

A Monoclonal Antibody Detaches Embryonic Skeletal Muscle from Extracellular Matrices

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ABSTRACT We have described a monoclonal antibody that rounds and detaches chick skeletal myoblasts and myotubes from extracellular substrata. The antibody also inhibits the attachment of myogenic cells to a gelatin-coated substratum but has no detectable effect on myoblast fusion. The cellular response to antibody treatment varies with differentiation and cell type. Young myoblasts and myotubes are rapidly rounded and detached by the antibody. Older myotubes require longer incubation times or higher antibody titers for rounding and detachment. Chick embryo fibroblasts, cardiac cells, and neurons are not similarly rounded and remain attached. Since the antibody also detaches cells from embryonic muscle tissue explants, the cell-substratum interaction perturbed by the antibody appears relevant to the in vivo interaction of myogenic cells with their extracellular matrices. Binding studies using iodinated antibody revealed $2-4 \times 10^5$ sites per myoblast with an apparent K_d in the range of $2-5 \times 10^{-9}$ molar. Embryo fibroblasts bind antibody as well and display approximately twice the number of binding sites per cell. The fluorescence distribution of antigen on myoblasts and myotubes is somewhat punctate and particularly bright along the edge of the myotube. The distribution on fibroblasts was also punctate and was particularly bright along the cell periphery and portions of stress fibers. For both cell types the binding was distinctly different than that reported for collagen, fibronectin, and other extracellular molecules. The antigen, as isolated by antibody affinity chromatography, inhibits antibody-induced rounding. SDS PAGE reveals two unique polypeptides migrating in the region of ~ 120 and 160 kilodaltons (kd). The most straightforward mechanism for the antibody-induced rounding and detachment is the perturbation of a membrane molecule involved in adhesion. The hypothesized transmembrane link between extracellular macromolecules and the cytoskeleton provides an obvious candidate.

Extracellular macromolecules that comprise the basal lamina and extracellular matrix play a central role in development, tissue maintenance, and regeneration. They are thought to provide the substratum on which cells adhere and migrate, serve as a scaffold for tissue structure, contribute to histospecific cellular adhesions, and provide environmental cues for cellular differentiation (2, 60). In skeletal myogenesis and regeneration, putative roles for extracellular macromolecules have been identified. Collagen markedly stimulates myogenesis in vitro by providing a substrate conducive to cell attachment (24). Extracellular macromolecules are also implicated in the accumulation of acetylcholine receptors and differentiation of nerve terminals at neuromuscular junctions (7, 9, 51). In contrast to this accumulating knowledge and understanding of the com-

ponents and functions of extracellular macromolecules, membrane components that regulate and participate in the interaction of cells with extracellular matrices remain to be isolated and characterized. One approach to identifying these molecules is through antibodies that perturb adhesion. Gerisch and co-workers have exploited this approach most fully, and have identified molecules participating in *Dictyostelium* aggregation (23). More recently, this approach has been extended to several different adhesive processes including that of cells to substrata (3, 8, 18, 20, 21, 29, 30, 43, 61).

We report a monoclonal antibody CSAT that rounds and detaches skeletal myogenic cells in culture. The antigen with which this antibody reacts is potentially highly significant because it is involved in regulating adhesion and morphology.

MATERIALS AND METHODS

Cell Culture

Breast muscle from 11-d-old chicks was cleaned of large pieces of connective tissue, and myoblasts were prepared as described previously (34) except that 0.1% Dispase II (neutral protease from *Bacillus polymyxa*, Boehringer Mannheim Biochemicals, Indianapolis, IN) was used to dissociate the tissue. Cells were grown in Ca⁺⁺-free Dulbecco's modified Eagle's medium (Ca⁺⁺-free DME) containing 10% horse serum (HS), 2.5% embryo extract (38), and 50 µg/ml gentamycin (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) (basic medium). Cells were pre-plated at high density on uncoated 100-mm tissue culture plates for 1.5 h to reduce the number of contaminating fibroblasts (45). Medium from these plates containing the myoblasts was seeded into gelatin-coated (0.1%) 35-, 60-, or 100-mm tissue culture petri dishes at a density of 1.2×10^5 cells/mm². Ca⁺² (1.8 mM) was added to cultures at seeding or, when unfused myoblasts were desired, EGTA (0.3 mM) was added to the cultures 24 h after seeding (46). Chick embryo fibroblasts were prepared as described previously (34) and passaged four times before use in experiments. Cultures of cardiac cells were prepared from 5-d-old embryos as described elsewhere and used after 24 h (15). Cultures of 6-d-old neural tube and human muscle were gifts of G. Bennett and T. Heiman, respectively.

Monoclonal Antibody Production

Hybridomas secreting monoclonal antibodies were produced (37) from the fusion of a mouse plasmacytoma cell line (SP 2/0-Ag 14), obtained from the University of Pennsylvania Cell Center (Philadelphia, PA), and spleen cells from an immunized 8-wk-old male BALB/C mouse. The mouse was injected with 100 µg of plasma membrane blebs prepared from 52-h myoblasts using the method of Scott (53) as modified by Sessions and Horwitz (54). Aliquots of blebs in Ca⁺⁺- and Mg⁺⁺-free phosphate buffered saline (CMF PBS) containing 100 µg of protein were stored in liquid N₂ and thawed immediately before injection. The first three injections were intraperitoneal, 1 wk apart, the first in complete Freund's adjuvant, the second in incomplete Freund's adjuvant, and the third in 0.15 M NaCl. This was followed by three daily intravenous (tail) injections in saline starting 1 wk after the last intraperitoneal injection (57). One day after the last intravenous injection the mouse was killed by cervical dislocation, the spleen removed, and the fusion performed (33). Supernatants from resulting hybridomas were screened for their morphologic effects (i.e. rounding, detachment, etc.) on myogenic cell cultures. For this assay, chick myoblasts were seeded at 4×10^6 cells in 0.5 ml of basic medium in 24-well Linbro tissue culture plates. EGTA (0.3 mM) was added 24 h after seeding. At 52 h after seeding, 150 µl of hybridoma supernatants were added to each well, and the cells were observed microscopically at 6, 12, and 24 h, subsequently. Out of the 160 clones screened from this fusion, several showed morphologic alterations but only CSAT rounded and detached cells. The hybridoma producing this antibody was recloned to homogeneity (i.e. all subclones produced rounding antibody).

Typing, Concentration, and Purification of CSAT

The immunoglobulin type of CSAT was determined to be an IgG_{2b} by Ouchterlony double immunodiffusion using rabbit anti-mouse immunoglobulin classes obtained from Cappel Laboratories Cochranville, PA.

CSAT hybridoma was grown in 150 cm² T flasks in 50 ml of HY medium containing 10% fetal calf serum (FCS), 0.15 mg/ml oxaloacetate, 0.5 mg/ml pyruvate, and 0.2 U/ml bovine insulin, 50 µg/ml gentamycin, and 2.5 µg/ml fungizone, and seeded at a density of 5×10^5 cells/ml. When desired, tissue culture fluid from CSAT hybridoma was concentrated by precipitation with 50% ammonium sulfate followed by dialysis against CMF PBS. CSAT was purified from the tissue culture medium on a Sepharose CL-4B-Staph protein A (Pharmacia Fine Chemicals, Piscataway, NJ) column by a modification of the procedure of Ey et al. (19). Briefly, the column was washed with 50 ml of Britton-Robinson buffer, (citric acid, 28.5 mM; KH₂PO₄, 29 mM; barbitol, 5.26 g/liter; and boric acid, 18.8 mM) adjusted to pH 8.5. CSAT hybridoma tissue culture medium (500 ml to 1 liter) was adjusted to pH 8.5 and loaded onto the column which was eluted with a gradient of Britton-Robinson buffer (pH 8.5–2.8). Fractions were assayed for absorbance at 280 nm and the pH was recorded. Peak protein fractions were pooled and assayed in an enzyme-linked immuno-adsorbent assay [ELISA] for binding activity. CSAT eluted at a pH of 3.8 as expected for antibodies of the IgG_{2b} class (19). The pooled fractions were neutralized and concentrated by dialysis against CMF PBS and stored at –20°C. Purified CSAT was titered for biological activity (rounding of myogenic cells). Purity of the preparations was examined by SDS gel electrophoresis (7% running gels) according to the method of Lammeli (39). Only two Coomassie Blue-stained bands, which comigrated with the heavy and light chains of a marker mouse IgG, were observed.

Purified CSAT was iodinated using Chloramine T according to the method described by Kennett et al. (33), except that free iodine was separated from the

iodinated protein on a previously calibrated Bio-Gel P-6 column (Bio-Rad Laboratories, Richmond, CA) equilibrated with 20 mM sodium phosphate pH 7.4. Iodinated CSAT was stored at –20°C.

Binding Assays

Cells were grown in 100-mm petri dishes; myoblasts were cultured in basic medium containing EGTA or exogenous calcium Ca⁺⁺ depending upon the experiment, and fibroblasts were used at log and confluent stages of growth. Monolayers were washed twice with warm (37°C) versene (1.48 mM EDTA in CMF PBS) and harvested in the same solution. The pellet was resuspended in Hanks's Balanced Salt Solution containing 20 mM HEPES, pH 7.2, 0.5% bovine serum albumin (BSA), and 19 mM NaN₃ (HH-BSA-NaN₃). Between 10⁵ and 10⁶ cells were then aliquoted into Eppendorf tubes (Beckman Instruments, Fullerton, CA). For quantitation of background binding, cold CSAT at a concentration previously determined to be above saturation was added (20 µg/ml in HH-BSA-NaN₃) and tubes were kept at 4°C for 1 h. At the end of this period, cells were spun in an Eppendorf microfuge for 15 s and the pellet was washed once in PBS. ¹²⁵I-CSAT (10⁴ Ci/mol) in HH-BSA NaN₃ was then added; the final assay volume was 40 µl. The tubes were incubated for 1 h at 4°C, at which time the binding had reached saturation for both fibroblasts and myoblasts. Each point was done in duplicate. At the end of the hour, the reaction volume was diluted to 200 µl with PBS and the tubes were spun for 40 s in the microfuge. The pellet was washed once in 200 liters of PBS and the final pellet was dissolved in 100 µl of 0.1 N NaOH-0.1% SDS. This was transferred to another tube and the volume brought to 300 µl with H₂O. For determination of total binding, the procedure was identical except that prior incubation of cells with cold CSAT was omitted. Radioactivity was counted in a Beckman gamma counter (Beckman Instruments), and protein concentration was determined by the method of Lowry et al. (41) using BSA as a standard. Specific binding values were determined by subtracting counts bound in the presence of saturating cold antibody from total counts bound.

The number of antigenic sites and their affinity for CSAT were estimated, assuming monovalent binding, using two methods: (a) from the plateau and half-maximal binding values observed in hyperbolic plots of antigen binding versus antibody concentration; (b) from a closed, linearization of the binding data (17):

$$\text{antibody bound} = (\text{Max antibody bound}) - K_d \frac{(\text{antibody bound})}{(\text{total antibody added})}$$

The binding at very low antibody concentrations appeared to deviate somewhat from a simple hyperbola. This was paralleled in the linear representations of the data. The omission of the anomalous data did not significantly affect estimates of the number of sites (i.e. maximum antibody bound), and it affected (decreased) the K_d estimates less than twofold. Since these low concentration data were not available for all determinations, they were not included in any of the estimates.

Rounding and Detachment Assays

For microscopic observation of rounding by CSAT, the antibody at various concentrations was added to myogenic or fibrogenic monolayers in DME containing 1% HS. For quantitative measurements of cell detachment, cell monolayers in 35-mm plates were incubated with the indicated amount of antibody in 1 ml of DME containing 1% HS. Control cultures with mouse IgG or other purified monoclonal antibodies or with no antibody received the same medium and underwent identical manipulations. At indicated times, medium containing detached cells was aspirated from triplicate cultures. The cells remaining attached to the dish were removed by incubation for 5 min in 0.25% trypsin in versene. Each of these fractions, representing cells detached or cells remaining after antibody treatment, were diluted into 8 ml of isotonic buffered saline (Scientific Products, McGraw Park, IL) and counted in a Model F Coulter cell counter (Coulter Electronics, Hialeah, FL). Data were expressed as the fraction of cells detached by CSAT (50–60% maximally) minus the fraction of detached cells in control (10–20%) cultures multiplied by 100.

Creatine Phosphokinase Assay

Creatine phosphokinase (CPK) activity was determined colorimetrically using a hexokinase/glucose-6-phosphate dehydrogenase reagent kit supplied by Sigma Chemical Co. (St. Louis, MO). Cells were grown on 35-mm tissue culture dishes in EGTA containing basic medium. 48-h cultures were treated with CSAT. The cells remaining attached to the tissue culture dishes after this treatment were refed, and the detached cells replated with calcium-containing basic medium. Control cultures were treated identically except that no CSAT was added. At the desired time, monolayers were rinsed 3 × with Hanks's Balanced Salt Solution containing 20 mM HEPES pH 7.4 and frozen at –20°C for up to 3 wk. After

thawing, cells were then scraped in 0.5 ml of 0.5 M glycylglycine buffer, pH 6.7, containing 2 mM dithiothreitol, sonicated on ice for 10 s and spun for 30 s in an Eppendorf microfuge. The supernatants were used for determination of enzyme activity. Protein concentration was determined by the method of Lowry et al. (41). Data are expressed as International Units of CPK activity per milligram protein.

Measurement of Fusion

Fusion was measured in suspension as detailed elsewhere (44). In brief, cells were grown in suspension in basic medium containing EGTA in agarose-coated plates to produce fusion-competent cells (48–52 h). Antibody and calcium were added at the indicated times. At appropriate times after the addition of calcium, cells were trypsinized to single cells and sized qualitatively by counting at two different thresholds in a Model F Coulter cell counter (Coulter Electronics). Data are expressed as percent control (i.e. + Ca^{++} cultures).

Immunofluorescence Microscopy

Myoblasts were plated onto collagen-coated glass coverslips in medium with normal calcium levels (1.8 mM) and used either 48 or 72 h later. Fibroblasts were plated onto glass coverslips in the same medium and used 24 h later. Two different fixation protocols were used. (a) Cells were fixed with 2 paraformaldehyde in PBS (pH 7.2) for 20 min. After rinsing in PBS containing 0.15 M glycine and PBS alone, cells were permeabilized with acetone at -20°C for 3 min. The cells were incubated with 50 $\mu\text{g}/\text{ml}$ affinity-purified CSAT in PBS containing 2 mg/ml normal goat globulin (PBS NGG) for 1 h at 25°C . After rinsing with PBS NGG, cells were stained for 1 h with a 1:64 dilution in PBS NGG of rhodamine-conjugated goat anti-mouse IgG (Cappel Laboratories: R-GAM) which had been absorbed on monolayers of fixed permeabilized chick fibroblasts (50) and on gelatin-coated dishes to remove nonspecific staining. (b) Living cells were incubated with CSAT at 25°C for 30 min. Cells were then fixed and processed as described above.

Labeling of Embryo Fibroblasts

Chick embryo fibroblasts were grown to near confluence in 490 cm^2 plastic roller bottles (Corning Glass Works, Corning, NY) in basic medium containing calcium. The monolayers were then washed three times with PBS. 10 ml of Minimal Essential Medium (MEM) made up in Hanks' salts, containing no methionine and supplemented with ITS Premix (a mixture of insulin, transferrin, and selenium purchased from Collaborative Research, Inc., Waltham, MA), was added to each roller bottle. 100 μCi of L- ^{35}S methionine (1026.9 Ci/mmol translation grade, New England Nuclear, Boston, MA) was added to the roller bottles, and cells were incubated for 6 h at 37°C .

Extraction of Antigen

Antigen was extracted from 10 roller bottles containing $\sim 1 \times 10^8$ cells. After labeling with L- ^{35}S methionine, the monolayers were washed five times with PBS. Cells were harvested with 1 mM EDTA in PBS and washed three times in cold PBS. The final pellet was resuspended in 20 ml of 0.01 M tris acetate buffer pH 8.0 containing 0.002 M phenylmethylsulfonyl fluoride and 0.5% Nonidet P-40 (NP-40). Extraction was carried out at 4°C for 15 min. The extract was centrifuged, and the supernatant subjected to acetic acid precipitation and subsequent acetone precipitation as described by Knudsen et al. (36). The final acetone precipitate was dissolved in 1.5 ml of PBS containing 0.5% NP-40.

Antibody Affinity Chromatography

CSAT (1 mg) was conjugated to 0.5 mg of cyanogen bromide activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) according to the manufacturer's instructions. A control column was prepared by conjugating 1 mg of antibody from the ascites fluid of a mouse injected with a hybridoma producing an unrelated antibody directed against an antigen on hamster cells. Water-jacketed columns were packed with each preparation. The dimensions of the bed were $\sim 1 \times 0.5$ cm. 200 ml of an acetone precipitate dissolved in PBS containing 0.5% NP-40 was loaded onto each column and allowed to stand at 4°C for 20 min. Each column was then washed exhaustively with 20 times the bed volume of PBS NP-40 until the radioactivity eluting from the column was near background. Bound radioactivity was eluted with 0.2 M acetic acid containing 0.5% NP-40. 400 μl were added to each column, and allowed to stand for 10 min. The released counts were then eluted by continued washing of the column with 0.2 M acetic acid containing 0.5% NP-40; 400- μl fractions were collected. The fractions containing the most radioactivity were pooled, the pH was adjusted to 7.0 and the protein precipitated overnight at -20°C in 6 vol of acetone. The precipitates

were harvested by centrifugation and redissolved in 100 μl of PBS NP-40. Samples were taken for SDS PAGE and for testing of blocking activity.

Blocking Assay

The antigen of interest was monitored during NP-40 extraction and fraction-

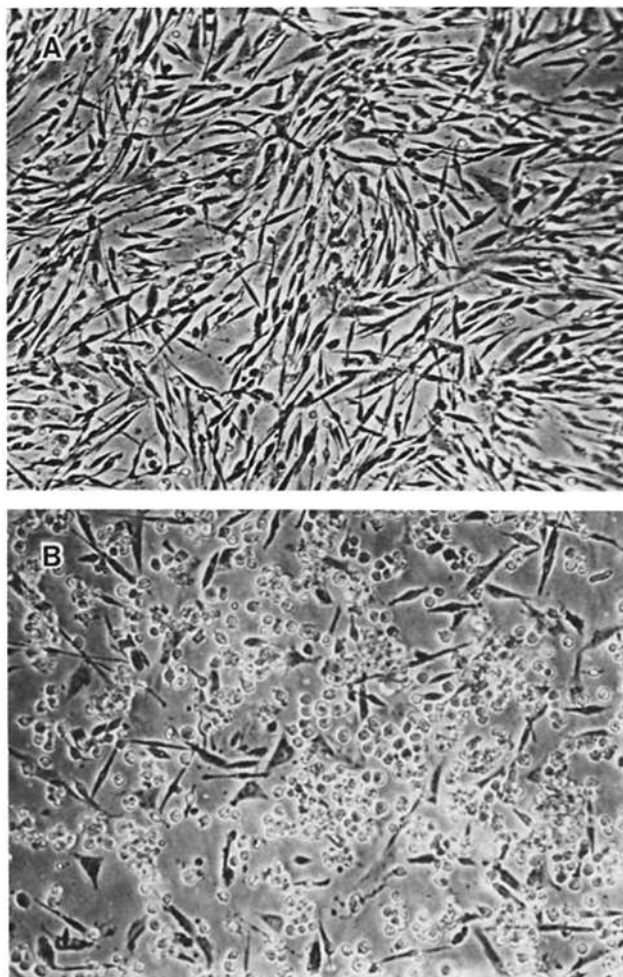


FIGURE 1 48-h myoblasts cultured in the presence of EGTA 0.3 mM. (A) Cell monolayer incubated for 4 h in DME + 1% horse serum and (B) cell monolayer incubated in the same medium containing CSAT for 4 h. $\times 90$.

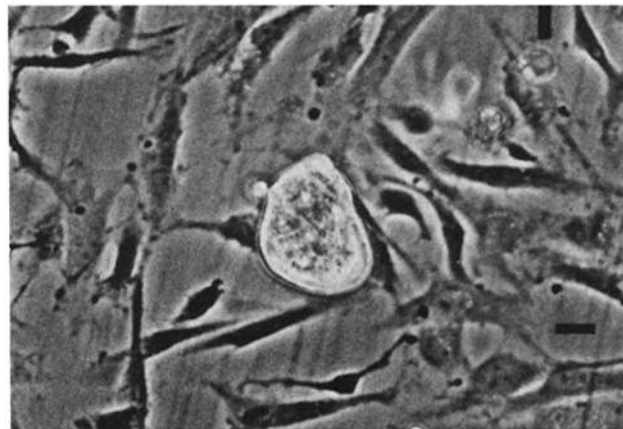


FIGURE 2 Myoblast formation in the presence of CSAT. CSAT and Ca^{++} (1.8 mM) were added to 48-h EGTA grown myoblast monolayer cultures. Picture was taken 12 h later. Bar, 20 μm . $\times 375$.

ation by following its ability to interfere with CSAT-induced rounding and detachment of myoblasts in a blocking assay similar to that described by Knudsen et al. (36). Myoblasts were plated in 96-well microtiter plates (Linbro Scientific, Hamden, CT) at a concentration of 1×10^4 cells per well in basic medium. Cells were used in the blocking assay 48 h after plating. To detect blocking activity, 30, 15, and 7.5 μ l of antigen were mixed with 30 μ l of MEM containing 20% FCS, 2 \times vitamins and amino acids, and 0.6 μ g CSAT. The mixture was brought to a total volume of 60 μ l with PBS where necessary. Detergent was removed by

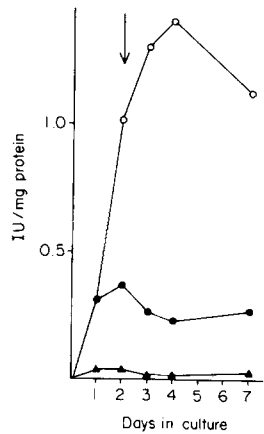


FIGURE 3 Creatine phosphokinase specific activity of cells either removed or left adhering to the tissue culture plate after incubation of 48-h myoblasts in DME containing CSAT (1:2 dilution of hybridoma supernatant) for 4 h. Detached cells were spun out of antibody-containing medium, washed, and replated in basic medium containing calcium. Cells remaining attached were fed with the same medium. Fibroblasts were cultured as described in Materials and Methods. CPK activity was assayed, as described in Materials and Methods, at the indicated times. Arrow indicates time of application of antibody.

Cells removed after application of antibody (○). Cells remaining after application of antibody (●); fibroblasts (▲).

extraction with SM-2 beads (Bio-Rad, Richmond, CA) as described by Knudsen et al. (36), and the supernatants from each tube were added to individual microtiter wells. Controls consisted of wells containing Biobead extracted PBS NP-40 in the 2 \times MEM mixture containing 0.6 μ g CSAT, and also PBS NP-40 in 2 \times MEM containing no CSAT. Test samples were judged to contain the antigen if the CSAT failed to round and detach the myoblasts within 4 h after addition of the test mixture.

SDS PAGE

Samples were run on SDS polyacrylamide gels and autoradiographs prepared exactly as described by Knudsen et al. (36).

RESULTS

CSAT Selectively Rounds and Detaches Myogenic Cells

The addition of hybridoma supernatant (or purified antibody) to a 52-h culture of chick pectoral myoblasts rounds and detaches a majority of the cells (Fig. 1 B). Although the photomicrographs in Fig. 1 are of cells grown in a low calcium medium for 52 h (to produce a population of mononucleate cells), analogous results were also observed for myogenic cells grown in medium containing normal calcium levels. These cultures contain populations of fibroblasts in addition to myogenic cells. Inspection of the morphology of the cells remaining on the tissue culture dish after antibody treatment shows an enrichment in cells possessing a fibroblastic morphology

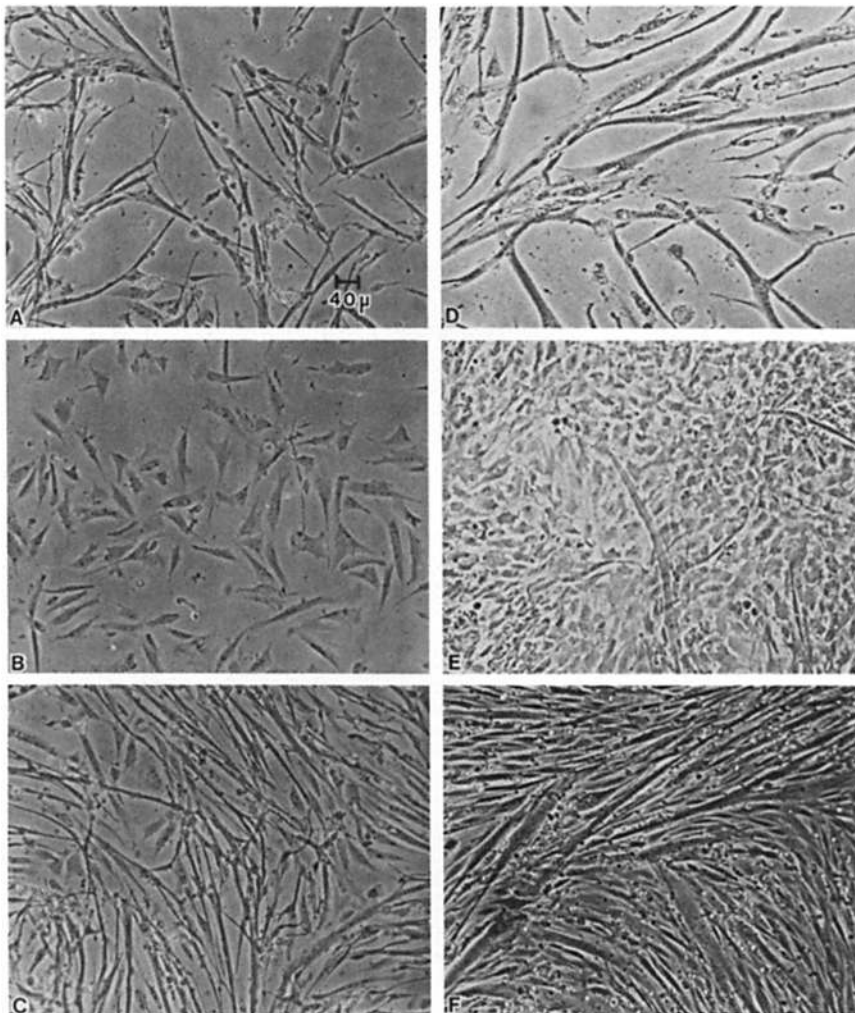


FIGURE 4 Morphology of cells remaining attached or removed after treatment of 48-h myoblast with CSAT. Conditions were as described in the legend to Fig. 3. Cells removed by the antibody and then replated 2 (A) and 5 d (D) after removal of with CSAT. Cells remaining attached 2 (B) and 5 d (E) after incubation of 48-h monolayer with CSAT. Control monolayers 4 (C) and 7 d (F) after seeding. $\times 75$.

(Fig. 1 B). In contrast, cells possessing the spindle shape characteristic of myoblasts are no longer present in significant quantities. When fusion-promoting (normal) calcium levels are added along with the antibody to fusion-competent cells, the rounded cells fuse rapidly in suspension, forming multinucleate "myoballs" (Fig. 2) (32).

The morphologic indication of selectivity by the antibody was confirmed by assays of two phenomena associated with myogenic cells: creatine phosphokinase (CPK) specific activity and fusion. 48-h cultures were incubated with CSAT. The detached cells were gently aspirated, washed, and replated; fresh medium was added to the cells remaining on the dish. The replated cells and the cells remaining on the dish were then scored, at subsequent time intervals, for CPK specific activity and fusion. The CPK activities of these two populations of cells are shown in Fig. 3. The CPK activity for the cells detached by the antibody was 4-5 times higher than that of the remaining cells. The CPK activity of cells remaining substrate attached after antibody treatment was higher than that of control fibroblast cultures. This is due, in part, to the presence of myogenic cells remaining after the gentle aspiration used to remove the rounded cells. Myogenic cultures that were not incubated with CSAT displayed an intermediate CPK specific activity reflecting the mixed population of myoblasts and fibroblasts present in the culture (data not shown).

Phase-contrast micrographs of cells remaining substrate-attached after antibody treatment along with those of detached and replated cells are shown in Fig. 4. After incubation with antibody, the two cell populations were cultured in normal, fusion-promoting calcium levels. The photomicrographs of the detached and replated cells cultured subsequently for 2 and 5 d show that nearly all of the nuclei reside in multinucleate myotubes (Fig. 4 A and D). In contrast, the cells remaining after antibody treatment did not show significant fusion, exhibited a fibroblastic morphology, and proliferated to form a confluent monolayer (Fig. 4 B and E).

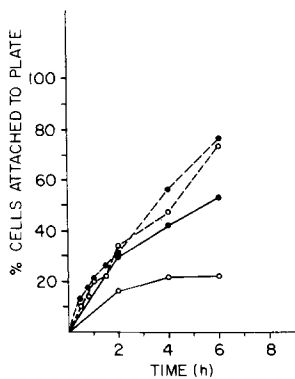


FIGURE 5 Attachment of fibroblasts and myoblasts to 0.1% gelatin-coated tissue culture dishes in the presence and absence of CSAT (1:10 dilution of hybridoma supernatant) in DME +10% HS. Fibroblasts were removed from stock plates by treatment with 5 ml of versene at 37°C for 5 min followed by gentle aspiration. The detached cells were incubated for 2 hr at 37°C in a 0.8% agarose-coated 100 mM petri dish in medium containing CSAT as described above. At the end of this

time, cells were centrifuged out of this medium and resuspended in fresh CSAT-containing medium and plated onto gelatin-coated 35-mm petri dishes. At the indicated times thereafter, medium containing unattached cells was removed, the monolayer washed 3X with Hanks' salts solution, and the attached cells were removed by incubation for 5 min in 0.25% trypsin in versene. The trypsinized cells were diluted into Isoton (Scientific Products) and counted in a Coulter cell counter. 48-h-EGTA-grown myoblasts were treated identically except that they were removed from stock plates with CSAT. Fibroblasts: -antibody (●---●) and +antibody (○---○) CSAT. Myoblasts: -antibody (●—●) and +antibody (○—○) CSAT.

CSAT Inhibits Attachment, But Not Fusion, of Myogenic Cells

The effect of CSAT on the initial adhesion of fibroblast and muscle cell cultures was determined by assaying the kinetics of their attachment to gelatin-coated tissue culture dishes (Fig. 5). In the absence of CSAT, both myoblasts and fibroblasts showed a monotonic increase in the fraction of cells adhering to the substratum during the 6- to 8-h incubation period. As anticipated, the rate of myoblast attachment was less than that of the fibroblasts.

Attachment in the presence of antibody provided a striking

TABLE I
Effect of CSAT on Fusion

Experiment	Condition	Control %
I	+Ca ⁺⁺ + CSAT (20 μg/ml)	107
II	+Ca ⁺⁺ + CSAT (4 μg/ml)	97
	+Ca ⁺⁺ + CSAT (40 μg/ml)	102
III	-Ca ⁺⁺ + CSAT (40 μg/ml)	0

Fusion was measured using the suspension assay described in Materials and Methods. Purified CSAT was added (at the indicated concentration) to EGTA-grown, 48-h myoblasts 12 h before addition of Ca⁺⁺ (1.8 mM). Control cultures received Ca⁺⁺ only.

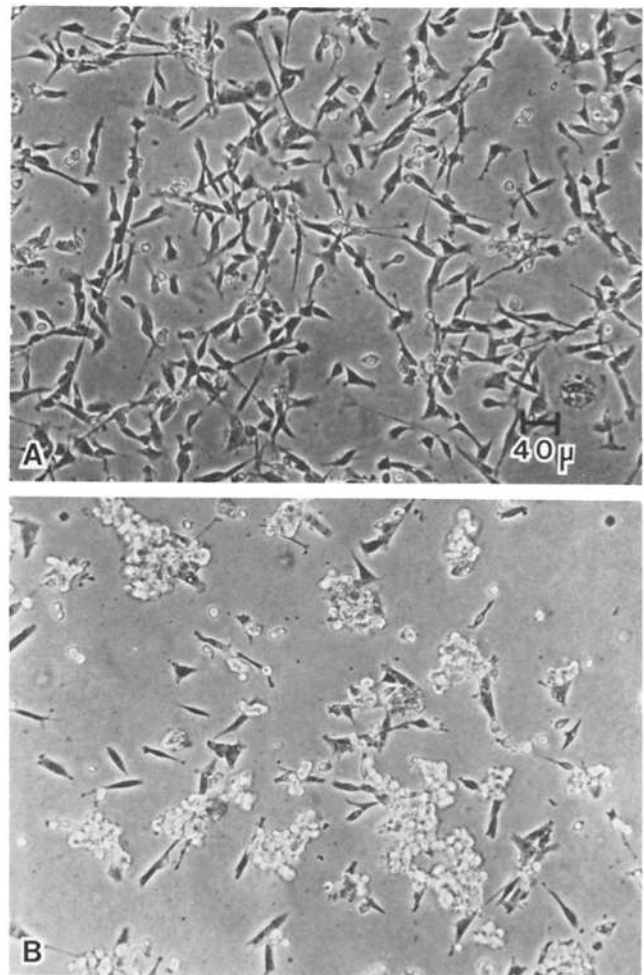


FIGURE 6 Effect of CSAT on myoblasts grown for 48 h in basic medium in the presence of PMA (1×10^{-7} M). (A) no antibody, DME + 1% HS. (B) CSAT, 5 μg/ml in DME + 1% HS for 1 h at 37°C. $\times 90$.

contrast between the two cell types. The attachment of fibroblasts was essentially identical to that of cells plated in the absence of antibody, whereas that of myoblasts was inhibited substantially (two- to threefold). The presence of contaminating fibroblasts likely accounts for some of the background adhesion.

The fusion of myoblasts into multinucleate myotubes is another adhesive property displayed by skeletal muscle. In one assay, myogenic cells were grown in a low calcium medium to produce a collection of fusion-competent myoblasts. CSAT was then added along with normal (fusion-promoting) levels of calcium. The myogenic cells detached and aggregated into clumps. Within a few hours, large cellular structures were apparent (Fig. 2). They are morphologically indistinguishable from the myoballs that form when myoblasts fuse in suspension (32, 41).

The influence of CSAT on fusion was measured directly using an assay of fusion in suspension. This assay is particularly sensitive to fusion after only short incubation times. The data in Table I reveal no detectable inhibition of fusion even using an antibody titer 16 times that required for rounding and detachment.

Effect of CSAT during Differentiation of Myogenic Cells and on Cells from Other Tissues and Species

CSAT produced morphologic changes in myogenic cells

throughout their differentiation in culture. In young, 24- to 48-h cultures, when little fusion has occurred, nearly all of the cells are readily rounded as described above. Myogenic cells seeded in low calcium medium and cultured for 48 h in the presence of phorbol-12-myristate-13 acetate (PMA) (Fig. 6) or 5-bromodeoxy uridine (1.6×10^{-4} M) (data not shown) do not differentiate normally and undergo extra rounds of replication (4, 28); they were rounded by CSAT at titers and incubation times similar to those of myoblast cultures.

Older cultures (3-11 d), in which extensive fusion has occurred, are also detached by CSAT; however, the details appear different. After addition of antibody, the initial morphologic change is a retraction and rounding of the longitudinal edges of the myotube (Fig. 7 *B* and *D*). Subsequently, one or both of the myotube ends round and detach. Entire myotubes were often seen floating in the medium. The kinetics of detachment of the older cultures were markedly slower than that of the younger cultures. For example, using $5 \mu\text{g}/\text{ml}$, a concentration of antibody sufficient to saturate all cellular sites (see below), 2-d myoblasts rounded in 15-30 min; in contrast, 4-d myotubes required about 2 h. Very high concentrations (40 times saturating levels) were required to detach the 11-d myotubes (Fig. 7). By culturing cells in a low calcium medium, we have assayed the effect of CSAT on myoblasts cultured for 96 h (data not shown). In contrast to the 96-h myotubes described above, the mononucleate cells were rapidly rounded and detached by the antibody.

The effect of CSAT on myogenic cells derived from other

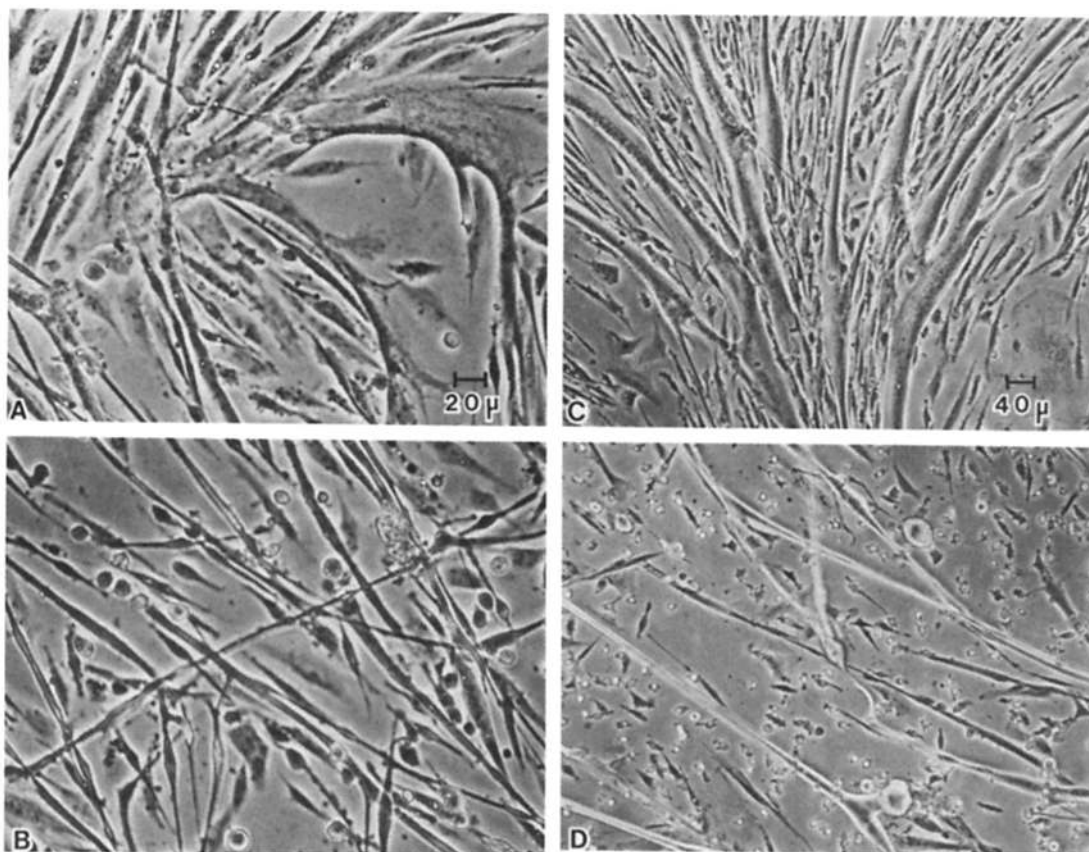


FIGURE 7 Myotube rounding and detachment in the presence of CSAT. (A and B) 72-h myotubes in absence (A) and presence (B) of CSAT ($50 \mu\text{g}/\text{ml}$, 4 h). (C and D): 10-day myotubes in absence (C) and presence (D) of CSAT ($100 \mu\text{g}/\text{ml}$, 4 h). All cells were in DME + 1% HS. $\times 200$. $\times 90$.

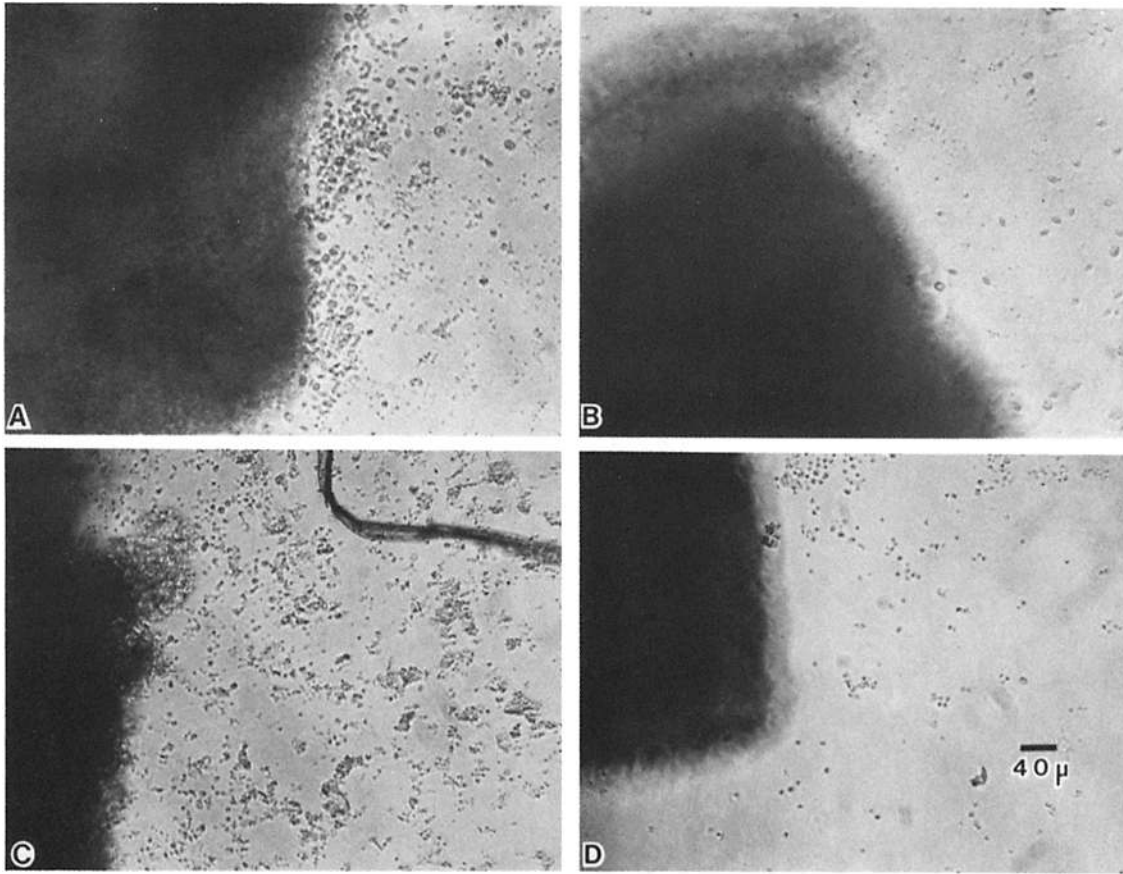


FIGURE 8 Release of cells from myo-aggregates and from pieces of 11-d chick embryo pectoral muscle. Myoblast aggregates: Myoblasts were grown for 48 h as aggregates in suspension culture in basic medium in the presence of 0.27 mM EGTA in 1.4-ml conical plastic tubes. Aggregates were washed in calcium- and magnesium-free Hanks's (CMF-H) and incubated at 37°C in the presence of 80 $\mu\text{g}/\text{ml}$ CSAT in DME + 1% HS. Control aggregates were incubated in the presence of 80 $\mu\text{g}/\text{ml}$ mouse IgG (Cappel Laboratories, Cochranville, PA). (A) 4 h after addition of CSAT. (B) Control. Explants: Pieces ($\sim 1 \text{ mm}^3$) of muscle were removed from 11-d chick embryo pectoral muscle and rinsed three times in CMF-H and incubated as described above. The edge of the muscle or myoaggregate is at the left side of each photograph. (C) 4 h after addition of CSAT and (D) control. $\times 125$.

TABLE II
Binding of CSAT to Myogenic and Fibrogenic Cells

Cell type	Ab Molecules bound per μg cell protein*	Ab Molecules bound per cell	Apparent K_d nM
Nonconfluent fibroblast	$45 \pm 1 \times 10^8$	$4.7 \pm 0.1 \times 10^5$	6 ± 2.5
48-h myoblast	$29 \pm 5 \times 10^8$	$1.8 \pm 0.3 \times 10^5$	3.2 ± 2.1

Binding was performed as described in Materials and Methods. The fibroblast data are the average of two experiments and the myoblast data the average of three.

* $103 \mu\text{g}/10^6$ fibroblasts, $62 \mu\text{g}/10^6$ myoblasts.

species and on other cell types was explored preliminarily (data not shown). Antibody titers used to round the chick myoblasts were without apparent effect on myoblasts derived from mouse and rat embryo or from human muscle explants; however, myogenic cells from quail were comparably rounded. Chick embryo fibroblast, 16-d tendon fibroblast, 5-d cardiac, and 6-d chick neural tube cultures were also treated with antibody. The neurones (cells with extensive processes) did not appear to round; however, an unidentified subpopulation (putative neural epithelial cells) did. The embryo fibroblasts and cardiac

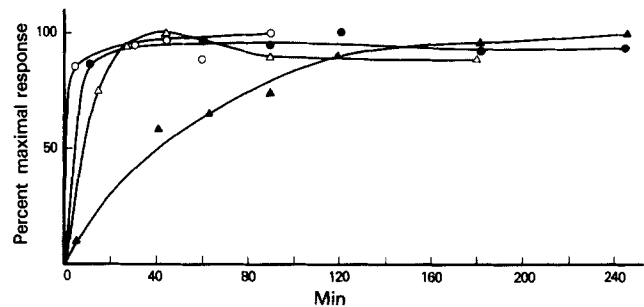


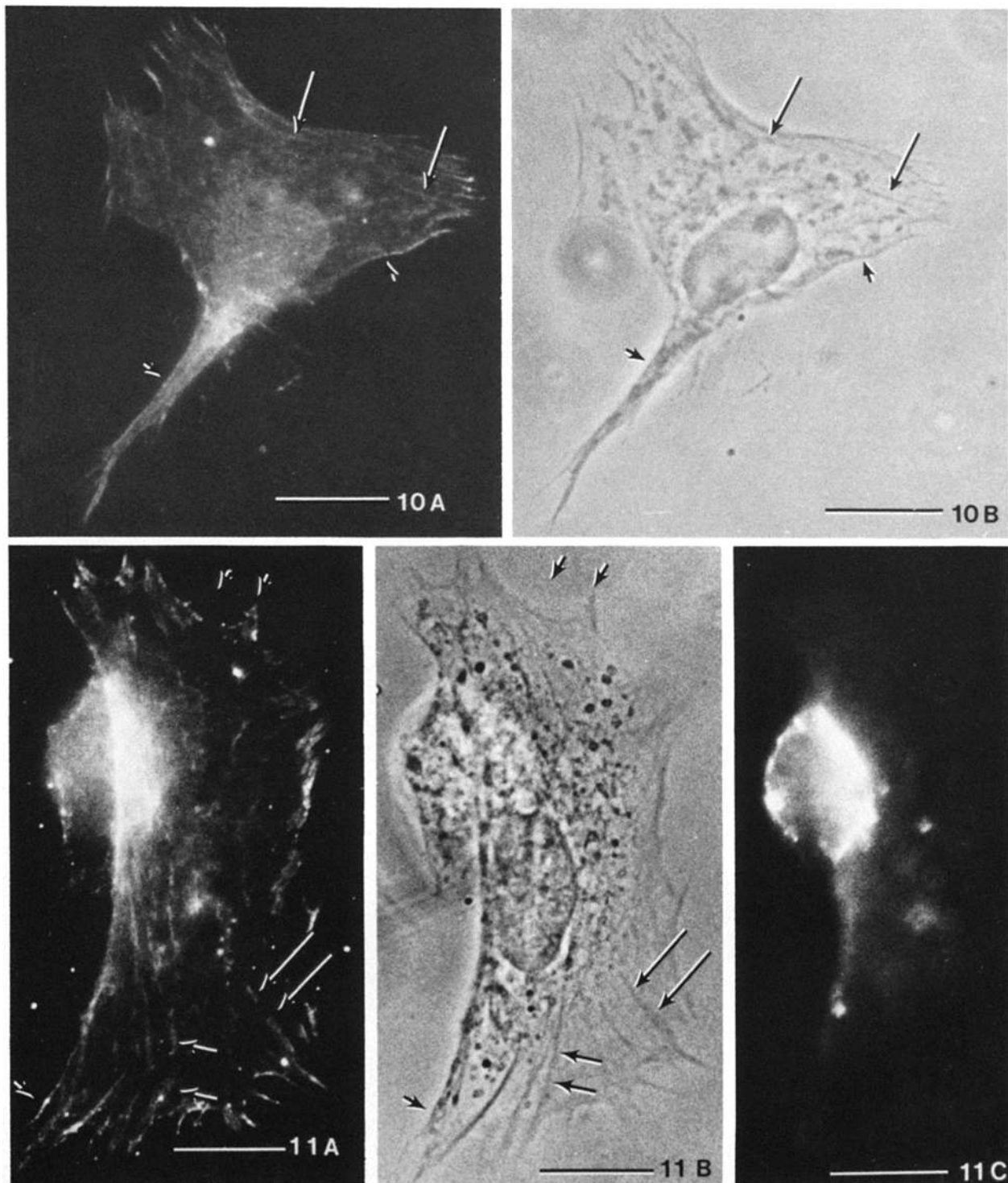
FIGURE 9 Kinetics of cell binding and cell detachment in presence of CSAT on 52-h EGTA grown myoblasts. Binding of ^{125}I -CSAT and cell detachment were assayed at two different antibody concentrations as described in Materials and Methods. Data are expressed as maximal response, i.e., highest number of antibody molecules bound at each concentration and total number of cells detached. 5 $\mu\text{g}/\text{ml}$ CSAT: (O) cell binding, (Δ) cell detachment; 0.6 $\mu\text{g}/\text{ml}$ CSAT, (\bullet) cell binding, (\blacktriangle) cell detachment.

cells were neither rounded nor detached by the antibody. However, embryo fibroblast cultures incubated at 4°C with CSAT appeared somewhat more refractile than did the control. The cardiac myoblasts, after a 24-h incubation with the antibody, appeared less well spread. In contrast to embryo fibro-

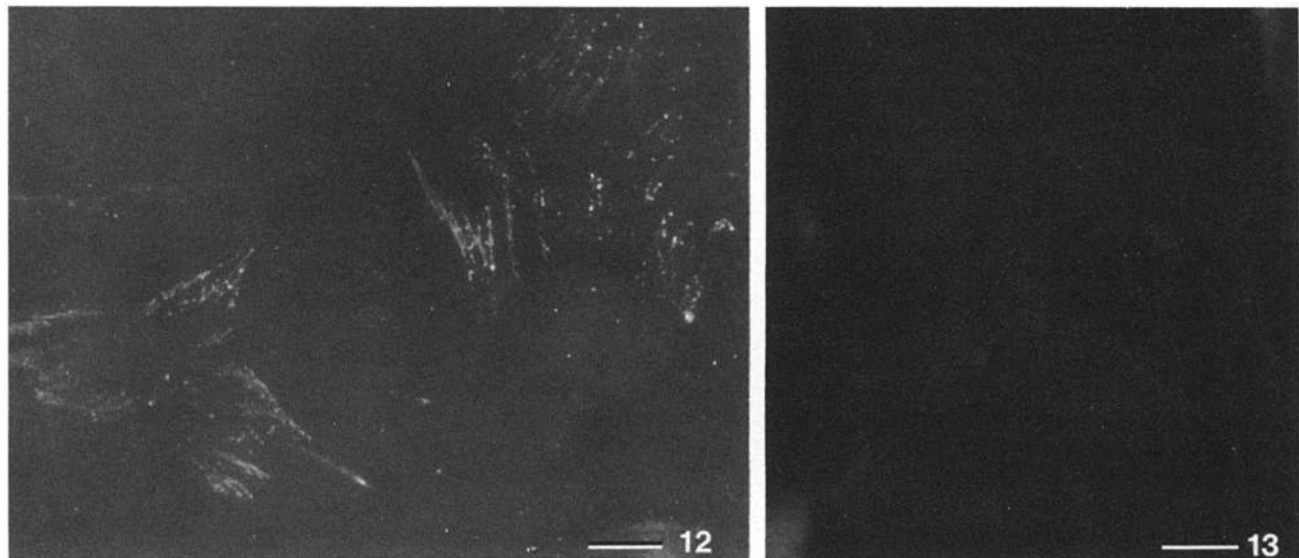
blasts, the tendon fibroblasts, at 37°C, were rounded by the antibody (J. Sasse, personal communication).

Small pieces of explanted tissue from 10-d embryo pectoralis

muscle were also treated with CSAT. Within a few hours, the tissue appeared partially dissociated; many cells and groups of cells separated from the tissue mass (Fig. 8 C). This was not



FIGURES 10 and 11 Fig. 10: Fluorescence (A) and phase-contrast (B) micrograph of a chick embryo fibroblast fixed with 2% paraformaldehyde and permeabilized with acetone at -20°C before staining with CSAT followed by Rhodamine-conjugated goat antimouse (R-GAM). Fluorescence staining is coincident with portions of stress fibers visible by phase optics (long arrows). Additional fine punctate staining appears along the edges of the cell (short arrows). $\times 1,900$. Fig. 11: A flat, well-spread chick embryo fibroblast and rounded cell stained for 1 h at 4°C with CSAT before fixation with 2% paraformaldehyde. R-GAM was applied after fixation. $\times 1,900$. Phase micrograph (B) shows prominent stress fibers within the thin, lamellar, fan-shaped portion of the cell photographed at the level of the substrate. The fluorescence micrograph (A) shows patches of fluorescence coincident with regions of the stress fibers (long arrows). Cell edges have punctate fluorescence (short arrows). (C) The same cell pair photographed in the focal plane level of the rounded cell. The fibroblast is out of the plane of focus. Bar, $12\ \mu\text{m}$. $\times 1,500$.



FIGURES 12 and 13 Fig. 12: Fluorescence micrograph of material left behind after detachment of cells which sometimes occurs during preparation by either method. The pattern of fluorescence is similar to that seen in Fig. 11 A. In many cases, material observed by fluorescence is coincident with bits of membrane visible by phase microscopy. $\times 1,000$. Fig. 13: Preimmune control. Cells were incubated with an affinity-purified monoclonal antibody directed toward a *Drosophila* protein before fixation and incubation with R-GAM. These cells were exposed twice as long as immune samples. $\times 1,000$.

observed in similar explants treated with a control antiserum (Fig. 8 D). Aggregates of myogenic cells cultured in suspension were similarly dissociated (Fig. 8 A and B).

Binding of CSAT to Myogenic and Fibrogenic Cells and Its Relation to Its Kinetics of Rounding and Detachment

The antigenic determinants for CSAT binding were characterized preliminarily by measuring the binding of iodinated antibody to isolated cells removed from their substratum. As Williams and Mason (42) have pointed out, antibody binding can be more complex than that expected for the simple association of a monovalent ligand with its receptor. However, our objective was only to estimate and compare the relative number of antigenic sites and their affinities on myoblasts and fibroblasts.

With 48-h cultures of myoblasts or nonconfluent fibroblasts, the number of sites/cell is 1.8×10^5 and 4.7×10^5 , respectively (Table II). This yields a myoblast-to-fibroblast binding ratio of 0.4 and agrees fairly well with the ratio of 0.5–0.6 computed using data from an enzyme-linked immunoassay on cell cultures fixed to their plastic substratum (data not shown). It implies a larger number of sites on fibroblasts than on myoblasts; however, the size of these two cells is not identical—rounded myoblasts and fibroblasts have diameters of $\sim 10 \mu\text{m}$ and $15 \mu\text{m}$, respectively. When the number of sites is normalized to cell surface area, the resulting antigen concentrations on the two cell types appear similar.

The number of cellular sites was measured at different culture ages and on cultures grown in different calcium concentrations (data not shown). Between 24 and 96 h in culture, the concentration of sites changed (increased) at most twofold. The calcium concentration in the growth medium also had only a small (<33%), if any, effect. Incubating myogenic cells with 0.1% trypsin for 10 min at 37°C reduced the number of binding sites by half. Measurements of antibody binding to the

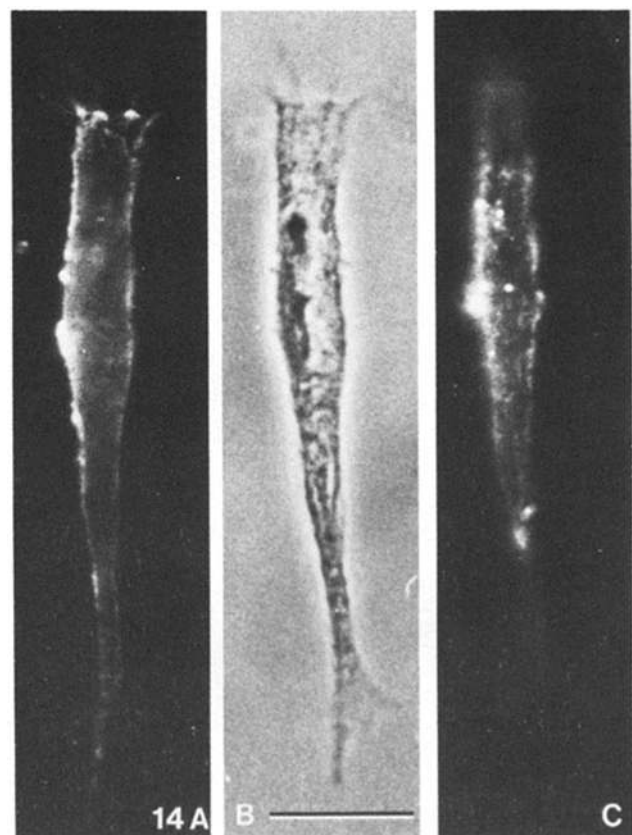


FIGURE 14 An elongated binucleate cell from a 48-hour myoblast culture plated onto collagen-coated coverslips and exposed to CSAT for 30 min. at 25°C before fixation. When photographed at the level of the substrate (A) fluorescent spots outline the edges of the cell. Punctate fluorescence is also present on the dorsal surface of the cell (C). A phase-contrast micrograph is shown in B. $\times 1,500$.

substrata from which myogenic cells had been removed using EDTA revealed no detectable binding, i.e. the number of

substrate-associated sites was <10% that seen on the dissociated cells.

The apparent K_d values (Table II) for both myoblasts and fibroblasts fell into a range of values between 2 and 5×10^{-9} molar. Much of this variation reflects experimental error.

The kinetics of cell detachment at fractional and saturating antibody concentrations are shown in Fig. 9. Using an antibody titer sufficient to saturate all cellular sites ($5 \mu\text{g}/\text{ml}$), the rounding and detachment is nearly complete after only 15–30 min. Fig. 9 also shows the kinetics of antibody binding which is somewhat faster than that for rounding and detachment. The kinetics of detachment using $0.6 \mu\text{g}/\text{ml}$ antibody, a titer sufficient to occupy ~10% of the cellular sites, are markedly slower than those using a high titer; roughly, 1.5 h are required to round and detach most of the cells

Distribution of Antigen on Myogenic and Fibrogenic Cells

To determine the cellular distribution of antigen to which CSAT binds, indirect immunofluorescence was performed on fibroblasts (Figs. 10–13) and on cultures containing primarily myogenic cells (Figs. 14 and 15). Fibroblasts, fixed and permeabilized before staining, showed a very fine punctate fluorescence which followed the edges of the cell at the level of the substratum (Fig. 10A). Linear patches of fluorescence were also present which were coincident with portions of stress fibers

visible by phase microscopy (Fig. 10B). Fig. 11 shows a well-spread fibroblast along with a rounded cell stained with CSAT before fixation and staining with R-GAM. Again, the staining pattern in the spread fibroblast (Fig. 11A) shows edge fluorescence and coincidence between portions of stress fibers, visible by phase microscopy (Fig. 11A and B). The rounded cell has bright staining over its entire surface (Fig. 11C). In cultures prepared by either of the above methods, patterns of fluorescence were sometimes observed that resembled the shapes of fibroblasts but for which there were no corresponding intact cells visible by phase microscopy (Fig. 12). Usually, these patterns corresponded to membrane remnants visible by phase microscopy that were left behind by cells that were detached by some means during preparation. The staining patterns observed for CSAT on fibroblasts (Fig. 10–12) and their relation to stress fibers are similar to those reported by other investigators for adhesion plaques and for adhesion-related molecules such as fibronectin and vinculin (6, 11, 12, 22, 31, 56).

Myogenic cultures incubated with CSAT before fixation showed cells in various stages of rounding. Fig. 14 shows an elongated, adherent binucleate cell. When the cell is photographed at the level of the substratum (Fig. 14A), a distinct punctate edge fluorescence is visible around the entire cell. Punctate fluorescence is also visible on the upper surface of the cell (Fig. 14C). Fig. 15 shows a field containing long myotubes as well as several single cells. The large branched myotube in

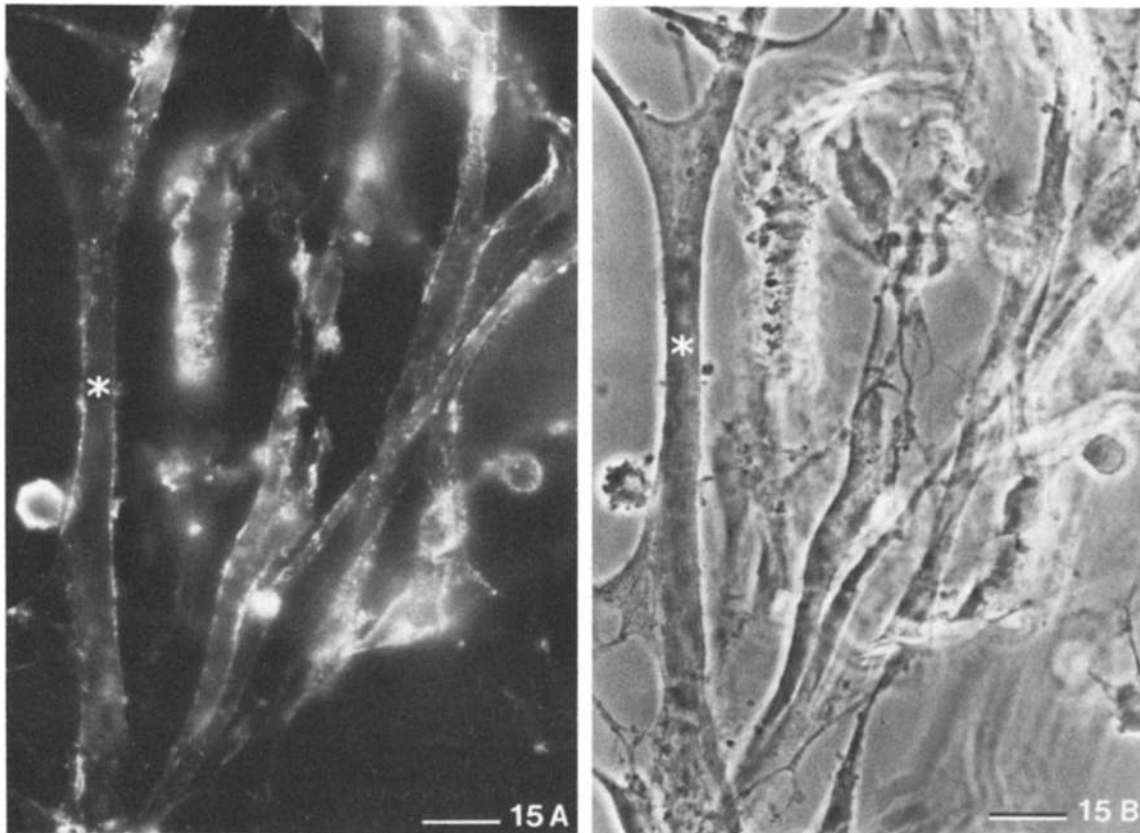


FIGURE 15 A field of mononucleate and multinucleate cells from a 72-h myogenic cell preparation plated onto collagen-coated cover slips and exposed to CSAT before fixation. Cells are present at all stages of rounding. The long branched myotube (*) appears still to be anchored to substrate. Other cells, presumably myoblasts, are completely rounded. There are also single mononucleate cells which range in morphology from flat to partially rounded. All cells display a punctate peripheral fluorescence. The large hazy structure in the field is probably a myotube which has become detached at one end and is draped over the field out of the plane of focus. A phase-contrast micrograph is shown in B. $\times 800$.

Fig. 15 appears to be anchored at both ends. Punctate fluorescence is present over the entire surface of the myotube. The other individual cells in the field show bright punctate fluorescence at all stages of rounding. Cells fixed and opened before staining also showed staining over their entire surface. The myotubes appeared flatter (as illustrated in Fig. 7) and the punctate nature of the staining, while evident, was not as dramatic as that seen when cells were incubated with CSAT before fixation (not shown).

Identification of the Antigen to Which CSAT Binds

The antigen to which CSAT binds was isolated by antibody-

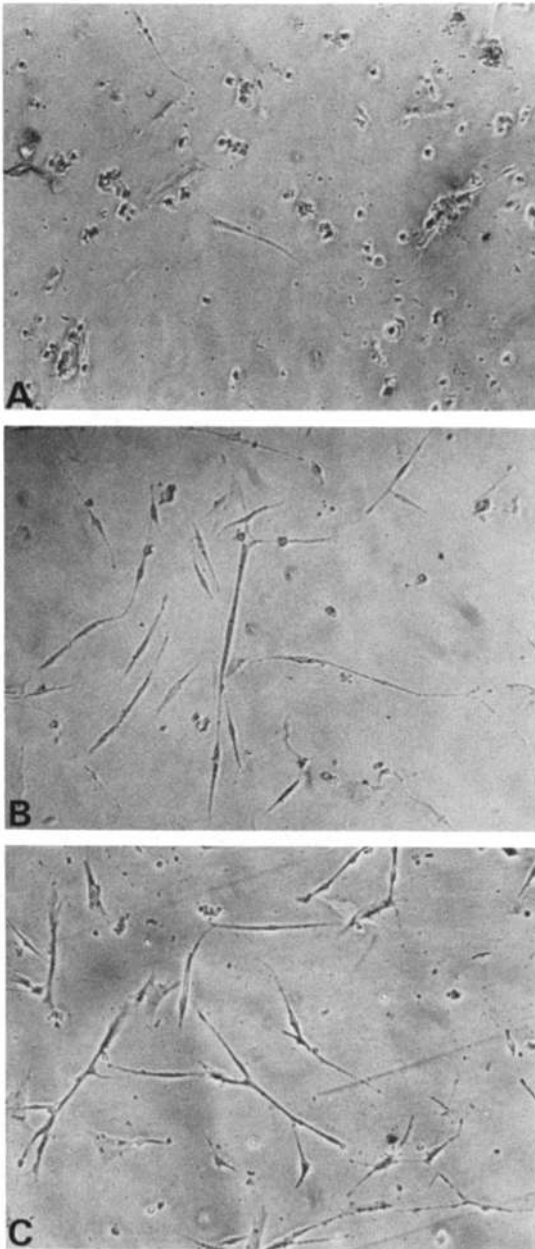
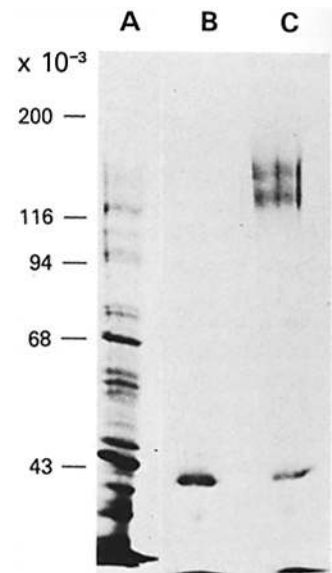


FIGURE 16 Blocking of CSAT rounding activity by a partially purified extract of chick embryo fibroblasts. Chick myoblasts were seeded and assayed for blocking activity as described in Materials and Methods. A, 1 μ g/ml CSAT; B, no CSAT; C, 1 μ g/ml CSAT + a partially purified extract of chick embryo fibroblasts treated as described in Materials and Methods. $\times 75$.

FIGURE 17 SDS PAGE profile of antigen bound to CSAT-antibody affinity column. NP-40 extract of L- 35 S]methionine-labeled chick fibroblasts was prepared and applied to antibody affinity columns as described in Materials and Methods. Control column was prepared using antibody isolated from the ascites fluid induced by an unrelated hybridoma. Lane A, autoradiogram of unfractionated extract; Lane B, autoradiogram of materials eluted at low pH from control column; Lane C, autoradiogram of material eluted at low pH from CSAT-affinity column.



affinity chromatography and identified by SDS PAGE. Chick embryo fibroblasts were labeled by growth in [35 S]methionine and harvested with EDTA. The membrane proteins were solubilized in NP-40, partially purified by solvent fractionation, and absorbed to a Sepharose column to which CSAT had been covalently derivatized. As a control, a similar column was prepared containing an identical amount of antibody protein from mouse ascites fluid formed by a hybridoma prepared against hamster cells. Identical portions of the NP-40 extract were passed over each column, and the material bound was eluted with acetic acid. Fractions of eluate were assayed for their ability to block the antibody-induced changes in morphology and adhesion. A typical blocking assay is shown in Fig. 16. When the columns were loaded with identical, initial antigen concentrations, the material eluted from the CSAT affinity column, but not that eluted from the control column, blocked effect of CSAT on myoblast cultures.

To identify the antigens possibly responsible for this blocking activity, unfractionated material (Fig. 17A) and samples of eluate from both the control (Fig. 17B) and CSAT columns (Fig. 17C) were analyzed by electrophoresis on SDS polyacrylamide gels. Both lanes B and C (Fig. 17) contain low molecular weight material in common; however, the material eluting from the CSAT column (Fig. 17C) contains, in addition, two clearly resolved and unique polypeptides with apparent molecular weights of 120 kd and 160 kd.

DISCUSSION

A number of mechanisms not related directly to adhesion or morphology could account for the rounding and detaching activity of CSAT. These indirect mechanisms, which include general metabolic or structural perturbations, appear unlikely. The antibody-treated cells exclude trypan blue, spontaneously contract and, when the antibody is removed, even after continuous exposures as long as 7 d, the cells reattach and continue to grow. This observation argues against a general cytotoxicity. Both adhesion and fusion of myoblasts into myotubes are reported to be sensitive to a large number of structural and metabolic alterations including perturbations of energy metabolism, protein synthesis, and treatment with proteases (16, 26, 27, 35). Since fusion is not affected by the antibody, these indirect mechanisms appear unlikely as well. Both adhesion

and fusion are also sensitive to drugs which enter the cell and affect the cytoskeleton (27). Treatment of myoblasts with cytochalasin B, for example, produces a rounded morphology. However, cytochalasin B also inhibits fusion and causes fibroblasts to arborize. The antibody does neither. Finally, the antibody is unique among ~200 that we have scored for detaching activity, and the number of cell surface sites to which it binds is similar to that of other monoclonal antibodies (42). This makes a general steric masking of sites an unlikely mechanism as well. The elimination of these nonspecific or indirect mechanisms as explanations for the action of CSAT suggests that the antigen to which CSAT binds is involved more directly in maintenance of cell substratum adhesion and cellular morphology. This is suggested further by the very short time interval between antibody binding and detachment.

We have identified the antigen(s) on chick embryo fibroblasts to which CSAT binds and which very likely are involved in regulating myoblast morphology and adhesion. The molecules in the 120 to 160-kd region identified on SDS gels are the only observable components retained on the CSAT, but not the control, antibody column. In addition, the eluate from the CSAT column inhibits the antibody-induced rounding and detachment whereas that from the control column does not. The migration of the column eluate on SDS gels indicates that two components may be retained on the CSAT column. Whether this reflects proteolysis, antigenic heterogeneity, coprecipitation with other molecules, or subunit composition remains to be determined. The identification, in this report, of antigens in the 120- to 160-kd range as being relevant to cell substratum adhesion in chick myoblasts is similar to results described for substrate adhesion-related integral membrane glycoproteins from mammalian fibroblasts (36) and epithelial cells (14).

The nature of the antigen is of particular interest. Extracellular molecules such as fibronectin provide obvious candidates; however, our evidence indicates that alternative molecules should be considered. The apparent molecular weight of the antigen differs from that of fibronectin. Furthermore, the distribution of antigen revealed by fluorescent antibodies is distinctly different from that of either fibronectin or collagen (5, 12, 52, 58). The fluorescence distribution also indicates that the antigen is associated primarily with the cell surface rather than with extracellular material. The apparent absence of antigen on substrata from which myoblasts have been removed using either antibody or EDTA further argues against an extracellular matrix locale. These observations along with resistance of a significant fraction of the antigen (as observed by indirect immunofluorescence) to extraction with 1 M urea (data not shown) are consistent with an integral membrane association.

The morphology and adhesion-modulating properties of CSAT is cell-type dependent. Skeletal myogenic cells, tendon fibroblasts, and an unidentified cell type present in cultures from neural tube are rounded and detached; however, cardiac, skeletal, and embryo fibroblasts, neurones, and cardiac myoblasts are not. The similar numbers of antibody binding sites and affinities on myoblasts, myotubes (data not shown), and embryo fibroblasts along with the monoclonality of CSAT and data from blocking experiments suggest that the same antigenic determinant is present (comparable assays have not yet been performed on the other cells). While the antigenic determinant, when present, may function differently on these cells, antigen accessibility and new or additional adhesive interactions provide alternative explanations for the differential effect of the

antibody on these cells. Our observation that the antibody does not inhibit embryo fibroblast attachment makes antigen accessibility a less attractive alternative. In any case, these observations demonstrate the complexity and diversity of adhesive processes.

The action of this monoclonal antibody demonstrates an intimate link, via a membrane protein, between morphology and adhesion in myogenic cells. The current literature on the adhesion and morphology of fibroblasts suggests that these phenomena are interrelated and controlled through a transmembrane coupling mechanism involving the cytoskeleton (6, 10, 11, 31, 55, 56), proteins which might anchor the cytoskeleton to the membrane (10, 21, 22), putative integral membrane proteins (11), and extracellular components with which these membrane proteins interact (6, 13, 49, 62). The most striking example of such a coupling occurs at the focal contact or adhesion plaque. These are sites of closest approach of the cell surface to the substratum and at which cytoplasmic microfilament bundles, or stress fibers, terminate (1, 25, 32, 48, 59). The close contact, a putative adhesion site that is somewhat farther separated from the substratum than the focal contact (25, 32), as well as other microfilament-membrane linkages with associated fibronectin provide further examples (10, 40, 47, 53). Although the sites of membrane-extracellular matrix and membrane-microfilament association in myogenic cells have not yet been well characterized, presumably there are parallels with those just described for fibroblasts. Thus, the most straightforward mechanism for the antibody-induced rounding and detachment is a perturbation of the hypothesized transmembrane link between extracellular macromolecules and the cytoskeleton at adhesion sites. However, due to the intimate relation between the cytoskeleton, adhesion, and morphology, other sites of membrane-cytoskeleton and membrane-extracellular matrix interaction could provide alternative candidates.

We are fortunate to have many generous colleagues who have contributed advice, discussion, and methods. They are Drs. H. Holtzer, R. Kennett, K. Bechtol, A. Sessions, V. Bedian, and K. Knudsen.

These studies were supported by National Institutes of Health (NIH) grants GM 23244, CD 53C, CA 19144, CA 10815, and by the H. M. Watts, Jr. Neuromuscular Disease Research Center. N. T. Neff was a postdoctoral trainee of the NIH (HD07152).

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Received for publication 28 May 1982, and in revised form 19 July 1982.

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