Use of Guinea Pig Embryo Cell Cultures for Isolation and Propagation of Group A Coxsackieviruses

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The isolation of group A coxsackieviruses from clinical specimens generally requires the use of suckling mice. By using guinea pig embryo cells, the following coxsackieviruses were isolated from throat swabs and stool samples obtained from patients with a variety of illnesses: two of type A2, one each of types A6 and A8, and four of type 10. Distinct cytopathic effects were produced in 3 to 5 days in the guinea pig embryo cells inoculated with the clinical specimens. In addition, a number of prototype group A coxsackieviruses, including types 2-6, 8, 10, and 12, were readily propagated in guinea pig embryo cell cultures. Thus, guinea pig embryo cells appeared to be a sensitive alternative cell culture system for the isolation and propagation of certain types of group A coxsackieviruses.

With few exceptions, the isolation of group A coxsackieviruses from clinical specimens requires the use of newborn mice. Over the past several decades, numerous attempts have been made to propagate group A coxsackieviruses in cell culture. As yet, cell culture systems are not commonly used for the propagation of this group of viruses, and adaptation is usually required before cytopathic effect (CPE) is produced. In 1961, Wenner and Lenahan (8) were able to propagate 15 of 23 group A serotypes and two subtypes (20a and 20b) of coxsackieviruses in human amnion cell cultures; however, no isolations were attempted from clinical specimens. In 1975, Schmidt et al. (7) used the rhabdomyosarcoma (RD) cell line and were able to propagate 14 of 23 serotypes. In the latter study, comparisons of isolations of group A coxsackieviruses in RD cells and suckling mice were made from clinical specimens previously known to contain group A coxsackieviruses.

During the course of isolating viruses from clinical specimens obtained from patients suspected of having viral illnesses, an unexpected isolate was obtained in guinea pig embryo (GPE) cell cultures inoculated with a throat swab. The isolate was subsequently identified as coxsackievirus A10. Other clinical specimens previously known to contain group A coxsackieviruses and mouse tissue suspensions containing prototype strains of group A coxsackieviruses were used to determine the usefulness of GPE cells as an alternative cell culture system for the isolation and propagation of group A coxsackieviruses. The results are included in the present report.

MATERIALS AND METHODS

Cell culture. GPE cell cultures were prepared in our laboratory as previously described (1). Briefly, 30-day-old guinea pig embryos were trypsinized in 0.005% trypsin diluted in phosphate-buffered saline. The cell pellets were resuspended in Eagle minimum essential medium with 10% newborn calf serum at a ratio of 1:400. This suspension was then seeded into roller bottles or flasks and incubated at 35°C until confluent monolayers were obtained. All cultures were maintained in Eagle maintenance medium with 5% calf serum. Secondary cultures grown in tubes or Corning Costar panels were used for virus isolation and virus assay. The procedure for the preparation of mouse embryo (ME), chicken embryo (CE), and rabbit kidney (RK) cell cultures were similar. For human embryonic lung fibroblasts (HEL), WI38 and IMR90 cells were used interchangeably. Human embryonic kidney (HEK), rhesus monkey kidney (RhMK), and WI38 cell cultures in tubes were purchased from M. A. Bioproducts (Walkersville, Md.). IMR90 cell cultures in tubes were purchased from Flow Laboratories (McLean, Va.). Human placental (HP) cell cultures were obtained from Warren Andiman of Yale University School of Medicine. HEp-2 cell cultures were prepared in our laboratory.

Clinical specimens. Throat swabs, rectal swabs, and stool samples were obtained from patients with a variety of illnesses. Throat swabs and rectal swabs were placed in viral transport medium containing Hanks balanced salt solution with antibiotics and 10% fetal bovine serum. Stool samples were resuspended in a similar medium at a 10% concentration (wt/vol).

The clinical specimens previously known to contain group A coxsackieviruses were obtained through the courtesy of M. H. Hatch of the Centers for Disease
Control (CDC), Atlanta, Ga., and R. Deibel of the New York State Department of Health, Albany, N.Y. These specimens were stored at $-70^\circ$C for various periods of time and shipped to us on dry ice.

**Prototype group A coxsackievirus stocks.** Through the courtesy of M. H. Hatch, 10% homogenates of infected mouse brain and skeletal muscle containing prototype group A coxsackievirus types 1-8, 10-15, 17-22, and 24 and subtypes 20a and 20b were received. A coxsackievirus A9 prototype, passaged in RhMK in our laboratory, was also utilized.

**Specimen inoculation and virus assays.** On original isolation, the throat swab obtained from a patient, PiCh, was inoculated into a variety of cell cultures, including GPE, HELF, HP, HEp-2, HEK, ME, and RK. Clinical specimens obtained from the CDC and the New York State Health Department were originally inoculated into suckling mice and several types of cell culture (see Table 1). Upon reisolation, clinical specimens were inoculated into GPE and HELF cell cultures (0.1 to 0.2 ml/tube, two tubes per sample). Virus infectivity titers were determined either by CPE in tube cultures as 50% tissue culture infectious doses or by plaque-forming units. The latter was done in Corning Costar panels with confluent GPE monolayers under 0.5% methyl cellulose overlay (1). Prototype group A coxsackievirus stocks consisting of 10% homogenates of mouse brain and skeletal muscle were inoculated into GPE cell cultures (0.1 ml/tube) and suckling mice as described below.

**Mouse inoculation.** Supernatants from GPE cell cultures infected with the PiCh isolate and 10% mouse tissue homogenates of the prototype virus stocks were each inoculated into 24- to 48-h-old suckling mice (0.01 ml intracerebrally and 0.03 ml intraperitoneally). The mice were observed daily for illness and death.

**Electron microscopy and buoyant density determination.** For electron microscopy, both negative staining of virus-infected GPE cell culture supernatants and thin sectioning of infected GPE cells were performed as previously described (3). Buoyant density determination in cesium chloride from infected GPE cell cultures of the new isolate was determined by a previously reported method (6). Virus infectivity titers of the gradient fractions were determined by plaque formation in GPE monolayers.

**Neutralization test.** Antiserum to group A coxsackieviruses were obtained from the American Type Culture Collection (types 13, 19, 20, and 24) and from the CDC (types 1-8, 10-12, 14-18, 21, and 22 and subtypes 20a and 20b). Neutralization tests were done either by inhibition of CPE or by plaque reduction. Equal volumes of serum and virus suspension, containing approximately 100 plaque-forming units, were mixed and incubated at room temperature for 1 h. The mixtures were inoculated into GPE cells. Absence of CPE or reduction of virus-induced plaques were indications of virus neutralization by antiserum.

**RESULTS**

**Isolation and characterization of PiCh virus.** (i) Isolation. A throat swab originally obtained from an 8-year old boy, PiCh, with a

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<tr>
<th>Laboratory</th>
<th>Specimen designation</th>
<th>Source of specimen</th>
<th>Suckling mouse assay</th>
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$^a$TS, Throat swab; TW, throat washing; Stool, 10% suspension.

$^b+$, Paralysis, death, or both; -, no effect; ND, not done.

$^c$RD, rhabdomyosarcoma cell line; MK, primary rhesus or cynomologous monkey kidney cell culture; HELF, human embryonic lung fibroblast cell culture; GPE, primary guinea pig embryo cell culture. Other cell cultures tested with PiCh virus were: human embryonic kidney, human placenta, HEp-2, mouse embryo, and rabbit kidney, ND, Not done; -, no growth; +, growth.

$^d$Reisolations were made from specimens which had been frozen and thawed three times.

$^e$Mice were inoculated with PiCh virus-infected GPE cell culture fluid; virus infectivity titer, 6.0 log$_{10}$ plaque-forming units per 0.1 ml.
noted in the other similarly inoculated culture tubes, including HELF, HEK, HP, HEP-2, RhMK, ME, and RK cultures. After one passage of the PiCh virus in GPE cells, the new isolate then induced CPE in HELF cells, and after eight passages in GPE cells, the PiCh virus induced CPE in HP cells. However, virus infectivity titers were higher in GPE cells (6.0 log<sub>10</sub> 50% tissue culture infectious doses) than in HELF cells (4.5 log<sub>10</sub> 50% tissue culture infectious doses) or HP cells (4.0 log<sub>10</sub> 50% tissue culture infectious doses). Under methylcellulose overlay, the PiCh virus also produced plaques in GPE cells, but not in CE cells.

(ii) Morphological characterization of the PiCh virus. Because the original observation that the PiCh virus induced CPE only in GPE cell culture was totally unexpected, additional techniques were applied to determine the morphological and physical properties of the new isolate as a means for preliminary identification. Electron microscopic examination of both negatively stained preparations of the infected GPE cell culture supernatant and thin sections of infected GPE cells revealed a picornavirus. Virus crystals in an infected GPE cell are shown in Fig. 2. Furthermore, isopycnic banding in CsCl of the PiCh isolate grown in GPE cell culture yielded a distinct band containing infectious virus particles with the peak of virus infectivity at a buoyant density of 1.34 g/ml, consistent with previous data for the picornavirus group (6).

Mouse pathogenicity. Suckling mice 24 to 48 h old inoculated with supernatants from GPE cell cultures infected with the PiCh virus developed a paralysis of the hindlimbs 2 days post-inoculation. By day 3, all eight inoculated mice had died. Reisolation of the virus from the infected mouse brain suspension was accomplished, i.e., 10% infected mouse brain suspension induced CPE in GPE cell cultures in 3 days. Histopathology of the mouse skeletal muscle revealed diffuse hyaline degeneration, loss of striation, and mononuclear cell infiltration, characteristic of group A coxsackievirus infection (4). Typical viral particles in crystal-like arrays were observed in thin sections of the mouse skeletal muscle and were similar to those previously reported (2, 5).

Serological identification. The PiCh virus was neutralized by the patient's convalescent serum to a titer of 1:80 (no acute serum was available for testing). Subsequently, coxsackievirus A10 type-specific antiserum was found to inhibit both plaque formation and CPE induced by the PiCh virus in GPE cell cultures. The PiCh virus was not neutralized by antiserum to group A coxsackievirus types 1-9 and 11-24,
Fig. 2. Electron micrograph of coxsackievirus A10 (PiCh virus) crystal-like array (arrow) in a GPE cell. Magnification, ×11,560. Insert shows at higher magnification (×48,000) a virus crystal from another infected GPE cell.

group B coxsackievirus types 1–6, poliovirus types 1–3, or echovirus type 9. Thus, the PiCh virus was identified as coxsackievirus A10.

Use of GPE cultures for isolating group A coxsackieviruses from clinical specimens. To determine the usefulness of GPE cell cultures for the primary isolation of group A coxsackieviruses, coded clinical specimens known to contain group A coxsackieviruses were obtained. Of the 12 additional clinical specimens tested, 7 induced distinct CPE in GPE cells within 3 to 5 days post-inoculation (Table 1). Of the specimens obtained from the CDC, two of the isolates were coxsackievirus A10, and one was coxsackievirus A8. These three isolates originally replicated in RD cells or in suckling mice, but not in RhMK or HELF cell cultures. A coxsackievirus A13 did not produce CPE in GPE cells, but grew in RD, RhMK, and HELF cell cultures upon original isolation and in HELF cells on reisolation. Of the specimens obtained from the N.Y. State Department of Health, two coxsackievirus A2, one coxsackievirus A6, and one coxsackievirus A10 were isolated in GPE cells in 3 to 5 days. These viruses were all originally isolated in suckling mice. All of the latter specimens were also originally inoculated into primary cynomologous monkey kidney...
(MK) and HELF cell cultures; in addition to three coxsackievirus A9 isolates which grew in MK cells, a coxsackievirus A10 grew in HELF cells. On reisolation the coxsackievirus A10 isolate produced distinct CPE in GPE cells, but not in HELF cells; the three clinical specimens known to contain coxsackievirus A9 did not produce CPE in either GPE or HELF cells. A coxsackievirus A16, originally isolated in suckling mice, did not induce CPE in MK, HELF, or GPE cell cultures.

**Propogation of prototype strains of group A coxsackieviruses in GPE cell cultures.** When the prototypes of group A coxsackieviruses in 10% mouse homogenates were each inoculated into GPE cell cultures, extensive CPE was produced by types 2-6, 8, and 10 in 4 to 5 days on first passage. Mouse homogenates containing coxsackievirus A12 also induced CPE in GPE cells, but only after 12 days. On subsequent passage in GPE cells, all eight types induced extensive CPE in 3 days. Virus titers in GPE cells increased with passage in tissue culture (Table 2). Coxsackieviruses A1 and A7 produced CPE after 2 blind passages in GPE cells, but even after 10 subpassages, infectivity titers were low. Other prototype group A coxsackieviruses tested, including types 9, 11, 13-15, 17-22, and 24, did not produce CPE in GPE cells despite seven blind subpassages at weekly intervals. All of these prototype stocks were pathogenic when inoculated into suckling mice or, in the case of coxsackievirus A9, produced CPE in RhMK cells.

**DISCUSSION**

Group A coxsackieviruses generally require the use of suckling mice for primary isolation. Most diagnostic laboratories no longer have mice available routinely; thus, cell culture systems which can support the growth of this group of viruses are needed.

Many cell cultures have been tested over the years for susceptibility to group A coxsackieviruses, but, to date, only human amnion cells and the RD cell line have demonstrated some success (7, 8). Although a number of prototype group A coxsackieviruses have been adapted to propagate in both of these cell types, only RD cells have been used for virus isolations from clinical specimens. However, Schmidt et al. showed that RD cells generally proved less sensitive than newborn mice. Eleven isolations were attempted: two isolates produced CPE within 4 or 5 days upon primary inoculation in RD cells; six isolates required a second or third passage before distinct virus-induced CPE was apparent; and three isolates failed to grow in RD cells, but grew in suckling mice (7).

In the present study, we found that a number of group A coxsackievirus types could be readily isolated from clinical specimens on first passage in GPE cell cultures. Of the 13 clinical specimens known to contain group A coxsackievirus that were tested, distinct CPE was produced in GPE cells within 3 to 5 days after the inoculation of 8 of these specimens (two of type A2, one each of types A6 and A8, and four of type A10). Thus, for the isolation of at least these coxsackievirus types, the sensitivity of GPE cells appears to be comparable to that of suckling mice.

To examine the susceptibility of GPE cells for the propagation of other group A coxsackieviruses, prototype virus stocks in 10% mouse tissue suspensions were tested. Group A coxsackievirus types 2-6, 8, 10, and 12 were capable of inducing CPE in GPE cells without blind passage, and virus titers increased with subsequent passage in GPE cell cultures.

Thus, the data obtained from the propagation of prototype group A coxsackievirus stocks in GPE cells supported the results obtained from the isolation of group A coxsackievirus types from clinical specimens. Since GPE cells are readily available and are also very sensitive for the isolation and propagation of herpes simplex virus (1), it is feasible to use these cell cultures routinely in a clinical laboratory. The data presented in the present study indicate that GPE cells appear to be a sensitive alternative cell culture system for the isolation and propagation of certain types of group A coxsackieviruses.

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LITERATURE CITED