Separation of Precursor Myogenic and Chondrogenic Cells in Early Limb Bud Mesenchyme by a Monoclonal Antibody

J. SASSE, A. HORWITZ,* M. PACIFICI, and H. HOLTZER
Departments of Anatomy and *Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia 19104

ABSTRACT We have addressed the problem of the segregation of cell lineages during the development of cartilage and muscle in the chick limb bud. The following experiments demonstrate that early limb buds consist of at least two independent subpopulations of committed precursor cells—those in (a) the myogenic and (b) the chondrogenic lineage— which can be physically separated. Cells obtained from stage 20, 21, and 22 limb buds were cultured for 5 h in the presence of a monoclonal antibody that was originally isolated for its ability to detach preferentially myogenic cells from extracellular matrices. The detached limb bud cells were collected and replated in normal medium. Within 2 d nearly all of the replated cells had differentiated into myoblasts and myotubes; no chondroblasts differentiated in these cultures. In contrast, the original adherent population that remained after the antibody-induced detachment of the myogenic cells differentiated largely into cartilage and was devoid of muscle. Rearing the antibody-detached cells (i.e., replicating myogenic precursors and postmitotic myoblasts) in medium known to promote chondrogenesis did not induce these cells to chondrify. Conversely, rearing the attached precursor cells (i.e., chondrogenic precursors) in medium known to promote myogenesis did not induce these cells to undergo myogenesis. The definitive mononucleated myoblasts and multinucleated myotubes were identified by muscle-specific antibodies against light meromyosin or desmin, whereas the definitive chondroblasts were identified by a monoclonal antibody against the keratan sulfate chains of the cartilage-specific sulfated proteoglycan. These findings are interpreted as supporting the lineage hypothesis in which the differentiation program of a cell is determined by means of transit through compartments of a lineage.

The concept of cell lineages has provided a model for understanding cell diversification in developing somites and limb buds (1, 20, 28–36). According to this view, the generation of terminal phenotypes follows an invariant pattern such that the fate of each descendent cell is predictable from its ancestry or position in the lineage tree. Early development involves the rapid emergence of a succession of early founder cells for multipotential lineages (31). These in turn yield secondary, tertiary, etc. lineages that proliferate extensively yielding first bipotential and finally unipotential lineages. This concept contrasts with other views that hypothesize the existence of populations in somites and limb buds of uncommitted, undifferentiated, and multipotent cells that, under specific environmental conditions, can be induced to differentiate into muscle, cartilage, or connective tissue cells (2, 3, 7–10, 22, 25, 40, 43, 50–52, 54, 56, 59, 60, 62).

There is much evidence supporting the lineage concept in the developing limb bud. In vitro cloning experiments first suggested this concept as a viable model. Single cells from early limb buds in the same culture dish yielded either chondrogenic-fibrogenic or myogenic-fibrogenic clones. Mixed clones of definitive chondrogenic and myogenic cells were never observed (19, 20, 28, 36). Subsequent in vivo experiments using quail-chick grafts demonstrated that cells from transplanted quail somites migrated into the chick limb buds when the latter first form. Somite-derived cells (e.g., myotome) appear to be the source of all future striated muscle that eventually appears in the mature limb, whereas all future cartilage and connective tissue cells appear to be derived from the somatopleure (13, 58).

The lineage hypothesis predicts that one should be physically able to separate and fractionate mesenchymal precursors

from the early limb into distinct subpopulations that, upon further differentiation, will express their unique predetermined phenotypes. In the present work, we fractionate the cell population from early chick limb buds into the major subpopulations—one containing predominantly precursor myogenic cells and the other precursor chondrogenic cells. This separation is based on the property of a monoclonal antibody, called CSAT, that has been shown to detach myogenic cells from extracellular matrices (17, 37, 38, 45). The basis for this separation appears to be a difference in the adhesion between these two subpopulations leading to a preferential rounding and detachment of the cells in the myogenic lineage by the CSAT antibody. These findings suggest that the ongoing differentiation program and phenotypic options of precursor limb bud cells are a function of their position in their respective lineages rather than the consequence of inductive interactions resulting from their position in the limb bud.

MATERIALS AND METHODS

Limb Bud Cell Cultures: Embryonated white Leghorn chick eggs (Shaw Farms, West Chester, PA) were used. Stage 20, 21, or 22 embryos (26) were selected and pooled separately. Wing and leg buds were cut away from the trunk region and were collected in Ca^{++},Mg-free saline. The tissue was dissociated into a cell suspension as previously described (5, 48) except that 0.1% Dispase II (neutral protease from *Bacillus polymyxa*, Boehringer-Mannheim Biochemicals, Indianapolis, IN) was used for a gentle dissociation. Cells were filtered through eight layers of lens paper to remove aggregates and were plated in 35-mm dishes (Falcon tissue culture dishes, Falcon Plastics, Oxnard, CA) at 5×10^6 cells/dish. The culture medium consisted of Dulbecco's modified Eagle's medium (DME,[1] high glucose) containing 10% fetal bovine serum and antibiotics. 3 h after plating the cultures were washed free of cellular debris and nonadhering cells, and fresh medium was added. The culture medium was changed daily.

The plating efficiency was roughly 50–70%. This estimate was based on seeding the cells at a density of 5×10^6/dish and after 6 h incubation, the cultures were washed, trypsinized, and counted. This was a very crude estimate and difficult to interpret, for over the next 48 h many cells died and many replicated. Whether the initial trypsinization of the limb bud preferentially affects the viability of cells in one or another lineage is being investigated. However, even if the culture procedures initially favored the survival of one or another subpopulation, this would not alter the interpretation of the data. Irrespective of plating efficiency, the basic observation was that invariably in all control day 3–6 cultures there were approximately equal numbers of definitive myogenic and chondrogenic cells. Failure to reliably determine the plating efficiencies does, however, preclude estimates of what percentage of the total population were in the myogenic, chondrogenic, fibrogenic, smooth muscle, etc. lineages.

Rounding and Detachment by the CSAT Antibody: In most experiments, cultured limb bud cells were treated with CSAT at a concentration of 2–8 µg/ml 3 h after plating at the time of the medium change. In another experiment, CSAT was added to 1-d-old cultures. To maintain the total population of cells after antibody treatment, only ~75% of the medium was carefully replaced every other day, thus preserving the majority of the cells. Roughly 30% of the cells at stages 22–23 were rounded by CSAT. In stages 24 and 25 the rounded fraction increased slightly to ~35%. Less than 5% of the cells from stage 19 and 20 limb bud were rounded by CSAT.

To isolate the CSAT-sensitive subpopulation of limb cells, the rounded and detached cells were recovered after 5 h antibody-treatment by three successive washes of the cell layer with PBS. The aspirated cells were pelleted, resuspended, and replated under conditions optimal for either chondrogenesis or myogenesis. Conditions promoting maximal cartilage differentiation were established by growing the cells in high-density micromass cultures (2). Accordingly, one portion of the cells was inoculated onto 35-mm Falcon tissue culture dishes in a volume of 25 µl DME with 10% fetal bovine serum which contained 5×10^5 cells. In contrast, to provide conditions conducive to muscle development the other part was plated at a density of 5×10^5 cells/dish in 8:1:1 medium (eight parts DME, one part horse serum, one part embryo extract) on collagen-coated

35-mm petri dishes. Under both culture conditions the medium was changed every other day.

Purification of CSAT Monoclonal Antibody: The characterization and purification of CSAT, an antibody of the IgG2b class, has been described previously (45). Briefly, the supernatant from CSAT hybridoma cells was concentrated by precipitation with 50% ammonium sulfate and then was applied to a column of Sepharose CL-4B-Staphylococcus protein A (Pharmacia Fine Chemicals, Piscataway, NJ), equilibrated with Britton–Robinson buffer (citric acid, 28.5 mM; KH_2PO_4, 29 mM; barbitol, 5.26 g/liter; boric acid, 18.8 mM; pH 8.5). The column was eluted with a gradient of Britton–Robinson buffer (pH 8.5–2.8). The fractions eluting at a pH of 3.8 consisted of pure IgG as revealed by SDS PAGE (41).

Histochemical Methods: Sulfated glycosaminoglycans accumulated in the cartilage matrix were detected by fixing the cultures at the times indicated in Kahle's fixative, rinsing once in distilled water, once in 0.1 N HCl, and then staining with alcian blue at pH 1.0 (42).

Immunohistochemistry: Cultures were fixed with 2% formaldehyde for 5 min at 25°C and rinsed with PBS at pH 7.4. Subsequently, the cells were permeabilized and treated with hyaluronidase as previously described (43). Briefly, the cultures were immersed in absolute methanol at −15°C for 10 min and air dried. To assure unrestricted antibody penetration, the extracellular proteoglycans were then removed in some cases by treatment with 200 U/ml of hyaluronidase (2,500 U/mg, from bovine testes, Calbiochem-Behring Corp., Los Angeles, CA) for 30 min. The cultures were incubated with a 1:200 dilution of antivimentin or antidesmin antiserum for 2 h, rinsed for 30 min with three changes of PBS, and then exposed to a 1:250 dilution of rhodamine-conjugated goat anti-rabbit IgG (Cappel Laboratories, West Chester, PA) for 30 min. In some experiments the cultures were counterstained with a 1:2,000 dilution of ascites fluid obtained from the IB2-D mouse hybridoma line secreting a monoclonal antibody against keratan sulfate, followed by three washes with PBS and exposure to a 1:250 dilution of fluorescein-conjugated sheep anti-mouse IgG (Cappel Laboratories). The cultures were mounted in glycerol-PBS (7:3) and examined with an epifluorescence Zeiss photomicroscope (Carl Zeiss, Inc., New York).

Antibodies: Preparation and characterization of rabbit antibodies against muscle-specific light meromyosin (anti-LMM) and the muscle-specific, intermediate filament protein desmin (antidesmin) have been described previously (4, 21, 23, 30). Both of these proteins are present in postmitotic mononucleated myoblasts and in multinucleated myotubes and are thus markers of cells in the terminal stages of skeletal myogenesis (32, 36). Cells that are LMM-negative, but desmin-positive, are tentatively assigned to the smooth-muscle lineage (5). The isolation of the IB2-D mouse hybridoma cell line secreting monoclonal antibodies against keratan sulfate will be described elsewhere. Briefly, the IB2-D antibody reacts in an enzyme immunosorbent assay both with skeletal and corneal keratan sulfate, whereas it does not recognize chondroitin sulfate A, B, and C, hyaluronic acid, heparin, and heparan sulfate. The antigen first appears in frozen limb bud sections at stage 25 in parallel with type II collagen and it localizes in prospective cartilage regions. In older limb bud sections, only the cartilage regions stain.

As a further control of specificity, cultured chick sternal cartilage cells or day 4 limb cell cultures containing numerous cartilage nodules were stained directly with a 1:2,000 dilution of IB2-D antibody, or with antibody preincubated with the panel of glycosaminoglycans. In all such experiments, preincubation of the diluted antibody with different concentrations (10–200 µg/ml) of chondroitin sulfate A, B, C, hyaluronic acid, heparan sulfate (Miles Laboratories, Elkhardt, IN), or heparin (Sigma Chemical Co., St. Louis, MO) did not affect the immunofluorescence staining, whereas preincubation with 10 µg/ml of corneal keratan sulfate (Miles Laboratories) or skeletal keratan sulfate (a generous gift of V. Hascall, National Institute of Dental Research) abolished the reaction.

Immunofluorescence and Autoradiography: Autoradiography was combined with immunofluorescence to determine whether the CSAT-sensitive limb cells that bound muscle-specific antibodies after replating and subsequent growth had incorporated tritiated thymidine into their DNA. Cell cultures from stage 21 limb buds were established as described above, and the CSAT-sensitive cell population was detached by the treatment with 5 µg/ml of CSAT-antibody for 5 h. Subsequently, the detached cells were collected by repeated rinsing with PBS and washed free of antibody. The pellet was resuspended in DME with 10% fetal bovine serum containing 0.1 µCi/ml tritiated thymidine (specific activity 50 Ci/mmol, New England Nuclear, Boston, MA) and replated at a density of 5×10^6 cells/dish on 35-mm Falcon tissue culture dishes. The cultures were fixed after 48 h and stained with anti-LMM or antidesmin followed by the incubation with the appropriate rhodamine-conjugated secondary antibodies as described above. The DNA-binding fluorochrome Bisbenzimide H 33258 was used as a nuclear counterstain. The cultures were then coated with a 1:1 dilution of NTB2 nuclear track emulsion (Eastman Kodak Co., Rochester, NY). After air drying, the cultures were stored in light-

[1] *Abbreviations used in this paper:* anti-KS, monoclonal antibody against the keratin sulfate regions of the cartilage proteoglycan; anti-LMM, rabbit antibodies against muscle-specific light meromyosin; DME, Dulbecco's modified Eagle's medium.

tight boxes for 5–10 d. Autoradiographs were developed using D-19 developer (Kodak) for 2 min, rinsed with distilled water, fixed for 5 min with rapid fix (Kodak), washed for 15 min with several changes of distilled water and mounted in glycerol:PBS (9:1). Cells with greater than 40 grains overlying the nuclei were scored as positively labeled. Usually most of the nuclei were covered by grain deposits.

RESULTS

High-density Cultures of Early Limb Cells Yield Terminally Differentiated Myogenic and Chondrogenic Cells

Cell suspensions were prepared from the limb buds of stage 20, 21, and 22 chick embryos, and plated at high cell density in DME with 10% fetal calf serum. Under these conditions the appearance of the cultures changed from apparent homogeneity on day 1 (Fig. 1 a) to heterogeneity by day 4, when cultures displayed definitive cartilage, muscle, and fibroblast-like cells (Fig. 1 b). Most work with high-density limb cultures has emphasized emerging chondroblasts (7, 9, 10, 40, 60). Immunofluorescent staining with anti-LMM after 2 d in culture revealed the presence of numerous terminally differentiated, mononucleated myoblasts and multinucleated myotubes, many with well-organized striated myofibrils (Fig. 2, a and b). Muscle cells were found distributed throughout the culture with the exception of focal areas of extensive multilayering. These multilayered areas invariably differentiated
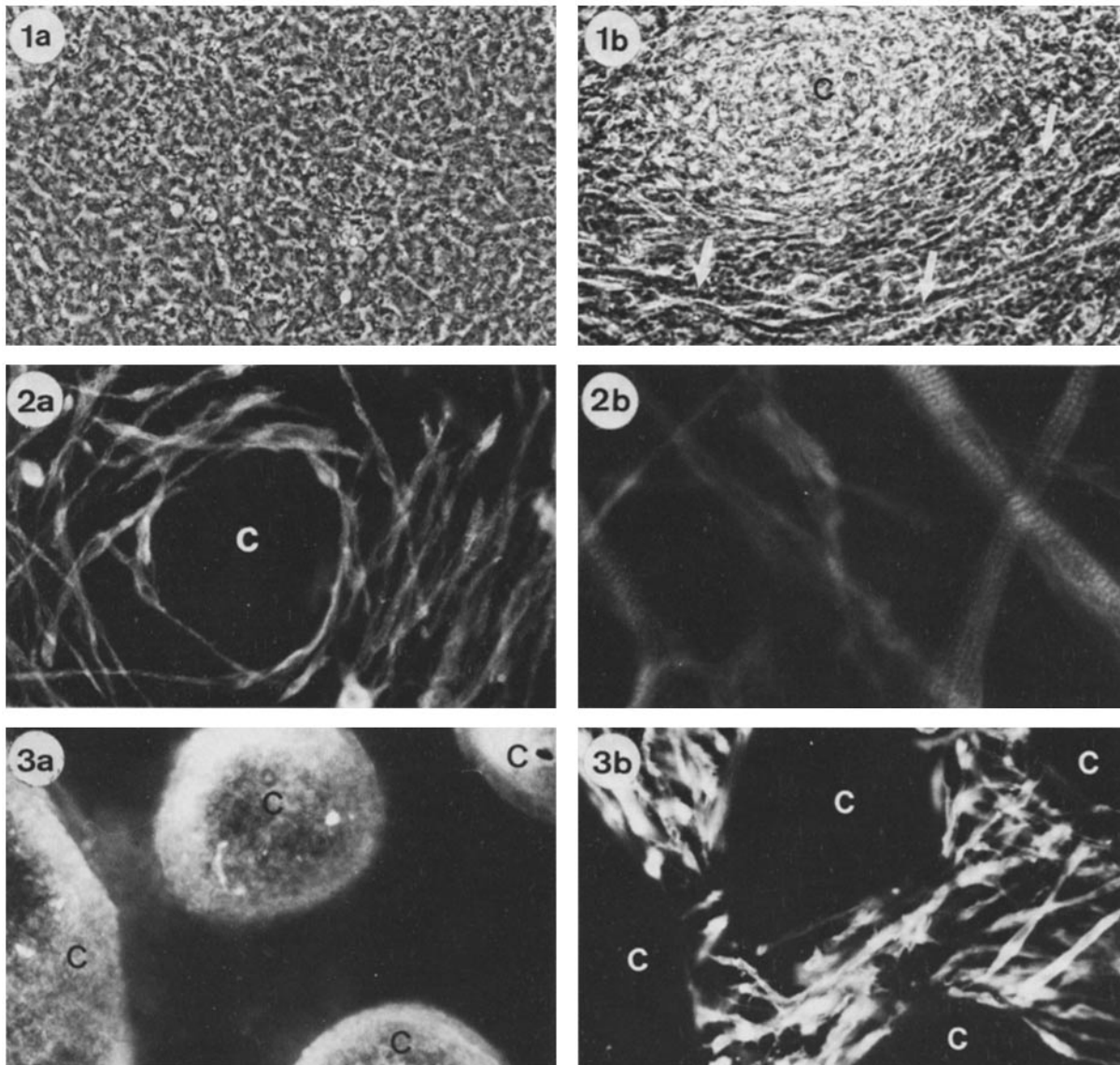

FIGURES 1–3   Differentiation of stage 21 limb cells in high-density cultures. Fig. 1: Phase-contrast micrograph of a (a) day 1 and (b) day 4 culture. Note the emergence of cartilage nodules (C) surrounded by fibroblast-like cells and muscle (arrows, b). × 2200. Fig. 2, A day 2 culture of stage 21 limb cells stained with antibodies against muscle specific LMM (anti-LMM). Terminally-differentiated, mononucleated myoblasts, and multinucleated myotubes are abundant throughout the culture except for precartilage regions. Both postmitotic mononucleated myoblasts and multinucleated myotubes reveal typical cross-striated myofibrils. C, cartilage nodule. (a) × 220. (b) × 650. Fig. 3: Immunofluorescence micrograph of a day 5 culture. The same microscopic field has been stained to localize the cartilage-specific KS using rhodamine optics (Fig. 3a) and the muscle-specific LMM (Fig. 3b) using fluorescein optics. Note that the dense cartilage matrix in this 5-d-old culture resists antibody penetration into the chondrogenic nodule and thus prevents the staining of the surface of individual cartilage cells. As illustrated in Figs. 12–14, brief pretreatment of these cultures with hyaluronidase allows visualization of the individual chondroblasts in these nodules. Note, too, that many of these cells in this micrograph and in Fig. 2, a and b do not bind the muscle-specific or cartilage-specific antibodies. × 220.

into cartilage nodules by day 4 of culture. The emerging chondroblasts deposited an abundant extracellular matrix that could be visualized by its reaction with a monoclonal antibody against the keratin sulfate (KS) regions of the cartilage proteoglycan (anti-KS) (Fig. 3a). The cartilage nodules were surrounded by muscle cells (Figs. 2a and 3b) and by fibroblast-like cells that did not stain either with the anti-LMM or the anti-KS. Currently, we have no way of determining which early compartment of the myogenic, chondrogenic, fibro-

genic, or smooth muscle lineage these unstained cells occupy. Pending the availability of satisfactory markers, these unstained cells will be referred to as "fibroblastic cells" (27, 29, 34, 44).

The relative and absolute numbers of myoblasts and myotubes that emerged in these cultures varied considerably depending on (a) the composition of the culture medium and (b) the age of the cultures at the time they were monitored (not shown). For example, lowering the serum concentration
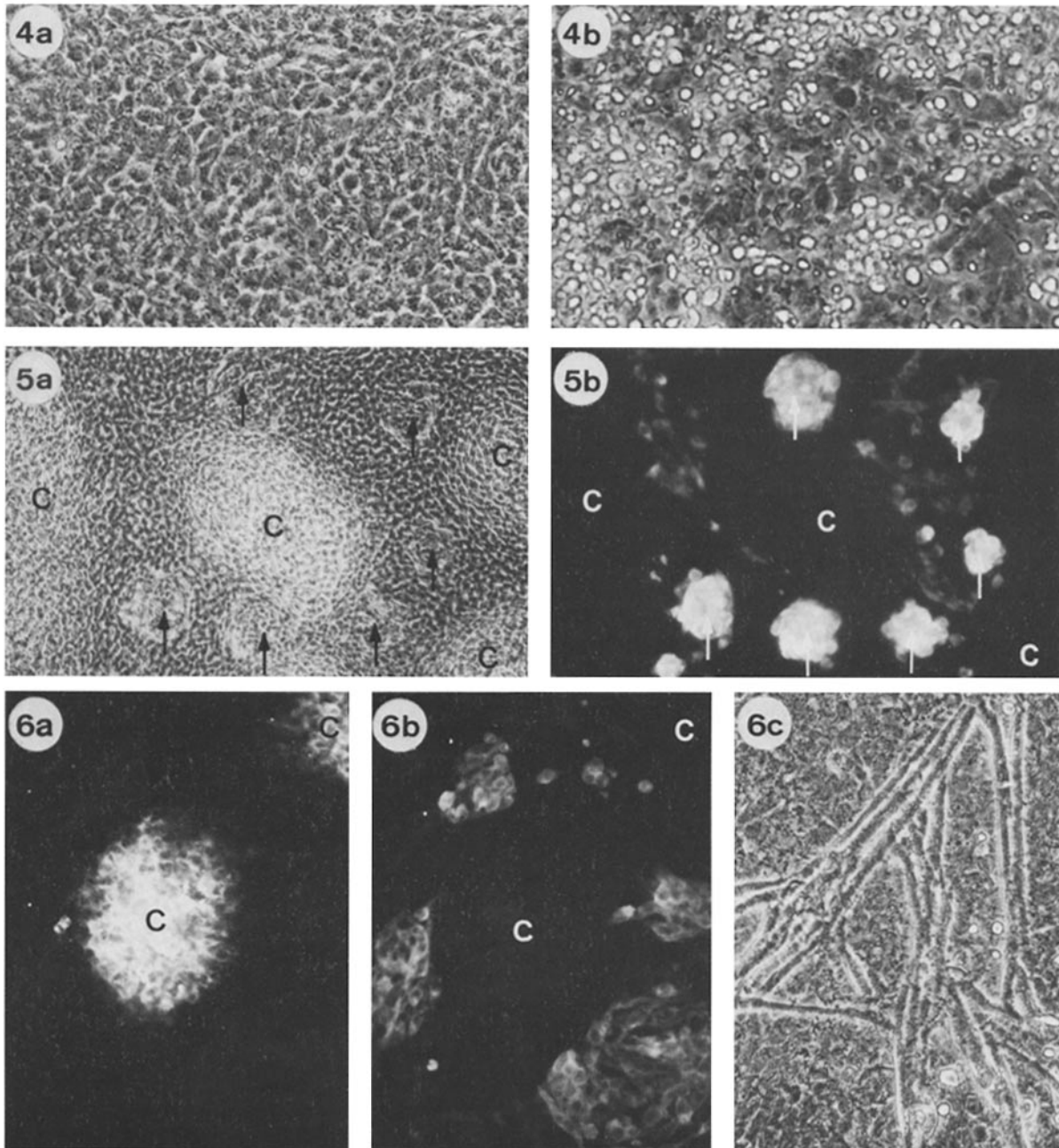
Figures 4-6 Influence of the CSAT monoclonal antibody on cultured stage 21 limb cells. Fig. 4: Phase-contrast micrograph of a day 1 culture before (a) and 5 h after (b) the addition of 5 μg/ml of CSAT-antibody. Note the rounding up of many cells in b. × 220. Fig. 5: The same culture as in Fig. 4 but maintained for another 3 d in medium with CSAT. The same microscopic field is shown under phase contrast (a) and under the fluorescent microscope after staining with anti-LMM (b). Note the formation of clumps of differentiated muscle cells. C, cartilage; arrows, muscle. × 150. Fig. 6: Immunofluorescence double-staining with anti-KS (a) and anti-LMM (b) of a parallel culture to that shown in Fig. 5a, but 12 h after removal of the CSAT antibody. a illustrates a chondrogenic nodule stained with anti-KS; b shows a spreading cluster of anti-LMM-positive mononucleated and oligonucleated muscle cells; c is of a parallel culture 2 d after removal of CSAT. Note the typical elongated myotubes atop a confluent layer of not readily-identifiable cells. × 150 (a and b); × 220 (c).

to 2% or below has little effect on the number of definitive myogenic cells present on day 2 of culture. However, after 4 d in a low-serum medium, their number was reduced roughly to one tenth that illustrated in Figs. 2 and 3.

Influence of CSAT on Cultured Limb Cells

The addition of CSAT hybridoma supernatant or the purified antibody exerted a striking effect on the attachment of cultured limb cells. We observed that a fraction of the limb cells was prevented from attaching and spreading if plated in medium containing 2-8 μg of purified antibody. CSAT at concentrations of >30 μg/ml completely blocked initial attachment (see below). Because studies dealing with different initial plating efficiencies involving potentially different cell populations were difficult to interpret, in subsequent experiments, we allowed cells to attach and spread for 3-20 h in normal medium before adding CSAT antibody.

When CSAT, at a concentration of 2-8 μg/ml, was added to attached and spread day 1 limb cell cultures (Fig. 4a), within 3-5 h, ~30% of the cells rounded and either detached completely or remained only loosely connected with the underlying monolayer (Fig. 4b). Prolonged incubation with the antibody did not significantly increase the fraction of rounded cells. When the cultures were kept continuously in medium containing the antibody, care was taken to minimize loss of rounded cells during subsequent medium changes. After 3 d many cells in the dense population had undergone extensive

chondrogenesis forming numerous cartilage nodules (Fig. 5a). However, in the same cultures, the cells rounded by CSAT had formed clumps of cells loosely attached to the cells remaining spread on the substrate. As shown in Fig. 5b the rounded, but loosely adhering, cells had differentiated into LMM-positive, mononucleated myoblasts and multinucleated myotubes. These tight clusters of striated myogenic cells often contracted spontaneously. When CSAT was removed and replaced with normal medium, these aggregates of swirled muscle cells flattened, spread, and eventually formed typical elongated, richly striated, multinucleated myotubes (Fig. 6, b and c).

Cultures of freshly plated, well-spread limb cells (Fig. 7a) responded to the addition of CSAT antibody in a concentration-dependent manner. Low to medium concentrations of antibody (1-15 μg/ml) exhibited a differential effect by causing detachment of ~30% of the cell population within 2-5 h (Fig. 7b). The adherent (CSAT-insensitive) cell population seemed largely unaffected by the antibody except for a marginal change in morphology, which caused the cells to appear somewhat less well spread. In contrast, 100% of the cells in limb bud cell cultures treated with very high doses of >30 μg/ml antibody were rounded and detached (Fig. 7c). The latter finding served—in addition to the experiments in which limb cells were exposed to the antibody for several days (compare Figs. 5 and 6)—as a check for a possible influence of CSAT treatment on the developmental potential of the whole population of limb cells, i.e., the ability to differentiate into
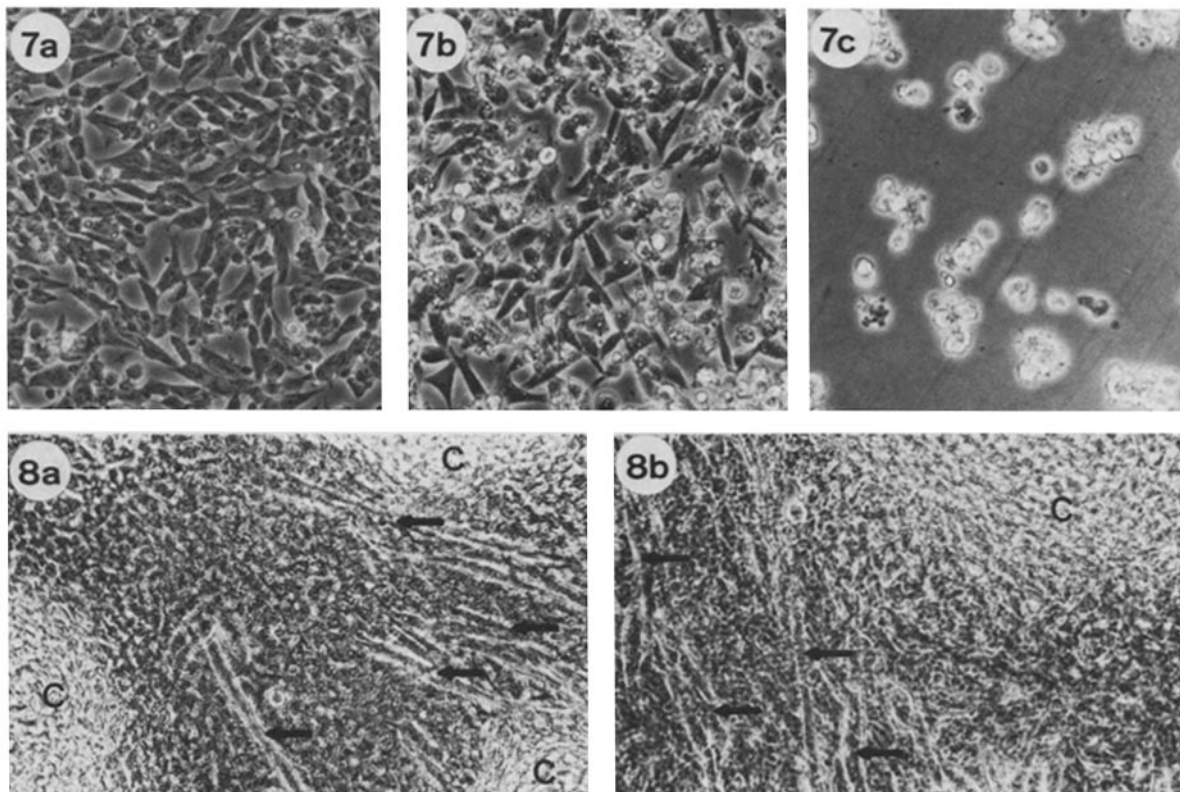
FIGURES 7 and 8 The effect of different concentrations of CSAT monoclonal antibody on freshly plated limb cells. Fig. 7: Phase-contrast micrograph of a cell culture of stage 21 limb cells 3 h after plating in normal medium (a). 5 h after the addition of 5 μg/ml CSAT antibody 30-40% of the cells are rounded and detached (b) whereas the addition of 40 μg/ml of antibody for 5 h has caused complete detachment (c). × 220. Fig. 8: Comparison between the differentiation pattern of a control population of limb cells and one that had been completely detached by CSAT. Phase-contrast micrographs of a control day 4 culture of stage 21 limb cells (a) and a culture that was completely detached by 40 μg/ml CSAT on day one (compare Fig. 7c) and then replated in medium without antibody and cultured for 4 d (b). Note that both cultures display cartilage nodules (C) and noncartilage cells (fibroblastlike cells) surrounding the nodules and muscle (arrows). × 220.

muscle and cartilage. Accordingly, populations of limb cells that had been completely detached by high CSAT concentration (Fig. 7c) were washed free of antibody, replated, and kept for 4 d in normal medium (Fig. 8b). A comparison with an untreated control culture of the same age (Fig. 8a) revealed the presence of cartilage nodules, muscle cells, and fibroblastic cells in both cases, arguing strongly that CSAT treatment and subsequent detachment did not alter the developmental potential of the whole population of limb cells.
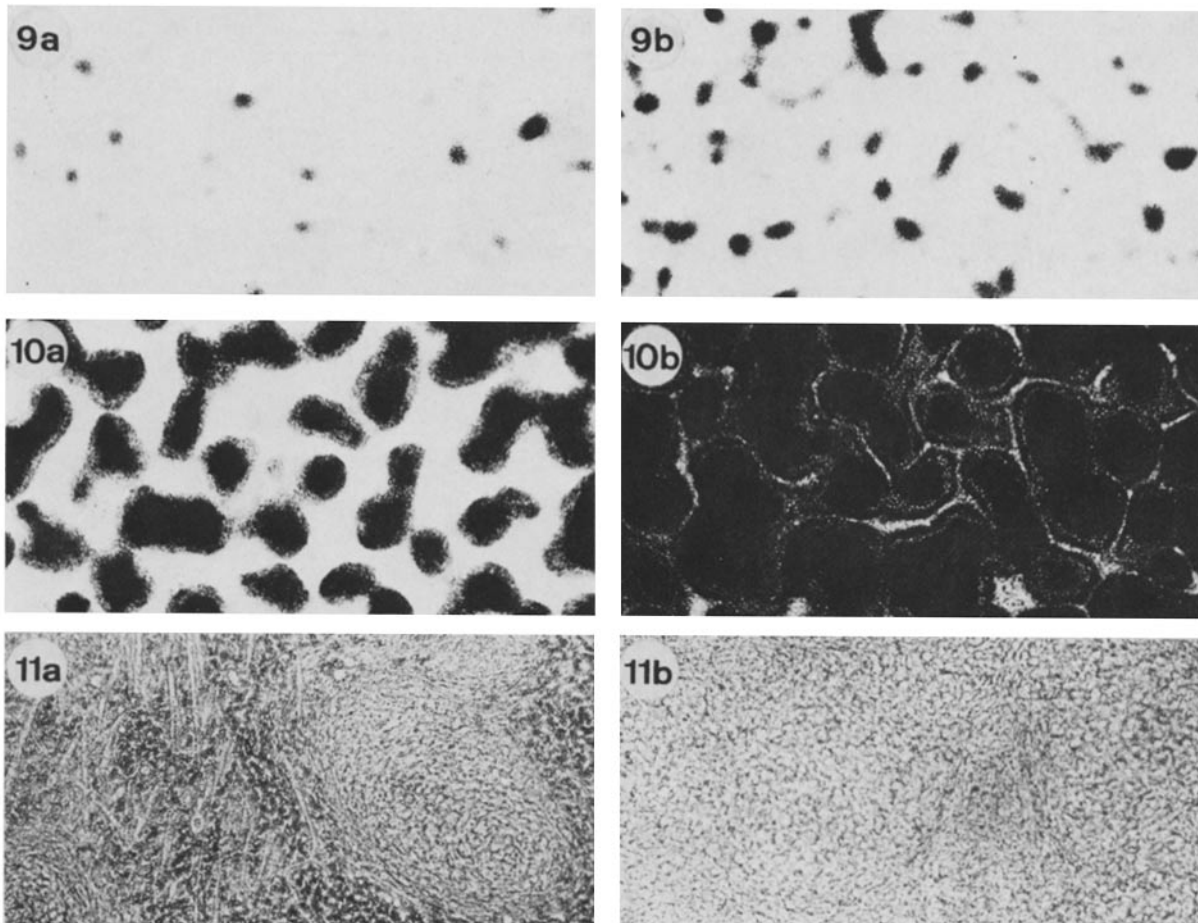
Adherent (CSAT-insensitive) Cell Population Is Highly Enriched in Chondrogenic Cells

Limb cell cultures depleted of the CSAT-sensitive cell population were markedly different from both control cultures and cultures in which the whole cell population had been detached and replated. Control cultures grown in normal medium gave rise to metachromatic cartilage nodules (Figs. 9a and 10a) surrounded by a substantial number of myogenic and fibroblastic cells (Fig. 11a). In contrast, the CSAT-insensitive cell population (i.e., those remaining attached after CSAT treatment) displayed a marked relative increase in

cartilage (Figs. 9b and 10b). Areas in some cultures appeared as an almost continuous sheet of cartilage with patches of varying sizes of interspersed fibroblastic cells (Figs. 10b and 11b). Quantitative data on the type IV cartilage proteoglycan that the type II collagen chains synthesized in these cultures will be published elsewhere.

To visualize the extent of chondrogenesis and myogenesis, the cultures were double-stained with anti-KS and anti-LMM. These antibodies reacted with terminally differentiated chondrogenic (Figs. 12a, 13a, and 14a) and myogenic cells (Figs. 12b, 13b, and 14b), respectively. Control cultures and reconstituted cultures (i.e., those in which antibody treatment had been used to isolate the CSAT-sensitive population, which was subsequently washed free of antibody and added back to the original plate) gave rise to both cartilage and muscle (Figs. 12, a and b). Cultures depleted of CSAT-sensitive cells, formed virtually no muscle (Fig. 13a). Fig. 13b was chosen from a panel of randomly selected fields—most of which did not reveal a single muscle cell and exhibited more extensive chondrogenesis than exhibited in this particular micrograph.

The removal of the CSAT-sensitive cell population produced a nearly complete depletion of muscle precursor cells under all culture conditions tested. The adherent cell popu-
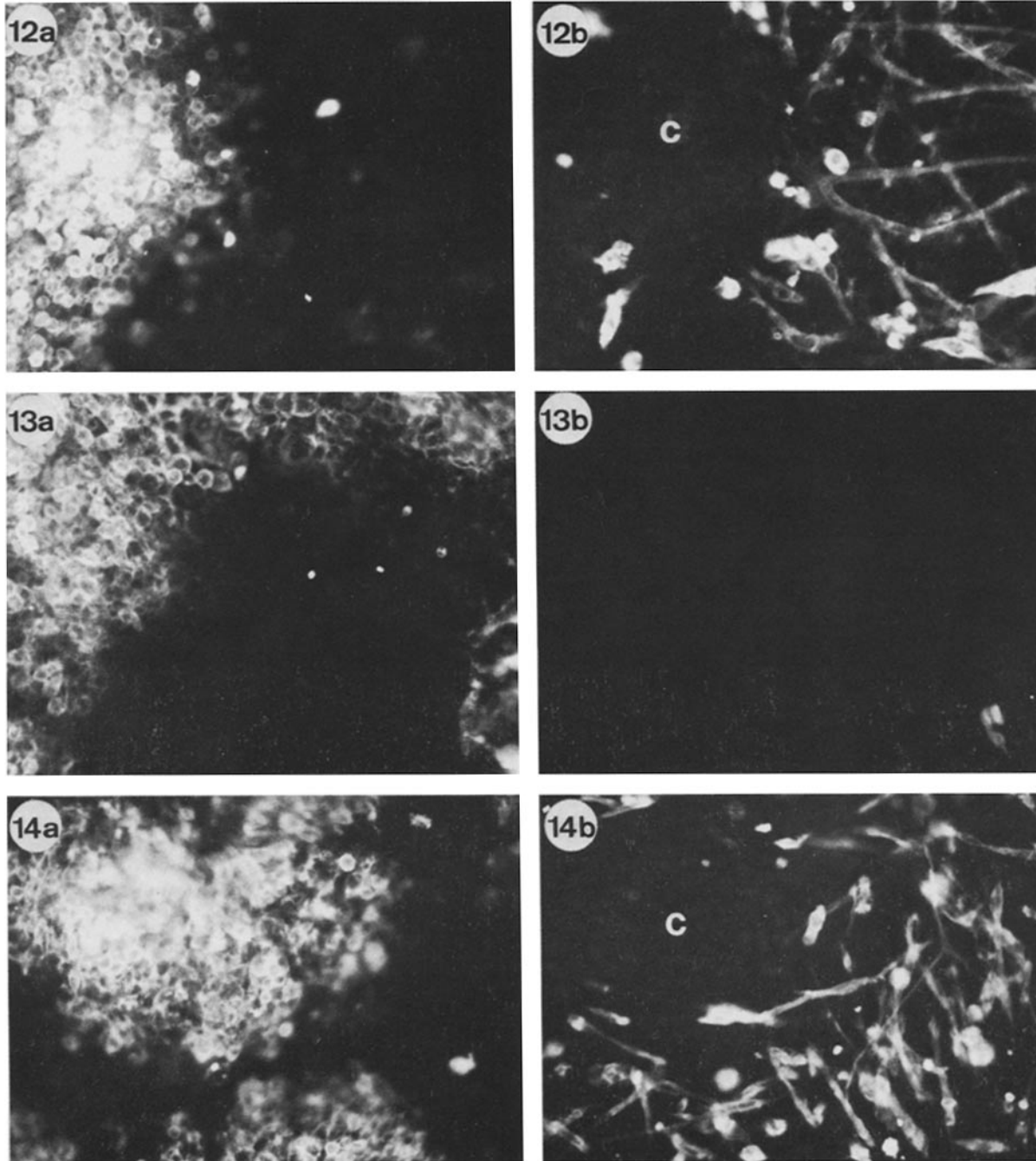

FIGURES 9–11   Cartilage differentiation in control stage 21 limb cell cultures and in cultures depleted of the CSAT-sensitive cell population. Photomicrographs of day 2½ (Fig. 9) and day 5 (Fig. 10) cell cultures stained with alcian blue, at pH 1, to stain the sulfated glycosaminoglycans deposited in the cartilage matrix, and phase-contrast micrographs of day 5 cultures (Fig. 11). Control cultures (Fig. 9a, 10a, and 11a) developed from the whole population of stage 21 limb cells whereas the treated cultures (Fig. 9b, 10b, and 11b) were depleted on day 1 of the subpopulation of cells responding to the addition of 5 µg/ml of CSAT-antibody (compare with Fig. 7b). Note the high cartilage yield in the cultures depleted of the CSAT-sensitive population. The numerous definitive muscle cells present in Fig. 11a cannot be appreciated in this phase-contrast micrograph due to the high cell density and multilayering of the cells. Virtually no muscle cells are present in Fig. 11b. (Figs. 9 and 10) × 30; (Fig. 11) × 110.

lations could not be induced to differentiate into muscle even if cultured under conditions known to promote the replication, survival, and differentiation of myogenic cells (e.g., culture at medium cell density on collagen-coated dishes, in medium containing embryo extract). The effect of the muscle-promoting environment on the adherent cell population was limited to lowering the yield of chondroblasts and increasing the number of fibroblastlike cells in the cultures. It will be of interest to determine the origin of this lower yield of definitive chondroblasts. Two possibilities are a reduced replication of the committed chondrogenic cells or a failure to express their chondrogenic differentiation program (1, 27–29, 34, 44).

Detached (CSAT-sensitive) Cell Population Is Highly Enriched for Myogenic Cells

The developmental potential of the CSAT-sensitive population (i.e., cells rounded after 5 h of treatment with 5 μg/ml
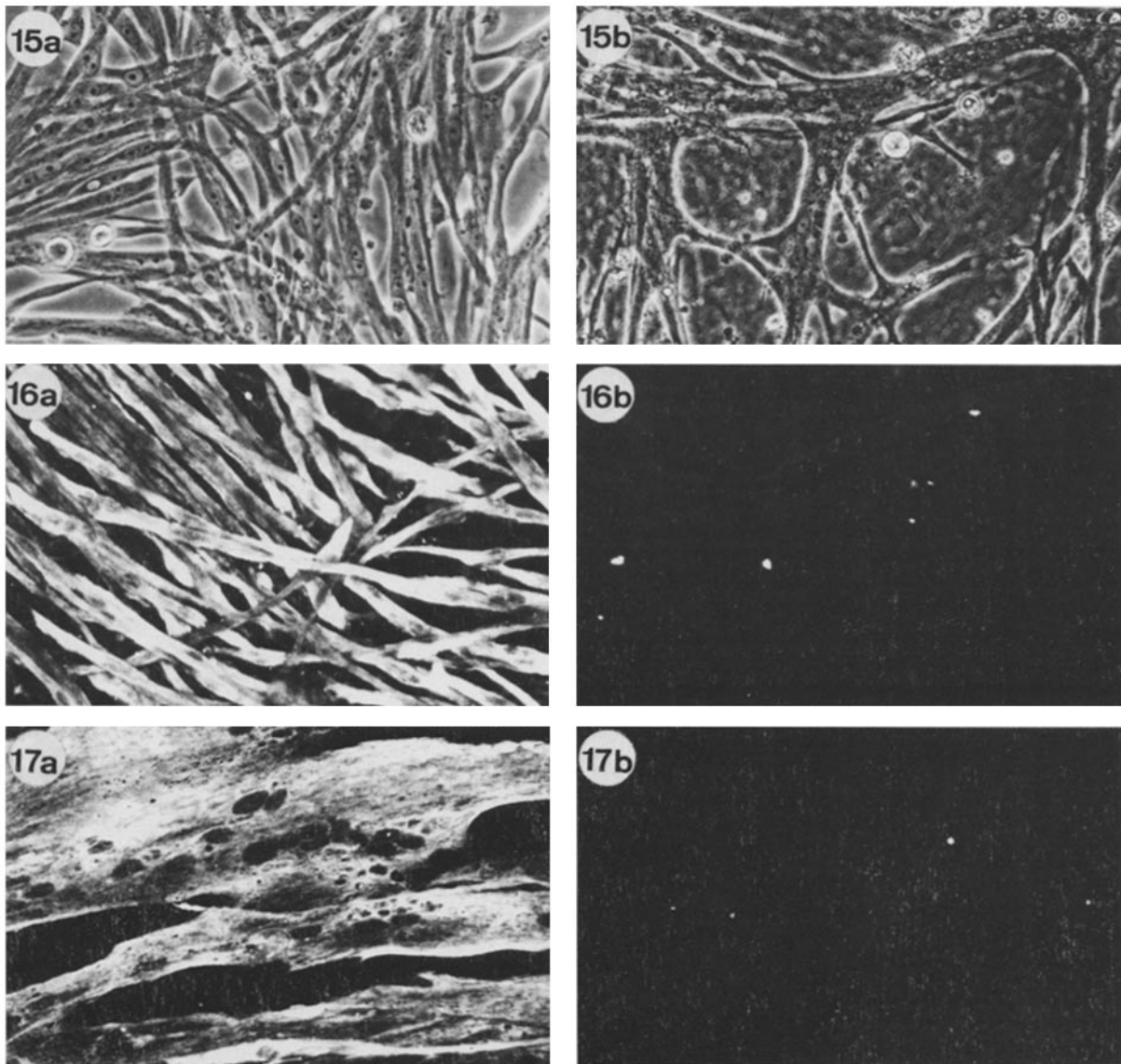
FIGURES 12–14   Cartilage and muscle differentiation in control stage 21 limb cell cultures and in cultures depleted of the CSAT-sensitive cell population. Immunofluorescence double-staining of the same microscopic field of day 4 cultures. Figs. 12a, 13a, and 14a are viewed using rhodamine optics to localize the cartilage-specific KS, whereas Fig. 12b, 13b, and 14b are viewed using fluorescein optics to visualize the LMM. The cultures were treated with hyaluronidase before antibody staining to facilitate penetration of the anti-KS. × 300. Fig. 12: Control culture. Fig. 13: Culture depleted of the CSAT-responsive cell population. 3 h after attachment 5 μg/ml CSAT-antibody was added for 5 h. The detached cells (compare Fig. 7c) were then washed off the plate and the adherent cells were fed normal medium. Fig. 14: Reconstituted culture. A parallel culture to that in Fig. 13 was depleted of the CSAT-responsive cell population using the same protocol. The detached cells were then collected, washed free of antibody, and readded in normal medium to the plate from which they originated.

of antibody) was assayed after the cells were collected, washed, and replated under conditions selectively supporting either myogenesis or chondrogenesis. If the detached cells were repleted under "muscle-promoting conditions" (collagen-coated substratum and growth medium containing embryo extract), they differentiated into a network of interconnected muscle fibers (Fig. 15a). Approximately 90% of the cell nuclei in these cultures were found in myoblasts and myotubes. No chondroblasts were recognized in these cultures. If replated under "cartilage-promoting conditions" (micromass culture at very high cell density) the cells still gave rise to 60% muscle. They displayed a network of myotubes resting on a monolayer

of fibroblastic cells; however, there were still no recognizable chondroblasts (Fig. 15b).

The differentiation of chondroblasts in these cultures was further investigated by replating the CSAT-sensitive cells under cartilage-promoting conditions. The cultures were fixed at different times in culture and stained with anti-KS and with antidesmin, using the immunofluorescence double-label technique. Desmin, the muscle-specific intermediate filament protein, is not present in replicating myogenic precursors and first appears as longitudinal filaments in the mononucleated, terminally differentiated myoblasts and multinucleated myotubes (4, 23, 24, 34). Stage 21 cells detached with CSAT and

Figures 15-17 Muscle differentiation and lack of cartilage differentiation in cultures derived from the CSAT-sensitive limb cell population. CSAT-responsive cells were detached by 5 µg/ml antibody from day 1 limb cell cultures (compare with Fig. 7b), washed free of antibody, and replated under various conditions. Fig. 15: Phase contrast of day 4 cells replated under muscle-promoting conditions (a) on collagen coated dishes in medium containing embryo extract, and under cartilage-promoting conditions (b) at high density on tissue culture dishes in F12 medium lacking embryo extract. Note in Fig. 15b the numerous "unidentifiable" cells adhering to the substrate. × 250. Figs. 16 and 17: Immunofluorescence double-staining of CSAT-detached cells with antibodies against desmin (16a and 17a) and KS (16b and 17b), 2 d (Fig. 16) and 6 d (Fig. 17) after replating under cartilage-promoting conditions. Note the prominent muscle differentiation and the complete lack of cartilage formation in the CSAT-sensitive cell population even when cultured under conditions promoting chondrogenesis. The desmin-positive cells in Fig. 16a are mono-, bi- and trinucleated cells. After another 4 d in culture these cells have fused further forming huge, multinucleated myotubes. × 350.

grown for 48 h under conditions promoting chondrogenesis displayed numerous mono- and bi-nucleated desmin-positive cells (Fig. 16a). By day 6 many of these cells had fused into multi-nucleated myotubes containing dozens of nuclei (Fig. 17a). Even though the CSAT-sensitive cells were grown under conditions promoting chondrogenesis, staining with anti-KS (Figs. 16b and 17b) did not reveal any cartilage cells in these 2- or 6-d-old cultures.

Our failure to detect appreciable numbers of LMM and desmin-positive cells in stage 21 limb bud cell cultures during the first 24 h argued that many CSAT-sensitive cells were replicating, skeletal myogenic precursors. The following experiment demonstrates that this was indeed so. The CSAT-sensitive cells were collected and replated under either muscle- and cartilage-promoting conditions in medium containing tritiated thymidine. After 2 d the cultures were fixed, incubated with antidesmin, counterstained with the nuclear dye bisbenzimide, and processed for autoradiography. A comparison between the immunofluorescent pattern identifying terminally-differentiated myoblasts and myotubes by using antidesmin, and their associated nuclei by using bisbenzimide is shown in Fig. 18. Grain deposits over nuclei of the terminally-differentiated myoblasts and myotubes showed that during the 48 h in medium containing tritiated thymidine, ~60% of the cells cycled at least once before becoming postmitotic myoblasts. The results were the same irrespective of whether the CSAT-sensitive cells were cultured under muscle- or cartilage-promoting conditions. These findings strongly suggest that the CSAT antibody rapidly rounds both terminally-differentiated myoblasts as well as their replicating precursors.

Many cultures displayed varying numbers of desmin-positive, myosin-negative cells. These are probably presumptive and/or definitive vascular smooth muscle cells (5, 21).

DISCUSSION

Cells of the early limb bud can be fractionated into two major subpopulations consisting primarily of cells in the myogenic and chondrogenic lineages, respectively. The separation was effected by using a monoclonal antibody (CSAT) that preferentially rounds and detaches the myogenic cells present in limb bud cultures (17, 45). Many of the cells in the CSAT-insensitive population (i.e., cells that remain attached after

antibody treatment) have the properties of immediate precursor cells in the chondrogenic lineage. After 3 d in culture the majority of CSAT-insensitive cells differentiate into chondroblasts interspersed with clusters of fibroblastic cells. Occasionally some muscle cells are observed in these cultures. Their presence is almost certainly due to the incomplete separation of small numbers of precursor myogenic cells from the relatively more adherent chondrogenic cells. This interpretation is suggested by experiments showing that the CSAT-insensitive subpopulation, even when cultured under conditions favoring myogenesis, fails to increase significantly the number of terminally-differentiated myogenic cells (i.e., those staining with antidesmin and anti-LMM).

In contrast, the CSAT-sensitive cell subpopulation has properties expected of replicating precursors and terminally-differentiated cells in the myogenic lineage. When plated under conditions optimal for myogenesis, >90% of the nuclei are found in myoblasts and myotubes—cells binding antidesmin and anti-LMM. The failure of this cell population to develop chondrogenic cells when reared under conditions known to be optimal for chondrogenesis is especially noteworthy. Under these conditions, which are suboptimal for terminal myogenesis, the cultures still yielded ~60% terminally-differentiated myogenic cells after 4 d in culture. The remaining cells displayed a fibroblastic phenotype. It is not surprising that many of these CSAT-sensitive cells may not express their myogenic option because the omission of even one permissive factor (e.g., collagen, Ca^{++}, etc.) is known to decrease the yield of readily recognizable myogenic cells.

The position in the myogenic lineage of the CSAT-sensitive cells is of considerable importance. Radioautographic studies combining uptake of tritiated thymidine and staining with anti-LMM demonstrated that the majority of terminally-differentiated, postmitotic myoblasts and myotubes that emerged from replated CSAT-sensitive cells from stage 20–22 limb buds were the in vitro progeny of replicating myogenic precursors. The CSAT-sensitive cells were reared for a total of 56 h in culture: the first 8 h—during attachment and CSAT-treatment—in the absence of tritiated thymidine; then—after collecting and replating—for 48 h in the presence of tritiated thymidine. The cell cycle time for these cells in vivo and in vitro is <8 h (6, 28). Our observation that >60%
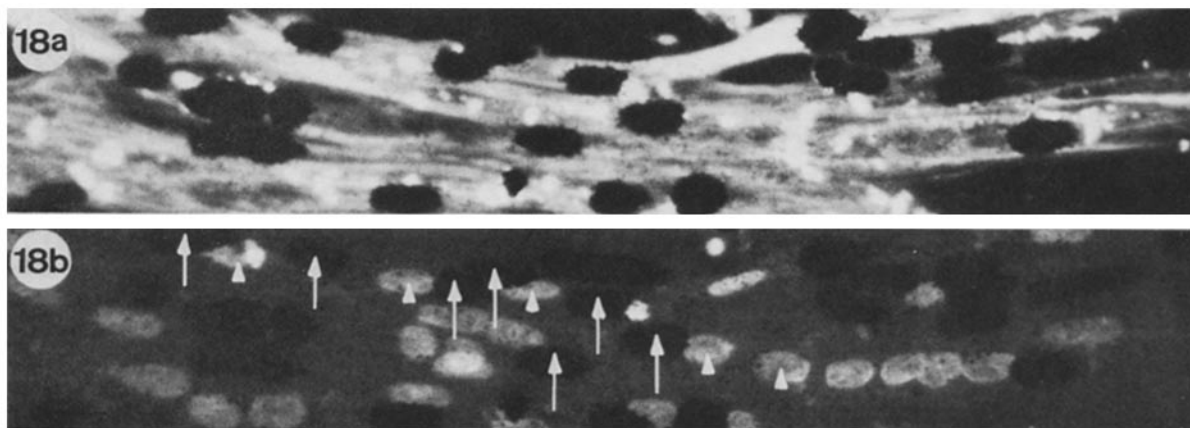

FIGURE 18   Cell divisions in the population of CSAT-sensitive cells. The CSAT-sensitive population of cells was detached on day one with 5 μg/ml of antibody (compare with Fig. 7c), the cells washed free of antibody, and replated under cartilage promoting conditions in medium containing 0.1 μCi/ml of [³H]thymidine. The culture was fixed on day 2, double-stained with antibodies against desmin (Fig. 18a), the nuclear stain bisbenzimide (Fig. 18b), and then was processed for autoradiography. Note that the majority of nuclei present in muscle cells are labeled with [³H]thymidine. a is photographed using rhodamine optics; b is photographed using fluorescein optics. Many of the nuclei are in multinucleated myotubes. Such nuclei tend to line up in long rows. Long arrows indicate [³H]thymidine-labeled nuclei; arrowheads indicate unlabeled nuclei, both in the same myotubes.

of the CSAT-sensitive cells had taken up tritiated thymidine after replating underestimates the fraction of replicating myogenic precursors for it fails to account for those that became postmitotic during the first 8 h in culture. Therefore, it seems likely that most of the stage 20–22 limb cells responding to CSAT treatment are replicating presumptive myoblasts rather than terminally differentiated postmitotic myoblasts. When limb cells are cultured from progressively older embryos— stages 23–25—CSAT rounds and detaches a cell population containing an increasing fraction of such mononucleated, postmitotic myoblasts (data not shown). During the time period that separates a stage 22 from a stage 25 limb bud (24 h), there is a significant increase in the proportion of cells shifting from the replicating presumptive myoblast compartment into the postmitotic compartment of the myogenic lineage.

Could CSAT be acting as an "inducing" agent? The following observations argue strongly against the alteration of phenotypic options of the cells by CSAT: (a) Cultured chick limb bud cells differentiate into definitive chondroblasts, myoblasts, and/or myotubes and fibroblastic cells either in the presence or absence of antibody. (b) Separation of the CSAT-responsive subpopulation from the remaining adherent cells followed by their subsequent recombination yields cultures that give rise to chondroblasts and/or myotubes and fibroblastic cells in a pattern and proportion similar to that of untreated controls. (c) The treatment of cultured limb bud cells with very high concentrations of CSAT at these early times in culture results in the rounding and detachment of all cells. After removal of antibody from these cells and replating in normal medium, they differentiate into a pattern of chondroblasts, myoblasts, and/or myotubes and fibroblastic cells, also indistinguishable from untreated controls. From these observations, we conclude that CSAT does not appear to alter the subsequent phenotypic expression of the treated cells.

The ability of CSAT to fractionate different cell types has been reported for other cell cultures. Neff et al. (45) reported that CSAT fractionates cultures from avian pectoral muscle into fibrogenic and myogenic cells by a selective rounding and detaching of the latter. They also reported that CSAT rounded and detached 5-bromo-2-deoxyuridine treated myogenic cells—replicating precursors. More recently, CSAT has been reported to fractionate cardiac myocytes from contaminating fibroblasts by selectively delaying the attachment of the myocytes (17). These selective effects are not limited to muscle but have also been observed among a group of different kinds of fibroblasts when plated under appropriate conditions (17). The mechanism by which CSAT distinguishes among different cell types appears to be due to intrinsic differences in their adhesion to extracellular materials. The proliferation of fibroblasts, differentiation of cardiac myoblasts, the fusion of skeletal myoblasts, and the synchronous beating of cardiac myocytes are all not detectably perturbed by CSAT (17, 37, 45). These observations, along with the presence of the CSAT antigen and the selectivity of the response on all of the different cell types discussed above, argue for a specific effect of CSAT. The likely locus of CSAT action is on adhesion. This is supported by immunofluorescence that localizes the CSAT antigen in the adhesion sites of fibroblasts and by the ability of CSAT to inhibit adhesion to extracellular matrix materials (16, 33, 40).

These findings suggest that limb bud cells are fractionated by a differential effect of CSAT on the adhesion of the two major cell types present—those in the myogenic and chon-drogenic lineages. Myogenic cells appear to be more sensitive than chondrogenic cells to disturbances of cell-matrix adhesion or cytoskeletal structure. In preliminary experiments we have found that cytochalasin B, which is known to interfere with cytoskeletal organization, mimics the effects of CSAT on early limb bud cells. This finding extends earlier studies in which cytochalasin B was used to separate myoblasts and myotubes from cultures of embryonic breast muscle cells (16, 33, 38).

The present study confirms and extends our earlier investigation which, by the use of cloned limb bud cells, had provided circumstantial evidence for the existence of at least two separate lineages, a chondrogenic and a myogenic (19, 20). The additional data in this study together with the evidence provided by the quail-chick grafting experiments (11–13, 39, 58) strongly suggests that the population of CSAT-sensitive cells (forming >90% muscle under suitable conditions in vitro) constitutes the population of somite-derived cells (giving rise to 100% muscle in vivo). The CSAT-insensitive cell population (differentiating predominantly into cartilage in vitro) presumably consists of the population of limb somatopleural cells (13, 58).

Finding that the cells in a stage 21 limb bud have already bifurcated into myogenic and chondrogenic lineages contributes nothing as to the origin(s) of the fibroblasts that constitute the tendons, endo- and epimysium, dermis, or of the smooth muscle cells that eventually appear in the limb. Presumably, the founder cells for the unipotential myogenic, chondrogenic, fibrogenic and smooth muscle lineages are all derived from an earlier common founder cell. The data from experiments with somite cells (1, 19, 20, 28, 44), coupled with the quail chick chimeras suggests that the common founder cell for all these lineages is a transitory phenotype. If the myogenic cells of the limb bud are derived from the same unipotential lineage that supplies the myogenic cells of the myotome, then the transitory common founder cell for all these lineages must cease self-renewing during the early stages of somiteogenesis (34, 44).

Our data support the concept of cell lineages, in which cell diversification in the limb follows invariant cell lineages where the differentiation program, as well as the phenotypic options of each cell, are predictable from its ancestry and position in the lineage tree. The intracellular, lineage-dependent mechanisms that predictably specify the differentiation program in every cell in the nematode (18, 53) also operate in every cell in the chick (28, 31). This hypothesis predicts that embryonic cell populations contain replicating precursor cells in different lineages that should be identifiable and separable. This view stands in opposition to hypotheses that stress the instructional role of microenvironmental influences on the process of cell diversification. We propose that microenvironmental cues (e.g., fibronectin, collagen chains, glucosaminoglycans [GAGS]), growth factors, etc.) play major roles for such processes as differential replication and survival, cell–cell adhesion, directed migration, the maintenance of cytoarchitecture, and morphogenesis (27, 34, 35). There is little evidence, however, that such cell-cell or cell-matrix inductive interactions play primary roles in generating the unique, but invariably limited, differentiation program of any cell. Whereas exogenous molecules influence which myosin isoform or which neural transmitter (55, 61) will ultimately be synthesized, only definitive postmitotic myogenic or neurogenic cells possess the ongoing differentiation program that permits these specific cell types to respond to such "inducing" molecules.

There is now evidence for cell lineages in the development of other embryonic chick systems including neural crest (14, 15, 55, 61) and metanephros (49). As discussed in Holtzer et al. (31, 32, 36) even a 12-h chick blastodisk is probably segregated into multipotential founder cells which by rapid and repeated bifurcations, yield unipotent founder cells for at least five different lineages.

The concepts of "determination" and "commitment" were derived, in part, from studies on limb buds. Most of these studies (see Introduction) concluded that such cells were multi-potential and that they first became determined and then committed to myogenesis or chondrogenesis 24–30 h later than those used in our experiments. Given our findings, it is pertinent to ask how in a lineage model the mechanisms responsible for determination can be distinguished from those responsible for commitment, and how both of these can be distinguished from those responsible for differentiation.

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