anti-M-96X), multinucleated myotubes retracted and lifted off from the substratum (Fig. 13). When these floating, rounded myotubes were treated with GAR-FITC 12-24 h after the addition of Anti-M-96X and observed under fluorescence optics, the stain was uniformly distributed over the cell surface with little evidence of patching or capping (Fig. 14). Surface blebbing was occasionally observed on these retracted myotubes, and such surface herniations closely resembled those observed with cells placed in hyposmotic medium. Surface membrane outpocketings such as these were never fluorescent after exposure to absorbed or unabsorbed Anti-M-24 or Anti-M-96X followed by GAR-FITC (Fig. 14), thus suggesting that these regions of the cell membrane are free of any antigens detected by these antisera. An occasional mononucleated cell also lifted off from the substratum in these cultures. These cells were rounded in suspension and exhibited patchy distributions of fluorescence.

DISCUSSION

In this report, we have presented several lines of evidence for the presence of tissue- and development stage-specific antigens on the surface of embryonic chick skeletal muscle cells. From these data, we have concluded that: (a) all embryonic chick tissues share a class of antigenic determinants at the cell surface; (b) embryonic chick skeletal myocytes, at all stages of in vitro development, share antigenic determinants that are not detectable serologically on the surfaces of cells from other embryonic chick tissues; (c) antigens are present on prefusion myoblasts that are not detectable on muscle cells at later stages of development or on cells from other embryonic chick tissues; (d) muscle tissue-specific antigens are free to diffuse laterally in the plane of the surface membrane of single myoblasts, but are restricted from doing so in multicellular aggregates of unfused myoblasts or in multinucleated myotubes; and (e) the surface antigens detected with Anti-M-24 are membrane components that are exposed to the extracellular milieu and potentially available for intracellular transduction of environmental cues during myogenesis. While no biological function has been ascribed to these antigens, our results rule out the possibility that they are an experimental artifact of tissue culture: (a) absorption of Anti-M-24 with culture medium does not effect the binding of Anti-M-24 to muscle cells; (b) all binding of Anti-M-24 can be removed by absorption with fresh tissue; (c) immune serum
Immunofluorescent staining of myotube monolayers with Anti-M-24. 96-h-old monolayers were treated with sterile antiserum and GAR/FITC, replaced in a culture incubator, and examined with fluorescence optics at (a and b) 1, (c and d) 12, or (e and f) 24 h after the addition of antibody and GAR-FITC. Myotubes were first photographed under phase-contrast (a, c, and e) and then fluorescence (b, d, and f) optics. Bar, 50 µm.
binds to intact cells that have never been exposed to serum or tissue culture; and (d) frozen sections of intact embryos can be stained with Anti-M-24 and GAR-FITC. Before discussing the conclusions presented above, we would like to comment on the use of cytotoxicity assays in the serological characterization of antisera to whole cell surfaces.

4 Bayne, E., M. Friedlander, and D. A. Fischman. Unpublished observations.

The Physiological Basis for the Myoblast Specificity of Anti-M-24

The antigenic differences we have detected between myoblasts and other cell types of the chick embryo are based largely on cytotoxicity data. While it is well recognized that the complement-dependent $^{3}^{1}$Cr release cytotoxicity assay is extremely sensitive, it is semiquantitative at best.
FIGURE 14 (a) Phase-contrast, and (b) fluorescence photomicrograph of retracted “myoball” treated with GAR-FITC immediately after Anti-M-96X-induced myotube retraction. Fluorescence in such structures was uniformly distributed over the cell surface with no indication of antigenic redistribution or loss. Membrane blebbing (arrow) was frequently observed in these cells, but such outpocketings of the cell membrane never exhibited fluorescence when treated with antiserum and GAR-FITC. Bar, 50 μm.

because there is no simple relationship between isotope release and the number of immunoglobulin molecules bound. Specific conditions must be met for effective cell lysis after complement fixation, and the interpretation of cytotoxicity data may be further complicated by the heterogeneity of cell populations used in the study of embryonic systems.

The differential susceptibility to complement-mediated lysis seen between myoblasts and other cells in the presence of absorbed antiserum may result from several factors not necessarily related to antigen density or serological specificity of the antiserum. As revealed in studies of other systems, these parameters include: differential membrane fragility (42), variability in antigen-associated lytic-sensitive membrane regions (40), cell cycle-associated variations in antigen density (8), steric arrangement within the cell membrane of several antigens (35), absence of cross-reacting surface antigens (34), physiological shedding (61), or antigenic modulation (29). Tissue culture conditions are also believed to influence detection of surface antigens (55).

This study makes no attempt, other than the use of relatively synchronous fusion conditions (12, 41) and the consideration of the well established cell cycle kinetics during myodifferentiation (4), to correlate antigenic expression with the cell cycle. While the cell cycle-dependence of antigen expression is supported by studies of some cultured cell systems (8), we do not believe that the phase of a cell's growth is the basis for the antigenic specificities observed with Anti-M-24. Prefusion myoblasts, under our culture conditions, are a dividing population of cells with cycle times very similar in length to, and in phase with, those of fibroblasts within the same culture dish (14). Because, after appropriate absorption, nearly all myoblasts and very few fibroblasts bind Anti-M-24, as assessed by cytotoxicity assays and immunohistochemical procedures, we conclude that the antigenic differences between these two populations are not a reflection of cell cycle-associated

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For discussion see: Friedlander, M. 1976. Doctoral dissertation. The University of Chicago, Chicago, Illinois.
variations in expression of antigens common to both cell types. Similarly, appropriately absorbed antiserum to myotubes did not detect antigenic characteristics associated with postmitotic cells only, because absorption with neonatal or adult brain or retina (both of which are largely postmitotic) lowered the cytotoxicity for myotubes only slightly. Differential cellular ability to fix or activate complement could not account for these differences either, because unabsorbed antiserum is highly cytotoxic for all embryonic chick tissues tested.

The surface morphology of cells is known to change during the course of the cell cycle (44), and during myodifferentiation in vitro a sarcolemma develops with structural and compositional characteristics considerably different from those of the sarcolemma of the prefusion myoblast (54). Despite such changes in surface morphology, any of which could effect antibody and/or complement binding (and thus prevent cytolysis), there did not appear to be any decrease in sensitivity of myotubes to antibody-complement-induced cytoxicity with progressive differentiation. Thus, the serological distinctions between different stages of myogenesis are not the result of stage-dependent variations in membrane fragility or lytic-sensitive regions.

The length of time in tissue culture has been reported to affect the stability of HL-A determinants on human fibroblasts (49). Whether this is an artifact of tissue culture or a reflection of antigenic loss correlated with normal senescence of cells is unresolved, but others have been able to consistently detect these same antigens throughout the lifespan of the cultures in vitro, thus suggesting that different experimental conditions can influence antigen detection (5). Many other cell types have also been observed to retain (55) and/or acquire (60) characteristic surface antigens with progressive time in tissue culture. Our own studies have revealed that myotubes and fibroblasts bound complement-fixing antibody even after 3 wk in culture. Thus, we do not believe any selective loss of antigenicity occurred as a result of tissue culture-associated artifacts.

The tissue- and stage-specific antigens detected with Anti-M-24 were also found in vivo; homogenized 8- or 10-d-old embryonic chick hindlimb muscle effectively removed all reactivity of the sera for cultured muscle. In addition, frozen sec-

The question of whether the serologically detected stage and tissue specificities reflect qualitative, quantitative, or distributional differences between cells remains unresolved. It is tempting to speculate that the tissue- and stage-specific differences detected by using Anti-M-24 might be related to different densities of similar antigens on the surfaces of developing muscle and other embryonic tissues. Although high correlation coefficients exist in certain systems between complement-mediated cytoxic sensitivity and the concentration of antigenic receptors (35), exceptions have also been noted in which sensitivity does not relate directly to antigen content (40, 42). Use of strict quantitative procedures such as the determination by radiolabeling techniques of the number of antibody-binding sites per cell (based on uptake of immunoglobulin with a radioactive tag of known specificity) should help assess the relative quantitative differences between cell types. The distribution of these antigens at the ultrastructural level has been examined by the immunoperoxidase technique. It will also be important to clarify the antigenic "relatedness" between myogenic and nonmyogenic cells in cultures of embryonic chick hindlimb muscle. We have observed, in addition to the myoblast specificity of Anti-M-24, several broader classes of specificities that support the earlier observations of Goldscheider and Moscona (18); antiserum to mesodermally-derived tissues (e.g., heart and skeletal muscle) may be absorbed exhaustively with tissues from other germ layers and still react with mesodermally-derived tissues. Only after additional absorptions with other striated muscle tissue (e.g., heart) were we able to detect stage-specific cytoxicity of whole embryos could be stained by Anti-M-24 and GAR-FITC; the myotome regions of early embryos, and developing breast and hindlimb muscle in later ones, exhibited bright immunofluorescence. While we have no evidence to suggest that these antigens play a role during myogenesis in vivo, it is clear that they are present, and therefore it may be possible to examine their possible function by using immunological probes in intact embryos.

* Friedlander, M. Unpublished observations.

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*** Holoday, M. Z. 1978. Doctoral dissertation. The University of Chicago, Chicago, Illinois. Friedlander, M. 1976. Doctoral dissertation. The University of Chicago, Chicago, Illinois.
observe skeletal muscle specificity in Anti-M-24. Thus, Anti-M-24 absorbed with cardiac muscle still reacted with myoblasts, myotubes, and a number of the fibroblasts found in cultures of skeletal muscle. To remove all reactivity of Anti-M-24 with fibroblasts, it was necessary to absorb with cultures of well differentiated muscle. These results imply that there is an antigenic difference between cardiac and skeletal muscle fibroblasts. While we have not fully explored the antigenic relationships between fibroblasts from different tissue sources, such differences may exist and will require additional investigation before we understand how surface antigens may serve to distinguish heterologous cell types within a particular developing tissue. Our results support the existence of such antigenic distinctions.

**Differences in Antigen Mobility on Myoblasts and Myotubes**

In recent years, it has become clear that the membranes of many cell types are fluid enough to permit the lateral and rotational mobility of component macromolecules (10, 56). Differences in membrane fluidity may reflect different cellular activities or genetic programs. In at least two nonmuscle systems, there appears to be a general decrease in lateral mobility of cell surface lectin receptors with progressive differentiation. During chick erythroid cell development, concanavalin A (Con A) and Ricinus communis agglutinin I (RCA I) receptors of more mature erythroid cells were nearly completely immobilized while those on proerythroblasts patched and capped extensively (7). During continuous culture of embryonic chick retina, the cells progressively lost their capacity to patch and cap Con A receptors concomitant with the ability to become agglutinatable by a tissue-specific component (32).

Because the muscle cell membrane undergoes dramatic alterations concomitant with myogenesis, much attention has recently been focused on the dynamics of its component molecules. The fluidity of lectin-binding sites within the myoblast membrane has been demonstrated in several laboratories (43, 48, 51) by the use of different experimental techniques. Quantitative studies of local membrane dynamics by fluorescence relaxation microscopy (21) or more general observations of membrane microviscosity (45) both suggested that localized and transient increases in myoblast membrane fluidity precede fusion and subsequent myodifferentiation. A slight increase in microviscosity of the myotube membrane relative to that of the mononucleated myoblast was also noted. While it is clear from other studies that there is lateral diffusion of surface antigens (11) and acetylcholine receptors (3, 20) within membranes of cultured myotubes, the dynamics of molecular movements and distributions are complex and may reflect topographical organization of cell membrane components into biologically significant, yet diverse, domains.

Our observations of rapid patch and cap formation of tissue-specific surface antigens in prefusion myoblasts, followed by sloughing of these complexes into the extracellular milieu, are consistent with the fluidity measurements cited above. Preliminary work in our laboratory (15) indicated that the covering up of these antigens with specific antibodies can block tissue-specific cell aggregation, thus suggesting that these molecules may be involved in the regulation of intercellular recognition during myogenesis. The finding that myotubes do not exhibit such dynamic capping responses to treatment with immune serum is not surprising in light of the observation by others that there may be a generalized decrease in fluidity of the cell membrane which accompanies differentiation (21, 45). While the antigens of the myotube surface that are detected with Anti-M-24 and Anti-M-96X are free to diffuse laterally within the myotube membrane; they do so very slowly and the mechanism whereby the antigen-antibody complex is removed from the cell surface appears to be different from that observed for myoblasts. Such different modes of surface antigen redistribution have been observed for Con A receptors in cultures of fibroblasts (57). We believe that the myoblast sheds its capped antigens into the medium and rapidly restores such molecules to its surface as evidenced by (a) the presence of small fluorescent vesicles in the medium of cultures with capped cells, and (b) the reattachment of the cells to the substratum after capping and the reappearance of detectable antigens with fresh antibody and fluorescein conjugate. Myotubes were observed to patch muscle surface antigens only after several hours, and still exhibited surface fluorescence and fluorescent cytoplasmic vesicles as many as 72 h after antibody-fluorescence conjugate treatment. It appeared that these cells removed antigen by endocytosis of small regions of
the cell membrane. Ultrastructural studies will be required to confirm this hypothesis. Even when myotubes were induced to retract from the substratum with antiserum to myotubes, no capping of antigens was observed.

We would like to emphasize that retracted myotubes frequently exhibited one or two surface blebs that were not fluorescent after treatment with antiserum to muscle cell surface antigens and GAR-FITC (Fig. 14). By light microscopy, these structures were very similar to those described by others as being representative regions of myotube membranes obtained by hyposmotic treatment of muscle cultures with dilute paraformaldehyde (38, 53). We suggest that membrane vesicles obtained by such treatment may not be representative of the myotube membrane as evidenced by the absence of detectable tissue-specific surface antigens. Intramembrane particle-free blisters, similar in light microscopical appearance to those described above, have been observed by electron microscopy in cells fixed with glutaraldehyde and are believed to be fixation-induced (19). While we recognize that such membrane "blisters" are not identical in ultrastructural characteristics to those observed in paraformaldehyde-treated muscle (53), we would caution against the use of sarcolemmal isolation procedures that depend on membrane blebbing or blistering to obtain regions of "representative" muscle surface membrane.

We conclude that muscle tissue-specific surface antigens are free to diffuse laterally, patch, and cap on the myoblast, while the identical class of antigens does not exhibit comparable dynamics within the myotube surface. Such differences in fluidity of a class of surface molecules would be consistent with the idea that gene expression followed by fusion and/or differentiation may be mediated via mobile surface receptors (e.g., tissue-specific all ligands) (9, 36). Such a hypothesis would be supported by studies on Con A receptor mobility during myoblast recognition and fusion (48).

Well characterized antisera that are specific for muscle cells at different stages of development should be useful in the analysis of surface located macromolecules that may be involved in facilitating intercellular recognition and transduction of environmental cues back to the level of the genome during histogenesis. We believe that an immunological approach, coupled with biochemical analysis, may provide the sensitivity necessary to detect compositional changes in the cell surface that may accompany differentiation. Anti-M-24 is currently being used to trace myogenic cell lineage during embryogenesis and to isolate and identify those molecules that serve to distinguish the pre-fusion myoblast cell surface.11 With the advent of more sophisticated immunological techniques, such as monoclonal antibody production (27), it should be possible to raise antibodies of higher titer and specificity with respect to the various components of differentiating embryonic cell membranes. Such studies are currently in progress.

The authors gratefully acknowledge the expert technical assistance of Lovenia Williams, Furman Davis, Marian Daniels, and Yukio Hamada. We also thank Dr. Frank Fitch for the generous use of his laboratory for certain phases of this work and Sally Hoskins for invaluable assistance in the preparation of the antisera. Our discussions with Doctors Ted Steck, Michael Edidin, Aron Moscona, Tom McKearn, Sheila Fallon, Denyes van der Westhuyzen, and Linda Marton were most helpful and greatly appreciated. Doctors Rosemary Ginzburg and Ted Lawrence provided valuable criticism of this manuscript. We also wish to thank Kathryn Wall for expert assistance in the preparation of art work and photography and Madeleine Naylor and Susan Friedlander for their patience and typing of this manuscript.

Martin Friedlander was a predoctoral fellow in the National Institutes of Health Developmental Biology Training Program (grant 3T1010297) and also received support from American Cancer Society Institutional grants 1N-41-0 and 1N-41-P. This research was funded by United States Public Health Service grant NHLI-13505, the Muscular Dystrophy Association of America, the University of Chicago Cancer Center (grant 1 P01 CA 14599-02), the Harry Levine Memorial Foundation, and the Esther Dymannt Memorial Fund.

Received for publication 25 August 1978, and in revised form 12 December 1978.

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