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Sensitivity of Rhabdomyosarcoma and Guinea Pig Embryo Cell Cultures to Field Isolates of Difficult-To-Cultivate Group A Coxsackieviruses

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Forty-two difficult-to-cultivate group A coxsackieviruses (i.e., group A types other than A7, A9, and A16), collected primarily from throat swab specimens of patients suffering from fever, pharyngitis, lymphadenopathy, and cough during the 1986 enterovirus season, were isolated in <24-h-old suckling mice. Thirty-six moribund mice were sacrificed and autopsied, and then their brains and back musculature were inoculated into rhabdomyosarcoma (RD), guinea pig embryo (GPE), rhesus monkey kidney (RhMk) and human carcinoma of the larynx (HEp-2) cell cultures. Twelve of the 36 suckling mice isolates were adapted to grow in RD and GPE cells after two passes and have been identified in RD cells by type-specific antisera as group A coxsackievirus types A2, A4, and A8. Three passes in RhMk or HEp-2 cell cultures were insufficient to affect a discernible cytopathic effect. Coxsackievirus types A1, A19, and A22, unable to grow in any of the four cell cultures tested, were identified by virus neutralization in suckling mice. These data denote the efficacy of suckling mice for the isolation of difficult-to-cultivate group A coxsackieviruses.

The isolation and identification of most group A coxsackievirus serotypes are difficult to perform, because many group A serotypes cannot be grown in cell culture or often require more than one pass to develop a discernible cytopathic effect (CPE) (10, 13, 16). Solid-phase enzyme-linked immunosorbent assays have been developed for the detection and identification of antigens to the group A coxsackieviruses. However, the preparation of an enzyme-linked immunosorbent assay to detect all group A coxsackievirus serotypes is impractical; the use of antibody pools in such a system has not been thoroughly evaluated and may adversely effect the sensitivity and specificity of the reaction (20, 21).

Suckling mice have been suggested as the most sensitive system for the cultivation of most group A coxsackievirus serotypes (13). Few clinical virology laboratories, however, utilize the mouse system and consequently do not identify many of the group A coxsackievirus serotypes which infect their patient populations. The failure of the Centers for Disease Control, for example, to predict the appearance on a national level of most group A coxsackievirus serotypes probably reflects the lack of surveillance laboratories to employ an appropriate (e.g., suckling mouse) assay system (2, 3, 17).

Two host systems, the rhabdomyosarcoma (RD) and guinea pig embryo (GPE) cells, have been suggested as an alternative to suckling mice. Although these cells types are sensitive to many group A coxsackievirus serotypes, they have been only partially evaluated by separate workers using different group A coxsackievirus strains. A need exists to evaluate in parallel the sensitivities of both cell types to suckling mice with a single large group of coxsackievirus field isolates obtained during the course of an enterovirus season.

The purpose of this study was multifactorial and included (i) the evaluation of RD and GPE cell cultures as well as two additional commonly used cell types for the isolation of group A coxsackieviruses, (ii) the determination of an approximate prevalence in our population of the difficult-to-cultivate group A coxsackieviruses during the 1986 enterovirus season, and (iii) the determination of a rationale for the inoculation of suckling mice with specimens obtained from patients suspected of suffering from a group A coxsackievirus illness.

MATERIALS AND METHODS

Clinical specimens. Throat, nasopharyngeal, and rectal swab specimens were collected during the 1986 enterovirus season from patients suffering from a variety of respiratory and gastrointestinal illnesses. All swab specimens were placed into viral transport medium, consisting of Hanks balanced salt solution containing 0.5% gelatin and antibiotics. Specimens were received by the virology laboratory within 24 to 48 h of collection.

Routine virus isolation. All specimens were inoculated into primary rhesus monkey kidney (RhMk) and primary human embryonic kidney (HEK; Whittaker MA Bioproducts, Walkersville, Md.) cell cultures. Briefly, specimens were treated with gentamicin and amphotericin B and vortexed vigorously, and 0.2 ml of the suspension was inoculated into commercially available cell cultures (12- by 75-mm screw-cap glass tubes). The cell cultures were observed every 2 days and sometimes daily for a period of 14 days for the appearance of a CPE. After the 14-day incubation period, tubes suspected of displaying a CPE were passed a second time into cultures of the same kind.

Mouse inoculation of original specimens. Three hundred sixty-two specimens, obtained from patients suffering from a variety of respiratory and gastrointestinal illnesses including but not limited to fever, headache, lymphadenopathy, pharyngitis-exudative tonsillitis, cough, and nausea and vomiting, were inoculated into <24-h-old suckling Swiss Webster

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mice (Charles River Breeding Laboratories, Wilmington, Mass.; Ticonic Farms, Germantown, N.Y.). Samples of 0.3 to 0.5 ml of each specimen were inoculated into the dorsal fat pads (subcutaneously, approximately 1 to 1.5 mm in depth) of at least three suckling mice. With appropriate markings (e.g., subcutaneous inoculation of a vital stain), two specimens plus a control (medium alone) were inoculated into a litter of nine pups. The mice were observed for a period of 14 days. Flaccid paralysis of the hind legs was indicative of a group A coxsackievirus infection (8). Autopsy tissues from positive mice were reinoculated into the same animal type to confirm the presence of the virus. The same tissues were used to inoculate in parallel RD, GPE, human carcinoma of the larynx (HEp-2), and RhMk cell cultures. The autopsy tissues were ground in the presence of 2 ml of phosphate-buffered saline and stored at −78°C until use.

Isolation and identification of virus from mouse autopsy material. (i) Cell culture. RD (passage no. 59) and HEp-2 cells were purchased from ViroMed Laboratories, Inc. (Minneapolis, Minn.) and Whittaker MA Bioproducts, respectively. GPE cells were kindly supplied as monolayer cultures in 16-oz. (ca. 473-ml) glass bottles by G. D. Hsiung (Veterans Administration Medical Center, West Haven, Conn.). The growth and maintenance of the cells were performed as described previously (11). Briefly, the RD, RhMk, and HEp-2 cell monolayers were digested with a 0.2% trypsin solution (in the absence of CA^{2+} and Mg^{2+}) and seeded into each of 96 wells of flat-bottom microdilution plates (Costar). The growth medium consisted of Hanks minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine, and antibiotics. The maintenance medium for the RD, HEp-2, and GPE cells was the same as the growth medium, except that 2% fetal bovine serum was used. Serum was not present in the medium used to maintain the RhMk cells. GPE cells, extremely sensitive to trypsin, were subcultured into 96-well plates by the procedure of G. D. Hsiung (personal communication). The monolayer of GPE cells was rinsed once in prewarmed phosphate-buffered saline without Ca^{2+} or Mg^{2+}. Prewarmed 0.125% trypsin (3 ml) was added to the monolayer (16-oz. bottle), and the bottle was immediately turned upside down to free the cells from the trypsin. After 45 s to a few minutes, when the cells appeared to be sloughing off, the trypsin was removed, and growth medium was added. Up-and-down pipetting was performed to remove cells from the bottle and to disperse clumps. The cells were washed once and seeded into wells of 96-well microdilution plates and 25-cm^3 plastic flasks. A 1:2 split was performed.

(ii) Virus identification. Group A coxsackievirus mouse isolates (other than types A7, A9, and A16) which produced a CPE in RD or GPE cell cultures were identified by neutralization (inhibition of CPE) in monolayer cultures of RD cells grown in 96-well plates. The group A coxsackieviruses which grew in suckling mice only were neutralized (inhibition of animal death) in these animals.

For neutralization in cell cultures, virus isolates from RD cells were serially diluted from 10^{-1} to 10^{-3} in maintenance medium and inoculated with an equal volume of monospecific antisera to group A coxsackieviruses (A1-A21 from Telcolab [Immunitalia] Corp., New York, N.Y.; A22 from the American Type Culture Collection, Rockville, Md.). Antisera to group A types 1 through 21 were diluted 1:5 in sterile physiologic saline. Lyophilized antiserum to coxsackievirus type A22 was reconstituted in sterile distilled water, followed by additional dilution to 1:10 in physiologic saline. Samples of each antiserum were stored at −78°C until use. The control consisted of the viral isolate diluted with an equal volume of phosphate-buffered saline. The isolates and their controls were incubated for 2 h at 36.5°C, followed by duplicate inoculation of 0.2 ml of each mixture into RD cell cultures. The cell cultures were observed daily or every 2 days for 14 days. Inhibition of CPE was indicative of neutralization by the antiserum.

For neutralization in suckling mice, mouse autopsy material was diluted from 10^{-1} to 10^{-3} in maintenance medium, added to an equal volume of monovalent antiserum, vortexed vigorously, and incubated as described above. The control consisted of the same virus which was incubated with an equal volume of phosphate-buffered saline. Inoculation of the suckling mice was performed as described above. Survival of the animal was indicative of neutralization by the monovalent antiserum.

(iii) Preparation of guinea pig embryo cell culture. A pregnant (26-day term) guinea pig was purchased from Hazelton Laboratories, Inc. (Denver, Pa.). Three embryos were aseptically removed from the anesthetized (ketamine hydrochloride-acepromazine maleate) animal and then minced in 10 ml of sterile phosphate-buffered saline. The minced embryo was subjected to digestion in 50 ml of 0.2, 0.02, and 0.002% trypsin, with mechanical mixing (magnetic stir bar) at 37°C for 30 min. After the 30-min treatment, each digest was allowed to stand at room temperature for approximately 15 min or until the larger tissues settled. The largest cell yield, identified in the 0.2% trypsin digest, was washed in phosphate-buffered saline, suspended in growth medium, and seeded into two 25-cm^2 tissue culture flasks and 48 wells of a 96-well flat-bottom tissue culture plate. Cell confluency occurred after 5 days at an incubation temperature of 37°C. GPE cells, grown in a 25-cm^2 flask, were trypsinized and seeded into a flat-bottom 96-well plate to permit a second passage of the specimen inocula. Growth and passage of each cell type were performed as described above. Erythrocytes were removed by washing GPE cell cultures 2 days after seeding.

(iv) Isolation of difficult-to-cultivate group A coxsackieviruses in cell cultures from original specimens. All 16 1987 original specimens, positive in suckling mice but not in RhMk, HEK, or HEp-2 monolayer cultures, were tested in primary GPE and RD cultures (GPE and RD monolayers were prepared in the Virology Laboratory). Two passages of each specimen inoculum in GPE and RD cell cultures were performed. Final readings took place after day 8.

RESULTS

Isolation in suckling mice of coxsackieviruses from field specimens. Of 362 specimens tested, 42 (8.5%) were group A coxsackievirus positive in suckling mice. The number of days required for the appearance of virus-induced paralysis in the suckling mice ranged from 3 to 8 (mean of 4) days. Six of the 42 moribund mice were not removed in time from their cages and were consumed by the nursing female.

Isolation of group A coxsackieviruses in cell culture. None of the 36 mouse isolates produced a CPE in the RhMk or HEp-2 cell cultures; two additional passes failed to effect a lytic infection in these cell types. Twelve of the 36 mouse isolates grew in RD and GPE cells. However, a clearly defined CPE was apparent in these cell cultures only after a second passage was performed. An obvious CPE was subsequently realized by 6 to 7 and 1 to 2 days earlier in the RD and GPE cell cultures, respectively. The CPE of group A coxsackievirus-infected RD cells is shown in Fig. 1. The

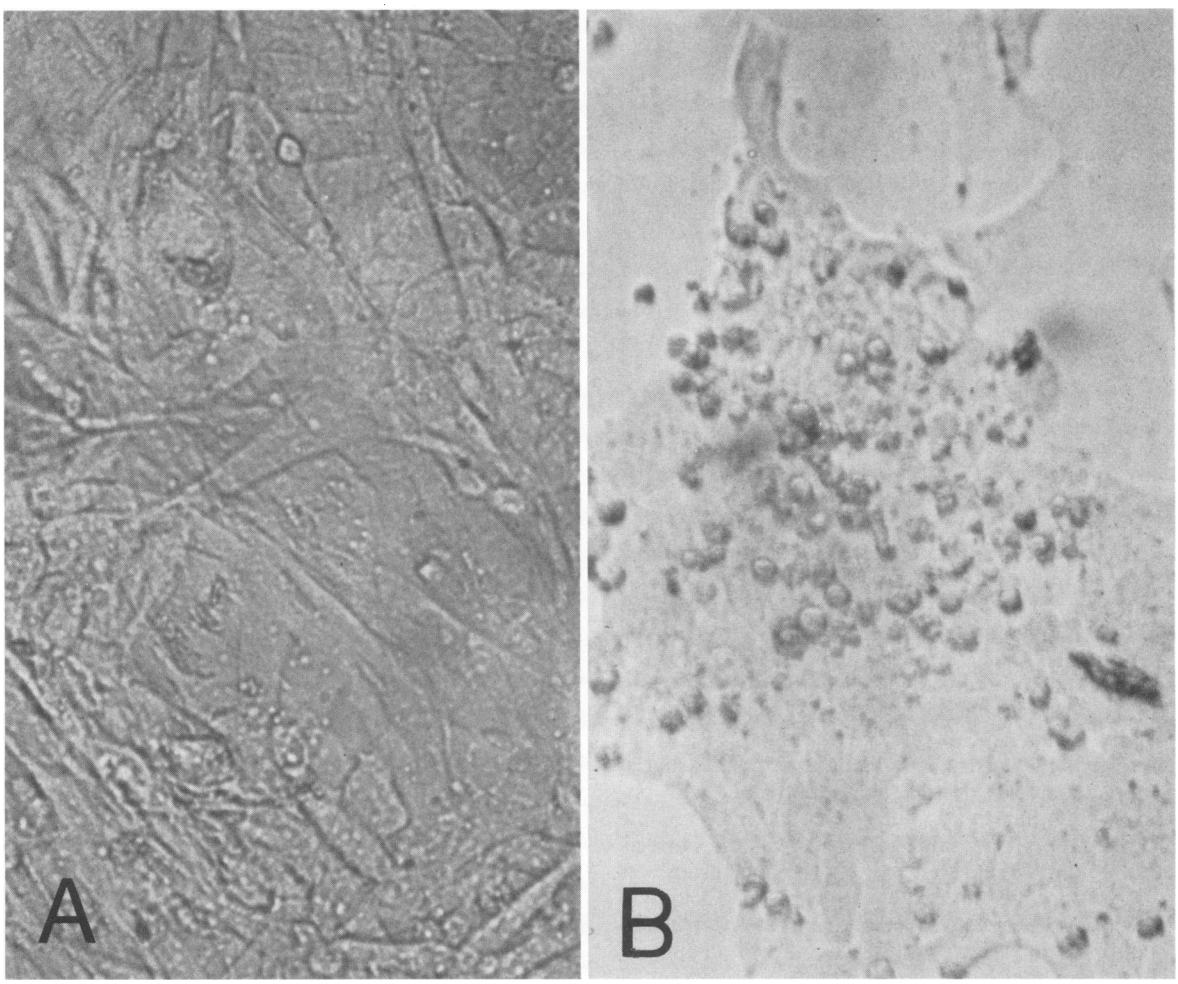

FIG. 1. CPE of coxsackievirus type A8 in RD cells. (A) Uninfected RD cells; (B) coxsackievirus type A8-infected RD cells, 6 days (second passage) after inoculation. Cellular degenerations of RD cells by coxsackievirus types A2, A4, and A8 were indistinguishable. Magnification, ×250.

virus-induced degeneration of a GPE cell culture was previously reported (10).

Identification of group A coxsackievirus isolates. All of the group A coxsackieviruses which produced a CPE in RD and GPE cells were serologically identified by using monovalent antiserum (Table 1). Of the 12 group A coxsackieviruses which grew in both RD and GPE cells, 5, 4, and 3 were identified as serotypes A2, A8, and A4, respectively. Of the remaining 24 mouse isolates which did not produce a cellular change in any of the cell cultures tested in this study, 11 were identified by neutralization in suckling mice as type A1, A19, and A22. The 11 isolates were chosen at random from frozen (−78°C) autopsy specimens.

Association of clinical symptoms with the isolation of difficult-to-cultivate group A coxsackieviruses. Group A coxsackievirus infections during the 1986 enterovirus season were most frequently identified among patients suffering from pharyngitis and exudative tonsillitis (81%), fever (79%), upper respiratory illness (40%), otitis media (33%), cough (26%), and lymphadenopathy (24%). All specimens except one were obtained from throat swabs. More than half of the 42 patients in question were started on a regimen of antibiotic therapy before laboratory results reporting the absence of a bacterial pathogen were received.

Isolation of difficult-to-cultivate group A coxsackieviruses: 1980 and 1982 through 1987. The number of group A coxsackieviruses isolated in suckling mice during the enterovirus seasons in 1980 and 1982 through 1987 are shown in Fig. 2. The largest number of isolates occurred during the months of June, July, and August. The viruses appeared sporadically in May, September, and October, although an equal number of specimens was inoculated into suckling mice throughout each month in question.

Growth of coxsackieviruses from original specimens in GPE and RD cell cultures. The ability of primary GPE and RD cell cultures to support the growth from original specimens of our 1987 difficult-to-cultivate group A coxsackieviruses is reported in Table 2. After two passes in RD cells, a clearly defined CPE occurred in 8 of 16 (50%) original specimens which were positive in <24-h-old suckling mice. After a single pass in the RD cells, virus-induced cell degeneration was apparent in only 2 of the 16 inocula. The isolation rate of the 1987 difficult-to-cultivate group A coxsackieviruses from original specimens in GPE cell culture monolayers failed to approach the sensitivity of that identified in suckling mice. After one passage in the culture, only 1 of 16 original specimens produced a CPE. A second passage in GPE cells

TABLE 1. Susceptibility of RD and GPE cell cultures to field isolates of difficult-to-cultivate group A coxsackieviruses[a]

Specimen no.	CPE in: RD cells P1	RD cells P2	RD cells P3	GPE cells P1	GPE cells P2	GPE cells P3	Serological identification
3881	−	−	−	−	−	−	A1
4243	−−+	+	+	−+	+	+	A2
4314	−−+	+	+	−+	+	+	A8
4478	−	−	−	−	−	−	A1
4501	−	−	−	−	−	−	A1
4512	−−+	+	+	−−+	+	+	A4
4579	−−+	+	+	−−+	+	+	A2
4596	−−+	+	+	−−+	+	+	A2
4635	−	−	−	−	−	−	A19
4637	−	+	+	−+	+	+	A4
4644	−−+	+	+	−+	+	+	A8
4749	−	+	+	−+	+	+	A8
4761	−−+	+	+	−+	+	+	A2
4769	−	−	−	−	−	−	A1
4846	−−+	+	+	−+	+	+	A4
4877	−	−	−	−	−	−	A22
5038	−−+	+	+	−+	+	+	A2
5066	−	−	−	−	−	−	A22
5376	−	−	−	−	−	−	A19
5401	−	−	−	−	−	−	A1
5543	−−+	+	+	−+	+	+	A8
5731	−	−	−	−	−	−	A22
5907	−	−	−	−	−	−	A1

[a] All specimens grew in suckling mice but not in RhMk or HEp-2 cell culture monolayers. P1, P2, and P3 indicate one, two, and three viral passages, respectively, in the indicated cell culture.

TABLE 2. Isolation from original specimens of 1987 difficult-to-cultivate group A coxsackieviruses[a]

Specimen no.	Age of patient (yr)	Isolation of virus from: RD cells P1	RD cells P2	GPE cells P1	GPE cells P2	Mice
3936	4	−+	+	−+	−	+
4360	6	−+	+	−	+	+
4418	2	−+	+	−+	+	+
4626	4	+	+	−+	−	+
4781	2	−	−	−	−	+
4874	16	−+	+	−	+	+
4944	0.7	−	−	−	−	+
4967	2	+	+	−	−	+
5330	3	−+	+	+	+	+
5403	7	−+	−	−+	−	+
5580	0.4	−	−	−+	−	+
5581	1	−	−	−	−	+
5684	1	−+	−	−+	−	+
5869	1	−+	+	−+	+	+
5883	1.3	−	−	−	−	+
6882	1	−	−	−	−	+

[a] Isolates were obtained from throat swab specimens. Isolates grew in suckling mice but not in RhMk or HEp-2 cell culture monolayers. The numbers of viral passages in the respective cell culture are indicated as in Table 1.

increased the rate of isolation from 13 to 31% but still lacked the sensitivity of the suckling mouse system.

DISCUSSION

Most group A coxsackieviruses do not grow readily or produce a discernible CPE in cell cultures that are routinely employed in the clinical virology laboratory. African green monkey kidney cells and human embryonic fibroblasts, for example, are effective for the isolation of many coxsackieviruses. However, these cell types lack the ability to support the growth of many of the difficult-to-cultivate group A coxsackievirus types (e.g., A1, A4, A8, A19) (21).

The use of RD and GPE cells as an alternative host system to suckling mice for the isolation of certain difficult-to-cultivate group A coxsackieviruses has been proposed (10, 16). Our study, with 36 field isolates obtained during the 1986 enterovirus season, does not support the replacement of suckling mice by these cell types. Importantly, only 12 of 36 group A coxsackieviruses which grew in suckling mice were able to grow in monolayers of RD and GPE cells. Furthermore, all 12 of the 36 coxsackievirus field isolates required a second passage in both RD and GPE cells before the appearance of a discernible CPE. Earlier studies similarly

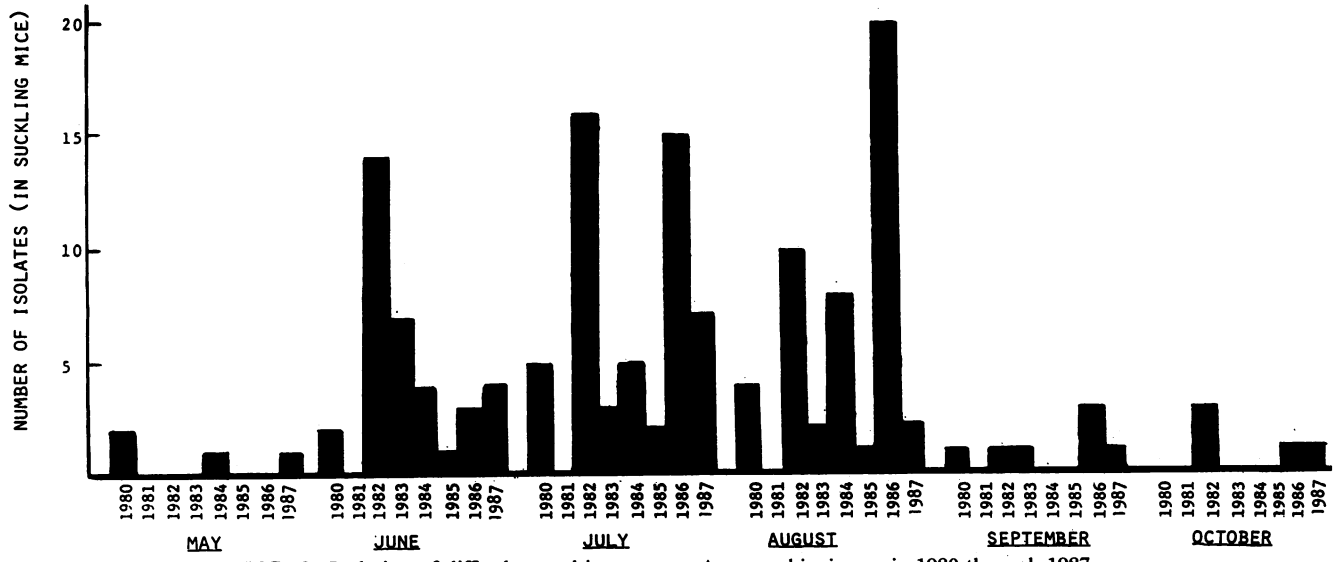

FIG. 2. Isolation of difficult-to-cultivate group A coxsackieviruses in 1980 through 1987.

reported that an initial passage was insufficient to identify most group A coxsackieviruses in RD cell cultures (16). Landry et al. (10), on the other hand, reported "extensive" CPE after a single passage in GPE cells of several group A coxsackievirus types (i.e., types 2 through 6, 8, and 10). In our studies, only a suggestive CPE in GPE cells was identified after an initial passage of mouse isolate types A2, A4, and A8; a second blind passage was required to identify a clear virus-induced cellular degeneration. Blind passages, parenthetically, are not generally performed in large clinical virology laboratories (7). Consequently, failure to isolate large numbers of group A coxsackieviruses, even with the use of an appropriate cell system, is not unexpected (5).

The isolation rates of our 1987 difficult-to-cultivate group A coxsackieviruses from original specimens in RD or GPE cell cultures failed to approach that identified in suckling mice. These data not only denote the necessity of a second blind passage in either RD or GPE cells, but also confirm the superiority of <24-h-old suckling mice for the isolation of the difficult-to-cultivate group A coxsackieviruses.

Some workers may still choose to incorporate RD or GPE cells into their panel of cell types during the summer (temperate climate) enterovirus season. This approach has validity, because large numbers of group A coxsackieviruses may be isolated which otherwise might be missed by the use of inappropriate cell cultures (i.e., RhMk or buffalo green monkey cells). However, extended technician time is required to perform and monitor two passages in RD or GPE cell cultures. Consequently, the turnaround time needed for group A coxsackievirus isolation in these cells might perhaps exceed 14 days. Furthermore, primary GPE cells are not commercially available and must be prepared de novo from the embryo. The maintenance of GPE cells also presents difficulty; final readings are problematic due to some granulation and rounding of the cells in monolayer culture. This effect does not occur in RD cell cultures. In the current study, isolation in mice of the difficult-to-cultivate group A coxsackieviruses occurred after a mean of 4 days. In addition, the isolation rate in mice of most difficult-to-cultivate group A coxsackievirus serotypes may be increased by inoculation of selected specimens based on the symptoms of the patients and the diagnoses of the physicians. Previous observations in this laboratory have identified gastrointestinal rather than respiratory specimens as less relevant to the clinically compatible illness (4; unpublished observations).

Of 36 randomly chosen autopsy specimens from which the viruses failed to grow in cell culture, 11 were identified by neutralization in suckling mice as serotypes A1, A19, and A22. The growth of serotype A1 viruses in GPE cells by Landry and co-workers, but not by us, is difficult to explain (10). Perhaps the prototype strain used by the Yale group differed sufficiently from our type 1 field isolates that the former was able to be adapted to grow in the cell culture. Remaining mouse autopsy specimens from our 1986 enterovirus season were not serotyped, because the number of suckling mice needed to perform the neutralization assays would be prohibitive. The remaining specimens, however, were probably either one or some combination of A19, A22, or possibly A1, because our cell culture panel supports the growth of other group A coxsackievirus types (10, 16). In our study, the failure of serotypes A1, A19, and A22 to grow in RD or GPE cell cultures confirms the intrinsic need of the mouse system for those workers attempting to isolate group A coxsackieviruses from clinical specimens (13, 19, 21).

Suckling mice are simple and inexpensive to breed and maintain. Maintenance of mice, including equipment, technician time, and food (based on our usage), approximates 95 cents per litter per day (John Selig, Supervisor, Animal Research Laboratory, Department of Animal Research, Nassau County Medical Center). Mice may be inbred for a period of 3 years. After that time outbred animals are recommended to replenish the stock.

The difficult-to-cultivate group A coxsackieviruses appear to be present in our population in numbers greater than that reported and predicted on the national level (2, 3). Our group A coxsackievirus isolates for example, represent 32% (42 of 130) of all nonpolio enterovirus isolates reported in 1986 (unpublished observations). The low incidence of group A coxsackievirus isolates reported by Centers for Disease Control surveillance laboratories probably reflects the fact that the laboratories do not use suckling mice (9, 17).

Suckling mice may also be an appropriate animal system for virologists performing water pollution and environmental impact studies. Difficult-to-cultivate group A coxsackieviruses, for example, are not commonly isolated from virus-laden sewage, shellfish, or receiving waters by using cell cultures alone (1, 6, 12, 18). However, the virus group has been identified in environmental samples when the availability of suckling mice prevailed (14, 15).

Our data indicate that the difficult-to-cultivate group A coxsackieviruses may be commonly isolated from patients suffering from a variety of upper respiratory illnesses, especially pharyngitis. The main presenting symptoms of these illnesses were fever, cough, and lymphadenopathy. Importantly, the two routinely used cell cultures and the RD and GPE cell cultures lacked the sensitivity of suckling mice to support the growth of many of our group A coxsackievirus isolates during the 1986 and 1987 enterovirus seasons. According to our data, for example, 50 to almost 70% of our 1987 difficult-to-cultivate group A coxsackievirus field isolates (group A types other than A7, A9, and A16) would have been undetected if suckling mice had not been incorporated into our screening panel.

Inoculation of selected specimens, coupled with the low cost and simplicity of handling and maintaining mice, makes this animal system appropriate for virologists interested in the isolation of the difficult-to-cultivate group A coxsackieviruses.

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