Eclipse of Coxsackievirus Infectivity: the Restrictive Event for a Non-fusing Myogenic Cell Line

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SUMMARY

Coxsackieviruses A2, A5 and B3 did not replicate in LsCL3-U cells (a non-fusing variant of the rat Ls myogenic cell line) although these cells possessed a common receptor for coxsackieviruses A2 and A5, and a different receptor for coxsackievirus B3. The restriction in replication was identified as a block in viral eclipse, since 6 M-LiCl treatment permitted recovery of the coxsackievirus A2 inoculum from LsCL3-U cells after 2 h at 37 °C, and the cells could be transfected by viral RNA. Cellular fusion which was induced in LsCL3-U cultures by herpes simplex virus type 1 (HF strain) facilitated coxsackievirus A2 and A5 replication. Differentiating myogenic Ls cells acquired full susceptibility to infection concurrently with the appearance of acetylcholine receptors, the muscle-specific isoenzyme of creatine phosphokinase, prominent myotube formation and the acquired capacity of the cells to eclipse virus.

INTRODUCTION

Several differentiating cell systems in culture have been described which have shown a pattern of changing viral susceptibility during the various stages of cellular maturation. From the early studies of Vainio et al. (1963), who made initial observations in this area, to the recent use of viruses as probes to study differentiation events (Maltzman & Levine, 1981), a large number of reports have focused attention on identifying the restrictive event which limits virus replication at different stages of cellular differentiation. In most virus–cell systems, the restricted event has been found to occur after virus attachment, penetration and uncoating. For example, Segal et al. (1979) noted that the restrictive interaction of simian virus 40 (SV40) with non-differentiated teratocarcinoma cells was related to failure in viral mRNA splicing. For polyoma virus (Vasseur et al., 1980) and cytomegalovirus (Dutko & Oldstone, 1981), a block in replication at the transcriptional level was reported in undifferentiated embryonal carcinoma cells (ECC). D’Auriol et al. (1981) suggested that the Rauscher murine leukaemia virus proviral DNA failed to integrate into the genome of ECC to account for the restriction in virus replication.

In contrast, the acquired susceptibility of differentiating murine skeletal myoblasts to group A coxsackieviruses (Goldberg et al., 1969; Goldberg & Crowell, 1971) appears to be regulated by a cell surface event associated with readiness for cell fusion (Schultz & Crowell, 1980). This cell surface event has now been found to correlate with the capacity of differentiating myogenic Ls cells to eclipse cell-bound virus. This communication also reports that a non-fusing variant (LsCL3-U) of the Ls myogenic cell line is resistant to infection by certain coxsackieviruses because of a restriction in virus eclipse. Furthermore, data are presented which reveal that cell fusion induced by infection with herpes simplex virus type 1 (HSV-1), HF strain, eliminates the block in coxsackievirus replication.

METHODS

Cell lines. The Ls rat muscle cell line (Yaffe & Saxel, 1977) and a non-fusing variant of the Ls line, designated LsCL3-U, were routinely propagated in growth medium (Ls-GM) consisting of Eagle’s minimal essential medium (MEM) prepared in Earle’s balanced salt solution (EBSS) supplemented with 10% horse serum as described...
previously (Schultz & Crowell, 1980). The origin and method of propagation of RD cells for growth and plaque assay of group A coxsackieviruses have been described previously (Schultz & Crowell, 1980). The maintenance of monolayer cultures of HeLa cells (JJH strain) for coxsackievirus B3 plaque assay (Crowell & Syverton, 1961) and the growth of suspension cultures of HeLa cells (Mandel strain) for coxsackievirus B3 propagation have also been described previously (Crowell & Philipson, 1971).

**Virus and virus purification.** The origin of coxsackievirus A2 (F.L. 49190 strain), coxsackievirus A5 (5134 strain) and coxsackievirus B3 (Nancy strain) has been described elsewhere (Crowell & Syverton, 1955). HSV-1 (HF strain) was obtained from Dr G. H. Cohen (University of Pennsylvania School of Dental Medicine, Philadelphia, Pa., U.S.A.) and the Seibert strain of HSV-1 was obtained from Dr J. J. Docherty (Department of Microbiology, Cell Biology, Biochemistry and Biophysics, The Pennsylvania State University, University Park, Pa.). High titre virus pools for purification of group A coxsackieviruses were prepared in RD cells in 32 oz prescription bottles. Infected cells were pooled at 37 °C for 9 h, followed by three successive cycles of freezing and thawing to release intracellular virus, and further processed as described previously (Schultz & Crowell, 1980). Group A coxsackieviruses and coxsackievirus B3 were purified by banding on two consecutive CsC1 gradients as described by Crowell & Philipson (1971).

Stock pools of HSV-1 (HF strain) were prepared in HeLa cells and HSV-1 (Seibert strain) stocks were prepared in Vero cells (Gilmian et al., 1980).

**Virus antisera.** Specific antisera to purified coxsackieviruses A2, A5 and B3 were prepared in female New Zealand white rabbits as described by Beatrice et al. (1980). The antisera had titres greater than 1:5000 (plaque reduction 50%) and did not neutralize heterotypic viruses at a dilution of 1:100. HSV-1 rabbit antiserum was kindly provided by Dr R. J. Goldberg (National Cancer Institute, Bethesda, Md., U.S.A.).

**Virus assay.** RD cell monolayers for plaque assay of group A coxsackieviruses were overlaid with agar medium containing DEAE-dextran (100 μg/ml) and 5 mM-MgCl₂ for coxsackievirus A2 or 10 mM-MgCl₂ for coxsackievirus A5, plaques were allowed to develop at 37 °C for 72 h for coxsackievirus A2 or 48 h for coxsackievirus A5 as described previously (Schultz & Crowell, 1980). Coxsackievirus B3 plaque assay on HeLa cells was described previously (Crowell & Syverton, 1961). HSV-1 (HF and Seibert strains) titres, expressed as TCID₅₀ per ml, were determined by using HeLa or L₈ CL₃-U cell monolayers respectively.

**Preparation and use of coxsackievirus A2 viral RNA.** Pellets from the ultracentrifugation of purified coxsackievirus A2 were resuspended in extraction buffer (100 mM-NaCl, 50 mM-Tris–HCl, 5 mM-EDTA, pH 7.4) and the viral RNA extracted with buffer-saturated redistilled phenol by a modification of the method of Wilson et al. (1979). 0-1 vol. of 300 mM-NaCl and 2 vol. of cold (-20 °C) 95% ethanol were added to the final aqueous phase to precipitate the RNA. The precipitate was resuspended in MEM in EBSS, 0-02 M-HEPES, pH 7-5 containing 300 μg/ml DEAE-dextran, and stored at -70 °C until used. Infectivity of the viral RNA was determined by the method of Holland et al. (1959). Ribonuclease (100 μg/ml) (Millipore) treatment of the RNA for 30 min at 25 °C prior to inoculation served as a control for the viral RNA preparation.

**Assay for acetylcholine receptor (AChR).** The AChR was assayed by determining the number of ¹²⁵I-labelled α-bungarotoxin (¹²⁵I-α-BuTx) (sp. act. 534 cpm/mg/micromol; Amersham) binding sites present on L₈ and L₈ CL₃-U cell cultures (Prives et al., 1976). Specificity was established by measuring the ability of 1 × 10⁻⁴ M-d-tubocurarine chloride (d-TC) (Sigma) to inhibit competitively the binding of toxin to cell cultures.

L₈ and L₈ CL₃-U cultures were seeded at 5 × 10⁵ cells/60 mm culture dish daily for 7 days; cultures were removed from the incubator and rinsed once with MEM-EBSS, 0-02 M-HEPES, pH 7-5, 0-5% bovine serum albumin (BSA) (toxin medium). The cells, which were in culture for various time periods, were overlaid with toxin medium and incubated at 25 °C for 10 min. Cultures were drained of spent fluids and incubated with 2 ml of toxin medium with or without 1 × 10⁻⁴ M-d-TC for 30 min at room temperature. ¹²⁵I-α-BuTx was then added to a final concentration of 1 × 10⁻⁸ M and the cultures kept at 25 °C for 30 min. The incubation medium was removed and the cultures rinsed three times with 2 ml EBSS, 0-02 M-HEPES, pH 7-5, 0-5% BSA, to remove unbound radioactivity. To determine the amount of ¹²⁵I-α-BuTx bound, cells of each culture were dissolved in 1 ml M-NaOH and radioactivity measured in a Beckman Gamma 8000 counter. The amount of ¹²⁵I-α-BuTx bound per culture was determined as a function of days in culture for L₈ and L₈ CL₃-U cells.

**Determination of creatine phosphokinase (CPK).** L₈ and L₈ CL₃-U cells were seeded into 60 mm culture dishes at 1 × 10⁵ cells/ml in 5 ml L₈-GM and incubated at 37 °C. After various time intervals, cultures were drained of spent fluids, washed once with phosphate-buffered saline (PBS), and stored at -70 °C. After thawing, cells were scraped from the plates in 0-05 M-glycylglycine pH 6-7, and sonicated using a Branson Sonifier Cell Disrupter fitted with a tapering micro-tip at setting 6-5 for 25 s at 4 °C. To assay CPK activity, CPK reagents in kit form (Stat-Pack; Calbiochem-Behring) were utilized, as based on the methods of Oliver (1955) and Rosalki (1967). The rate of ATP formation from ADP and creatine phosphate was determined by coupling the reaction with hexokinase and glucose-6-phosphate dehydrogenase. One enzyme unit was expressed as the amount of enzyme that catalysed the formation of 1 μmol of NADPH per min at 30 °C, pH 6-7. The rate of change of absorbance at 340 nm was directly proportional to CPK activity. Specific activity was expressed in milli-units (mU) of CPK.
activity per µg protein. Cell protein content was determined for L₈ and L₈CL3-U cell cultures with the Folin phenol reagent as described previously (Schultz & Crowell, 1980).

RESULTS

Growth of coxsackieviruses in L₈ cell cultures during myotube formation

The susceptibility to infection of the established rat L₈ cell line by 16 of the 23 immunotypes of group A coxsackieviruses (A1 to 5, 7, 10 to 13, 15 to 18, 22, 24) and coxsackievirus B3 was found to coincide temporally with the stage of myogenesis characterized by syncytia formation (Schultz, 1981). The results in Table 1 show the comparative yields of representative coxsackieviruses following serial passage in well-fused L₈ cultures. The viruses also produced a characteristic cytopathic effect in post-fusion L₈ cells after 24 h incubation at 37 °C. In contrast, no virus replication or cytopathic effect was detected in the fusion-arrested clonal subline of L₈ cells designated L₈CL3-U, which confirmed and extended previous results (Schultz & Crowell, 1980).

Absence of viral replication in fusion-inhibited L₈ cultures

Experiments were conducted to determine whether inhibition of fusion of differentiating L₈ cultures would also inhibit coxsackievirus replication. L₈ cultures were grown in the presence of diazepam, a benzodiazepine compound known to inhibit myotube formation (Bandman et al., 1978). Standard monolayer cultures of L₈ cells were treated with 0, 10, 50 or 100 µM-diazepam suspended in L₈-GM 24 h after being plated at 5 × 10⁵ cells/60 mm culture dish. Tissue culture fluids were exchanged for fresh medium containing the respective concentration of diazepam every 48 h. Table 2 shows the virus yields 24 h post-infection of diazepam-treated cultures 192 h after plating. L₈ cultures which had been treated with 100 µM-diazepam did not display myotube formation and gave a 2 log₁₀ decrease in virus yield as compared to fused cultures (0 and 10 µM-diazepam-treated). To help control for drug cytotoxicity, diazepam-treated RD cell cultures were tested in parallel. RD cells are highly susceptible to group A coxsackieviruses in the absence of cell fusion and diazepam did not inhibit replication of coxsackievirus A2. Thus, diazepam inhibition of coxsackievirus propagation in myogenic cells provides further evidence for a differentiation event controlling cell susceptibility to virus infection.

Table 1. Comparative yields of coxsackieviruses A2, A5 and B3 following serial passage in L₈ (post-fusion) cells and L₈CL3-U (non-fusing) cells*

<table>
<thead>
<tr>
<th>No. of serial passages</th>
<th>L₈</th>
<th>L₈CL3-U</th>
<th>L₈</th>
<th>L₈CL3-U</th>
<th>L₈</th>
<th>L₈CL3-U</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1·0 × 10⁶</td>
<td>7·7 × 10⁷</td>
<td>1·3 × 10⁶</td>
<td>1·3 × 10⁸</td>
<td>2·1 × 10⁷</td>
<td>2·1 × 10⁸</td>
</tr>
<tr>
<td>1</td>
<td>3·5 × 10⁴</td>
<td>2·4 × 10⁵</td>
<td>1·8 × 10⁵</td>
<td>2·2 × 10⁵</td>
<td>1·4 × 10⁷</td>
<td>1·5 × 10³</td>
</tr>
<tr>
<td>2</td>
<td>7·8 × 10⁵</td>
<td>2·0 × 10⁶</td>
<td>3·7 × 10⁵</td>
<td>1·4 × 10⁷</td>
<td>4·0 × 10⁶</td>
<td>&lt;1·0 × 10⁴</td>
</tr>
<tr>
<td>3</td>
<td>3·8 × 10⁶</td>
<td>&lt;1·0 × 10⁵</td>
<td>1·5 × 10⁶</td>
<td>&lt;1·0 × 10⁴</td>
<td>4·6 × 10⁶</td>
<td>&lt;1·0 × 10⁴</td>
</tr>
<tr>
<td>4</td>
<td>2·4 × 10⁷</td>
<td>&lt;1·0 × 10⁶</td>
<td>ND</td>
<td>&lt;1·0 × 10⁷</td>
<td>2·7 × 10⁶</td>
<td>&lt;1·0 × 10⁴</td>
</tr>
</tbody>
</table>

* 5 × 10⁵ cells were plated in 60 mm Petri dishes, incubated at 37 °C and the medium exchanged every third day. After 168 to 192 h in culture (post-fusion) L₈ cells and non-fusing L₈CL3-U cells were inoculated with 0·2 ml of the respective coxsackievirus. Following a 60 to 90 min attachment period at 25 °C, cultures were overlaid with 5 ml of L₈-GM containing 3% horse serum, 0·02 M-HEPES, pH 7·5 (L₈-VGM) and incubated at 37 °C for 1 day (L₈ cells) or 3 days (L₈CL3-U cells). Virus was recovered from the fluid phase after three freeze-thaw cycles and removal of cell debris by centrifugation. A 0·2 ml amount of undiluted fluid was used to initiate each serial passage. Supernatant fluids were stored at −20 °C until assayed.

† Total virus content was assayed by plaque formation on monolayer cultures of RD cells for coxsackieviruses A2 and A5, and HeLa cells for coxsackievirus B3.

‡ No virus was detected in the undiluted sample.

§ ND, Not done.
Fig. 1. Evidence for virus group specificity for receptors on L₈CL3-U cells as measured by virus attachment interference assays. (a) Attachment interference of coxsackievirus A2 with receptor-saturating amounts of coxsackievirus A5. ○, Unattached coxsackievirus A2 from normal L₈CL3-U cells; ●, unattached coxsackievirus A2 from coxsackievirus A5-saturated L₈CL3-U cells; △, coxsackievirus A2 stability control. (b) Lack of attachment interference of coxsackievirus A5 with receptor-saturating amounts of coxsackievirus B3. ○, Unattached coxsackievirus A5 from normal L₈CL3-U cells; ●, unattached coxsackievirus A5 from coxsackievirus B3-saturated L₈CL3-U cells; △, coxsackievirus A5 stability control.

Table 2. Inhibition of L₈ cell fusion by diazepam limits replication of coxsackievirus A2*

<table>
<thead>
<tr>
<th>Cell culture</th>
<th>Diazepam concn. (µM)</th>
<th>Yields of virus 24 h post-infection (p.f.u./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L₈</td>
<td>0</td>
<td>2.2 × 10⁷</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2.6 × 10⁷</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>3.9 × 10⁶</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>2.9 × 10⁵</td>
</tr>
<tr>
<td>RD</td>
<td>4.0 × 10⁸</td>
<td>5.0 × 10⁸</td>
</tr>
<tr>
<td></td>
<td>5.0 × 10⁸</td>
<td>4.6 × 10⁸</td>
</tr>
<tr>
<td></td>
<td>1.0 × 10⁸</td>
<td>3.6 × 10⁸</td>
</tr>
</tbody>
</table>

* Diazepam-treated L₈ cultures (192 h after plating) and RD cultures were washed once with PBS and inoculated with 0.2 ml of coxsackievirus A2 containing 3 × 10⁷ p.f.u./ml. Following a 90 min attachment period at 25 °C, cultures were washed twice with 5 ml cold EBSS, 0.02 M-HEPES, pH 7.5, containing 3% calf serum, then 5 ml of L₈-GM was added and the cultures incubated at 37 °C for 24 h. Virus was recovered as described in Table 1.

Attachment specificity of group A and B coxsackieviruses to fusion-arrested cells

Virus attachment experiments indicated the presence of cell surface receptor sites for coxsackieviruses A2, A5 and B3, even though L₈CL3-U cultures were refractory to productive infection (data not shown). Therefore, the specificity of these coxsackievirus receptors on L₈CL3-U cells was determined by virus attachment interference assays (Crowell, 1966).

L₈CL3-U cells were seeded at 3 × 10⁵ cells/16 mm multiwell culture dish in L₈-GM. After 24 h incubation at 37 °C washed monolayer cultures were inoculated with purified coxsackievirus A5 at an input multiplicity of 100 p.f.u./cell in 150 µl to saturate the receptors. The cells were rocked at room temperature for 24 h to permit virus attachment, unattached virus was removed, and the challenge coxsackievirus A2 was added to give an input multiplicity of 1 p.f.u./cell in 150 µl. During the second virus attachment period (24 h, 25 °C) the supernatant fluids were removed at intervals to determine the amount of unattached virus. Fig. 1(a) shows that saturating amounts of interfering coxsackievirus A5 blocked the binding of coxsackievirus A2 to L₈CL3-U cells.

In a similar experiment, saturating amounts of interfering coxsackievirus B3 did not interfere with the attachment of challenge coxsackievirus A5 (Fig. 1(b)), reflecting the virus group specificity for receptors on cells (Crowell, 1976).
Table 3. Yields of coxsackievirus A2 from insusceptible and susceptible cell cultures inoculated with viral RNA

<table>
<thead>
<tr>
<th>Cell culture</th>
<th>Yield of virus (20 h post-infection, p.f.u./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insusceptible</td>
<td></td>
</tr>
<tr>
<td>LsCL3-U</td>
<td>$9.0 \times 10^{3}$</td>
</tr>
<tr>
<td>HeLa</td>
<td>$4.5 \times 10^{4}$</td>
</tr>
<tr>
<td>Susceptible</td>
<td></td>
</tr>
<tr>
<td>RD</td>
<td>$7.7 \times 10^{5}$</td>
</tr>
<tr>
<td>L8 (192 h)</td>
<td>$3.6 \times 10^{6}$</td>
</tr>
</tbody>
</table>

* Monolayer cultures of LsCL3-U, HeLa, RD and post-fusion L8 cells were rinsed three times with PBS and treated with 0.1 ml of coxsackievirus A2 RNA diluted in MEM in EBSS, 0.02 M-HEPES, pH 7.5 containing 300 μg/ml DEAE-dextran. Following incubation for 20 h, yields of virus were determined as described in Table 1. No virus was recovered from cultures similarly treated with viral RNA that had been incubated for 30 min at 25 °C with ribonuclease (100 μg/ml) prior to use.

Table 4. Virus eclipse as the event restricting replication of coxsackievirus A2: recovery of cell-associated virus from L8 and L8 CL3-U cells by 6 M-LiCl

<table>
<thead>
<tr>
<th>Incubation temperature</th>
<th>Percentage recovery of cell-associated virus by LiCl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L8 CL3-U cells</td>
</tr>
<tr>
<td>2 h (37 °C)</td>
<td>100</td>
</tr>
<tr>
<td>2 h (6 °C)</td>
<td>115</td>
</tr>
</tbody>
</table>

* Monolayer cultures of post-fusion L8 (192 h) and L8 CL3-U cells in 60 mm Petri dishes were rinsed twice with PBS, 0.02 M-HEPES, pH 7.5, after being preincubated at 6 °C for 40 min. Cultures were inoculated with 0.2 ml of coxsackievirus A2 (at an input multiplicity of 10) diluted in EBSS, 0.02 M-HEPES, pH 7.5, containing 3% calf serum (EBH-3% CaS) and the fluids pooled and assayed for unattached virus. Parallel cultures were overlaid with 5 ml L8-GM and either maintained at 6 °C or shifted up to 37 °C for a further 2 h incubation, after which the culture fluids were removed and stored at −20 °C until assayed. The cultured cells were treated with 6 M-LiCl for 10 min at 25 °C to dissociate virus and a sample of the fluid overlay was removed, diluted in EBH-3% CaS, centrifuged at 2000 rev/min for 15 min at 4 °C, and the supernatant fluids were collected and stored at −20 °C until assayed for virus.

Localization of the restriction event in coxsackievirus replication in L8 CL3-U cells

To localize the level of restriction in coxsackievirus replication in the resistant L8 CL3-U cells, transfection experiments were conducted with infectious RNA that was phenol-extracted from purified coxsackievirus A2.

Table 3 shows the yields of infectious virus obtained 20 h after the cultures were transfected. The results showed that both the L8 CL3-U and HeLa cells produced comparable amounts of infectious coxsackievirus A2. The higher yields from RD and post-fusion L8 cell cultures probably represented reinfection in these susceptible cell lines. Pretreatment of viral RNA with ribonuclease completely abolished the production of p.f.u. The evidence suggests that cell resistance to infection is not due to a translational block in viral replication, but to a restrictive event between attachment and translation.

To determine whether the restriction of susceptibility for the L8 CL3-U cell line was at the level of viral eclipse, post-fusion L8 and L8 CL3-U cells were treated with 6 M-LiCl, a virus-receptor bond-dissociating reagent (Zajac & Crowell, 1969). Total recovery of input virus was obtained for L8 CL3-U cultures incubated at both 6 °C and 37 °C for 2 h (Table 4). In contrast, substantially less virus was recovered from the differentiated L8 cultures following incubation at 37 °C for 2 h. These results suggest that the well-fused L8 cultures eclipsed input infectious virus, whereas non-fusing L8 CL3-U cultures were restricted at this phase of infection.

Induction of membrane fusion in L8 CL3-U cells

Numerous attempts were made to facilitate virus infection by promoting cell differentiation and membrane fusion in the non-permissive L8 CL3-U cell line. Unfortunately, all attempts to
induce L₉ CL3-U cells to undergo the sequential stages of skeletal muscle growth with dimethyl sulphoxide (Friend et al., 1971), N,N'-hexamethylene bisacetamide (Reuben et al., 1978), 5-azacytidine (Constantinides et al., 1977), retinoic acid (Strickland & Mahdavi, 1978) and other commonly used differentiation-inducing reagents were unsuccessful (data not shown).
Fig. 3. Time of appearance of AChR and CPK in L₈ and L₈ CL3-U cell cultures. AChR activity is shown in L₈ (Δ) and L₈ CL3-U (▲) cell cultures. CPK activity is shown in L₈ (○) and L₈ CL3-U (●) cell cultures.

Table 5. Fusion of L₈ CL3-U cells by HSV-1 (HF strain) facilitates replication of coxsackieviruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Yields of superinfecting virus 24 h post-infection (p.f.u./ml) after intervals (h) of primary infection with HSV-1 (HF strain)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Coxsackievirus A2</td>
<td>1·0 × 10⁷†</td>
</tr>
<tr>
<td>Coxsackievirus A5</td>
<td>4·5 × 10⁷</td>
</tr>
<tr>
<td>Poliovirus T2</td>
<td>&lt;1·0 × 10⁷</td>
</tr>
</tbody>
</table>

* Confluent L₈ CL3-U cell monolayer cultures were infected for 1 h at 37 °C with HSV-1 at an input multiplicity of 0·05 TCID₅₀/cell. The cultures were washed twice and incubated in 5 ml of L₈-VGM at 37 °C for 1, 4 or 10 h before coxsackievirus superinfection at an input multiplicity of 5 p.f.u./cell. After adsorption for 1 h at 37 °C, cultures were washed twice and overlaid with 5 ml of L₈-VGM and incubated at 37 °C. Control cultures, not infected with HSV-1, were inoculated with the respective virus and treated in parallel. Cultures were processed for virus content 24 h post-infection as described in Table 1. Samples were neutralized for 90 min at 25 °C with HSV-1 rabbit antisera prior to the plaque assay of superinfecting virus.

† The yields of virus shown represent the difference between the amounts of virus in dually infected cultures and singly infected cultures.

However, cell membrane fusion was achieved by use of HSV-1 (HF strain) (Person et al., 1982). Fig. 2 shows the characteristic appearance of cellular fusion induced by HSV-1 (HF strain)-infected L₈ CL3-U cultures. These cultures were assayed for their capacity to eclipse and replicate coxsackieviruses A2 and A5 at intervals after primary HSV-1 infection.

Table 5 shows that both coxsackieviruses A2 and A5 replicated to high titres following superinfection of L₈ CL3-U cells infected with HSV-1. Interference with coxsackievirus A2
superinfection was observed when superinfection was delayed until 10 h; however, no such interference of A5 virus occurred. Poliovirus T2 did not replicate in L₈ CL3-U cells, regardless of HSV-1-induced cell fusion, and served as a negative control.

In a similar experiment to determine whether the enhancement of virus replication was due to the fusion capacity of HSV-1, the non-fusing Seibert strain of HSV-1 was examined. The results showed that there was no significant replication of coxsackievirus A2 and A5 in the dually infected L₈ CL3-U cells (data not shown). It was concluded that induction of cell fusion by HSV-1 (HF strain) enabled L₈ CL3-U cells to eclipse virus.

**Controlled expression of muscle-specific markers for L₈ and L₈ CL3-U cell cultures**

The acquisition of the capacity for virus eclipse appeared to be coordinated with the expression of extensive cell fusion, which is under developmental control during muscle cell differentiation. Two specific biochemical markers of skeletal muscle differentiation, AChR (Prives et al., 1976) and the muscle-specific isoenzyme of CPK (MM-CPK) (Caravatti et al., 1979), were also investigated for their controlled expression in L₈ and L₈ CL3-U cultures.

Fig. 3 shows the comparative development of CPK activity and AChR expression in L₈ and L₈ CL3-U cell lines at intervals following their subcultivation. A sharp rise in MM-CPK enzyme and ¹²⁵I-α-BuTx binding to the L₈ cell line was noted at the time of fusion. This revealed a pattern of developmentally regulated gene expression for the differentiating L₈ cells. The non-fusing L₈ CL3-U cell line failed to demonstrate AChR or CPK activity during the course of these experiments. These results provide evidence that each of the biochemical events associated with muscle cell development, i.e. the appearance of AChR, transition to the muscle-specific isoenzyme (MM-CPK) and the morphological marker of overt myotube formation, could be correlated temporally with the expression of a factor(s) which is essential for coxsackievirus eclipse.

**DISCUSSION**

The studies reported here represent an extension of earlier work from our laboratory which described the capacity of differentiating murine skeletal muscle cells to replicate coxsackieviruses of group A (Goldberg et al., 1969; Goldberg & Crowell, 1971; Landau et al., 1972). It was found that pre-fusion myoblasts and fully contracting muscle fibres were resistant to infection, whereas myoblasts capable of fusion acquired susceptibility. In a related study of the rat L₈ myogenic cell line, the acquisition of cell susceptibility was not related to receptors for binding virus, since resistant (pre-fusion) cells attached virus (Schultz & Crowell, 1980). Earlier workers (Hsiung & Melnick, 1958; Holland, 1962) reported that enteroviruses will adsorb to resistant cells, although virus receptor specificity was not demonstrated. In the present study, the receptors on the resistant cells were shown to have specificity by virus saturation and competition experiments (Crowell, 1966, 1976). Virus attachment interference assays have been utilized to determine receptor families for other non-enveloped viruses including rhinoviruses, polioviruses, group B coxsackieviruses, adenoviruses (Lonberg-Holm et al., 1976), foot-and-mouth disease viruses (Baxt & Bachrach, 1980) and reoviruses (Lee et al., 1981).

The L₈ CL3-U cells, a non-fusing clonal derivative of the L₈ cell line, have been shown here to have a specific receptor for coxsackieviruses A2 and A5 which was different from that binding coxsackievirus B3. Nevertheless, these cells were non-permissive to infection by each of these viruses. The cellular resistance to infection was found to be due to a restriction in virus eclipse, since the cells could be transfected with viral RNA, and cell-associated virus could be recovered by dissociation with 6 M-LiCl, even after 2 h incubation at 37 °C.

It has been suggested that receptors have a dual function, namely to attach virus and to eclipse virus (Crowell & Siak, 1978). The acquisition of virus susceptibility by the differentiating rat L₈ myogenic cell line and the virus-resistant L₈ CL3-U subline provide a novel in vitro system to determine specific requirements for virus eclipse. It should be possible to ascertain whether the inability of cells to eclipse virus is due to a relatively low receptor density in the plasma membrane, or to the delayed expression of a virus eclipsing factor which functions as an integral part of the receptor complex (Holland, 1962). Virus eclipse was facilitated by cellular fusion,
either in the normal course of muscle cell development or induced by HSV-1 (HF strain). Whether the HSV-1-specified fusion factor (Person et al., 1982) increased membrane fluidity (Herman & Fernandez, 1978) or induced a host factor responsible for the growth of coxsackieviruses is not known. Other fusogenic and membrane fluidity-promoting agents which might assist coxsackievirus replication in the non-fusing, restricted LsCL3-U cells are under investigation. Additional examples of the influence of differentiation on virus susceptibility have been documented for differentiating skeletal muscle (Cox et al., 1977), keratinocytes (Laporta & Taichman, 1981) and ECC (Dutko & Oldstone, 1981).

The acquired susceptibility to coxsackievirus infection by post-fusion Ls-skeletal muscle cell cultures was correlated temporally in our study with myotube formation, an increase in the number of AChR on the developing muscle cell surface, and a sharp rise in the cytoplasmic muscle-specific isoenzyme MM-CPK. In contrast, the non-fusing LsCL3-U cells were negative for these characteristics which are associated with muscle cell development (Prives et al., 1976; Caravatti et al., 1979). These data provide further evidence for the regulated expression of functional membrane receptors in coxsackievirus infection during myogenesis. It is possible that a factor(s) which mediates virus eclipse, and whose expression is tied to differentiation, may serve as a determinant of acute or chronic virus-induced muscle disease.

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