The Permissiveness of Differentiating Mouse Muscle-Cell Cultures to Infection by Group A Coxsackievirus Types 1 and 5[1] (36865)

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(Introduced by Amedeo Bondi)

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Though many of the known wild-type Group A coxsackieviruses have been grown in a variety of cell cultures, the host range of several serotypes has been shown to be restricted (1). Recently, Goldberg and Crowell (2) demonstrated that coxsackievirus A13 grew better in primary fetal-mouse muscle-cell cultures undergoing myogenic differentiation than in non-differentiating cell cultures (3, 4). The present study was done to determine whether dfferentiating muscle-cell cultures would support the growth of wild-type myotropic strains of coxsackieviruses A1 and A5, as representative of those group A coxsackieviruses which reportedly do not replicate in cell cultures (1).

Materials and Methods. Primary cell cultures. Primary fetal-mouse muscle- (PFMM) cell cultures were derived and grown essentially as described by Goldberg and Crowell (2). Skeletal muscle from the hind limbs of near-term fetal mice (Huntingdon Farms, Conshohocken, Pa.) was removed by microdissection, minced and trypsinized. Monodispersed cells were suspended at a concentration of 3×10^5 cells per ml of growth medium (GM) containing Eagle's MEM (Grand Island Biological Co., Grand Island, N.Y.) solution with non-essential amino acids, 0.2% $NaHCO_3$, 0.02 *M* HEPES buffer and supplemented with 10% horse serum. Two ml of cell suspension was dispensed into 35 mm plastic petri dishes (Falcon Plastics, Div. of B-D Laboratories, Los Angeles, Cal.) and incubated at 37° in an atmosphere of 5% CO_2, 95% air. Growth medium was changed on days 1 and 4.

Continuous cell cultures. The origin and growth of ML cells have been described previously (3).

Virus strains. The origin of the strains of coxsackieviruses A1 and A5 and the preparation of virus pools from infected newborn mice have been described by Crowell and Syverton (5). The coxsackievirus A13 pool was prepared from infected ML cells by 2 cycles of freezing and thawing followed by centrifugation of the disrupted cell suspension at 600*g*. Supernatant fluids containing virus were pooled and stored at —20°.

Assay of virus. Quantitative assay of infectious coxsackieviruses A1 and A5 was done by intraperitoneal inoculation of newborn mice. During the 12 to 14 day test period, mice were observed daily for paralysis and death. LD_{50} end points were determined by the method of Reed and Muench (6). Coxsackievirus A13 infectivity was determined by plaque assay in ML cells as described elsewhere (3).

Growth studies of virus in PFMM cells. Five-day-old primary cultures of PFMM cells, grown as monolayers in 35 mm plastic petri dishes, were drained of growth medium. Replicate cultures were exposed to 0.1 ml of virus suspended in balanced salt solution supplemented with 3% calf serum (BSS-CaS3) for 1 hr at 37°. The cultures were washed three times with PBSa and 0.1 ml of homotypic monkey anti-serum added to each culture. Incubation of cultures at 37° was continued for an additional hour, and

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the cultures were washed successively 3 additional times with PBSa. Finally, each culture was overlaid with 2 ml of growth medium and incubated at 37°. Virus released spontaneously from infected cells was harvested by pooling the overlay medium from 2 replicate cultures and removing cellular debris from the fluid by centrifugation at 960*g* for 5 minutes. The supernatant fluid was aspirated and stored at —20° until assayed.

Cell-associated virus was obtained by replacing the overlay medium with 2 ml of fresh growth medium per culture. The cells were scraped from the plastic substrate into the fluid and subjected to 3 cycles of freezing and thawing to release intracellular virus. Cellular debris was removed by centrifugation at 960*g* for 5 min. The supernatant fluid was aspirated and stored at —20° until assayed.

Immunofluorescence studies. Detection of intracellular coxsackievirus antigen was accomplished by the indirect immunofluorescent procedure described elsewhere (2). PFMM cells grown on coverslips in 35 mm petri dishes were infected with 1 to 4 $\times$ 10^8 LD_{50} of either A1 or A5 virus. Following fixation in acetone, infected and uninfected PFMM cell cultures were exposed immediately or after storage at —20°, to virus type-specific monkey anti-serum previously absorbed with mouse liver powder (Sylvana, Millburn, N. J.) and then to fluorescein-conjugated goat anti-monkey IgG (Microbiological Associates, Inc., Bethesda, Md.) absorbed with mouse liver powder. Preparations were examined using a Zeiss standard microscope equipped for fluorescence microscopy.

Electron microscopy. PFMM cells were grown in 60 mm plastic petri dishes and inoculated with 7.8 $\times$ 10^8 LD_{50} of A5 virus. After 1 hr incubation followed by 3 successive washes with PBSa, the cell cultures were overlaid with GM. At appropriate time intervals, cell cultures were drained of GM, washed with PBSa, and 1% phosphate-buffered osmic acid was added. After 5 min, the cells were scraped into the fixative and centrifuged at low speed for 5 min. The cell pellet was dehydrated in ethanol and embedded in Spur low viscosity medium (7).

TABLE I. Growth of Coxsackievirus A13 in PFMM Cultures.

Hours post-infection	PFU/ml[a]	
	Cell associated	Supernatant
2	$<1 \times 10^1$	$<1 \times 10^1$
8	6×10^5	1×10^4
31	3×10^5	2×10^5

[a] Titrated in ML cells.

Sections were stained with lead citrate and uranyl acetate and examined using a Phillips 300 electron microscope.

Results. Five-day-old cultures of PFMM cells used in virus growth studies were characterized by formation of long multinucleated myotubes reflecting a high degree of cellular fusion activity. In addition, cultures were comprised of bipolar mononucleated cells and fibroblasts. Spontaneous rhythmic contraction of fully differentiated myotubes was observed 6 to 8 days after plating cells in culture.

To establish a reference for studies of the growth of coxsackieviruses A1 and A5, PFMM cells were grown and infected with 3 $\times$ 10^6 PFU coxsackievirus A13 at the same stage of cellular differentiation as in the experiments done by Goldberg and Crowell (2). As summarized in Table I, the data show that at 2 hr only trace amounts of virus was detected. The negligible amount of virus found at this time provided a measure of the effectiveness of procedures employed in reducing the amounts of residual unattached virus. Most of the virus produced by 8 hr remained cell-associated. By 31 hr, the amount of released virus had increased and was approximately that of cell-associated virus. These findings confirmed the presence of susceptible myogenic cells in PFMM cultures used in these experiments.

Using the same procedure described above, growth studies of coxsackievirus A5 were done. Replicate cell cultures were exposed initially to approximately 4 $\times$ 10^6 LD_{50} of virus. The data depicted in Fig. 1 show that about 10^5 LD_{50} of virus were produced during the first 24 hr; most of it was cell-associated. By 30 hr, the amount of infec-

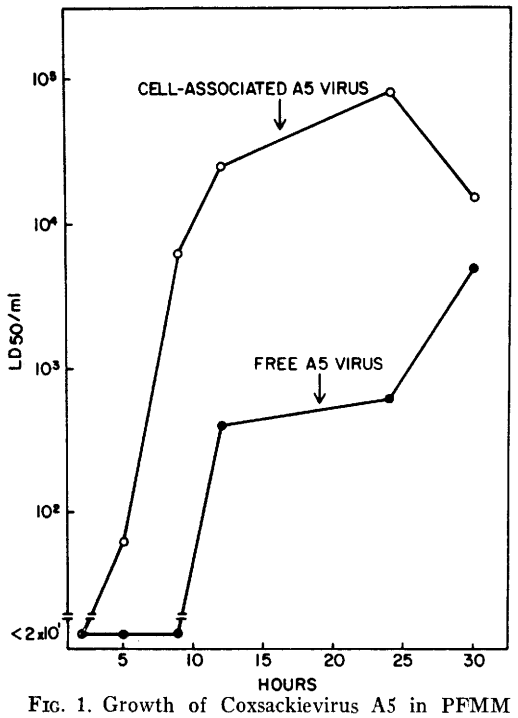

FIG. 1. Growth of Coxsackievirus A5 in PFMM cell cultures.

tious virus released into the supernatant fluid was approximately equivalent to the titer of cell-associated virus. The identity of the A5 virus produced by PFMM cells was confirmed by neutralization tests with homotypic anti-serum. Similar data were obtained from experiments designed to measure the growth of A1 virus.

To determine whether increases in virus titer measured over a 26 hr period were due to *de novo* synthesis, replicate cultures of cells were pretreated with puromycin (100 μg/ml), infected with virus, and then maintained in medium containing puromycin. In these experiments, puromycin-treated and untreated PFMM cells were compared for their capacity to support the replication of A1 and A5 viruses. Data from experiments in which replicate cultures of PFMM cells were infected with 1×10^6 LD_{50} of A1 virus are presented in Table II. A substantial increase in the amount of virus was found in the untreated cell cultures during the test period. In contrast, puromycin-treated cells produced no measurable levels of free virus over the 26 hr period. Amounts of cell-associated virus found at 2 and 26 hr, respectively, were approximately equivalent. Similar results were found also for coxsackievirus A5. Thus, the increase in titers of both A1 and A5 viruses grown in untreated PFMM cells represented synthesis of new virus.

Further evidence of the capacity of A1 and A5 viruses to replicate in PFMM cells was obtained by use of the indirect immunofluorescence procedure and by electron microscopy. Specific fluorescence in cells infected with either virus was observed as early as 10 hr post-infection. Uninfected cell cultures did not exhibit specific fluorescence. As shown in Fig. 2 for A5 virus-infected cells, fluorescence was most intense in the perinuclear region but was also found elsewhere in the cytoplasm. The cell nuclei did not stain. Specific fluorescence was detected only in the differentiating mononucleated myoblasts and multinucleated myotubes.

Electron microscopic studies on PFMM

TABLE II. Comparative Growth of Coxsackievirus A1 in PFMM Cultures in the Presence or Absence of Puromycin.

Hours post-infection	LD_{50}/ml[a]			
	Absence of puromycin		Presence of puromycin[b]	
	Cell-associated	Supernatant	Cell-associated	Supernatant
2	$<2 \times 10^1$	$<2 \times 10^1$	3×10^1	$<2 \times 10^1$
9	3×10^4	7×10^3	ND[c]	ND
26	7×10^4	7×10^4	8×10^1	$<2 \times 10^1$

[a] Titrated in newborn mice.
[b] 100 μg/ml.
[c] Not done.

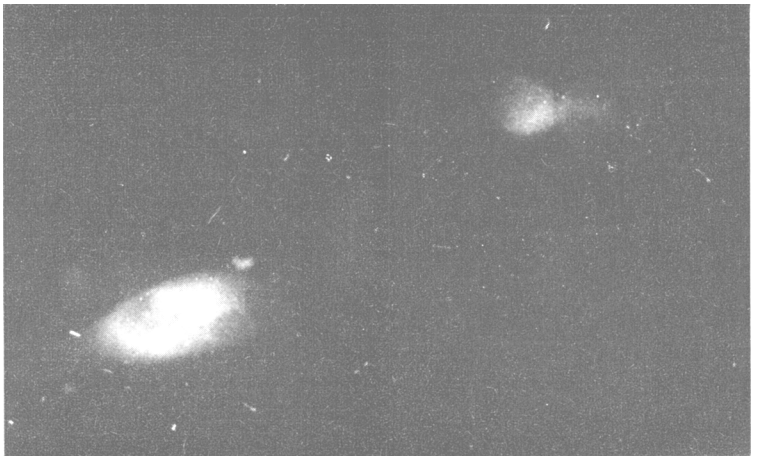

FIG. 2. Immunofluorescence of coxsackievirus A5-infected PFMM cells stained by the indirect procedure 24 hr post-infection, × 400.

cells infected with A5 virus revealed (by 10 hr post-infection) the presence of virus particles measuring about 22 to 24 nm. Virus particles were found only in those cells containing myofibrils. A direct correlation was found between the results of the immunofluorescence studies and those obtained from ultrastructure analysis in that only a limited number of cells was found to contain virus. Virus particles were found in the perinuclear region and elsewhere in the cytoplasm but not in the nuclei (Fig. 3). Neither virus particles nor virus antigens were observed in uninfected cells used as controls. The demonstration of A1 and A5 virus capsid antigen in infected cells by immunofluorescence and direct visualization of intracellular virus particles by electron microscopy confirmed the occurrence of *de novo* viral synthesis.

Discussion. Coxsackieviruses A1 and A5 have been shown to replicate in PFMM cultures as determined by infectivity assays and specific immunofluorescence. Also, virus particles, which resembled the morphology of coxsackieviruses (8) were observed by electron microscopy in the myogenic cells of PFMM cultures infected with coxsackievirus A5. Neither A1 nor A5 virus was observed in the fibroblastic elements present in the culture. The inability of A1 virus to grow in fibroblasts derived from mouse skeletal muscle tissue has been reported by others (9). The permissiveness of the myogenic cells in PFMM cultures to grow these viruses could depend upon the presence of specific viral receptors at the cell surface (10–12). The way in which differentiating cells in culture acquire virus receptors remains unknown. Studies utilizing clonally derived myogenic cell populations (13, 14) undergoing synchronous differentiation may provide a system to elucidate the nature of the restriction of virus replication to differentiating muscle cells in PFMM populations.

Finally, the finding of the growth of 2 additional coxsackieviruses of Group A extend the previous observations from our laboratory of the growth of coxsackievirus A13 in differentiating muscle cell cultures. Our interpretation of these data leads us to suggest that not only other Group A coxsackieviruses, but also unrelated viruses with equally limited host ranges (15) might be grown successfully using differentiated cell cultures that are consonant with the specific *in vivo* tropism of the test virus.

Summary. Differentiating primary fetal-mouse muscle-cell cultures have been shown to support the replication of prototype strains of coxsackieviruses A1 and A5. Heretofore, mouse cell cultures had been found to be restrictive to the growth of these agents. Myoblastic elements rather than fibroblasts

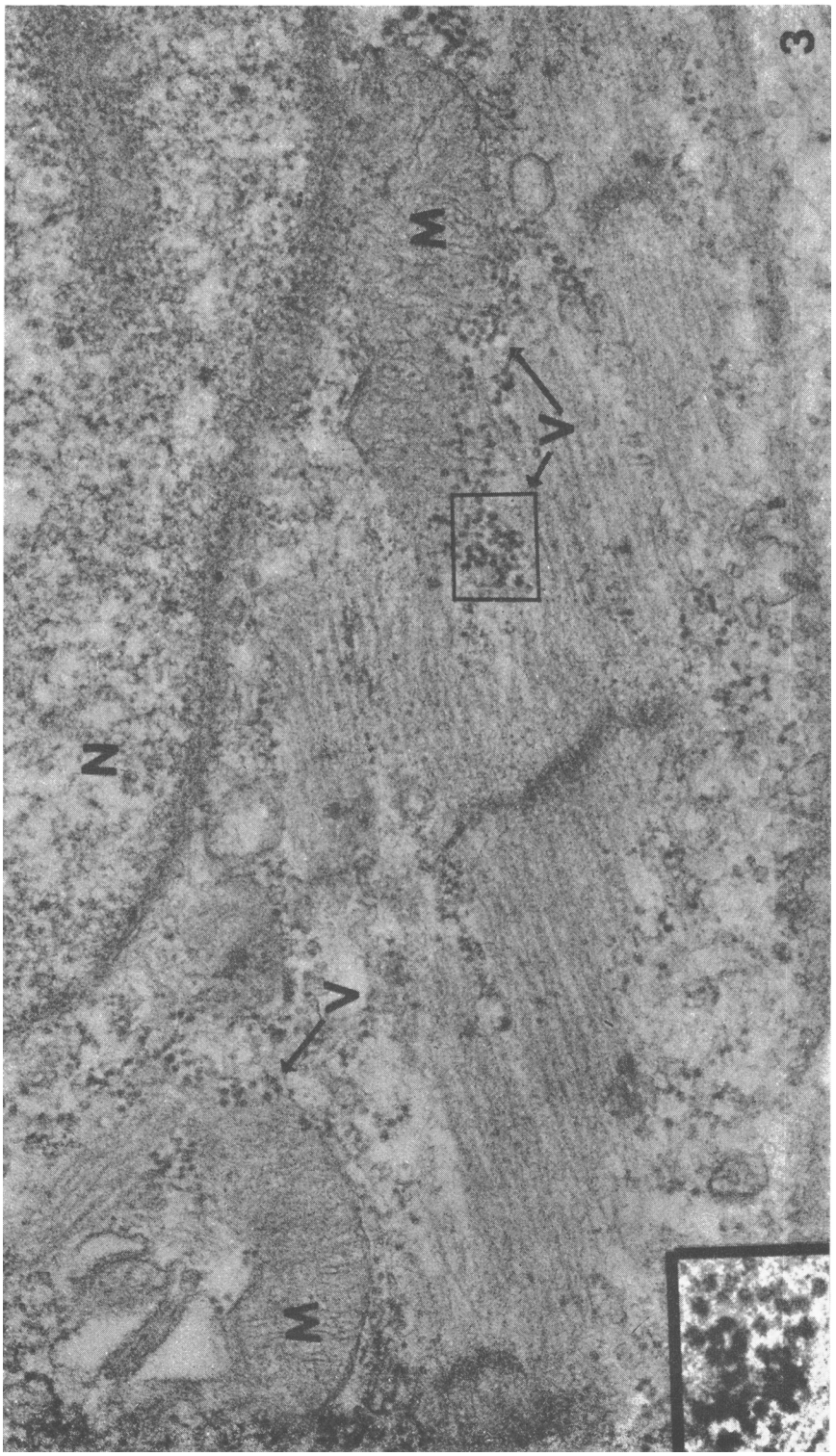

FIG. 3. Coxsackievirus A5 in PFMM cells 18 hr post-infection. Virus particles (V) can be seen between the myofibrils of infected cells, clustered around mitochondria (M) or scattered throughout the cytoplasm. N = nucleus. × 50,000. Inset: higher magnification of a cluster of virus particles × 100,000.

appeared to be the primary cell type susceptible to infection by those coxsackieviruses of Group A tested.

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