Replication of acute hemorrhagic conjunctivitis viruses in conjunctival-corneal cell cultures of mice, rabbits, and monkeys

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Acute hemorrhagic conjunctivitis (AHC) viruses—enterovirus type 70 (E70), and coxsackievirus type A24 (CA24)—infect the superficial cells of the conjunctiva and cornea. We examined the growth of several E70 and CA24 isolates in monkey, rabbit, and mouse conjunctival and corneal (C/C) cells. We found that E70 isolates grew well in monkey (10^6.5 TCID<sub>50</sub>/ml), rabbit (10^6.8 to 10^8.5 TCID<sub>50</sub>/ml), and mouse (10^5.8 to 10^6.6 TCID<sub>50</sub>/ml) C/C cultures and caused cytopathic effects (CPE). In contrast, CA24 isolates replicated well and caused CPE only in monkey C/C cultures (10^8.5 TCID<sub>50</sub>/ml). These results show that E70 isolates grow readily in monkey, rabbit, and mouse C/C cells whereas CA24 isolates replicated in monkey C/C cells only. This finding is consistent with the suggestion that a lower animal reservoir may be a source of human infection by E70. These cell culture systems may be useful for studying virus-cell interactions and host defenses operating at the surface of the eye and for evaluating the feasibility of prophylactic or therapeutic use of interferon, interferon inducers, and/or antibody for controlling AHC virus infections.

Key words: conjunctivitis, enterovirus, coxsackievirus, conjunctiva, cornea, eye, picornavirus

Enterovirus type 70 (E70)<sup>1</sup> and coxsackievirus type A24 (CA24)<sup>2</sup> are etiological agents of a newly recognized highly contagious eye infection of humans, called acute hemorrhagic conjunctivitis (AHC).<sup>3, 4</sup> Epidemics of AHC have occurred in Africa, Asia, and Europe<sup>5</sup> and have been recurrent in many countries of Asia.<sup>5</sup> The infection is primarily localized in the epithelial layers of the conjunctiva and cornea.<sup>6, 7</sup> However, the relative abilities of E70 and CA24 isolates to replicate in cultures composed of conjunctival and corneal cells of various animals have not been reported.

In this paper we describe methods for preparing relatively pure cultures of primary epithelial cells derived from mouse, rabbit, and monkey conjunctival and corneal tissue. These cultures can be used to study virus-cell interactions, host defenses, and possible modes of therapy for this and other virus infections of the eye. At this time we report the growth characteristics of several E70 and CA24 isolates in these cultures.
Fig. 1. Confluent monolayer of C/C cells from mouse (A), rabbit (B), and cynomolgus monkey (C). The pigmented epithelial cells observed in C are derived from the bulbar conjunctiva at the level of the limbus.

Materials and methods

**Culture medium.** Excised ocular tissues and cell cultures were grown and maintained in minimum essential medium containing Earle's salts (D-valine substituted for L-valine) (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 2% fetal bovine serum (FBS) and antibiotics (100 μg of streptomycin and 100 U/ml penicillin) (D-val medium). This medium has been reported to selectively inhibit growth of fibroblast cells. 8

**Mouse conjunctival and corneal (C/C) cell cultures.** The excised eyes of 6- to 8-week-old Swiss white mice (Timco Breeding Laboratories, Houston, Texas) were washed twice in culture dishes with ample volumes of medium and placed into tissue culture wells (two to three eyes per well) (24-well culture dish; Falcon Plastics, Oxnard, Calif.) containing 1.0 ml of D-val medium. Following 2 to 3 days' incubation at 33° C in 5% CO₂, the eyes and growing, loose cell sheets were removed from the wells and washed twice by centrifugation (200 x g for 10 min). The pelleted eyes and cells were trypsinized for 10 min in 0.25% trypsin, the eyes were removed from the cell suspension, and the remaining cells washed twice by centrifugation (1000 x g for 5 min) prior to being suspended in D-val medium at a concentration of 5 to 8 x 10⁵ cells/ml. Cell cultures were prepared by adding 0.1 ml of the cell suspension to microtiter plate wells (96-well culture dish; Costar, Cambridge, Mass.) and incubating at 33° C in 5% CO₂. Fresh medium was added after 3 days of incubation, and confluent monolayers of cells were obtained in 4 to 6 days. Fifty mice yielded about 30 ml of cell suspension.

**Rabbit and monkey C/C cell cultures.** Conjunctival and corneal tissues were harvested immediately after death from 6- to 20-week-old New Zealand white rabbits (Timco) or 4 to 6 lb juvenile cynomolgus monkeys as follows. Sufficient air was injected beneath both the palpebral and bulbar conjunctival membranes to separate them from underlying tissue. The conjunctival membranes were removed by first cutting and separating them from the edge of the eyelids and then cutting them free from the eye at the level of the limbus. The cornea with a 1 to 2 mm rim of sclera was removed with scissors. Conjunctival membranes and corneas were pooled and cut into small fragments (<3 mm), washed twice in D-val medium, and tryp-
Replication of AHC viruses in eye cells

Fig. 2. Growth curves for four E70 isolates in primary cultures of mouse C/C cells.

Fig. 3. Growth curves for two E70 isolates in cultures of primary rabbit C/C cells.

Viruses. E70 isolates (J670/71 and J648/71) were received from Dr. R. Kono (National Institute of Health, Tokyo, Japan). An E70 (SEC146/71) and a CA24 isolate (SEC24/70) were obtained from Dr. M. Yin-Murphy (University of Singapore, Singapore). An E70 isolate (AE/72) and a CA24 isolate (AE92/75) were received from Dr. N. Sanghawibha (Virus Research Institute, Bangkok, Thailand). TX77/77 (CA24) was isolated from an accidental human infection that occurred in our laboratory.8 The E70 isolates were propagated in human amnion WISH (American Tissue Culture Collection) cells or mouse L-cells,10 and CA24 isolates were propagated in human foreskin (HFS4) fibroblasts.

Multicycle growth curves. E70 and CA24 isolates previously adapted to WISH cells, L-cells, HFS4 fibroblasts, or rabbit C/C cells were diluted (1:10) in D-val medium, and 0.1 ml pipetted onto confluent microtiter plate cultures of mouse, rabbit, or monkey C/C cells. The input multiplicity of infection (MOI) in the experiments ranged from 0.3 to 8.0 pfu/cell. After 1 hr of incubation at 33°C the culture medium was removed, the cultures were washed twice with D-val medium, and 0.1 ml of fresh D-val medium was added to each well. The culture fluids from two or three wells were harvested at various times, pooled, and kept frozen at −70°C until assayed.

Virus assay. Fluids from infected cultures were diluted in minimum essential medium containing Earle's salts supplemented with 2% FBS and antibiotics. A 0.1 ml volume of each dilution was inoculated into four cultures of WISH cells,
L-cells, or HFS 4 fibroblasts (depending upon the cell source of the infecting virus) and incubated at 33°C with 5% CO₂ for 5 days. The cultures were observed microscopically for CPE, and the 50% tissue culture infectious dose (TCID₅₀) was determined by the Kärber method.¹¹

**Results**

**C/C cell cultures.** C/C cell cultures were prepared from mouse, rabbit, and monkey in the manner described in Materials and methods. Compared to rabbit and monkey C/C cultures, the mouse cultures contained a slightly higher percentage of contaminating fibroblasts, probably because whole eyes were used as the source of tissue (Fig. 1, A to C). C/C cell cultures derived from rabbit and monkey (Fig. 1, B and C) contained more than 90% epithelial cells. The method of preparation and the use of d-val medium to inhibit fibroblast growth probably accounted for the almost "homogeneous" epithelial cell population of the rabbit and monkey C/C cell cultures. d-val medium to inhibit the growth of fibroblasts in cultures derived from ocular tissues of all three species of animals, since colonies of fibroblasts were not observed. Routinely, 300 microtiter cultures could be prepared from 100 mouse eyes, and 60 to 80 microtiter cultures could be prepared from two rabbit or monkey eyes.

**E70 replication in mouse C/C cells.** The multicycle growth curves of four L-cell-adapted E70 isolates in mouse C/C cells are presented in Fig. 2. The AE/72 (Thailand) isolate replicated to high titer, and 100% viral cytopathic effect (CPE) was observed after the fourth day. In comparison, the J670/71 and J648/71 (Japan) isolates replicated moderately well and caused CPE, whereas the

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Fig. 4. Growth curves of two E70 isolates (A) and two CA24 isolates (B) in primary cultures of cynomolgus monkey C/C cells.
SEC146/71 (Singapore) isolate was the slowest to adapt and replicate in these cells. No CPE was observed. The differences in growth between the isolates is probably due to the MOI of the challenge virus or rate of adsorption rather than to origin of virus, since the MOI for SEC146/71 was the lowest whereas the MOI for AE/72 was the highest. Clearly all strains of E70 tested replicated in primary mouse C/C cells. CA24 isolates were not tested because they did not replicate in mouse L-cells or in primary mouse embryo cells (unpublished observation).

E70 and CA24 replication in rabbit C/C cells. The growth curves of two rabbit C/C cell–adapted E70 isolates (AE/72 and SEC146/71) are presented in Fig. 3. Input multiplicities of 5.0 and 8.0 pfu/cell, respectively, were used, and both isolates replicated to approximately \(10^5\) TCID\(_{50}\)/ml within 10 to 16 hr after infection, with 100% CPE in 24 hr. In contrast, the three CA24 isolates did not appear to replicate in rabbit C/C cultures, since virus yields did not exceed the input challenge (data not shown).

E70 and CA24 replication in monkey C/C cells. Primary cynomolgus monkey C/C cultures were infected with two E70 isolates (AE/72 and J670/71) that had been passaged in human WISH cells three times and two CA24 isolates (SEC24/70 and TX77/77) that had been passaged in HFS4 fibroblasts two times. The MOIs were 0.03 pfu/cell for the E70 isolates and 0.5 pfu/cell for the CA24 isolates. All isolates replicated to high levels by 72 hr after infection (Fig. 4, A and B); the two E70 isolates produced approximately \(10^6.0\) TCID\(_{50}\)/ml of virus whereas the two CA24 isolates produced approximately \(10^6.5\). These results indicate that E70 and CA24 isolates can adapt and grow to high titers in primary monkey C/C cell cultures.

Discussion

We have investigated the growth characteristics of several isolates of E70 and CA24 in easily prepared mouse, rabbit, and monkey C/C cell cultures. The cultures were prepared by the techniques described in Materials and methods, and D-val medium was used to restrict fibroblast growth. The mouse, rabbit, and monkey C/C cell cultures were composed of over 90% epithelial cells, as judged by microscopic examination. Colonies of fibroblasts were not observed in any of the cultures, indicating that the D-val medium was inhibiting the growth of fibroblasts.

We found that all isolates of E70 and CA24 grew relatively rapidly and to high titers in the monkey C/C cell cultures. The E70 isolates reached titers of \(10^5.0\) TCID\(_{50}\)/ml by the third day, whereas the CA24 isolates reached \(10^5.5\) during the same period. CA24 isolates did not grow in rabbit C/C cell cultures; however, rabbit cultures infected with E70 isolates produced high levels of virus \(10^6.0\) TCID\(_{50}\)/ml in less than 24 hr. E70 isolates also grew in mouse C/C cell cultures, but variable lag periods (6 to 72 hr) were observed for some isolates before rapid growth began. This may have resulted from the low MOI used to infect the cultures and/or the time required for adaptation.

Taken together, these data suggest that primary monkey, rabbit, and mice C/C cell cultures may be used as models to investigate growth characteristics and virus-cell interactions of E70 and/or CA24 that occur during the early infectious process in humans. These cultures do not represent the exact in vivo situation, as evidenced by the difficulty in developing an animal model (personal observations). This suggests the existence of an undefined host defense mechanism operating at the surface of the intact eye. Through the use of these C/C culture systems it may be possible to define this mechanism and also allow better evaluation of an early-appearing antiviral activity in tears during human CA24 eye infection. In addition, treatment of these cultures with interferon, antibody, and other possible early host defense factors should help define their biological importance and potential use for therapy or prophylaxis against AHC.

Furthermore, these studies support the view that there may be a lower animal reservoir for E70, since E70 has the ability to grow in a wide number of cell types, including primary monkey, rabbit, and mouse...
C/C cultures. Hopefully, continued passage of E70 and CA24 isolates will select for high-titered populations of viruses that can cause ocular infections in animals that mimic AHC infection in humans. Such an animal system would allow definitive study of this virus infection, including modes of therapy and prophylaxis, and of host defenses associated with the surface of the eye.

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REFERENCES