Surface Antigens of the Embryonic Chick Myoblast: Expression on Freshly Trypsinized Cells

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Using an antiserum raised in rabbits against embryonic chick skeletal myoblasts (Anti-M-24), we have examined the trypsin and neuraminidase sensitivity and physiological expression of myogenic cell surface antigens. It was found that trypsin-released muscle cells more effectively inhibited, on a cell to cell basis, the cytotoxicity of Anti-M-24 for 24-h-old myoblast monolayers than did identical cells that had received a 3–4 h suspension culture recovery period from trypsinization. There was no such difference in absorptive capacities observed for any other embryonic chick tissue tested (e.g. brain, retina, liver, heart, and red blood cells) when freshly trypsinized cells were compared to ones which were given a 3–4 h culture period. If freshly trypsinized muscle cells were treated with high concentrations (30,000 international units (IU)/0.1 ml packed cells) of trypsin or with neuraminidase (30,000 IU/ml packed cells), there was a selective loss of myoblast-specific surface antigens. When single cells that had been in suspension culture for 3.5 h were re-exposed to low concentrations (10,000 IU/0.1 ml packed cells) of trypsin, more antigenic sites were revealed on their surfaces as detected by an increased absorptive capacity in removing myoblast-binding antibodies from Anti-M-24. This increase in antigenic expression was time-dependent and inversely related to the length of culture time after trypsinization. Immunofluorescence studies revealed that tissue specific myoblast cell surface antigens are present on both muscle cells that were freshly dissociated and those that had been in suspension culture for 3–4 h. Furthermore, freshly trypsinized myoblasts possessed cell surface components that were highly antigenic; antiserum to such cells reacted extensively with both trypsinized and recovered muscle cells as detected by complement-dependent ^{51}Cr release cytotoxicity assays and immunofluorescence. We conclude that embryonic chick myoblasts possess surface antigens that may be selectively removed by neuraminidase or high concentrations of trypsin. These antigens may be progressively masked, with increasing time of culture after protease-dissociation, by molecules that are sensitive to low concentrations of trypsin. Such masking of tissue-specific cell surface antigens could result in the display of molecular mosaics which may play a role in facilitating intercellular recognition and subsequent differentiation and histogenesis.

Key words: embryonic muscle, cell surface antigens, myogenesis, cytotoxicity assays

Recently we reported the immunological detection of tissue and developmental stage specific surface antigens in embryonic chick skeletal muscle in vitro (1—3). These studies were initiated to detect subtle compositional changes at the cell surface which might accompany differentiation of a particular embryonic tissue. We chose to examine developing embryonic muscle because this tissue provides an excellent system for studying the relationship between cell surface modifications and the nuclear events which underlie the phenotypic modifications we term differentiation. Cell recognition prior to myotube formation is highly specific (4, 5), both prefusion myoblasts and multinucleated myotubes exhibit cellular migration and motility (6), and cell-cell and cell-substratum adhesions clearly influence the course of myodifferentiation (7, 8). The aggregation of myogenic cells and the formation of intercellular junctions at interfacing plasma membranes prior to cell fusion (9, 10) are poorly understood, but probably significant, steps in muscle differentiation. In addition, there are well established methods available for culturing muscle which produce a developing tissue that faithfully mimics in vivo myogenesis (11—13).

Antiserum to the prefusion myoblast (Anti-M-24) was produced in rabbits and extensively characterized by serological and immunohistochemical methods (3, 14). The results of these experiments are summarized in Table I. Such criteria have also been used to identify tissue specific surface antigens in other embryonic (15—18), transformed (19—22), or adult (23, 24) tissues. If the molecular basis for such operationally defined specificities is to be understood, we felt it would be necessary to examine the biochemical properties of the myoblast cell surface antigens. As a preliminary step towards immunochemically isolating and characterizing these antigens, we have examined their physiological expression and protease sensitivity. A number of proteases have been used in the isolation and characterization of histocompatibility antigens from mouse (25) and human (26) tissues. Chymotrypsin may be used to selectively expose tumor associated antigens on infected avian cell surfaces (27), while the major surface glycoprotein of fibroblasts is markedly trypsin sensitive (28, 29). Trypsin has also been used to explore the physiological expression (30, 31) and membrane fluidity (32—34) of lectin binding sites on embryonic cell surfaces. Others have used neuraminidase to examine the expression of a number of cell surface antigens (35, 36). While such enzymatic treatments of living cells is known to have marked effects on a number of cell properties (37, 38), it is still of interest to consider their use in the analysis of potentially cryptic, or masked, membrane antigens.

In this communication we wish to present new data we have obtained concerning the protease and neuraminidase sensitivity and the physiological expression of myoblast surface antigens. These data show that myoblast antigens may be selectively removed from the cell surface after treatment with neuraminidase or high concentrations of trypsin. Freshly trypsinized muscle cells apparently have greater quantities of these antigens on their surface than do cells permitted a period of culture after trypsinization; mild retrypsinization of such cells reveals the same amount of antigen as found on freshly dissociated cells. Parallel studies have involved the immunochemical analysis of detergent-extracted antigens by radioimmunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The results of those investigations will be reported elsewhere (39).

MATERIALS AND METHODS

Cell Culture

Muscle was obtained from the hindlimbs of 12-day-old embryonic White Leghorn chicks by methods routinely used in our laboratory (40). All visible nervous, vascular, and

TABLE I. Summary of the Characteristics of Antiserum (Anti-M-24) Prepared in Rabbits Against Embryonic Chick Prefusion Myoblasts

1. Complement-mediated ^{51}Cr release cytotoxicity experiments indicate:
   A. Absorption of Anti-M-24 with live cell monolayers, live cell suspensions, acetone powders, or freshly homogenized tissue from embryonic red blood cells, liver, brain, retina, or heart or adult skeletal muscle will remove all cytotoxicity of this antiserum for these tissues while lowering the cytotoxicity for embryonic muscle by only 20–60%. We interpret these results to demonstrate TISSUE SPECIFICITY.
   B. Absorption of Anti-M-24 with myotubes or skeletal muscle fibroblasts removes all cytotoxicity of this antiserum for these cell types at a point where there is still significant cytotoxicity remaining for myoblasts. We interpret these results to demonstrate DEVELOPMENTAL STAGE SPECIFICITY.
   C. Light microscopical observations of cultures treated with appropriately absorbed antiserum and guinea pig complement confirm the serological data; in such cultures only the spindle-shaped myoblasts are lysed.
2. Immunohistochemical staining (indirect immunofluorescence or immunoperoxidase) of monolayers or single cell suspensions further demonstrates that, after appropriate absorption, Anti-M-24 will stain only prefusion myoblasts. Shared, muscle tissue-specific, antigens may also be detected on embryonic muscle at all stages of differentiation.
3. Myoblast-specific antigens are cell surface components: 1) intact, viable cells inhibit cytotoxicity of Anti-M-24 for myoblasts and 2) observations by indirect immunofluorescence demonstrate that, under appropriate conditions, these antigens are mobile within the plasma membrane.
4. Myoblast cell surface antigens detected with Anti-M-24 are not an artefact of tissue culture: 1) fresh, homogenized chick tissues will absorb reactivity of Anti-M-24 with cultured cells or cells freshly released from the embryo and 2) Anti-M-24 binds to frozen sections of fresh embryonic hindlimb muscle as detected by indirect immunofluorescence.

connective tissue was removed from the limbs by careful dissection and the remaining muscle finely minced with scissors. After washing the tissue with calcium- and magnesium-free Tyrodes solution (CMF), it was dissociated into single cells by a 50 min incubation period at 37°C in 0.3% trypsin (Armour Pharmaceuticals, St. Louis, Missouri, "Tryptar") in CMF. Proteolytic dispersion of the cells was stopped by washing the cell suspension 3 times with Eagle's basal medium containing 10% heat inactivated (30 min at 56°C) horse serum (Grand Island Biologicals, Grand Island, New York), 1% glutamine, 1% penicillin (E-HS) and soybean trypsin inhibitor (10 μg/ml). After passing the cell suspension through a metal mesh filter, single cells suspended in E-HS with 5% embryo extract (complete medium, CM) were plated at varying densities on gelatin coated plastic culture dishes (Falcon Plastics, Oxnard, California). Such primary muscle cell cultures contained approximately 90% myoblasts as assessed by morphologically scoring 24-h-old monolayers established from comparable cell suspensions (47, 48). Other embryonic chick tissues (e.g. heart, brain, liver, retina) were obtained in similar fashion. Red blood cells were collected from the chorioallantoic vessels of the embryo, washed with phosphate-buffered saline, and used immediately for absorptions or cytotoxic assays.

Antiserum

Freshly dissociated embryonic muscle cells were cultured after trypsin dissociation for 3.5 h by placing $5-10 \times 10^6$ cells/4.0 ml E-HS into 25-ml Ehrlenmeyer flasks and rotating the flasks at 110 rpm on a New Brunswick Gyratory Shaker. Such a culture period has been operationally defined as "preaggregation" to emphasize the observation that during the 3–4 h time period multicellular aggregates do not form and greater than 95%

of the cells remain as single, viable cells. To monitor the state of aggregation during such a time period, parallel suspension cultures were established and samples taken at 1-h intervals from 0 to 6 h of culture. For each time point, the cells were pelleted by centrifugation, resuspended with a known volume of E-HS, and observed in a light microscope. Representative fields were scored for the number of single cells and multicellular aggregates. By 2–3 h small clusters of cells were observed, but these were easily dissociated into single cells by gentle pipetting. Only after 4–5 h were larger multicellular aggregates present that could not be easily dispersed into single cells by pipetting. Such aggregates rapidly increased in number from 6–24 h of culture, even at gyratory shaker speeds of 120 rpm. Such a period of suspension culture presumably permitted the renewal of cell surface materials which may have been denuded by tryptic treatment during the dissociation procedure (15, 41).

After 3.5 h of suspension culture, cells were collected, washed several times with Tyrodes solution, and injected into rabbits. The immunization protocol and bleeding schedule has been reported elsewhere and will not be detailed here (3, 14). Antiserum raised in rabbits against prefusion embryonic chick myoblasts will be referred to as Anti-M-24 and represents serum pooled from 3 individual rabbits.

Cytotoxicity Assay

The complement-dependent ^{51}Cr release cytotoxicity assay is a modification of that described by Wigzell (42) and Sanderson (43). Target cells were plated at $1-5 \times 10^4$ cells/ 0.1 ml CM/well on Falcon microtiter plates. Six hours later, 1.0 μCi of ^{51}Cr (New England Nuclear Corporation, Boston, Massachusetts) in 0.1 ml E-HS was added to each well and after an additional 12 h incubation at 37°C, the excess ^{51}Cr was removed by several washes of the wells with E-HS. Antibody (0.05 ml) and guinea pig complement (0.05 ml) diluted to a final concentration of 1/10 (Beckman Diagnostics, Fullerton, California) was added and the cultures incubated for an additional 50 min. The reaction was stopped by adding 0.1 ml of cold Tyrodes solution to each well. Following a 10 min centrifugation at 2,800 rpm to remove cellular debris, 0.1 ml samples from each of triplicate wells were taken to be counted on a Searle Autogamma Counter. The results were calculated as a percent cytotoxicity:

$$\text{Percent Cytotoxicity} = \frac{\text{cpm }^{51}\text{Cr released by antibody} - \text{background}}{\text{cpm }^{51}\text{Cr released by TX-100} - \text{background}} \times 100$$

For each set of triplicate samples the mean and standard deviation were determined. The variability of replicates in a single experiment was never greater than ± 10% cytotoxicity and generally was between 5 and 8. All experiments were repeated in their entirety at least twice and most were repeated 3–5 times. For Figs. 2–7, the mean values from 2 to 5 experiments were averaged and presented as a single data point.

For inhibition of cytotoxicity assays, 0.5 ml of antiserum diluted 1/10 or 1/100 with E-HS was absorbed with increasing numbers of cells overnight on a wrist shaker at 4°C. Cells used for absorption were removed by centrifugation and the absorbed serum used immediately. For trypsinization of absorbing muscle cells, 10^8 freshly dissociated or pre-aggregated myoblasts were suspended in 4.0 ml of trypsin-CMF (0.05–0.3%), rotated at 110 rpm for 50 min in a gyratory shaker bath at 37°C, washed extensively with E-HS and soybean trypsin inhibitor (10 μg/ml), and then used to absorb antiserum as described above. Neuraminidase treatment was conducted under identical conditions as for trypsinization;

30,000 IU/ml of neuraminidase Type I from Cl. perfinigens (Sigma Chemical Company, St. Louis, Missouri) was used.

Immunofluorescence

Cells to be stained by immunofluorescence were either live or fixed with 2.0% para-formaldehyde for 10 min at 50°C. All subsequent procedures were conducted on ice (for live cells) or at room temperature (for fixed cells). Five million cells were washed with E-HS, treated for 15 min with Anti-M-24 or preimmune serum from the same rabbit diluted 1/10 with E-HS, washed 3 times with E-HS, incubated for an additional 15 min with goat antirabbit IgG conjugated to fluorescein isothiocyanate (GAR-FITC) (Cappel Laboratories, Downingtown, Pennsylvania), washed 2 times with E-HS, mounted in glycerol under a glass coverslip and observed under an Apo 40X oil immersion lens mounted to a Zeiss epifluorescent microscope. Photomicrographs were taken after a 30 sec exposure on Kodak Tri-X film and subsequently developed in Diafine developer. All prints were processed under identical conditions.

RESULTS

Anti-M-24 was prepared by immunizing rabbits with myoblasts that had been allowed a 3–4 h culture period after primary trypsin-dissociation under the assumption that such a period of time was necessary for the cells to renew surface components that may have been denuded by the protease dissociation procedure (15, 41). To further test this assumption, we decided to compare the ability of freshly dissociated and preaggregated myoblasts to inhibit cytotoxicity of Anti-M-24 for 24-h-old myoblast monolayers. At this point, 2 observations should be emphasized: 1) muscle cultures consist of 2 predominant cell types, myoblasts and fibroblasts, the former accounting for approximately 90% of the cells present at 24 h of monolayer culture (40, 44) and 2) on a cell to cell basis, fibroblasts take up 3–4 times the amount of ^{51}Cr as do myoblasts (14). These are important considerations when attempting to interpret data obtained from ^{51}Cr-release experiments. For example, while myoblasts account for nearly 90% of the total cell population in a 24-h-old muscle culture, they only contain 40–60% of the ^{51}Cr bound within the cells of such a culture.

Single cells that had been cultured in suspension for 3.5 h inhibited cytotoxicity of Anti-M-24 for myoblast target cells in a linear fashion; 12×10^6 cells reduced the cyto-toxicity to 10% (Fig. 1). When freshly trypsinized cells were used, only 4×10^6 cells were necessary to obtain comparable inhibition (Fig. 1). Thus, antigenic sites detected by Anti-M-24 were present on freshly trypsinized cells and, on a cell to cell basis, there were fewer of them detected on muscle cells that had been cultured for 3.5 h. If freshly dissociated muscle possessed surface antigens also found on cells that had received a 3.5 h period of culture following trypsinization, then these cells should be immunogenic when injected into rabbits. If cells were injected into rabbits immediately following trypsinization, the resultant antiserum was highly cytotoxic for 24-h-old muscle monolayers (Fig. 2). Apparently, a 3–4 h culture period following trypsin dissociation was not necessary for the regeneration or expression of myoblast cell surface antigens as detected by Anti-M-24. We hypothesized that with time after trypsinization there may have been a progressive masking of myoblast surface antigens and to test this assumption the following experiments were conducted.
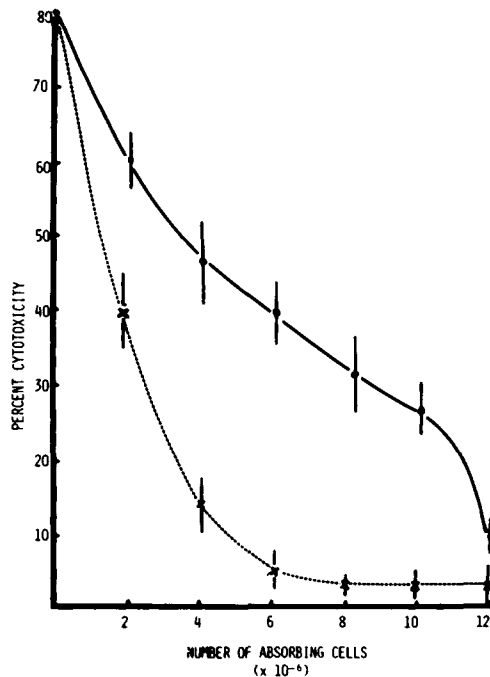
Fig. 1. Inhibition of cytotoxicity of Anti-M-24 for myoblast target cells after absorption with in-creasing numbers of suspension cultured (●——●) or freshly trypsinized (x- - -x) myoblasts. One-half milliliter of antiserum was diluted 1/100 with E-HS and absorbed overnight on a shaker at 4°C with either 2, 4, 6, 8, 10, or 12 million cells from 12-day-old embryonic chick hindlimb muscle that had been freshly dissociated with 0.3% Tryptar/ml of minced muscle or with identical cells that had been cultured for 3.5 h at 110 rpm on a gyratory shaker. The absorbed antisera were tested, in the presence of guinea pig complement, for cytotoxicity on 24-h-old muscle monolayers. Twice as many suspension cultured cells as freshly trypsinized ones were needed to remove all cytotoxicity of the antiserum for myoblast target cells. Vertical lines at each point represent the range of 1 standard deviation from the mean as calculated for 12 replicate samples obtained from 4 independent experiments.

Cells which had been cultured for 3.5 h following trypsinization ("preaggregated" cells) were treated with high (30,000 IU/0.1 ml packed cells) or low (10,000 IU/0.1 ml packed cells) concentrations of trypsin and then assessed for their ability to inhibit cytotoxicity of Anti-M-24 for 24-h-old muscle monolayers. Exposure of preaggregated cells to high concentrations of trypsin apparently removed most of the antigens while a comparable group of cells that had been exposed to low concentrations of this protease were as effective in the inhibition of cytotoxicity as freshly dissociated cells (Fig. 3). It should be emphasized that a single group of cells was used for the data obtained in Figs. 1 and 3; Anti-M-24 was absorbed with either the freshly dissociated cells or cells from the same dissociation which were then preaggregated or preaggregated and retrypsinized. Identical results were obtained when the experiment was repeated.

If freshly dissociated muscle was reexposed to higher concentrations of trypsin, the single cells were greatly reduced in the ability to inhibit cytotoxicity of Anti-M-24 for muscle monolayers (Fig. 4). However, if such absorbed antiserum was tested on pure fibro-blast monolayers, cytotoxicity for these target cells was removed entirely after absorption with 8×10^6 cells. These results suggested that trypsin may have selectively removed the
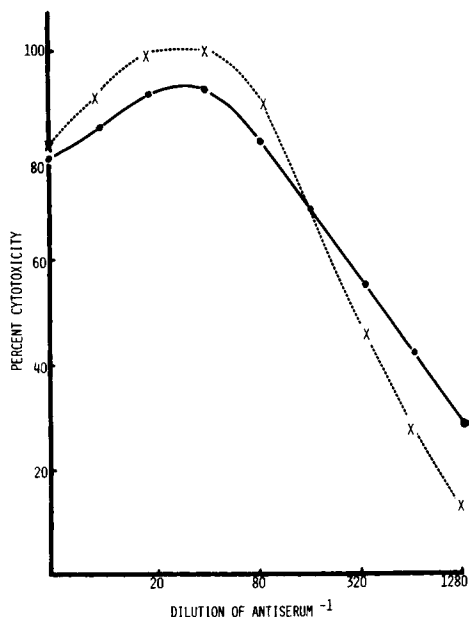
Fig. 2. Cytotoxicity for 24-h-old muscle monolayers of antiserum prepared in rabbits against freshly trypsin-dissociated embryonic chick hindlimb (Anti-M-24T, x- - -x) or identical cells that had been cultured in suspension for 3.5 h at 110 rpm before immunization (Anti-M-24, ●—-●). Each dilution curve represents pooled serum obtained from 2–3 rabbits. Preimmune serum from identical rabbits used to obtain immune serum induced less than 10% cytotoxicity at any dilution tested (1–1/800). See Materials and Methods in the text for discussion of variability of data and experimental repro- ducibility for data presented in this and subsequent figures.

myoblast-specific surface antigens. To further test this hypothesis, several other embryonic chick tissues were examined for the effects of trypsin treatment on the inhibition of Anti- M-24 cytotoxicity. Two representative tissues are presented in Fig. 5. For both embryonic brain and liver there was no difference in the ability of freshly dissociated or preaggregated cells to inhibit the cytotoxicity of Anti-M-24 for 24-h-old muscle monolayers. When such absorbed antiserum was tested back on the homologous tissue with which it was absorbed, all cytotoxicity was removed after absorption with $2-4 \times 10^6$ cells. Similarly, there were no differences in the inhibition of cytotoxicity curves of Anti-M-24 for myoblasts after absorption with either preaggregated or freshly trypsinized embryonic chick retina, heart, or red blood cells.

If indeed low concentrations of trypsin reexposed myoblast surface antigens which were progressively masked with culture after trypsinization, one might predict a correlation between antigen masking and the culture time. Such a correlation was observed when Anti-M-24 was absorbed with myoblasts that were either freshly trypsinized or preaggregated for 1, 2, 3, or 3.5 h and tested for cytotoxicity on myoblast monolayers (Fig. 6). Cells preaggregated for 1 h exhibited an equivalent inhibition of cytotoxicity in Anti-M-24 for myoblasts as did freshly dissociated cells. With increasing time in suspension culture there was a progressive decrease in the ability to inhibit antimyoblast cytotoxicity. Thus, when compared to freshly trypsinized cells, the myoblasts after 3 h in suspension culture ex- hibited fewer antigenic sites per cell that were available for binding to antibodies directed
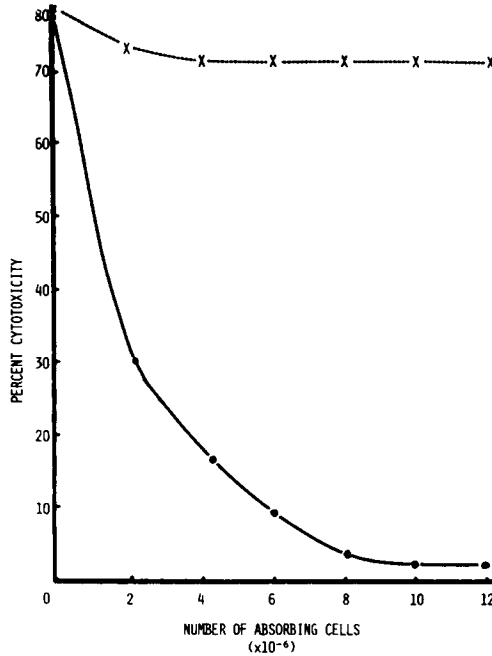
Fig. 3. Inhibition of cytotoxicity of Anti-M-24 for 24-h-old muscle cell monolayers after absorption with increasing numbers of myoblasts that had been suspension cultured for 3.5 h and then treated with high (x- - -x) or low (●——●) concentrations of trypsin as described in the text. Note 1) the lack of inhibition of cytotoxicity after treatment with high concentrations of trypsin and 2) the similarity in the inhibition curve of the low trypsin concentration treatment and the curve obtained after absorption with freshly dissociated cells as seen in Fig. 1.

against myoblast cell surface components. Periods of time longer than 3.5 h were not studied since these cells rapidly formed multicellular aggregates after approximately 4 h of rotary suspension culture. These varying sized aggregates would be very difficult to compare in absorption capacity to single cells since the exposed surface areas would not be equivalent.

Suspensions of freshly dissociated muscle cells were also incubated in neuraminidase and then assessed for the inhibition of cytotoxicity of Anti-M-24 for muscle or pure skeletal muscle fibroblast monolayers. After absorption with as many as 10^7 neuraminidase-treated cells, Anti-M-24 retained 75% cytotoxicity for 24-h-old muscle monolayers (Fig. 7). However, the cytotoxicity of this same antiserum for pure fibroblast monolayers began to fall precipitously after absorption with only 4×10^6 neuraminidase-treated cells (Fig. 7). Presumably, the myoblast-specific antigens are more susceptible to neuraminidase hydrolysis than those antigens shared with fibroblasts.

Immunofluorescence of Freshly Trypsinized and Suspension Cultured Muscle Cells

Both "preaggregated" and freshly trypsinized muscle cells stained intensely over the entire cell surface after incubation in unabsorbed Anti-M-24 and GAR-FITC (Fig. 8a–d). Absorption with preaggregated (Fig. 8e) or freshly trypsinized (Fig. 8f) embryonic liver cells diminished the intensity of the fluorescence and the pattern of staining became less regular. Unabsorbed Anti-M-24 intensely stained embryonic liver cells (Fig. 8g), but following absorption with liver cells no longer stained hepatocytes (Fig. 8h).
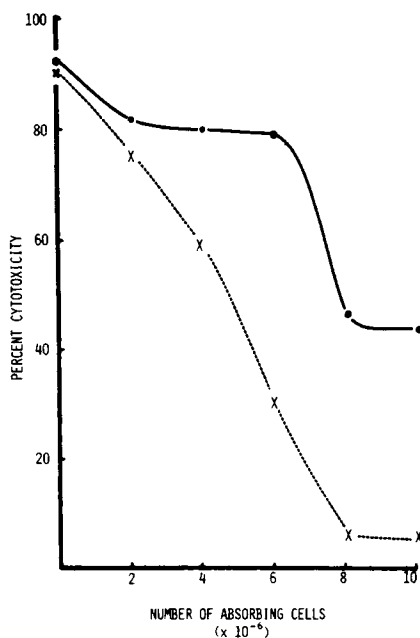
Fig. 4. Inhibition of cytotoxicity of Anti-M-24 for 24-h-old muscle (●——●) or pure skeletal muscle fibroblast (x- - -x) monolayers after absorption with increasing numbers of freshly dissociated myoblasts that had been retrypsinized. Muscle was dissociated into single cells by a 50 min incubation period at 37°C in 0.3% trypsin, washed 4 times with E-HS and soybean trypsin inhibitor, and then retrypsinized with a comparable amount of trypsin for an additional 50 min at 37°C in a gyratory shaker water bath. After 4 more washes with E-HS and soybean trypsin inhibitor, the cells were used to absorb Anti-M-24. Absorption with 8×10^6 treated cells removed all cytotoxicity of this antiserum for fibroblasts while as many as 10^7 only lowered the cytotoxicity for myoblasts to 50%.

Myoblasts incubated in neuraminidase or high concentrations of trypsin and subsequently stained with Anti-M-24 and GAR-FITC failed to demonstrate specific immunofluorescence (Fig. 8i). When freshly dissociated (Fig. 8j) or preggregated (Fig. 8k) myoblasts were stained with unabsorbed antiserum raised against freshly dissociated cells, a uniformly intense fluorescence pattern was observed around their periphery. Preimmune serum failed to stain any of the cell types used in this study (not illustrated). All immunofluorescence experiments were conducted on paraformaldehyde-fixed cells or on live cells at 4°C so the patterns of fluorescence could not be attributed to capping phenomena which occur as a result of mobile surface receptors (45). If cells were stained at 37°C, capping did occur, followed by the complete removal of surface antigen-antibody complexes (Friedlander, unpublished observations).

DISCUSSION

The role of surface glycoproteins as recognition molecules on developing embryonic cells has been explored in several systems and it is clear that such macromolecules are responsible for, or at least associated with, tissue specific aggregation phenomena of embryonic chick retina (46, 47) and the promotion of differentiation in cartilage (48), muscle (49), and glia (50). The manner in which such macromolecules exert their influence on cellular interactions and the promotion of differentiation is not clear; there have been
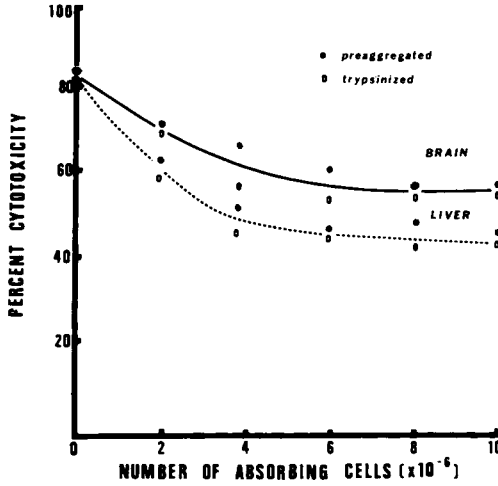
Fig. 5. Inhibition of cytotoxicity of Anti-M-24 for 24-h-old muscle monolayers after absorption with increasing numbers of 12-day-old embryonic brain or liver cells that had been freshly dissociated (o) or suspension cultured for 3.5 h (●). There was no significant difference in the rate at which freshly dissociated or preaggregated cells from liver or brain inhibited cytotoxicity.
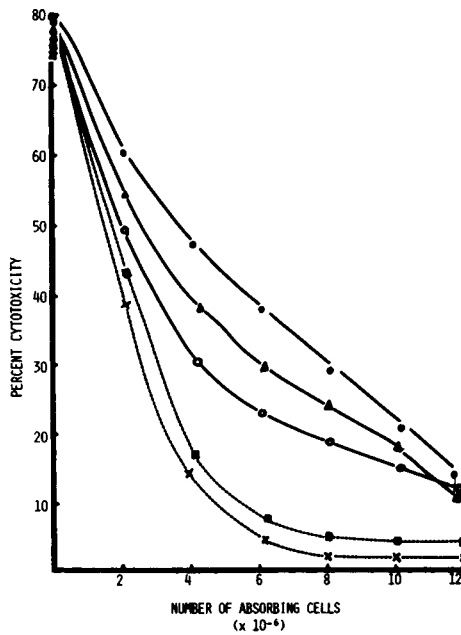

Fig. 6. Inhibition of cytotoxicity of Anti-M-24 for 24-h-old muscle cell monolayers after absorption with increasing numbers of myoblasts that were either freshly dissociated with trypsin (x- - -x) or cultured in suspension for 1 (■ - - - ■), 2 (o—o), 3 (▲—▲), or 3.5 (●—●) h. Cells to be used for absorption were divided into aliquots of 2, 4, 6, 8, 10, or 12 million and shaken gently overnight with 0.5 ml of antiserum diluted 1/100 with E-HS. After removal of the cells by centrifugation, the serum was assessed for cytotoxicity, in the presence of guinea pig complement, on myoblast target cells. Note that the cytotoxicity curves obtained after absorption of antiserum with cells preaggregated for 2 and 3 h were intermediate to those obtained with serum absorbed with cells preaggregated for 0 and 3.5 h.
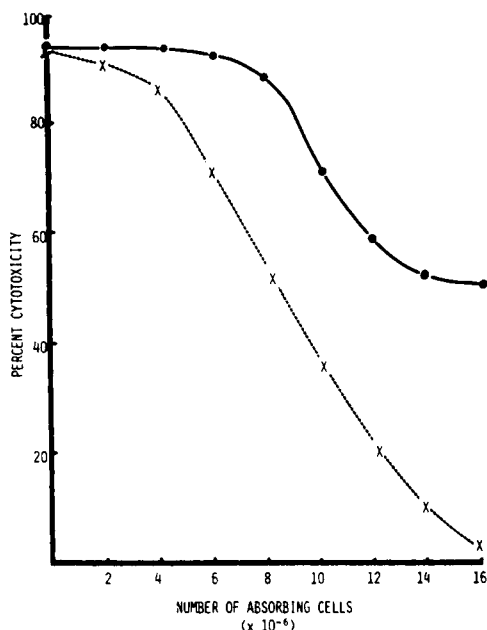
Fig. 7. Inhibition of cytotoxicity of Anti-M-24 for 24-h-old muscle (●—●) or pure skeletal muscle fibroblast (x - - - x) monolayers after absorption with increasing numbers of neuraminidase-treated myoblasts. Cells used for absorption were treated with enzyme as described in the text, washed with E-HS and then assessed for the ability to absorb out antibodies that normally bind to and, in the presence of complement, lyse myoblasts. Note that 16 million treated cells inhibited all cytotoxicity of this antiserum for pure fibroblast monolayers, but that this same number only reduced the cytotoxicity for myoblast monolayers to 50%. The fall in cytotoxicity for myoblast targets seen between absorption with 6 and 12 million cells reflects the loss of ^{51}Cr from fibroblasts that contaminate the 24-h-old muscle cultures and account for 40–60% of the ^{51}Cr in these cultures (see text for discussion).

suggestions that these glycoproteins may act as cell ligands (51), protease sensitive cell couplers (47), or enzymes that catalyze the transfer of sugars (52) and thus stabilize intercellular contacts. Our observations suggest that there is a class of trypsin- and neuraminidase sensitive molecules which serve to uniquely identify the prefusion myoblast cell surface and that these antigens are revealed in greater quantity by mild trypsinization of pre-aggregated muscle cells. Whether these antigens are involved in facilitating intercellular recognition is uncertain at this time, but it is tempting to consider this possibility.

Mild retrypsinization of cultured cells revealed 3–5 times the number of antigenic sites found on cells which, after dissociation, were placed in suspension culture for 3–4 h. Prior to such a culture period, these cells do not exhibit aggregation (53, 54) or histiotypic cell sorting (55) or possess surface glycoproteins detected by ruthenium red staining (56). Based on these biological constrictions, we found it surprising that myoblast surface antigens should be present in greater quantities at a time when the surface of these cells exhibited lower biological specificity than cells in culture for 3–4 h. Of interest was the failure to detect such differences in antigenic expression, as measured by the capacity to absorb out cross-reacting, species-related antigens, on other tissues of the chick embryo. Perhaps only the muscle-related antigens are exposed upon mild trypsinization. Furthermore, after suspension culture of freshly dissociated myoblasts, the original quantity of
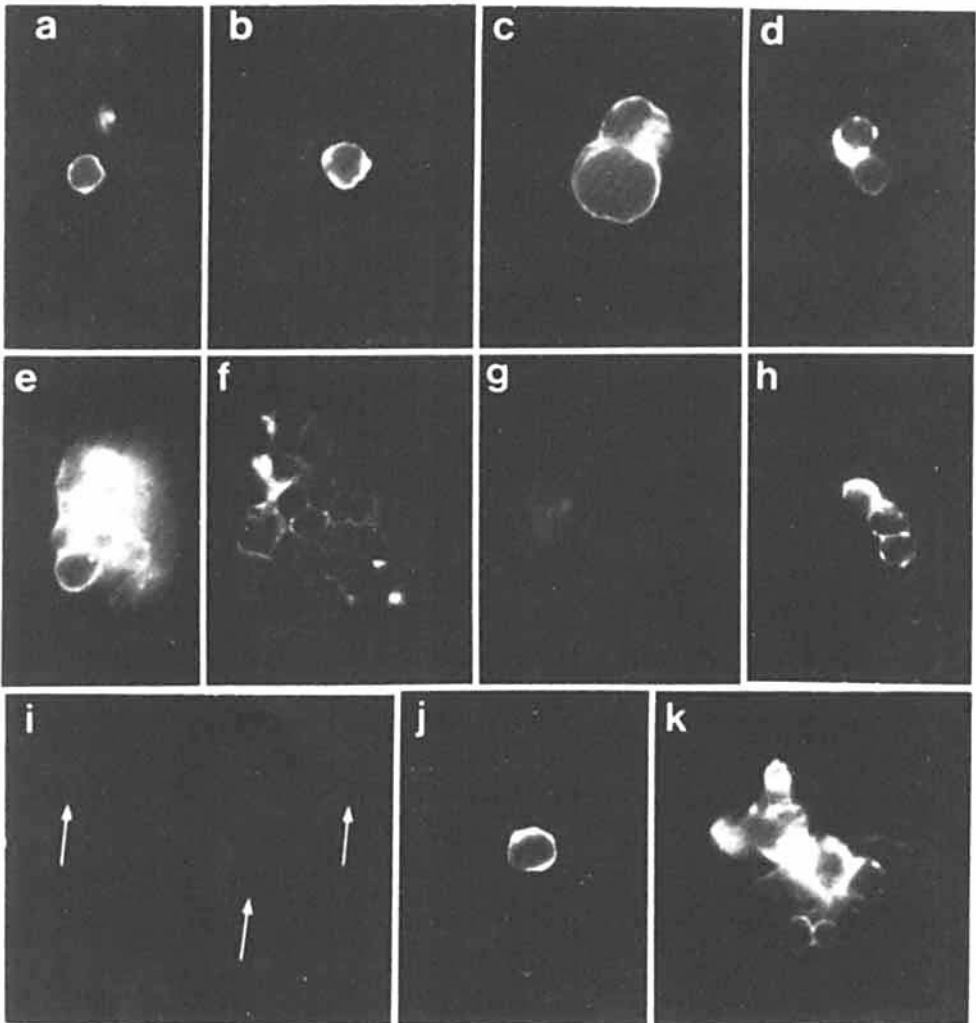
Fig. 8. Immunofluorescence staining of muscle or liver cell suspensions that had been treated with rabbit antiserum to freshly dissociated or trypsin-recovered (preaggregated) myoblasts. Unabsorbed Anti-M-24 diluted 1/10 with E-HS, followed by GAR-FITC, was used to stain either freshly dissociated (a, e) or preaggreated (b, f) muscle cells. This same antiserum intensely stained embryonic liver cells (c), but following absorption with embryonic liver homogenate or acetone powder no longer stained this cell type (g). If such absorbed antiserum was used to stain freshly dissociated (d) or preaggregated (h) muscle cells, a discontinuous pattern of surface staining was observed. When muscle cells were pre-treated with high concentrations of trypsin or neuraminidase and then stained with Anti-M-24 and GAR-FITC, no fluorescence was observed (i). Antiserum prepared against freshly dissociated myoblasts also intensely stained the surfaces of both freshly dissociated (j) and preaggregated (k) muscle cells.

these antigens could be reexposed by mild trypsinization. This uncovering of antigen was time dependent and suggested a progressive masking of myoblast antigens upon repair of the cell surface and concommittant myodifferentiation. Cell surface antigens of normal

and trypsinized myotubes must be compared before a statement can be made concerning the developmental significance of the trypsin effect. Such a study is in progress. Preliminary observations indicate that more muscle-specific antigens, as detected by inhibition of Anti-M-24 for myoblast monolayers, are exposed on mildly trypsinized myotubes than on untrypsinized cells (Friedlander, unpublished observations).

Our conclusion that only myoblast-specific surface antigens were enhanced by mild trypsinization, but eliminated by neuraminidase or extensive trypsinization, was based on experiments which warrant additional discussion. Myoblast-specific antiserum, as operationally defined, retained high cytotoxicity for 24-h-old muscle monolayers at levels of absorption where cytotoxicity was lost for other embryonic chick tissues. Thus, Anti-M-24 exhibited 40–60% cytotoxicity for myoblast monolayers after absorption, a 30–40% reduction from values obtained with unabsorbed antiserum. Although myoblasts account for 80–90% of the cells in 24-h-old monolayers, these cells only account for approximately 50% of the ^{51}Cr taken up in such cultures. Based on this observation and the morphological scoring of cell types that were specifically lysed in such heterologous cultures, we concluded that specifically absorbed Anti-M-24 binds predominantly to spindle-shaped myoblasts (3). Similarly, Fig. 4 and 7 are more readily interpreted when the heterogeneity of 24-h-old muscle cultures is considered. If myoblast-specific antigens were selectively removed with high levels of trypsin or neuraminidase, why was there an initial decline, then a plateau in the inhibition of cytoxicity of Anti-M-24 for myoblast monolayers after absorption with protease- or neuraminidase-treated muscle cell suspensions? Since the inhibition of cytoxicity plateaued between 40 and 60% cytoxicity in both series of experiments, and since myoblasts only account for 40–60% of the ^{51}Cr in such cultures, it seemed reasonable to conclude that the fibroblasts in such cultures were lysed by the antiserum and complement. In other words, absorption of Anti-M-24 with trypsin- or neuraminidase-treated muscle cell suspensions failed to remove antibodies reactive with both myoblasts and fibroblasts. However, we cannot be certain if the myoblast-specific antigens were totally removed by enzymatic treatment. Similarly, the shared antigens might also be removed upon prolonged exposure of the cells to sialidase or trypsin. To better understand the relative labilities of these 2 classes of antigens, enzymatic treatment for longer periods of time and with higher concentrations of trypsin and neuraminidase will be required. Furthermore, absorption with higher cell numbers will be required to establish if the observed differences in absorptive capacity of enzymatically treated cells are qualitative or quantitative phenomena. If cells after 3–4 h of suspension culture were incubated in high concentrations of trypsin, all surface antigens were apparently removed since these cells failed to inhibit the cytotoxicity of Anti-M-24 for myoblast targets. It is conceivable that residual trypsin, present during the preaggregation periods that preceded retrypsinization, rendered the cell surface more labile to a subsequent trypsin digestion. Such residual activity might explain the difference in the cytotoxicity curves observed after absorption of Anti-M-24 with myoblasts treated with high concentrations of trypsin either immediately following tissue dissociation (Fig. 4) or after 3.5 h of suspension culture (Fig. 3).

It has been suggested that with progressive differentiation various antigenic sites on embryonic cells surfaces become preferentially masked or unmasked, thus creating cell surface mosaics which are tissue and developmental stage specific (57, 58). Our data are consistent with the existence of protease-sensitive, developmentally regulated, cell surface mosaics. At this time we are uncertain as to how protease treatment preferentially exposes myoblast cell surface antigens; whether trypsin unmasks "cryptic" antigens or somehow

alters the organization of these molecules within the membrane, and thus affects their expression, remains unanswered. Our observation of differential staining patterns obtained with unabsorbed and absorbed Anti-M-24 suggest that there is a mosaicism to the distribution of the antigens detected by this antiserum. We previously reported that myoblasts possess surface antigens not detected on muscle of later stages of development (1, 2), but at that time could not rule out the possibility that these sites were simply masked or present in greatly diminished quantities on myotubes. We now present evidence that myoblast surface antigens were revealed in greater quantities by mild proteolysis and there are preliminary indications that similar treatment of myotubes makes these cells more effective inhibitors of cytotoxicity of Anti-M-24 for myoblast and myotube monolayers.

We conclude from these experiments that neuraminidase-sensitive antigens, which are also removed by high concentrations of trypsin, but revealed in greater quantities by mild proteolysis, may serve to distinguish the myoblast cell surface from other embryonic chick tissues and muscle at later stages of development. The potential biological roles for such developmental stage specific markers are many: intercellular recognition, the regulation of cellular proliferation, or the initiation of myofibrillogenesis by providing subplasmalemmal insertion sites for contractile filaments. All of these phenomena characterize the myogenic developmental program and probably involve surface associated events (59, 60). While the data presented here do not directly support any such role for the antigens we have detected, the information we now have about their neuraminidase and protease sensitivity should prove valuable in designing procedures for isolating and characterizing these molecules. We are currently exploring the immunochemical nature of these antigens, their fluidity within the cell membrane, and their possible relationship to cytoplasmic structures and physiological activities of the muscle cell.

ACKNOWLEDGMENTS

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