Preparation of a Monoclonal Antibody and Expression of its Antigen Associated with Myogenic Differentiation on Spontaneous and Artificial Myotubes Derived from Avian Myoblasts

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ABSTRACT. Quail myoblasts transformed with a temperature-sensitive mutant of Rous sarcoma virus (ts-RSV) proliferate and do not differentiate at 35.5°C, the permissive temperature for the virus, whereas their myoblast differentiation proceeds at 41.0°C, a non-permissive temperature. In this experimental system, myogenic differentiation is controlled by src gene products. Using QM-RSV cells as an antigen, a monoclonal antibody, Mb-N3, was prepared. Expression of Mb-N3 antigen was found to increase during differentiation. Therefore, in studies on the mechanism of myogenic differentiation, we examined the expression of Mb-N3 antigen on spontaneously forming myotubes formed at 41.0°C and fused myoblasts with hemagglutinating virus of Japan (HVJ, Sendai virus) disregarding programmed processes for myogenic differentiation.

When the myoblasts cultured at 35.5°C were treated with HVJ, they fused with each other. These fused myoblasts were elongated and were morphologically similar to spontaneously forming myotubes. Thus, we called fused myoblasts with HVJ "artificial myotubes." During culture at 35.5°C, the artificial myotubes did not show increased expression of Mb-N3 antigen and increase of creatine kinase activity, which are markers of normal biochemical differentiation. When artificial myotubes were cultured at 41.0°C, expression of Mb-N3 antigen and creatine kinase activity increased. These results suggest that the expression of the antigen is regulated by kinase activity derived from src gene products even after compulsory cell fusion. Moreover, compulsory fusion does not cause myogenic differentiation and expression of Mb-N3 antigen. Thus it seems that the differentiation program must proceed in order for myogenic differentiation and expression of Mb-N3 antigen to take place.

In myogenesis, myoblasts that have been proliferating continuously stop replicating and pass through the series of steps of cell recognition, cell interaction, cell adhesion and cell arrangement (15, 33). Simultaneously, the myoblasts fuse to form myotubes. During these morphological changes, the cells begin to express muscle-specific proteins, and the myotubes differentiate into muscle fibers. As these very remarkable phenomena can be reproduced in vitro, studies on myogenic differentiation are very useful for analyzing the mechanism of differentiation of higher animal cells.

We have studied the mechanism of myoblast differentiation, especially myoblast fusion, using quail myoblasts transformed with a temperature-sensitive mutant of Rous sarcoma virus (ts-RSV), QM-RSV cells (13, 14), because with these cells, myogenic differentiation can be controlled by change of the culture temperature. When QM-RSV cells are cultured at 41.0°C, they become fusion-competent within 12 hr and the appearance of myotubes starts about 18 hr and reaches a plateau within 48 hr (13, 14). This control is caused by change in tyrosine kinase activity of the src gene products of ts-RSV (13). The control of differentiation by src gene products is relaxed and cells differentiate into myotubes at 41.0°C; however, at 35.5°C, which is a permissive temperature for the virus, differentiation is inhibited (4, 8, 13, 22).

As myoblast differentiation involves a series of steps, as described above, the expression of various genes is thought to increase or decrease in order during the differentiation process. During myogenic differentiation, many morphological and biochemical changes are observed in cells (1, 2, 7, 9, 21, 25, 26, 32, 34, 35). Some characteristic monoclonal and polyclonal antibodies for antigens that are associated with myogenesis have been prepared and used in studies on myogenic differentiation (3, 5, 6, 10, 11, 17, 18, 19, 23, 28). Analysis of the dynamic expressions of cell surface antigens during differentiation including myoblast fusion seems significant to the understanding of the differentiation process.
Therefore, in this work we first tried to produce monoclonal antibodies associated with myogenic differentiation. We obtained a monoclonal antibody, named Mb-N3, whose antigen was expressed more strongly on myotubes than myoblasts. To obtain information of the relation between the order of differentiation events and myogenic differentiation, we prepared artificial multinucleated cells with hemagglutinating virus of Japan (HVJ, Sendai virus) and compared the expressions of Mb-N3 antigen as a marker of differentiation on spontaneous myotubes and artificial multinucleated cells.

MATERIALS AND METHODS

Cell culture. Quail myoblasts transformed using a temperature-sensitive mutant of Rous sarcoma virus (ts-RSV), QM-RSV cells, were prepared as described previously (13). Dulbecco's modified Eagle's medium (DMEM) plus 2 mM glutamine (Nissui Pharmaceutical Co., Tokyo, Japan), penicillin (100 U/ml) and streptomycin (0.1 mg/ml) was used as culture medium. This medium was supplemented with fetal bovine serum (FBS) at 20% for use as growth medium and at 5% for use as differentiation medium. Differentiation was initiated by replacing the growth medium by differentiation medium and shifting the culture temperature from 35.5°C to 41.0°C. After these changes, myoblast fusion occurred within 24 hr. Primary cultures of quail and chick myoblasts, and quail fibroblasts were prepared and cultured as described previously (13). QM-RSV cells, primary QM cells and primary chick myoblasts were cultured in plastic dishes coated with collagen (Cell matrix, Type I-P, Nitta Gelatin, Yao City, Osaka, Japan). Glass dishes were used for fibroblasts. The mouse skeletal muscle cell line C2C12 formed myotubes in the differentiation medium consisting of DMEM containing 5% horse serum, whereas for growth of these cells, DMEM containing 10% FBS was used. B3H1 cells, which have been used as a model of smooth muscle, were cultured in DMEM containing 10% FBS. C2C12 cells and B3H1 cells were cultured in gelatin-coated plastic dishes. Quail heart cells were prepared from 6-day-old quail embryos. These cells were cultured in DMEM containing 10% calf serum (CS). MDCK cells derived from dog kidney were cultured in minimum essential medium (MEM) containing 10% CS.

For treatment of QM-RSV cells with tunicamycin (Sigma Chemical Co., St. Louis), the cells were preincubated for 24 hr at 35.5°C, and then cultured at 41.0°C in differentiation medium for 6 hr. Thereafter, the cells were cultured for 18 hr in medium containing tunicamycin (1 µg/ml), which blocks protein glycosylation and expression of glycoproteins on the cell surface (24). We confirmed by lectin blotting that the synthesis of sugar chains was inhibited under these conditions. Tunicamycin dissolved in dimethyl sulfoxide (10 mg/ml) was used as stock solution.

For treatment of QM-RSV cells with herbimycin A (a gift from Dr. Y. Uehara, National Institute of Health, Tokyo, Japan), the cells were preincubated and then cultured at 35.5°C in differentiation medium containing a final concentration of 0.05 µg/ml herbimycin A. Herbimycin A inhibits the tyrosine kinase activity of src gene products (29, 30), and allows myotube formation by QM-RSV cells even at 35.5°C (13). Herbimycin A dissolved in dimethyl sulfoxide (1 mg/ml) was used as stock solution.

Preparation of monoclonal antibody. Monoclonal antibodies against QM-RSV cells were obtained by a procedure using polyethylene glycol. After culture of QM-RSV cells in growth medium at 35.5°C for 24 hr, the medium was changed to Ca2+-deficient differentiation medium and the culture temperature was shifted up to 41.0°C. Myoblasts do not fuse but show biochemical differentiation in Ca2+-deficient differentiation medium (14). QM-RSV cells cultured in Ca2+-deficient differentiation medium at 41.0°C for 18 hr were scraped off with a rubber policeman, suspended in 0.25 M sucrose, and homogenized with a Dounce homogenizer. The homogenate was centrifuged (820 × g) for 10 min at 4°C, and the supernatant was centrifuged (270,000 × g) for 20 min at 4°C. The pellet obtained was used as membrane fraction. This pellet was resuspended in Ca2+-free phosphate buffered saline (PBS (−); 8 g/l NaCl, 0.2 g/l KCl, 1.15 g/l Na2HPO4, 0.2 g/l KH2PO4) and used as an antigen. Spleen cells from mice immunized with this antigen were fused with X63-Ag8-6.5.3 myeloma cells by treatment with 50% polyethylene glycol (#4000), 10% dimethyl sulfoxide and 40% DMEM. The hybridomas obtained were cultured at 37.0°C in DMEM containing 15% FBS. About 250 hybridomas were obtained, and one, named Mb-N3, whose culture supernatant reacted strongly with myotubes, was used in this study.

Assay of creatine kinase. Creatine kinase (CK) activity was assayed as a marker of biochemical differentiation. Preparation of samples and the assay procedure were performed as described previously (13).

Indirect immunofluorescence staining. Cells were grown on glass coverslips. For study of expression of the antigen only on the cell surface, living cells were incubated with the first antibody, Mb-N3, for 60 min at 4°C, and then washed three times with PBS and incubated for 30 min at 4°C with FITC-conjugated antibody against mouse immunoglobulins (IgG+IgM+IgA) (Cappel Organon Teknika Co. West Chester) as the second antibody. Finally, the cells were fixed with 3.7% formalin. The cells were washed with PBS (−), mounted in glycerol containing p-phenylenediamine, and then observed with a fluorescence microscope (Carl Zeiss. Inc., Oberkochen, Germany). For study of expression of the antigen in the cytoplasm, the cells were fixed in acetone : methanol (1 : 1) at −20°C for 12 min before treatment with antibodies.

Immunoblotting. QM-RSV cells scraped from culture dishes and pectoral muscles of quail embryos were extracted with 0.5% sodium dodecylsulfate (SDS) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetamide, 1 µg/ml pepstatin, 10 µg/ml trypsin inhibitor, 1

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To confirm that the Mb-N3 antigen was associated at 35.5°C. Cultured mononucleated cells were stained as weakly as cells that remain unfused. The staining pattern of myoblasts cultured at 35.5°C showed little, if any, staining. At 41.0°C, myoblasts were intensely stained, while almost all remained unstained at 35.5°C. Equal amounts of protein, measured by the method of Lowry et al. (20), were applied. The sample solution was not treated with a reductant nor boiled, thus the antigen was not reduced. The proteins were transferred electrophoretically onto nitrocellulose membranes (Bio-Rad Laboratories, CA) by transfer buffer (0.1 M Tris, 0.192 M glycine), which did not contain methanol. After Western blotting, blocking was carried out with 5% skim milk in Tris-buffered saline (TBS; 20 mM Tris, 500 mM NaCl, pH 7.5) for 1 hr at room temperature, and then the nitrocellulose membranes were incubated overnight with Mb-N3 at room temperature. Horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad Laboratories, CA) was used as the second antibody. The buffer used for washing the membranes was TBS containing 0.5% Tween-20 (TTBS). The first and second antibodies were diluted with TTBS containing 3% skim milk.

Fusion of myoblasts with HVJ (Sendai virus). HVJ for fusion was propagated in chick embryos, concentrated by ultracentrifugation as described previously (12) and inactivated with UV light. QM-RSV cells were cultured at 35.5°C for 24 hr in growth medium seeded at 5.9 × 10^5 cells per collagen-coated dish (60 mm diameter), washed once with PBS (−) and treated with 400 μl of inactivated HVJ solution (500 HAU/ml) per dish. They were then left to stand on ice for 30 min, washed once with PBS (−), and cultured in 3 ml of fresh differentiation medium at 35.5°C. "Artificial myotubes" appeared within 12 hr in these cultures.

RESULTS

Preparation of monoclonal antibodies. We have tried to obtain monoclonal antibodies that are useful for investigating myoblast differentiation and prepared about 250 antibodies. These antibodies can be classified as those expressed 1) mainly on myotubes, 2) mainly on myoblasts, and 3) on both myotubes and myoblasts and 4) antibodies inhibiting myoblast fusion. It has, however, been difficult to obtain antibodies of group 4. In the present study, we used Mb-N3 which was classified under group 1. To examine the expression of Mb-N3 antigen, QM-RSV cells cultured at 35.5°C (Fig. 1a, b) and 41.0°C (Fig. 1c, d) were stained by immunofluorescence staining. Clear differences were seen between the cells cultured at 35.5°C and 41.0°C. Myoblasts cultured at 35.5°C showed little, if any, staining. At 41.0°C, myotubes were intensely stained, while almost all remaining mononucleated cells were stained as weakly as cells cultured at 35.5°C.

To confirm that the Mb-N3 antigen was associated with myogenic differentiation, we next examined the expression of the antigen in 4-13-day-old quail embryos. Extracts prepared from individual embryonic muscles were subjected to immunoblotting. As shown in Fig. 2, expression of the antigen increased during development to a maximum in 12-day-old embryos (lane 9 in Fig. 2), and then began to decrease. These results strongly suggest that the Mb-N3 antigen is associated with myogenic differentiation.

Relationship of expression of Mb-N3 antigen with kinase activity. As described above, Mb-N3 antigen was highly expressed on QM-RSV cells at 41.0°C, a non-permissive temperature.

We next examined the time course of its expression on QM-RSV cells after increasing the culture temperature. QM-RSV cells were cultured at 41.0°C after preculture at 35.5°C, and cell lysates were prepared at different intervals. These cell lysates were then subjected to immunoblotting and the quantity of the antigen expressed was measured by densitometry (Fig. 3). After increasing the temperature to 41.0°C, expression of the antigen was not detectable for the initial 6 hr, but a rapid increase was detected between 6 to 12 hr. This result suggests that expression of the Mb-N3 antigen increases rapidly during the 6-12 hr period after the increase in temperature. As reported previously, incubation for 10-12 hr at 41.0°C is required for QM-RSV cells to become fusion-competent (14). Next, expression of Mb-N3 antigen on QM-RSV cells cultured for 10 hr at 41.0°C was examined by indirect immunofluorescence staining to clarify its relationship with fusion-competence. Most of the cells cultured at 41.0°C for 10 hr stained intensely with Mb-N3 (Fig. 4). From these observations, it seems that expression of the Mb-N3 antigen is associated with acquisition of fusion-competence.

Mb-N3 antigen was expressed intensely on the myotubes 24 hr after the increase in temperature to 41.0°C when the myotubes were formed as well as the cells with fusion-competence. However, at this time, unlike after culture for 10 hr, remaining unfused mononucleated cells were scarcely stained with Mb-N3 (see Fig. 1d). These results suggest that this antigen disappears from cells that remain unfused. The staining pattern of myoblasts cultured at 41.0°C for 10 hr and that of myotubes after 24 hr were also somewhat different: that of myoblasts after 10 hr being diffuse and that of myotubes after 24 hr appearing as nondiffuse staining with some aggregates (Fig. 1d). Thus the expression and distribution of Mb-N3 antigen seem to change dynamically with the incubation time during differentiation at 41.0°C.

The finding that expression of Mb-N3 antigen on QM-RSV cells increased at 41.0°C but was suppressed at 35.5°C suggests that expression of Mb-N3 antigen is regulated by the tyrosine kinase activity of src gene pro-
Fig. 1. Expression of Mb-N3 antigen on QM-RSV cells.
Immunofluorescence staining of QM-RSV cells was performed with Mb-N3. Unfixed QM-RSV cells were treated first with Mb-N3, and then with FITC-conjugated goat anti-mouse immunoglobulins. The cells were cultured at 35.5°C for 24 hr in growth medium (a and b) or at 41.0°C in differentiation medium for 24 hr (c and d). (a) and (c) are phase-contrast micrographs, and (b) and (d) are immunofluorescence micrographs. Mb-N3 antigen was mainly expressed on myotubes cultured at 41.0°C. Bar, 100 μm.

ducts. To examine this point, QM-RSV cells were treated with herbimycin A, which inhibits the tyrosine kinase activity of src gene products (29, 30), at 35.5°C for 48 hr and then cell lysates were subjected to immunoblotting with Mb-N3 (Fig. 5). Mb-N3 antigen was expressed even at 35.5°C on QM-RSV cells treated with
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Fig. 2. Expression of Mb-N3 antigen in quail embryos.
Extracts were prepared from 4-13-day-old quail embryos and immunoblot analysis was performed with Mb-N3. Extracts from 4-13-day-old quail embryos were applied in lanes 1–10, respectively. Expression of Mb-N3 antigen increased with the developmental stage to a maximum in 12-day-old embryos, and then decreased.

Fig. 3. Quantitative expression of Mb-N3 antigen during differentiation of QM-RSV cells.
Cells were grown at 35.5°C in growth medium for 24 hr and then cultured for 48 hr at 35.5°C or 41.0°C in differentiation medium. Cells extracts were prepared at 0, 6, 12, 24, and 48 hr, respectively, and subjected to immunoblot analysis. Expression of Mb-N3 antigen was measured by densitometry. The level of expression at 41.0°C after 48 hr was taken as 100%. QM-RSV cells were cultured at 35.5°C (-O-), or at 41.0°C (-●-) after change to differentiation medium. At 35.5°C, expression was suppressed continuously. At 41.0°C, expression increased with the culture time to attain a plateau after 24 hr.

The results suggest expression of the Mb-N3 antigen is regulated by the activity of src gene products.

We also stained primary QM cells with Mb-N3 to confirm the correlation between expression of Mb-N3 antigen and src gene products. For this, primary QM cells were prepared from 10-day-old quail embryos and cultured under the same conditions as QM-RSV cells. Primary QM cells begin to fuse forming myotubes within about 24 hr after increasing of culture temperature, and many typical myotubes are formed within 72 hr. Myotube formation occurs at 35.5°C on primary culture (13).

In QM-RSV cells, no distinct expression of Mb-N3 antigen was observed at 35.5°C. Then, expression of Mb-N3 antigen on primary QM cells that had been cultured at 35.5°C for 24 hr was examined by immunofluorescence staining (Fig. 6). Mb-N3 antigen was expressed as intensely by primary QM cells at 35.5°C as at 41.0°C. We found that kinase activity was scarcely detectable in primary QM cells, unlike in QM-RSV cells (unpublished data). It seems that Mb-N3 antigen is expressed intensely on cells with low kinase activity. These results suggest that the expression of Mb-N3 antigen on QM-RSV cells is regulated by the kinase activity of src gene products.

Characterization of Mb-N3 antigen. As described above, results of immunofluorescence staining demonstrated that the Mb-N3 antigen was strongly expressed on the surface of cells at the competent stage. Next, we examined expression of Mb-N3 antigen in the cytoplasm. After the cell was rendered permeable by fixation in acetone : methanol (1 : 1), expression of this anti-
Fig. 4. Expression of Mb-N3 antigen on QM-RSV cells cultured at 41.0°C for 10 hr.
QM-RSV cells cultured at 41.0°C for 10 hr were stained with Mb-N3. (a), phase-contrast micrograph, (b), immunofluorescence micrograph. Mb-N3 antigen was already expressed on the cells, although the cells were still unfused. Most of the cells were stained diffusely, unlike myotubes cultured for 24 or 48 hr at 41.0°C. Bar, 50 μm.

Fig. 5. Expression of Mb-N3 antigen on QM-RSV cells treated with herbimycin A.
Extracts of cells cultured for 48 hr at 35.5°C (lane 1), at 41.0°C (lane 2), at 35.5°C in the presence of herbimycin A (lane 3). Mb-N3 antigen was expressed at 41.0°C but not at 35.5°C, the non-permissive temperature. In the presence of herbimycin A, the antigen was expressed even at 35.5°C, suggesting that the expression is regulated by kinase activity of viral src gene products.

Fig. 6. Expression of Mb-N3 antigen on QM-RSV cells treated with herbimycin A.
Extracts of cells cultured for 48 hr at 35.5°C (lane 1), at 41.0°C (lane 2), at 35.5°C in the presence of herbimycin A (lane 3). Mb-N3 antigen was expressed at 41.0°C but not at 35.5°C, the non-permissive temperature. In the presence of herbimycin A, the antigen was expressed even at 35.5°C, suggesting that the expression is regulated by kinase activity of viral src gene products.

To obtain an idea of the biological significance of the antigen, we examined some characteristics of the Mb-N3 antigen. Lysates of QM-RSV cells cultured at 35.5°C and at 41.0°C were subjected to 12.5% SDS-PAGE and Western blotting (lanes 1, 2 and 3 in Fig. 7). Under nonreducing conditions, a clear band was detected at about 41 KDa in lysates of cells cultured at 41.0°C (lane 3 in Fig. 7). At 35.5°C, the antigen was hardly expressed, as observed from immunofluorescence staining (lanes 1 and 2 in Fig. 7). The antigen was not detectable under reduced conditions. We also investigated whether the Mb-N3 antigen had a sugar chain. For this, QM-RSV cells were treated with tunicamycin, which blocks protein glycosylation and expression of glycoprotein, and then lysates of the cells were subjected to immunoblotting with Mb-N3. A clear band was detected with Mb-N3 (lane 4 in Fig. 7), but the molecular weight of this material was less than that of the antigen of control cells. This result suggests that Mb-N3 antigen has a sugar chain, but the recognition site of Mb-N3 is in its protein moiety. To confirm the location of the recognition site of Mb-N3, cell lysates were trypsinized at 37.0°C for 3 hr and then subjected to immunoblotting with Mb-N3. No Mb-N3 antigen was detected in the trypsinized lysate, indicating that the Mb-N3 antigen is a glycoprotein and its recognition site is not in its sugar chain but in its protein moiety.

We also examined the effects of Mb-N3 on some physi-
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Fig. 6. Expression of Mb-N3 antigen on primary QM cells.
Primary QM cells were prepared from 10-day-old embryos and cultured at 35.5°C for 24 hr after seeding. At this time the cells were still mononucleated. These primary QM cells were stained with Mb-N3. (a), phase-contrast micrograph, (b), immunofluorescence micrograph. Mb-N3 antigen was strongly expressed on primary QM cells even at 35.5°C. Bar, 50 μm.

Physiological functions of myogenic differentiation of QM-RSV cells, namely, creatine kinase activity, fusion and adhesion to dishes. No distinct effects of Mb-N3 on these physiological properties were detected.

Next, we examined the expression of Mb-N3 antigen on the other cells (Table I). Mb-N3 antigen was strongly detected on quail primary myogenic cells, quail heart cells and chick primary myogenic cells, but slightly detected on other cells, if at all. Thus the Mb-N3 antigen seems to be specific to avian myogenic cells.

Biochemical differentiation in Ca²⁺-deficient medium

Table I. Expression of Mb-N3 antigen on various cells.

<table>
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<th>Cell Type</th>
<th>myoblasts</th>
<th>myotubes</th>
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<tr>
<td>QM-RSV</td>
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<tr>
<td>QM-primary</td>
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<td>Quail Heart</td>
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<td>Chick-primary</td>
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<td>Quail Fibroblasts</td>
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<td>BC3H1</td>
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<td>C2C12</td>
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<td>MDCK</td>
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Mb-N3 antigen was detected by immunofluorescence staining. "+" indicates expression of the antigen and "−" indicates non-expression of the antigen. Number of "+" signs indicates level of intensity of expression. (+++ > ++ > +)
and expression of Mb-N3 antigen. Although Mb-N3 did not inhibit the increase of CK activity or myoblast fusion, experiments with quail embryonic pectoral muscles strongly suggested that it is associated with myogenic differentiation. Therefore, we examined the biochemical differentiation of cells and their expression of Mb-N3 antigen under low Ca\(^{2+}\) conditions to obtain some ideas on the biological significance of this antigen.

In general, myoblast fusion, as well as fusion of other cells, does not occur under low Ca\(^{2+}\) conditions (27, 31). However, myoblast fusion and biochemical differentiation can be distinguished under different experimental conditions (14). We examined the expression of Mb-N3 antigen by cells cultured in Ca\(^{2+}\)-deficient differentiation medium at 41.0°C or 35.5°C by immunoblotting. At 41.0°C, Mb-N3 antigen was expressed in the same degree of intensity in both the Ca\(^{2+}\)-deficient medium (lane 5 in Fig. 8) and the normal differentiation medium (lane 3 in Fig. 8), although formation of myotubes was inhibited in the former medium. Similar results were obtained by the immunofluorescence staining.

**Characteristics of artificial myotubes induced by HVJ.** As described above, biochemical differentiation could be distinguished from myoblast fusion under certain experimental conditions. However, myoblast fusion and biochemical differentiation usually proceed simultaneously, and there seems to be a close correlation between the two events. Furthermore, differential events are thought to proceed in a programmed order. To clarify the relationship between myogenic differentiation and this programmed order, we prepared multinucleated fused cells by fusion using HVJ under conditions that disregard programmed processes for differentiation. QM-RSV cells cultured at 35.5°C were treated with HVJ and cultured at 35.5°C, not 41.0°C, to prevent spontaneous myoblast fusion. Fused multinucleated cells began to appear after about 12 hr treatment with the virus (Fig. 9a) and were formed appreciably after 48 hr (Fig. 9b). These multinucleated cells formed with HVJ showed morphologically elongated “tubes” like normal myotubes formed spontaneously at 41.0°C. We used these multinucleated cells as “artificial myotubes.” Furthermore, when artificial fusion was induced by HVJ, Phase-contrast micrographs of QM-RSV cells (a), (b), cells cultured at 35.5°C for 12 hr and for 48 hr, after HVJ treatment, respectively. Artificial myotubes were morphologically similar to normal myotubes. Bar, 500 μm.

**Fig. 8.** Expression of Mb-N3 antigen under low Ca\(^{2+}\) conditions. Extracts of cells cultured at 35.5°C for 24 hr after seeding (lane 1), for 24 hr after medium change to differentiation medium at 35.5°C (lane 2), or at 41.0°C (lane 3), or for 24 hr after medium change to Ca\(^{2+}\)-deficient differentiation medium at 35.5°C (lane 4), or 41.0°C (lane 5). Under low Ca\(^{2+}\) conditions, expression of Mb-N3 antigen at 41.0°C was as strong as on cells cultured in normal differentiation medium.

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Fig. 10. CK activity assay of QM-RSV cells treated by HVJ. QM-RSV cells were treated with HVJ and then cultured at 35.5°C for 24 hr in differentiation medium. The culture medium was then changed to Ca2+-deficient differentiation medium to prevent spontaneous myotube formation. Fused QM-RSV cells were cultured at 35.5°C (-O-), or 41.0°C (Æf) after medium change. CK activity did not increase at 35.5°C.

We next investigated whether biochemical differentiation proceeded in artificial myotubes prepared in this way by measuring CK activity as a marker of biochemical differentiation (Fig. 10). QM-RSV cells were cultured at 35.5°C for 24 hr, then fused by addition of HVJ. After another 24 hr, the cells were cultured further at 41.0°C in Ca2+-deficient medium to prevent spontaneous myoblast fusion at 41.0°C. Lysates of QM-RSV cells were prepared at various times. Artificial multinucleated cells had a tubelike shape but showed no increase of CK activity on culture at 35.5°C. On the other hand, when the multinucleated cells were cultured at 41.0°C, their CK activity increased with the incubation time. These results suggest that myoblast fusion alone is not sufficient to increase CK activity, but that CK activity is regulated with kinase derived from src gene products.

We also examined the extent of expression of desmin on spontaneous myotubes and artificial myotubes. Since desmin is a muscle-specific protein, it has often been used as a marker of biochemical differentiation of muscle. As expected, desmin was detectable on spontaneous myotubes at 41.0°C. On artificial myotubes, however, it was not expressed at 35.5°C in spite of myoblast fusion. These results suggest that formation of artificial myotubes from presumptive myoblasts does not cause biochemical differentiation.

Expression of Mb-N3 antigen on artificial myotubes. To investigate the relation between expression of surface antigen and the fusion, we examined the expression of Mb-N3 antigen as a marker of differentiation on artificial myotubes. After treatment with HVJ, QM-RSV cells were cultured at 35.5°C for 24 hr and then their immunofluorescence staining was examined. Expression of Mb-N3 antigen on artificial myotubes was as weak at 35.5°C as that on myoblasts cultured normally at 35.5°C, as shown in Fig. 1b (Fig. 11a and b).

When these artificial myotubes were cultured at 41.0°C for 24 hr, their expression of Mb-N3 antigen became as intense as that of spontaneous myotubes shown in Fig. 1d. (Fig. 11c and d). This experiment was performed under low Ca2+ conditions to prevent spontaneous myotube formation at 41.0°C, thus, the myotubes observed were induced by HVJ treatment. These results suggest that expression of the Mb-N3 antigen gene is controlled by the kinase activity of src gene products even after formation of artificial myotubes, and also suggest that myoblast fusion alone is not enough to induce expression of Mb-N3 antigen.

DISCUSSION

We have tried to prepare monoclonal antibodies against components associated with myogenic differentiation to analyze the mechanism of myotube formation. As described in this paper, a monoclonal antibody named Mb-N3 was isolated using QM-RSV cells as an antigen. Expression of its antigen increased during myogenic differentiation both in vivo and in vitro, although this antibody did not block either fusion or biochemical differentiation. Therefore, Mb-N3 appears to be useful for investigating the dynamic state of the antigen that is associated with myogenic differentiation.

During differentiation, the antigen was found to be strongly expressed by myoblasts immediately before myoblasts became fusion-competent. This finding suggests that the molecule recognized by Mb-N3 is associated with the event of becoming fusion-competent. We think that Mb-N3 recognizes a molecule in a non-active epitope that is associated with myogenic differentiation. On the other hand, after culture at 41.0°C for 24 hr, when the cells had differentiated sufficiently to form myotubes, Mb-N3 antigen was expressed strongly and specifically on the surface of myotubes. Expression of Mb-N3 antigen starts before the fusion-competent step, and persists in the myotubes, but disappears from remaining mononucleated cells that do not form myotubes. These findings suggest that the Mb-N3 gene associated
Fig. 11. Expression of Mb-N3 antigen on artificial myotubes formed by HVJ.
Immunofluorescence staining was performed with Mb-N3. QM-RSV cells fused artificially by HVJ were cultured at 35.5°C for 24 hr. (a), phase-contrast micrograph, (b), immunofluorescence micrograph. (a) and (b) show the same field. Expression of Mb-N3 antigen on "artificial myotubes" at 35.5°C was as weak as that of myoblasts cultured at 35.5°C in spite of formation of "myotubes" (b). Then, the culture medium was changed to Ca\(^{2+}\)-deficient medium and culture was continued at 41.0°C for 24 hr. (c), phase-contrast micrograph, and (d), immunofluorescence micrograph of the same field. When "artificial myotubes" were cultured at 41.0°C, Mb-N3 antigen became intensely expressed (d). Bar, 100 μm.
with differentiation is expressed transiently at an essential step and then is suppressed. However, its expression on the myotubes may be necessary to maintain the myotubes functionally and/or morphologically. A little expression of Mb-N3 antigen at 35.5°C suggests that the expression of Mb-N3 antigen is necessary for myoblasts intrinsically. It also seems that the increase of the expression of this antigen is necessary for myogenic differentiation. Mb-N3 antigen seems to be associated with several events in myogenic differentiation. Recently, we found that the expression of Mb-N3 antigen was completely blocked by treatment with doxorubicin, which is an inhibitor of myoblast fusion (unpublished data).

As described above, myoblast fusion and biochemical differentiation can be distinguished under certain experimental conditions. However, in general, the two events proceed simultaneously and are closely correlated. To investigate the correlation between myoblast fusion and biochemical differentiation and the necessity of the programmed differentiation process, the expression of Mb-N3 antigen on artificial myotubes formed by fusion of undifferentiated myoblasts was examined, excluding the differentiation program. Mb-N3 antigen was not expressed on the artificial myotubes at 35.5°C, but was strongly expressed when the artificial myotubes were cultured at 41.0°C. It seems that expression of the Mb-N3 antigen on these artificial myotubes is still controlled by the kinase activity of src gene products. Expression of Mb-N3 antigen, biochemical differentiation and cell fusion were inhibited at 35.5°C, in spite of myotube formation, when myoblasts were fused with HVJ. These results suggest that orderly progress of the differentiation program, as well as myoblast fusion, is necessary for myogenic differentiation. Furthermore, it seems that phosphorylation of some protein(s) by kinase at 35.5°C is more closely concerned with earlier events than the events that control the expression of Mb-N3 antigen, biochemical differentiation and cell fusion.

Now, we are purifying the Mb-N3 antigen by affinity chromatography to prepare the polyclonal antibody of the antigen for further examination of the correlation of the antigen with myogenic differentiation. We are also investigating the Mb-N3 antigen at a molecular level.

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