Evidence for the Susceptibility of Primary Fetal Mouse Cells in Culture to Coxsackievirus A13[1] (34301)

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The central histopathologic feature resulting from infection of newborn mice by members of coxsackievirus group A is the extensive loss of striated muscle fiber definition culminating in paralysis and death of the animal (1). In addition, histologic changes have been observed in the lungs, intestines, liver, and brain of newborn mice following infection by coxsackieviruses A5 or A7 (2). The multiplication of infectious virus in neonatal (3,4) and fetal (5, 6) mice has clearly demonstrated the competence of the cells of these animals to support group A coxsackievirus replication *in vivo*. With the exception of the positive results reported by Slater and Syverton (7) and by Kantoch and Sieminska (8), however, attempts to propagate the group A coxsackieviruses in tissue culture systems derived from infant or fetal mice have been unsuccessful (5, 9–11). We now report evidence for the limited attachment and replication of coxsackievirus A13 in cultures of primary fetal mouse cells.

Materials and Methods. Primary fetal mouse (PFM) monolayer cultures. Fetuses near term were removed aseptically from mice sacrificed previously by cervical dislocation. The fetuses were washed in calcium–and magnesium-free phosphate-buffered saline (PBS-a) (12), decapitated, eviscerated, and weighed. The torsos were minced into small fragments of approximately 1 mm^3 with Simm's uterine scissors and washed three times with PBS-a. The mince was transferred to a Bellco trypsinizing flask, containing 10 ml/g of tissue of 0.2% trypsin (Difco 1:250) in PBS-a with 0.03% $NaHCO_3$. The mixture was gently rotated on a magnetic stirrer for 2 hr at 37°, the contents were filtered through 16 layers of gauze and the filtrate was centrifuged at 600 *g* for 15 min at 4°. The supernatant fluid was discarded, the packed cells were resuspended by gentle pipetting in approximately 20 ml of growth medium (GM), and the cells dispersed from 2 g of tissue were dispensed into a 32-oz prescription bottle (Duraglas, Owens Ovals, Owens-Illinois, Toledo, Ohio) containing 50 ml of GM. Confluent cell monolayers were attained following incubation at 37° in 48–72 hr without replenishment of GM. The GM contained 10% calf serum with Eagle's essential and nonessential amino acids and vitamins (BME) (13), penicillin, 100 units/ml; and streptomycin, 100 μg/ml; and 0.03% added sodium bicarbonate in Hanks' balanced salt solution (BSS).

Primary chick embryo fibroblast (CEF) monolayer cultures. Monolayer CEF cultures were prepared from trypsinized 9–11-day-old decapitated chick embryos. The tissue was processed for cell cultivation in a manner similar to that described above for fetal mouse tissues.

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Cell lines. A cell line designated ML cells was obtained as a frozen suspension from Dr. D. A. Buthala, Upjohn Co., Kalamazoo, Michigan and passaged serially as monolayer cultures in this laboratory. A subline of Earle's strain L cells, designated MCN, was obtained from Dr. Barbara Zajac, Childrens Hospital, Philadelphia, Pa., and grown also as monolayer cultures.

Virus strains. The origin and preparation of stock virus pools of coxsackievirus strains A13, and B1 in newborn mice have been described (5). The respective identity of the virus preparations was confirmed by neutralization with homotypic antiserum.

Poliovirus type 1 (Mahoney) was serially passaged as the supernatant fluids of infected monolayer cultures of HeLa cells (strain JJH) (14).

Vesicular stomatitis virus (VSV), Indiana strain, was generously supplied by Dr. Barbara Zajac, Childrens Hospital, Philadelphia, Pa. and propagated in 2-to-4 day old CEF monolayer cultures for preparation of virus pools.

Assay of virus in cell cultures. Plaque assays for coxsackievirus types A13 and B1 and poliovirus type 1 were done in ML cells using methods described previously for titration of enteroviruses in HeLa cells (14) except that 0.075% $NaHCO_3$ was used in the agar overlay medium.

Plaque assays of VSV in MCN cells were performed in a manner similar to that described for assay of viruses in ML cells except that horse serum was substituted for calf serum in the agar overlay medium and the final concentration of $NaHCO_3$ was 0.06%.

Assay for interferon. Test tube cultures of 2-day-old confluent monolayers of MCN cells were washed with serum-free GM prior to receiving 1-ml volumes of each test preparation. The cultures were incubated overnight at 37° and challenged with approximately 400 PFU of VSV in 0.2 ml. The presence of interferon activity in test preparations was indicated by a protective effect of cells challenged with VSV following an additional incubation at 37° for 2 days. Control cultures challenged with VSV, in the absence of interferon test preparations, showed a marked cytopathic effect (CPE) at this time.

Results. Propagation of virus in PFM cultures. In initial experiments, primary monolayer cultures derived from trypsinized fetal mouse tissues were tested to ascertain whether these cells would support multiplication of coxsackievirus A13. A 3-day-old PFM monolayer culture in a 32-oz bottle was washed and inoculated with 1 ml of virus containing approximately 5×10^6 PFU of coxsackievirus A13. A replicate culture was exposed to a similar concentration of coxsackievirus B1, to serve as a positive control, since this virus has been shown to multiply in PFM cells. The cultures were incubated at 37° for 90 min with intermittent rocking to allow virus attachment, after which time the unattached virus was reduced by washing. The cultures were overlaid with 50 ml of GM and incubated at 37° for production of virus. At intervals, samples of the extracellular fluids were removed for determination of the amount of free virus present by titration in ML cells. Virus stability was determined by adding A13 virus to a culture of heat-killed cells, and portions of the fluid overlay assayed for virus as above.

The results presented in Fig. 1 show that the PFM cultures produced a small, but measurable, amount of coxsackievirus A13, whereas an overall pattern of virus inactivation at 37° was observed in the control culture containing heat-killed cells. Coxsackievirus B1 multiplied in the PFM monolayer as expected. No CPE was observed in either the coxsackievirus A13 or the B1 infected cultures. In subsequent experiments, the addition to the GM of sodium bicarbonate at concentrations between 0.07 and 0.22% was found to enhance the yield of A13 virus by as much as tenfold, and was used as routine in the studies to be described.

Inhibition of the synthesis of coxsackievirus A13 and B1 by puromycin. Since, the PFM cultures produced relatively low amounts of coxsackievirus A13, it was important to exclude the possibility that the increase in virus yields was due to elution of virus from the cells. Puromycin, an inhibitor of protein synthesis, was used to inhibit virus replication in the system, thereby permitting

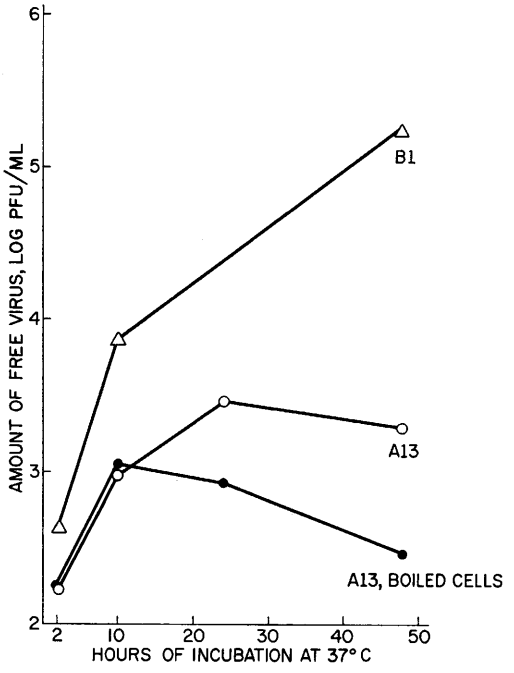

FIG. 1. Comparative production of coxsackieviruses A13 and B1 by cultures of primary fetal mouse cells.

a distinction to be made between that virus which was newly synthesized and that which was eluted from the cells. Coxsackievirus A13 growth was followed in PFM cultures which were pretreated for 1 hr and infected in the presence and in the absence of puromycin (Nutritional Biochemicals Corp.) at a final concentration of 10 μg/ml. In addition to assaying for the extracellular virus as done previously, the amount of total virus content was determined following disruption of cells by alternate cycles of freezing and thawing of replicate cultures. As a positive control, in an experiment of similar design, it was shown that puromycin at a concentration of 10 μg/ml inhibited coxsackievirus B1 synthesis (Fig. 2).

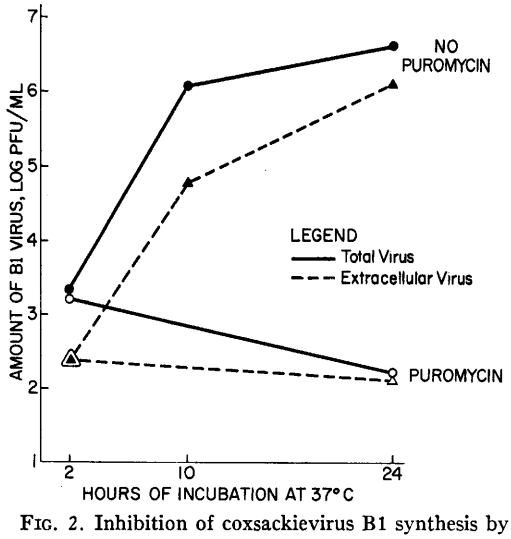

FIG. 2. Inhibition of coxsackievirus B1 synthesis by puromycin in cultures of primary fetal mouse cells.

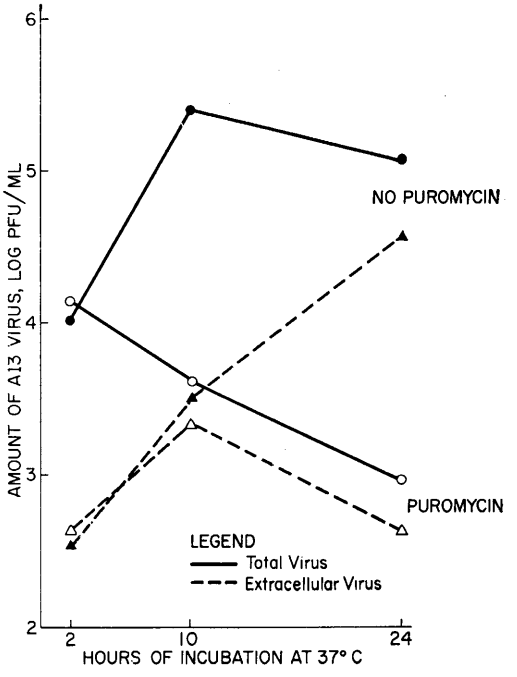

FIG. 3. Inhibition of coxsackievirus A13 synthesis by puromycin in cultures of primary fetal mouse cells.

The results illustrated in Fig. 3 clearly demonstrate that puromycin inhibited the synthesis of coxsackievirus A13 in replicate monolayer cultures of primary fetal mouse cells. It should be noted that although the extracellular and total virus assays showed the same trend of virus synthesis, a measure of total virus gave more striking results. The identity of the newly synthesized virus as coxsackievirus A13 was confirmed by neutralization with type specific antiserum. Coxsackievirus A13 titers obtained in control cultures, which were heat inactivated prior to inoculation, disclosed a similar pattern of vi-

rus inactivation at 37° in the presence or absence of puromycin. Thus, these data provided evidence that the low yields of coxsackievirus A13, which had been obtained repeatedly, represented a specific replication of the virus, and not elution of virus from the cells.

Attempts to detect interferon in PFM cultures infected with coxsackievirus A13. A series of experiments were performed to determine whether interferon was limiting coxsackievirus A13 growth in PFM cultures. The PFM monolayers in 32-oz bottles were infected with 1 ml of undiluted stock A13 virus and sampled for interferon production at 37° as described. Replicate infected cultures were overlaid with 10 ml of GM instead of 50 ml to increase the chance for recovery of small amounts of interferon.

No interferon activity was detected 24-hr postinfection in the fluid phase of any of the test or noninfected control cultures. In separate assays, interferon was not found in three different coxsackievirus A13 pools prepared from neonatal mice. The ability of the system to measure interferon was demonstrated by showing protection of MCN cells to challenge with VSV with a 1:960 dilution of a known mouse interferon preparation (kindly supplied by Miss Mary Donigian, Smith, Kline and French Laboratories, King of Prussia, Pa.). The next experiment was performed to test the possibility that a direct VSV challenge of PFM cells, infected previously with coxsackievirus A13, might be a more sensitive indicator of interferon activity. Twenty-four hr following A13 infection of a PFM monolayer, the fluid phase was sampled for assay of A13 virus, the pH was adjusted to approximately 7.6 with $NaHCO_3$ and the culture was superinfected with 2×10^6 PFU of VSV. A PFM culture not exposed to coxsackievirus A13, was also challenged with VSV to serve as a control. After an additional 23 hr of incubation at 37°, the extracellular fluids of both cultures were assayed for VSV production by the plaque assay in MCN cells. It should be noted that coxsackievirus A13 did not infect MCN cells and therefore the presence of A13 virus in the test sample did not interfere with the VSV assay.

The results of this experiment showed that the PFM monolayer infected with coxsackievirus A13 produced A13 virus as found previously; the extracellular titer of A13 virus increased from a postadsorption background level of 4.9×10^2 PFU/ml to a titer of 3.4×10^4 PFU/ml after 24 hr. Assay of the fluid phase of this culture and of the control culture, obtained 23 hr following challenge with VSV, revealed titers of 7.26×10^6 and 8.23×10^6 PFU/ml of VSV, respectively. These values were considered as evidence that viral interference was not manifested in the system. Thus, no interferon activity or viral interference was detected in the experimental system to account for the restriction of coxsackievirus A13 growth.

Determination of virus receptor activity of fetal mouse tissues and disaggregated cells. Because of the finding that coxsackievirus A13 replicated in PFM cells, it was decided to reinvestigate the results of an earlier report from our laboratory (5). In this report the resistance of PFM cultures to coxsackievirus A13 was attributed to the failure of the cells to attach demonstrable amounts of virus. The findings of initial experiments were in close agreement with these results in that minced fetal mouse tissues and suspensions of PFM cells failed to attach demonstrable amounts of coxsackievirus A13. However, since Crowell (15) has recently demonstrated a requirement for increased cell concentrations to demonstrate significant attachment of certain coxsackievirus to highly susceptible cells, experiments were performed to determine if fetal mouse tissues, in concentrations much higher than those tested previously, were capable of attaching coxsackievirus A13.

Cell suspensions were prepared from trypsin dispersed minced fetal mouse tissues as described in "Materials and Methods." The trypsinized cells from 2 g of tissues were distributed to screw-cap test tubes, centrifuged at 250*g* for 15 min at 4° and the supernatants were discarded. The packed cell pellets, each occupying a volume of approximately 0.3 ml and containing approximately 1.8×10^8 cells, were mixed separately with 0.1 ml of coxsackievirus A13, or poliovirus

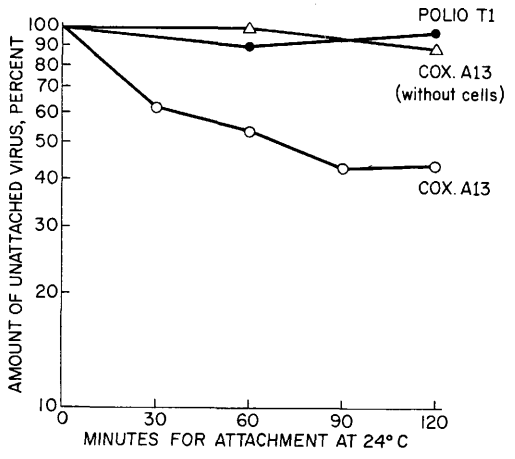

FIG. 4. Comparative rates of attachment of coxsackievirus A13 and poliovirus T1 to suspensions of trypsinized fetal mouse cells.

T1, each containing 1×10^4 PFU and incubated, respectively, at room temperature with intermittent shaking to allow virus attachment. At intervals of incubation, the reactions were stopped by the addition of 10 ml of cold GM to each of the cell–virus mixtures, the suspensions were shaken vigorously followed by centrifugation at 1000*g* for 15 min at 4°. The supernatant fluids were removed and stored at —20° until titrated for unattached virus in ML cells. Poliovirus type 1 was included in these experiments as a negative control, since it has been shown that this latter virus fails to attach in significant quantities to cultured mouse tissues (16).

Figure 4 shows that freshly trypsinized fetal mouse cells attached coxsackieviruses A13, whereas, poliovirus T1 remained unattached. A virus stability control, consisting of an equivalent amount of coxsackievirus A13 suspended in GM, showed little inactivation at room temperature. Similar results were obtained also for PFM cells grown in monolayer cultures and tested at equivalent concentrations for ability to attach coxsackievirus A13.

Infection of PFM Cells by viral RNA. Experiments were performed to determine whether RNA, with infectivity for PFM cells, could be extracted from coxsackievirus A13, since RNA extracted from coxsackievirus A6 was reported to be noninfectious for cultured mouse cells (11). These experiments also offered an approach whereby the yields of A13 virus by PFM cultures might be increased, since viral RNA could bypass the limitation imposed by the low receptor activity of these cultures for A13 virus.

A protocol similar to that described previously (17) was used to obtain a preparation of coxsackievirus A13 of high titer (2.2×10^9 PFU/ml) following infection of ML cells. The RNA was extracted from the fluid phase of the A13 virus preparation, following centrifugation (900*g*), by the cold phenol method of Wecker *et al.* (18). Infection of PFM monolayers was facilitated by use of diethylaminoethyl-dextran (DEAE-D; Pharmacia, Inc.) (19) at a concentration of 100 μg/ml. Synthesis of infectious virus was followed in replicate monolayers by plaque assay of samples on ML cells. In control experiments, the RNA preparation was shown to have a titer of 8.7×10^5 PFU/ml when assayed directly on ML cells. The infectivity of the RNA preparation was shown to be inactivated completely with pancreatic ribonuclease A (Worthington Biochem. Corp.) at a concentration of 50 μg/ml in 30 min at room temperature, whereas, an equivalent concentration of deoxyribonuclease I (Worthington Biochem. Corp. was without measurable effect.

The results of this experiment and of others of similar design showed, that when infection of PFM cultures was initiated with viral RNA preparations, the cultures yielded amounts of 10^6 PFU of complete A13 virus. These yields were not significantly different from those obtained from cells infected with complete virus. The virus progeny was not inactivated with RNase and was shown to be coxsackievirus A13 by neturalization with homotypic antiserum.

Discussion. Contrary to previous reports (5, 10, 11), the present findings revealed a pattern of limited, but significant, replication without CPE of a member of coxsackievirus Group A (A13) in cells cultured from fetal mouse tissues. Although a definitive explanation for these findings cannot be made at this time, it is speculated that the procedures employed for the preparation of PFM cultures enhanced the selection of a small num-

ber of cells susceptible to A13 virus infection.

Present results showed that equivalent virus titers were obtained in PFM cultures with either coxsackievirus A13 RNA or complete virus. Whether the same cell types were infected by each type of virus preparation remains unknown. The finding of A13 RNA infectivity is consistent however, with the knowledge that infectious RNA derived from enteroviruses is capable of replicating in many mammalian cell populations (20), and is in contrast to the negative findings of St. Geme *et al.* (11) for coxsackievirus A6 RNA.

The only factor found to enhance coxsackievirus A13 growth in fetal mouse monolayers was the addition of $NaHCO_3$ to the medium of infected cultures. The mechanism of this enhancement remains to be determined for this system. Many attempts by us to enhance coxsackievirus A13 synthesis by growing and infecting mouse cells under conditions of reduced oxygen supply or utilization were unsuccessful, and are in contrast to the results reported by Kantoch and Sieminska (8).

The requirement for high concentrations of cells to detect virus attachment in some systems has been demonstrated previously (15, 21). By using high cell concentrations the results of attachment studies of coxsackievirus A13 to mouse cells showed a limited, but specific, amount of virus attachment. This finding was consistent with the observation that mouse cells supported limited growth of this virus.

Summary. The limited attachment and replication of coxsackievirus A13 by cultures of primary fetal mouse cells was demonstrated for the first time. These results, taken together with the findings that CPE and interferon were undetectable in infected cultures support the hypothesis that a small number of cells in the population were susceptible to A13 virus infection. Efforts are now being made to determine the identity of the susceptible cell and to develop methods for increasing the proportion of susceptible cells in primary fetal mouse cultures.

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