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Susceptibility of Differentiating Muscle Cells of the Fetal Mouse in Culture to Coxsackievirus A13[1]

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The interaction of coxsackievirus A13 with differentiating muscle cells, cultured from tissues of the fetal mouse, was studied. Cultures infected at that stage of myogenic differentiation characterized by the rapid formation of multinucleated myotubes produced maximum virus titers of over 10^7 plaque-forming units. Virus-induced cytopathic effect was characterized by a marked diminution in the number of multinucleated cells. The susceptibility of these cultures decreased appreciably when infection was initiated after the majority of the myotubes had formed. The demonstration of newly synthesized A13 virus antigen by immunofluorescence provided direct evidence that A13 virus replication occurred both in myoblasts and myotubes. The synthesis of A13 virus was markedly depressed in muscle cultures in which the formation of multinucleated cells was inhibited by BUDR or by fusion-inhibiting media. After reversal of this inhibition, the cultures acquired the increased susceptibility to A13 virus characteristic of cells undergoing myogenic differentiation. In contrast to the results obtained with coxsackievirus A13, the primary fetal mouse muscle cultures were resistant to poliovirus T1. It is suggested that changes in the surfaces of developing muscle cells may coincide with the formation and disappearance of specific virus receptors and thereby regulate the cell susceptibility to coxsackievirus A13.

Propagation of the group A coxsackieviruses in tissue culture systems derived from infant or fetal mice has met with limited success (24, 29). More characteristically, a pattern of complete insusceptibility has been observed in various murine tissue cultures exposed to different group A coxsackieviruses (2, 14, 25, 26, 29, 30) or to extracted coxsackievirus A6 ribonucleic acid (RNA; reference 26). A report (15) describing the replication of coxsackievirus A4 in explant cultures of infant mouse tissues, grown under relatively anaerobic conditions on reconstituted collagen, awaits confirmation.

Recent experiments conducted in our laboratory (11) have shown that primary fetal mouse monolayer cultures were susceptible to infection by coxsackievirus A13 as evidenced by a limited, but unequivocal, multiplication of virus. Whether infection was initiated with complete virus or with viral RNA, yields of A13 virus of 10^6 to 10^7 plaque-forming units (PFU) per culture were obtained in the absence of a discernible cytopathic effect (CPE). These observations, together with the finding that no interferon was demonstrable in either the virus inoculum or in the test system, suggested that a relatively small portion of the cell population was susceptible to productive A13 virus infection. The purpose of the present study was to obtain differentiated muscle cells in culture (17, 27, 31) in an attempt to increase the number of cells which would replicate this myotropic virus and to identify the developmental stage during differentiation when the cells could be infected.

MATERIALS AND METHODS

Primary fetal mouse muscle (PFMM) cultures. Fetuses of approximately 18 to 21 days of age were removed aseptically from mice (Swiss-Webster, random bred, Huntingdon Farms. Conshohocken, Pa.)

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sacrificed previously by cervical dislocation. The hind limbs were removed and the skeletal muscle was dissected from the surrounding tissues with the aid of a Zeiss dissecting microscope at a final magnification of 10×. The muscle tissue was minced, transferred to a 125-ml trypsinizing flask (Bellco Glass Inc., Vineland, N.J.) and suspended in 30 ml of 0.2% trypsin (Difco 1:250) diluted in calcium and magnesium-free phosphate-buffered saline containing 200 μg of penicillin and streptomycin (PBS-a) per ml. (5). The mixture was gently agitated on a magnetic stirrer for 1 hr at 37 C. The contents were filtered through two layers of lens paper, and 10 ml of nutrient growth medium was added to inhibit the tryptic digestion. The nutrient growth medium routinely employed for propagation of mouse cells (mouse-GM) consisted of Eagle's minimal essential medium (MEM; reference 7) with 10% horse serum (Grand Island Biological Co., Grand Island, N.Y.), penicillin and streptomycin (100 μg/ml), 0.02 M *N*-2-hydroxyethyl piperazine-*N*¹-2-ethanesulfonic acid (HEPES) buffer and 0.225% $NaHCO_3$ in Earle's balanced salt solution. The filtrate was centrifuged at 600 × *g* for 10 min at 4 C, the supernatant fluid was carefully withdrawn, and the packed cells were pipetted gently into 10 ml of mouse-GM. Cultures were initiated in plastic petri dishes (Falcon Plastic, Div. of B-D Laboratories, Inc., Los Angeles, Calif.) with 2 ml of a cell suspension containing 6.5×10^5 cells/ml for each dish (35 by 15 mm) or 5 ml of a cell suspension containing 8.0×10^5 cells/ml for each dish (60 by 15 mm). Each culture dish contained one glass cover slip (22 mm^2). The cultures were incubated at 37 C in a humidified gas mixture containing 95% air and 5% CO_2. The mouse-GM was exchanged for fresh medium every day until the cultures were used for virus growth studies.

ML cells. ML cells were grown as previously described (11).

Virus strains. Coxsackievirus type A13 was propagated in newborn mice as previously described (2).

The origin and method of propagation of poliovirus T1 (Mahoney) have been described (4). Poliovirus was passaged serially as the supernatant fluids of infected cultures of HeLa cells (strain JJH) or, where indicated, in ML cells.

A protocol similar to that described by Crowell (4) also was used to obtain high titer pools of coxsackievirus A-13 (HTP-A13) in ML cells. Virus preparations were centrifuged at 8,700 × *g* for 30 min at 2 C. The supernatant fluids were collected and centrifuged at 122,000 × *g* for 2 hr at 8 C. The supernatant fluids were discarded and the pellets were resuspended in approximately 3 ml of Hanks balanced salt solution (BBS). The partially purified virus preparations (PP-A13) were stored at 4 C.

Assay of virus in ML cells. Methods for the quantitative assay of infectious coxsackievirus A13 and poliovirus T1 by plaque formation in ML cells have been described (11).

Determination of virus replication in PFMM cultures. Replicate primary cultures of fetal mouse tissues grown as monolayers were drained and inoculated with 0.2 ml (60- by 15-mm plates) or 0.1 ml (35- by 15-mm plates) of mouse passaged coxsackievirus A13, containing 10^7 PFU per ml. The plates were incubated at 37 C for 60 min for virus attachment. The amount of unattached virus was reduced by rinsing three times with 2-ml volumes of mouse-GM, and the cultures were overlaid with mouse-GM and incubated at 37 C for production of virus. The total virus yield was obtained for assay by removing replicate cultures at intervals after infection and freezing and thawing the cells five times to release intracellular virus. The disrupted culture contents were centrifuged at 600 × *g* for 15 min at 4 C, and the supernatant fluid was frozen at −20 C until assay. A cell-free preparation containing coxsackievirus A13 diluted in mouse-GM was incubated in parallel with the test preparations and served as control of virus stability.

Infection of cultures for immunofluorescent studies. PFMM cultures in 35-mm dishes were drained and inoculated with 6×10^7 PFU in 0.1 ml of A13 virus grown in ML cells. The plates were incubated at 37 C for 60 min for virus attachment and overlaid with 2 ml of mouse-GM per plate. At intervals of incubation at 37 C, cultures were removed from the incubator and washed three times with PBSa. The cover slips were fixed in acetone for 5 min at 4 C, air-dried, and stored at −20 C until stained.

Antisera. White New Zealand female rabbits received four weekly subcutaneous injections of PP-A13 virus. Each rabbit received 2 ml of virus containing 10^{10} PFU/ml suspended in an equal volume of Freund's complete adjuvant (BBL) per inoculation. The rabbits were bled from the central ear artery before immunization and 12 days after the final immunization. The serum was recovered and stored at −20 C until used.

Rabbit sera, both immune and normal, were diluted 1:10 in 2.5% bovine serum albumin (BSA; (Pentex Inc., Kankakee, Ill.) dissolved in PBSa and absorbed overnight at 4 C with 20 mg of mouse liver acetone powder per ml (The Sylvana Co., Melburn, N.J.) on a wrist-action shaker. The absorbed sera were centrifuged at 8,720 × *g* for 20 min at 2 C and the supernatant phase was collected and stored at −20 C until used. These sera were used at a final dilution of 1:25 in a 2.5% BSA solution in the immunofluorescent assay.

Fluorescein-conjugated goat antirabbit IgG serum (Microbiological Associates, Inc., Bethesda, Md.) was reconstituted, diluted 1:6 in BSS, and absorbed overnight at 4 C with 25 mg/ml of mouse liver acetone powder as above. The material was centrifuged at 8,720 × *g* for 20 min at 2 C. The supernatant phase was reabsorbed with mouse liver powder as before, separated by centrifugation at 600 × *g* for 15 min at 4 C, dispensed in small volumes, and stored at −20 C.

Immunofluorescent staining. Cover slips from PFMM cultures were washed once with PBSa and drained of excess fluid. One drop of antiviral or normal serum was added to each cover slip and distributed over the surface of the cells. After 1 hr of incubation at room temperature, the cover slips were washed three times with PBS and treated with 2 drops of fluorescein-conjugated goat antirabbit IgG serum (Microbiological Associates) per cover slip for 30 min at room temperature. Finally, each cover slip culture was rinsed

three times with PBSa, partially air dried, and mounted with HFM Harleco Fluorescence Mountant (Hartman-Leddon Co., Phila., Pa.).

All fluorescent preparations were examined with a Zeiss standard microscope equipped with an Osram high-pressure mercury bulb (HBO 200 w) as light source, dark-field condenser, UGI heat-protection filter, BG 12 and BG 3 exciter filters, and 50/44 barrier filters. Microphotographs were taken with a 35-mm Zeiss attachment camera on Kodak Panatomic-X or Ektachrome type B film.

RESULTS

Myogenesis in PFMM cultures. Fetal mouse muscle tissue was disaggregrated, and the cells were plated as described above. The pattern of myogenesis was found to be similar to that described for cultures prepared from trypsinized skeletal muscle tissue of the developing chick embryo (17, 22) and the embryonic or newborn rat (31). A population of variably shaped mononucleated fibroblast-like and bipolar cells were seen in 1-day-old cultures. Cell multiplication resulted in the appearance of confluent monolayers by day 3, at which time fusion into syncytial multinucleated forms was a prominent feature. Active cell fusion during the next 3 days produced a network of long, branched myotubes among the mononucleated elements. Longitudinal striations were apparent in the cytoplasm of some myotubes by day 5 (Fig. 1), and cross-striated myofibrils generally could be recognized by the end of the first week in vitro. Striated myofibrils became more numerous with increasing development of the muscle fibers (Fig. 2), and on day 6 or 7 many of the multinucleated cells began to contract rhythmically.

Susceptibility of PFMM cultures to coxsackievirus A13 during myogenic differentiation. The possibility was considered that PFMM cultures might differ in susceptibility to A13 virus, depending on the stage of differentiation at the time of infection. To evaluate this possibility, virus growth studies were performed in fetal mouse muscle cultures on successive days during their period of in vitro development as described above.

The results of this experiment are depicted in Fig. 3 It was found that PFMM cultures infected at 3 to 6 days of age produced more virus than did the older cultures. Microscopic comparison of the infected (Fig. 4) and uninfected (Fig. 5) cultures revealed a relative reduction in the number of myotubes in the infected cultures by 48 hr postinfection. It was noticed that large numbers of myotubes alone could not account for the greater susceptibility of the differentiating cultures, since the highly differentiated 7- and 8-day-old cultures produced the least amounts of virus and showed no evidence of CPE. Thus, these preliminary observations suggested that an early stage in the differentiation of myogenic cells may

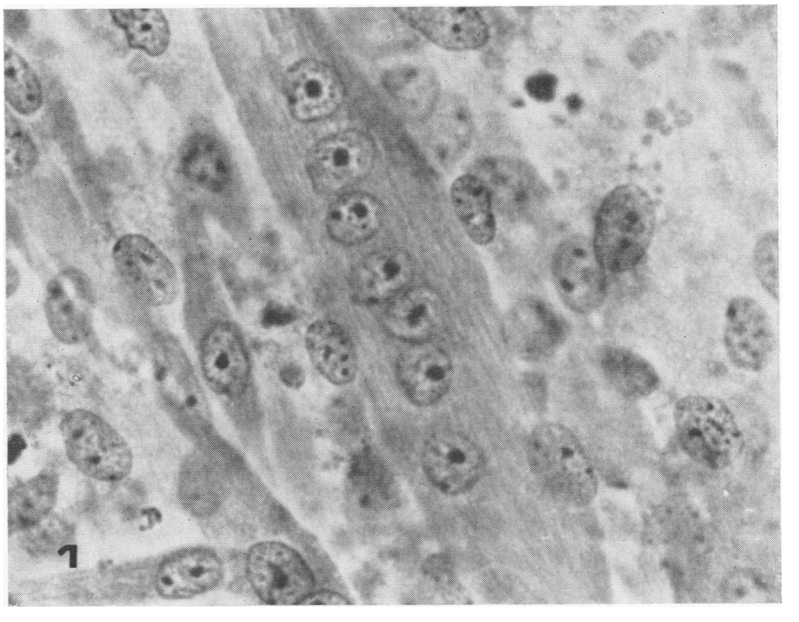

FIG. 1. *PFMM cells cultured for 5 days under optimal conditions for myogenic differentiation. Note the presence of longitudinal striations in the cytoplasm of the large multinucleated cell. Hematoxylin stain.* × *630.*

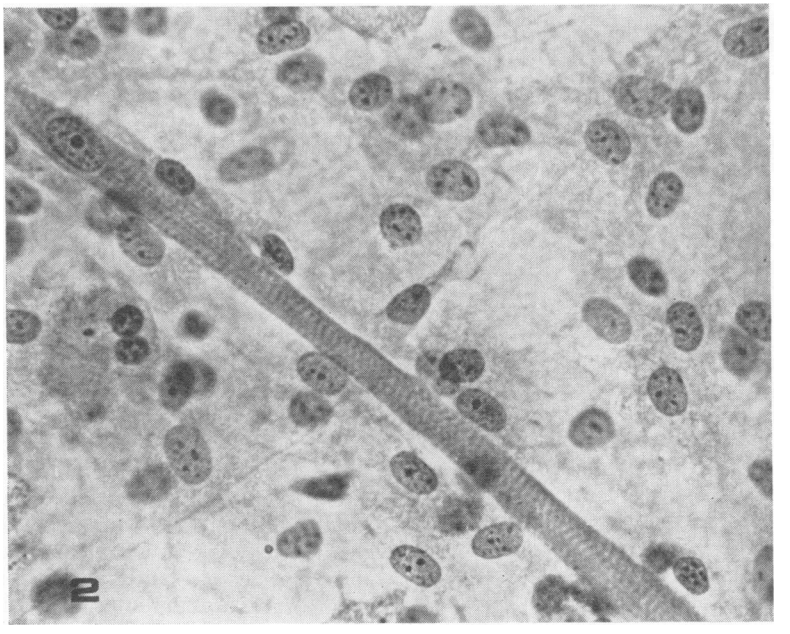

FIG. 2. *PFMM cells cultured for 12 days. A portion of a well differentiated, cross-striated muscle fiber is shown. Hematoxylin stain.* × *400.*

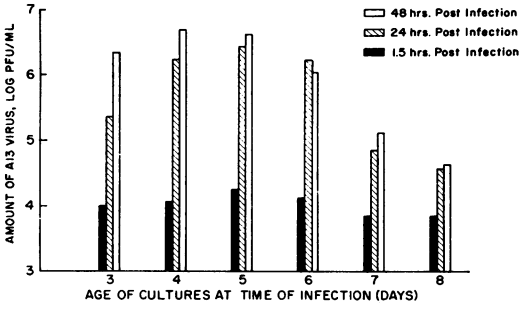

FIG. 3. *Influence of age of differentiating PFMM cultures on their susceptibility to coxsackievirus A13 infection.*

control the susceptibility of myotubes to coxsackievirus A13 infection.

Inhibition of fusion by various nutrient media. Nutrition was found to be a significant factor in determining the extent of myogenic differentiation in PFMM cultures. The various nutrient media tested included Eagle's basal medium (BME; reference 6) and MEM (7) with Hanks and Earle's BSS, respectively, M-199 (20) with Hanks BSS, medium F12 (12), CMRL 1066 (23), and GIB medium (Grand Island Biological Co., 1967 catalogue). All media were supplemented with 10% horse serum, 100 μg of penicillin per ml, 100 μg of streptomycin per ml, and 0.02 M HEPES buffer, with the exception that CMRL 1066 received no HEPES buffer. Sodium bicarbonate was added to M-199, GIB, and BME media to a final concentration of 0.075% and to MEM medium to a final concentration of 0.22%, whereas CMRL 1066 and F12 media contained 0.22% and 0.12% $NaHCO_3$, respectively. PFMM cultures were prepared as indicated above and observed for myotube formation over a 10-day period.

Large numbers of well differentiated myotubes developed when trypsin-dispersed cells were plated in MEM or CMRL 1066 media. The other media tested proved less satisfactory for myogenic differentiation; F12 and GIB media generally limited fusion to the formation of an occasional bi- or trinucleate unit in the respective cultures.

These observations offered another method for testing the hypothesis that differentiation in PFMM cultures enhanced their susceptibility to coxsackievirus A13. Thus, fetal mouse tissues were trypsinized and plated in 35-mm culture dishes, employing the different basal media supplemented with 10% horse serum. Replicate 4-day-old PFMM cultures were inoculated with A13 virus, overlaid with the respective media, and sampled at intervals over 3 days for production of total infectious virus as previously. Replicate cultures of ML cells were tested in parallel, as control, to determine the effect of the different media on A13 virus production in nonmyogenic cells.

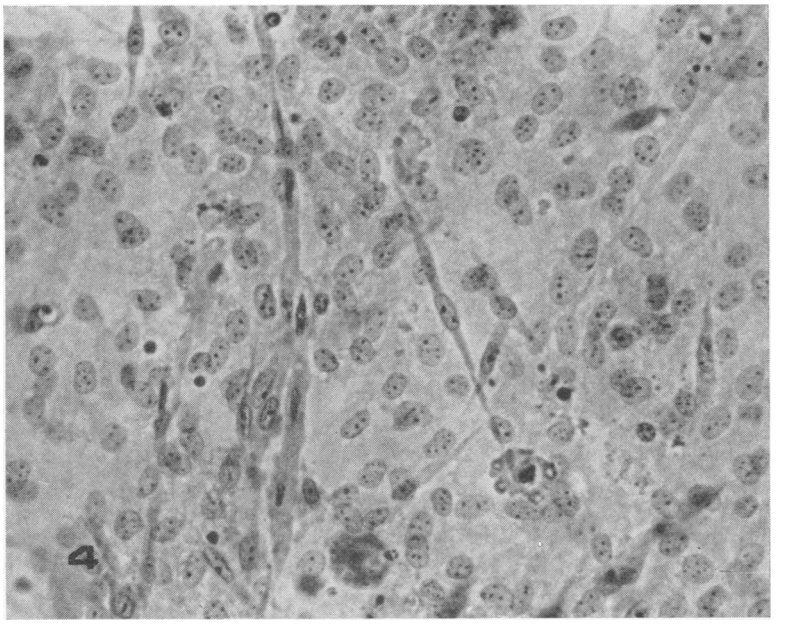

FIG. 4. *PFMM cells were cultured for 4 days, infected with coxsackievirus A13, then fixed, and stained at 48 hr postinfection. Note the presence of degenerating immature myotubes and the low number of multinucleated cells. Hematoxylin stain.* × *250.*

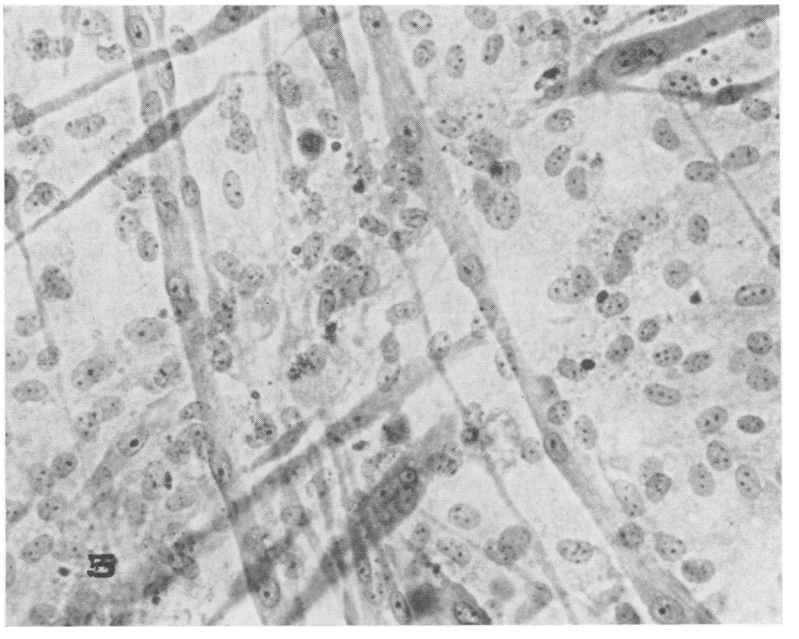

FIG. 5. *Uninfected PFMM cells cultured for 6 days. Hematoxylin stain.* × *250.*

The data in Table 1 show that the MEM-grown PFMM cultures produced high yields of A13 virus, whereas PFMM cultures grown in the media which gave poor myotube formation produced only minimal amounts of virus. In the control cultures of ML cells, high titers of comparable amounts of A13 virus were produced in each of the test media. There was no significant difference

TABLE 1. *Effect of different media on the ability of primary fetal mouse muscle cultures to produce myotubes and coxsackievirus A13*

Medium for cell cultivation	Amt of total virus at intervals postinfection (PFU/ml)[b]				Myotube formation in uninfected cultures
	2 hr	24 hr	48 hr	72 hr	
MEM[a]	5.3	870	2,000	1,500	Best
BME	4.3	130	450	370	Good
M-199	2.9	9.8	13	14	Poor
F-12	6.8	44	36	69	Poor
GIB	4.3	9.5	13	16	Poor

[a] Minimal essential medium.
[b] Values expressed $\times 10^4$.

in the rate of virus inactivation at 37 C among cell-free control preparations in each of the nutrient media tested.

Reversible inhibition of myogenesis. It was observed that the inhibition of myotube formation in 3- to 4-day-old cultures grown in F12 (fusion-inhibiting) medium was completely reversible within 48 hr after its replacement by MEM (fusion-promoting) medium. This observation permitted us to test further the hypothesis that differentiating cells acquired susceptibility to A13 virus.

Comparative virus growth studies were performed in PFMM cultures in 35-mm plates, grown in MEM and F12 media as described above, and in cultures which had the media exchanged. Three-day-old confluent cultures were washed two times with PBSa. MEM medium was added to all of the cultures grown in MEM medium and to half of the cultures grown in F12 medium. F12 medium was added to the remaining cultures. After an additional 24 hr of incubation at 37 C, the cultures received A13 virus as described above, except that F12 and MEM media were used for washing and for overlay in the respective cultures.

Microscopic examination of virus-free control cultures revealed that large numbers of multinucleated cells were found both in PFMM cultures grown in MEM medium and in cultures grown in F12 medium which were changed to MEM medium 48 hr previously. As expected, cell fusion was limited to the cytoplasmic coalescence of relatively few cells in the cultures continuously cultivated in F12 medium. The results presented in Table 2 clearly show that the nutritional induction of myogenic differentiation in PFMM cultures, which occurred after the replacement of the F12 culture medium with MEM, enhanced the susceptibility of these cultures to coxsackievirus A13.

Growth of coxsackievirus A13 in BUDR-treated PFMM cultures. BUDR has been shown to markedly restrict myotube formation in cell cultures derived from skeletal muscle tissue of the chick embryo (3, 28). Preliminary studies revealed that fusion of trypsin-dispersed fetal mouse cells also was markedly inhibited by concentrations of BUDR as low as 1 μg per ml (cf. Fig. 6 and 7).

Virus growth studies were performed in 5-day-old PFMM cultures grown in the presence or absence of BUDR at 1 μg/ml (Sigma Chemical Co., St. Louis, Mo.) in two separate experiments. In addition, parallel studies were conducted in nonmyogenic cell cultures (ML cells) to investigate the possibility of BUDR inhibiting A13 virus production by a mechanism independent of its effect on cell differentiation. Since the ML cells employed in this study were cultured routinely in a nutrient medium (GM-ML) other than that employed for the cultivation of mouse muscle cells (mouse-GM), virus growth studies were performed in ML cell cultures with both types of media to help account for any variations in response of this cell line to the different media.

Data presented in Table 3 demonstrate that A13 virus replication was inhibited by more than 90% in PFMM cultures which were grown in the presence of BUDR. However, BUDR at 1 μg/ml did not inhibit the virus yield by ML cells and did not change the rate of A13 virus inactivation in cell-free control preparations at 37 C. These data suggest that BUDR caused an increased resistance of PFMM cultures to coxsackievirus infection by inhibiting some stage of myogenic differentiation related to the formation of multinucleated myotubes.

Specificity of A13 virus infection in PFMM cultures. It has been established that fusion in myogenic cell cultures occurs between mononucleated cells, between mononucleated cells and multinu-

TABLE 2. *Enhancement of cell susceptibility of primary fetal mouse muscle cultures to infection by coxackievirus A13 after nutritional induction of myogenic differentiation*

Medium for cell cultivation	Amt of total virus at intervals postinfection (PFU/ml)[b]			
	1.5 hr	24 hr	48 hr	72 hr
MEM[a]	1.9	190	130	110
F12	1.7	7.1	5.6	4.5
F12 ↓ MEM	1.7	64	400	230

[a] Minimal essential medium.
[b] Values expressed $\times 10^4$.

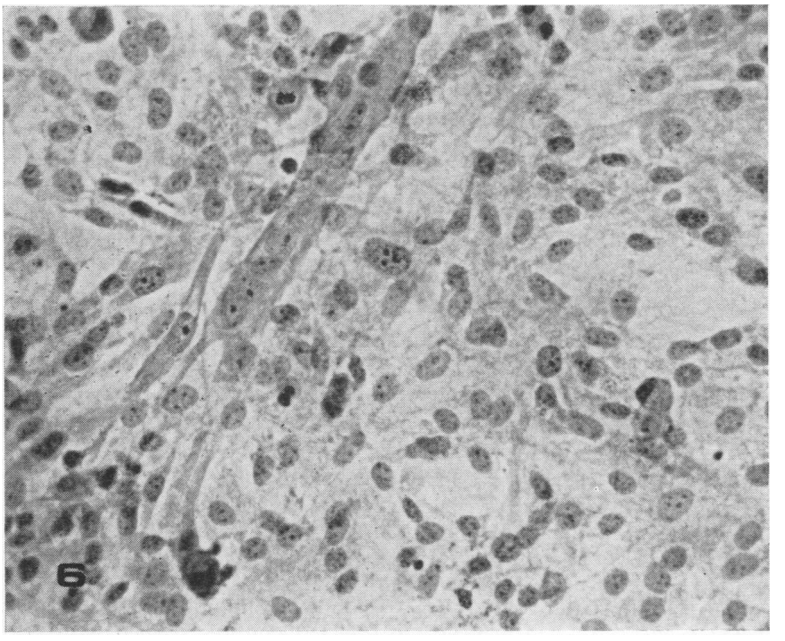

FIG. 6. *PFMM cells cultured in the presence of BUDR (1 μg/ml) for 5 days. Note the decreased number of myotubes as compared with that formed in the absence of BUDR. Hematoxylin stain.* × *250.*

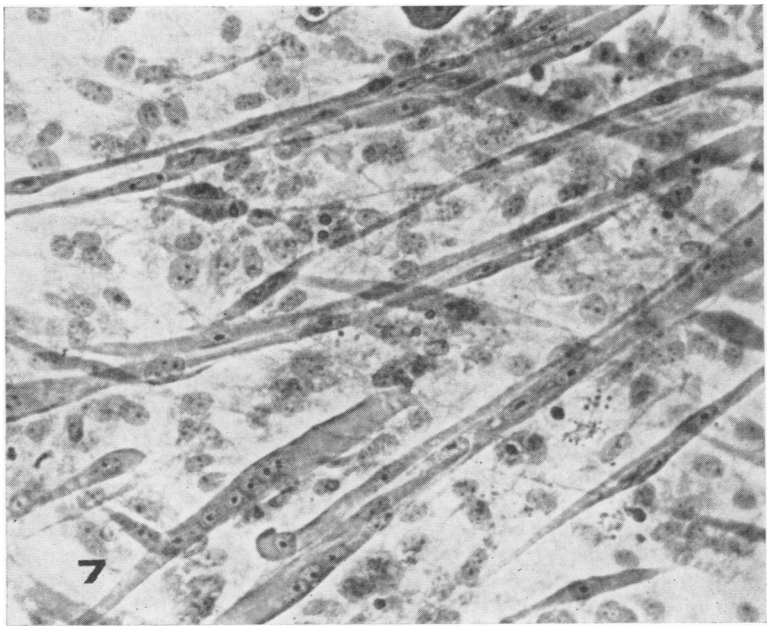

FIG. 7. *PFMM cells cultured for 5 days in medium without BUDR. Hematoxylin stain.* × *250.*

cleated myotubes, and between nascent myotubes. The possibility arose that the phenomenon of normal cell fusion may act nonspecifically to initiate A13 virus infection in a manner analogous to that of poliovirus in Sendai virus-induced fusion of normally resistant cells (8, 21). To evaluate this hypothesis, differentiating PFMM cultures were tested for their capacity to replicate poliovirus T1.

Virus production was followed in replicate 3-

TABLE 3. *Inhibition of coxsackievirus A13 replication in primary fetal mouse muscle cultures which were grown in the presence of BUDR (1 μg/ml)*

Expt no.	BUDR addition	Amt of total virus at intervals postinfection (PFU/ml)	
		2.5 hr	25.5 hr
1	−	1.6×10^4	3.2×10^6
	+	6.8×10^3	4.9×10^4
2	−	1.7×10^4	8.4×10^5
	+	7.2×10^3	7.6×10^4

day-old PFMM cultures which received 6.2×10^6 PFU of A13 virus or 1.9×10^7 PFU of poliovirus T1, respectively. These virus preparations had been prepared previously in ML cells. Similar conditions were employed to those described in Materials and Methods except that the cultures were washed six times to reduce the amount of unattached virus after the virus adsorption period. The amounts of virus produced by these cells are presented in Table 4. Three-day-old PFMM cultures produced over 10^6 PFU of A13 virus per ml, whereas no appreciable virus synthesis was measured in replicate cultures which had been inoculated with poliovirus T1.

Immunofluorescent assay. Immunofluorescence was used to determine directly the cell type in which A13 virus replicates. Replicate 5-day-old PFMM cultures grown on cover slips were infected with coxsackievirus A13 as indicated above. Virus synthesis was followed in replicate cultures which were washed six times, to remove the residual unattached virus, after the virus adsorption period. The infected cultures were incubated at 37 C and sampled at intervals for determination of virus titer and fluorescing cells. The large number of multinucleated cells in these cultures precluded an accurate quantitation of fluorescing cells in relation to the total number of cells present. Therefore, 30 fields on each of two slides from each time interval were scored for mononucleated and multinucleated fluorescing cells at a final magnification of 250×.

The results of a representative experiment are presented in Table 5. Fluorescing cells were first detected in the infected cultures at 6 hr postinfection. The ratio of the number of fluorescing mononucleated cells to fluorescing multinucleated cells decreased with continued incubation, most likely because of fusion and the more rapid viral-induced degeneration of infected mononucleated cells. Two morphological types of fluorescing mononucleated cells were observed in these cultures. Small round cells with nuclei partially or completely obscured by brightly fluorescing cytoplasm most likely were degenerating cells in the process of detaching from the substrate and longer bipolar cells; some in close proximity to myotubes could well represent infected myoblasts (Fig. 8). Fluorescence was usually most intense in the perinuclear region of infected multinucleated cells and could be observed either in isolated areas or throughout the entire cytoplasm (Fig. 9). No specific fluorescence was observed in the many flattened, stellate fibroblast-like cells in these cultures.

In a separate experiment of similar design, a comparison of cell susceptibility to A13 virus was made between 4- and 8-day-old PFMM cultures. As anticipated, infection of the older cultures revealed a significantly lower number of fluorescing cells (<10%) than were found when younger cultures were infected.

The specificity of the immunofluorescent reaction was established in control studies which showed that specific fluorescence was absent in uninfected PFMM cultures stained with A13 immune serum and conjugate, as well as in A13 infected cultures stained with normal serum and conjugate, or with conjugate alone.

TABLE 4. *Specificity of the replication of coxsackievirus A13 in primary fetal mouse muscle cultures*

Virus inoculated	Amt of total virus at intervals postinfection (PFU/ml × 10^3)		
	1.5 hr	21 hr	65 hr
A13	26	200	4,200
Poliovirus T1	26	5.5	1.6
A13-control[a]	34	1.4	0.10
T1-control[a]	42	2.0	0.33

[a] Cell-free virus stability control.

TABLE 5. *Detection of viral antigen by immunofluorescence in primary fetal mouse muscle cultures after infection with coxsackievirus A13*

Time postinfection (hr)	Avg. no. of fluorescing cells in 60 (250×) fields[a]		Amt of total virus (PFU/ml × 10^4)
	Mononucleated	Multinucleated	
1.25	ND[b]	ND	9
6	0.5	1	42
8	40	38	52
10	36	52	150
12	19	36	610
14	21	36	410
16	28	57	810
18	20	44	710

[a] Area per field = 0.32 mm^2.
[b] Not done.

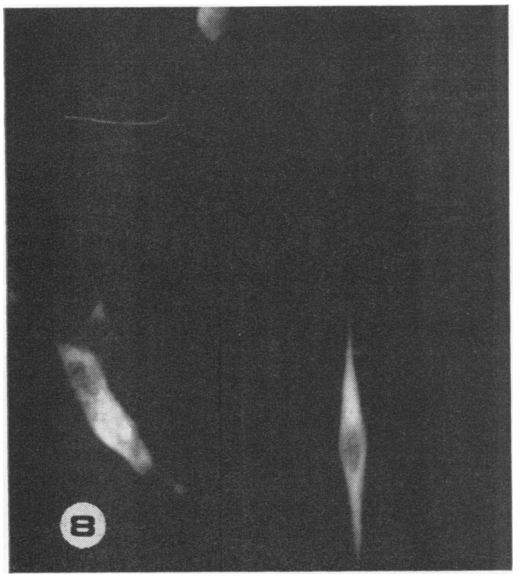

FIG. 8. *Coxsackievirus A13 antigen in the cytoplasm of a bipolar myoblast at 10 hr postinfection shown by immunofluorescent staining.* × *400.*

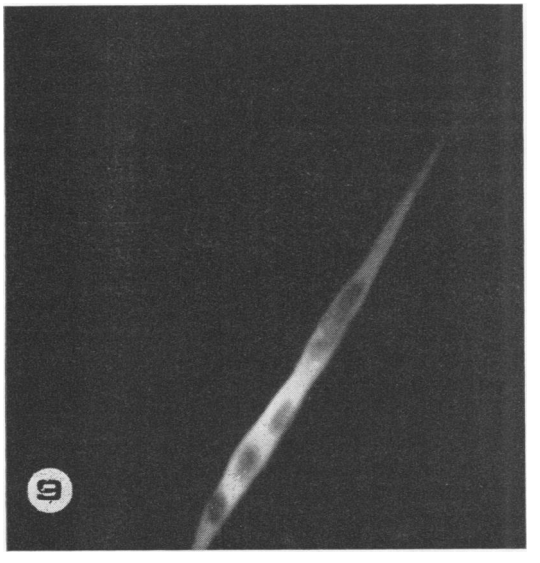

FIG. 9. *Coxsackievirus A13 antigen in the cytoplasm of a multinucleated myotube at 10 hr postinfection shown by immunofluorescent staining.* × *400.*

The results of the immunofluorescence experiments established that A13 virus capsid protein was synthesized in both mono- and multinucleated cells and confirmed the preceeding observation that these cells were most susceptible to virus infection during an early stage of myogenic differentiation.

DISCUSSION

The results of studies presented herein have revealed that the susceptibility of cultured fetal mouse cells to infection by coxsackievirus A13 was markedly enhanced, provided the infection was initiated when the cells were undergoing myogenic differentiation. Virus replication was accompanied by a CPE as evidenced by the presence of degenerating immature myotubes and a marked diminution of differentiated muscle cells within 48 hr post-infection, compared to noninfected control cultures. The demonstration of newly synthesized A13 virus antigen in differentiated mono- and multinucleated cells by immunofluorescence provided direct evidence that virus replication occurred in both myoblasts and myotubes. The susceptibility of myoblasts to coxsackievirus A4 also was suggested by Kantoch and co-workers (14, 15).

The efficiency of infection of multinucleated myotubes by coxsackievirus A13 occurred optimally during the stage of differentiation characterized by rapid cell fusion. In contrast, the susceptibility of these cells decreased appreciably when PFMM cultures were infected subsequent to the formation of the majority of myotubes. This latter observation was most likely due to the reduced number of myogenic stem cells and diminished fusion activity of mononucleated myoblasts in older myogenic cultures (1). Thus, we believe that the principal mechanism of infection of the myotubes probably occurred by fusion of infected myoblasts. Similar conclusions were reached by Fogel and Defendi (10) and Yaffe and Gershon (33) for polyoma and SV40 virus infection of differentiating muscle cell cultures of murine and human origin, although Lee et al. (19) concluded that fully developed myotubes cultured from chick embryo skeletal muscles were intrinsically susceptible to Rous sarcoma virus. A complete description of the determinants controlling the susceptibility of differentiating cells to various virus infections is needed.

The finding that events leading to cell fusion enhanced the susceptibility of PFMM cultures to coxsackievirus A13 infection prompted an extension of these studies to include poliovirus T1 to examine the specificity of the infectious process. Cells of murine origin were known to be resistant to polioviruses, although Neff and Enders (21) had demonstrated that resistant cells of an established hamster cell line acquired susceptibility to poliovirus T1 when the cells were fused by Sendai virus. Nevertheless, both CPE and significant virus replication were undetected after addition of

poliovirus T1 to PFMM cultures. Thus, a nonspecific entrapment of virus particles during normal cell fusion was not a major factor controlling the susceptibility of myogenic cells to coxsackievirus A13. More likely, a specific virus-receptor interaction accounted for the contrasting susceptibility of PFMM cells to the two enteroviruses tested (11).

Evidence has been presented by Okazaki and Holtzer (22) and Yaffe and Feldman (32) to indicate that normal cell fusion involves the formation of specific molecular configurations on the surfaces of myoblasts and enlarging myotubes. Likewise, the inhibition of cell fusion in older myogenic cultures may be the result of changes in the surface properties of the myotubes during maturation (1). Fogel (9) reported the absence of a cell surface antigen in multinucleated fibers which was present in surrounding mononucleated cells prior to fusion in cultured rat muscle cells. Even the surface of a given presumptive myoblast during the G_1 phase may differ from the surface of that same cell during the S, G_2, and M phases, since fusion occurred only during G_1 (22). Because virus-specific cell surface receptors are essential for initiating infections with enterovirus virions (13, 18), it seems reasonable to conclude that the changes in the surfaces of developing muscle cells may coincide with the formation and disappearance of specific virus receptors and thereby regulate the cell susceptibility to coxsackievirus A13.

It is also reasonable to suggest that the results of the present studies may provide an additional explanation for the diminishing susceptibility of postnatal mice to coxsackievirus infection. The relative resistance of older mice to these viruses may result from a decrease in the number of myoblasts which are formed and from a decline in cell fusion activity concomitant with the maturation of the skeletal muscles (16). Thus, the relative number of susceptible cells would diminish with age, at a time when antibody and interferon synthesis are increased. Studies are continuing in an attempt to determine whether the limiting factor controlling the susceptibility of differentiating muscle cells to coxsackievirus A13 infection is the development of specific receptors on the cell surface.

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