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Effect of Interferon, Elevated Temperature, and Cell Type on Replication of Acute Hemorrhagic Conjunctivitis Viruses

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Five strains of enterovirus type 70 (E 70) and four of coxsackievirus type A 24 (CA 24) were studied for their sensitivity to interferon (IF), ability to induce IF, replication at various temperatures, and adaptability to human and mouse cell cultures. We found that isolates ranged from 0.01 to 16 times as sensitive to fibroblast IF as vesicular stomatitis virus, depending upon the cell type used and the multiplicity of infection. Most of the isolates induced no detectable IF; however, when induction occurred the titers were relatively low (5 to 300 U). Only E 70 virus isolates were adaptable to growth in L-cells. Replication of all viruses was inhibited approximately 90% at 37 to 39°C depending upon the cell type. These results and the accessibility of the eye to application of IF and/or heat suggests the possibility of their use for treatment. The adaptation of certain E 70 viruses to mouse L-cells opens the possibility of development of a mouse model infection.

Acute hemorrhagic conjunctivitis (AHC) is a highly contagious viral infection of the eye characterized by a rapid onset of clinical symptoms and short incubation period (4, 6-8, 16-18, 26, 30, 33, 37, 38, 41). The initial infection occurs in the superficial epithelium of the conjunctiva causing intense pain, hemorrhage, and swelling, which may incapacitate a patient for 1 to 2 weeks. Sequential involvement of the preauricular lymph nodes (1 to 5 days postinfection) commonly has been reported (4, 6-8, 16-18, 26, 30, 33, 37, 38) followed by rare involvement of the central nervous system (1 to 4 weeks postinfection) where the virus causes a mild to severe radiculomyelitis (3, 4, 12, 21, 22, 32, 35, 36), which could be reproduced in monkeys (24).

Major epidemics involving millions of people have been reported in Africa and Asia (23), and small outbreaks have occurred in England (17), Russia (27), Yugoslavia (25), France (7), and New Caladonia (20) since 1969. The disease has become endemic in some countries where periodic outbreaks have occurred, the most recent one reported in Sri Lanka (15). In this regard, it is interesting that the disease has not been detected in the United States, even though there has been repatriation from endemic areas, and it appears that a large proportion of individuals living in the Southeastern United States are not immune to these agents (14).

Two antigenically different picornaviruses have been consistently isolated from the eyes of patients presenting with AHC: (i) enterovirus type 70 (E 70) and (ii) coxsackievirus type A 24 (CA 24). Strains of each of these viruses show intratypic antigenic variation (9, 16, 19, 39, 40). The conjunctival and neurological syndromes appear to be less severe in infections caused by CA 24 (26, 41).

Pain, incapacitation, potential neurological involvement, lack of effective therapy, low immunity in the United States, and possible importation of these viruses into the United States warrant their systematic study. Because the initial infection of the eye is superficial and local and precedes the occasional development of generalized infection, the opportunity presents itself for exploring the therapeutic effectiveness of local treatment by interferon (IF), IF inducers, and elevated temperature. The present studies were designed to help define certain biological characteristics of the two virus types, i.e., replication in different cell cultures, IF production, IF sensitivity, and replication at various temperatures. This type of information may be useful for developing animal model systems for studying the disease, designing approaches for control, and defining possible pathogenic mechanisms.

MATERIALS AND METHODS

Viruses. Five strains of E 70 and four of CA 24 isolated during epidemics of AHC or "picornavirus epidemic conjunctivitis" (38), respectively, were kindly donated to this laboratory. Sources and virus types are listed in Table 1. Vesicular stomatitis virus (VSV), Indiana strain (ATCC), was used as challenge in IF assays. Newcastle disease virus (NDV), strain B_1, was used as a control IF inducer. Echovirus type 5 (ECHO 5), recently isolated from cerebral spinal fluid, was

TABLE 1. *Description of virus isolates*

Virus isolate	Isolated in	Isolated from	Investigator	Passage history
E 70				
J 670/71	Japan	Eye	R. Kono[a]	HEK[b]
J 648/71	Japan	Eye	R. Kono	$HELA_3$, HEK_4[c]
SEC 146/71	Singapore	Eye	M. Yin-Murphy[d]	HELA
AE /72	Thailand	Eye	N. Sangkawibha[e]	KB[f]
260/74	Thailand	Feces	R. Kono[g]	MK_3, $MK\text{-}L_4$, MK[h]
CA 24				
SEC 24/70	Singapore	Eye	M. Yin-Murphy	HELA
SEC 1/75	Singapore	Eye	M. Yin-Murphy	HELA
AE 88/75	Thailand	Eye	N. Sangkawibha	KB
AE 92/75	Thailand	Eye	N. Sangkawibha	KB

[a] R. Kono, Central Virus Diagnostic Laboratory, National Institutes of Health, Tokyo, Japan.
[b] Three subpassages in human embryonic kidney (HEK) cells.
[c] Three subpassages in HELA cells and four in HEK.
[d] M. Yin-Murphy, Department of Bacteriology, University of Singapore, Singapore.
[e] N. Sangkawibha, Department of Medical Sciences, Virus Research Institute, Bangkok, Thailand.
[f] Isolated and passaged in human epithelial (KB) cells.
[g] Received from R. Kono who received it from Prasert Thongchareon, Siriraji Hospital, Mahidol University, Bangkok, Thailand.
[h] Three subpassages in monkey kidney (MK) cells, four in ML-L monkey cells and MK cells.

used to compare the effects of temperature on multiplication of AHC viruses (naturally adapted to grow at eye temperature) with ECHO 5 virus (naturally adapted to grow at body temperature).

Cell culture, media, and diluent. Human amnion WISH cells (Flow Laboratories, Rockville, Md.), human foreskin fibroblasts (HFS_4) (Jan Vilcek, New York University, N.Y.), and mouse L-929 cells were used for growing virus and assaying virus and IF. Cells were grown to monolayers in Eagle minimum essential medium containing Hanks salts supplemented with 10% fetal bovine serum and 100 μg of streptomycin and 100 U of penicillin per ml. Eagle minimum essential medium containing Earles salts supplemented with 2% fetal bovine serum and antibiotics (EMEM) was used as diluent for virus, IF, or as liquid-overlay medium.

Virus quantitation. Plaque assays of E 70 and CA 24 isolates were performed in WISH or L-cell cultures prepared in 96-well microtiter plates (Falcon) with EMEMs as the liquid overlay medium (1). Plaque assays for VSV were performed in L-cells by using a methylcellulose overlay (5).

IF quantitation. Depending on the experiment, IF was assayed by plaque reduction in WISH or L-cells (13), yield inhibition in WISH cells (2), or cytopathic effect inhibition in HSF_4 cells (34). VSV, E 70, and CA 24 viruses at high (3.0 plaque-forming units [PFU] /cell) and low (0.0003 PFU/cell) multiplicities of infection (MOI) were used as challenge, and cultures were read 24 h after infection. The IF titer in units was expressed as the reciprocal of the dilution that inhibited 50% of plaques, virus yields, or cytopathic effect. Relative sensitivities of the AHC viruses to VSV were determined from the ratio of the IF titer obtained with the AHC virus to the titer obtained by using VSV.

Virus stocks. Monolayer cultures of WISH or L-cells were infected with approximately 3.0 PFU/cell, incubated 24 to 30 h at 33°C, and then frozen and thawed three times. The harvested fluids were clarified by centrifugation at 6,000 × *g* for 15 min, $MgCl_2$ was added to a final concentration of 1 M, and portions were stored at −70°C.

IF and antisera to IF. Human fibroblast IF (5×10^5 international reference units), mouse fibroblast IF (5×10^3 international reference units), and rabbit anti-human and anti-mouse fibroblast IF antibody were obtained from the Antiviral Substances Program, National Institute of Allergy and Infectious Diseases, Bethesda, Md.

Characterization of antiviral activity (IF) present in supernatant fluids of cultures infected with AHC viruses. Virus suspensions, irradiated with ultraviolet (UV) light until no infective virus was detectable, were assayed for antiviral activity in HFS_4 cultures. Antiviral activity, when present, was not inactivated by treatment at pH 2 for 24 h, was not active in heterologous cells, and was neutralized by homologous antibody to human or mouse IF.

RESULTS

Relative adaptability of AHC viruses to human WISH and mouse L-cells. Nine AHC virus isolates were serially passaged 3 to 5 times in readily available human WISH or mouse L-cells. The results given in Table 2 show that all the E 70 and CA 24 isolates grew well and formed plaques in WISH cells by the second passage. Four of the five E 70 isolates, but none of the CA 24 isolates, replicated in L-cells. To our knowledge this is the first report of the adaptation of E 70 virus to mouse L-cells, a finding that may lead to the development of a suitable mouse model. It is interesting that the same two strains of E 70, which adapted poorly

TABLE 2. *Relative adaptability of AHC viruses to human WISH and mouse L-cells at 33°C*

Virus isolate	Virus yield (log_{10} PFU/ml) after passage in:					
	WISH cells			L-cells		
	1st	2nd	3rd	1st	2nd	3rd
E 70						
J 670/71	2.1	6.0	6.4	2.1	3.7	6.8
J 648/71	2.5	6.1	6.9	2.0	4.1	7.1
SEC 146/71	2.3	6.3	6.7	3.1	4.4	6.5
AE /72	<0.7	1.9	4.5	<2.0	2.3	5.4
260/74	<0.7	2.7	5.8	<0.7	<0.7	<0.7
CA 24						
SEC 24/70	<2.5	5.7	6.8	<0.7	<0.7	<0.7
SEC 1/75	2.1	5.9	6.3	<0.7	<0.7	<0.7
AE 88/75	<2.5	6.9	7.3	<0.7	<0.7	<0.7
AE 92/75	2.3	6.1	6.4	<0.7	<0.7	<0.7

to WISH cells, also adapted poorly or not at all to mouse L-cells.

Effect of incubation temperature on replication of AHC viruses in WISH and L-cells. WISH- or L-cell-adapted virus suspensions were studied for replication at the temperatures indicated in Fig. 1, 2, and 3. WISH cell, adapted E 70, and CA 24 isolates (Fig. 1 and 2) grew best between 33 and 37°C (normal eye and body temperatures, respectively); whereas, temperatures above 37°C (temperatures obtainable during a febrile response) inhibited E 70 and CA 24 virus replication 10- to 1,000-fold or greater. In comparison, ECHO 5, which naturally multiplies in the body tissues at 37°C, replicated more efficiently at higher temperatures than most of the E 70 isolates but similarly to the CA 24 isolates. The mouse L-cell-adapted E 70 strains, however, tended to have a lower optimal temperature of replication in L-cells (Fig. 3) than the WISH cell-adapted stains in WISH cells (Fig. 1). The ability to replicate at various temperatures by one E 70 isolate (SEC 146/71) was the same in both cell types. Thus, E 70 and CA 24 replication is inhibited by temperatures above normal body temperature in both human WISH and mouse L-cells. These results suggest that raising the surface temperature of the eye to slightly above 37°C may effectively inhibit virus replication.

Sensitivity of the AHC viruses to human and mouse IF. After 8 h of incubation of half-log_{10} dilutions of IF at 33 or 37°C, cultures were challenged with different MOI of each virus strain. The relative IF sensitivity of AHC isolates was compared to that of VSV under similar MOI and assay conditions. The comparative IF sensitivities of the AHC viruses at external eye temperature (33°C) and body temperature (37°C) are given in Table 3. Both E 70 and CA 24 appear to have similar sensitivities to human IF. In WISH cells at a high MOI, the E 70 and CA 24 viruses were 0.01 to 1.0 times as sensitive to human IF as VSV, whereas at a low MOI they were 2 to 16 times more sensitive than VSV at the same multiplicity. Thus, E 70 and CA 24 viruses appear to be most sensitive to IF at eye and body temperatures at a low MOI. However, in L-cells under similar conditions most of the L-cell-adapted E 70 viruses were only slightly more sensitive to mouse IF than VSV at a low MOI. CA 24 viruses were not tested since they did not grow in L-cells. Thus, at a low MOI and at normal eye or body temperatures, the E 70 and CA 24 viruses were

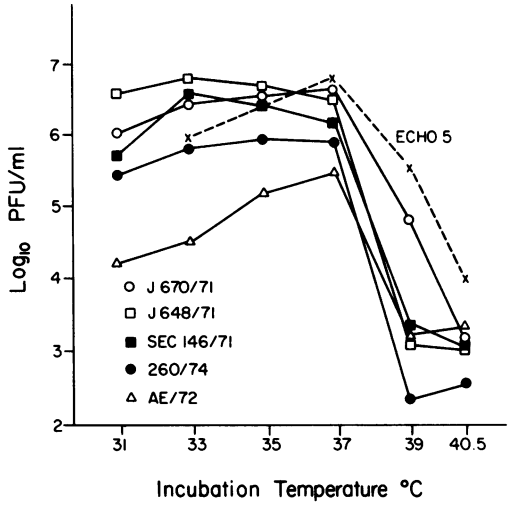

FIG. 1. *Replicative ability of five E 70 virus isolates in comparison with ECHO 5 virus at various temperatures in human WISH cells. Each point represents the mean of three assays, each performed in triplicate.*

sensitive to IF, which suggests that, under natural conditions of the disease, prophylactically applied IF may be effective in retarding replication of these viruses.

Induction of IF by AHC viruses in WISH and L-cells. To evaluate the relative IF-inducing ability of these viruses, the concentrations of IF in supernatant fluids of infected WISH and L-cell cultures were determined. Cultures were infected with approximately 3 PFU/cell, and fluids were harvested at 30 h or when 75% of the cells showed cytopathic effect. Infectious virus was inactivated with UV irradiation since the virus was resistant to acid. IF induced by NDV in both cell types and standard IF suspensions were similarly treated with UV radiation and tested for antiviral activity. No loss of IF

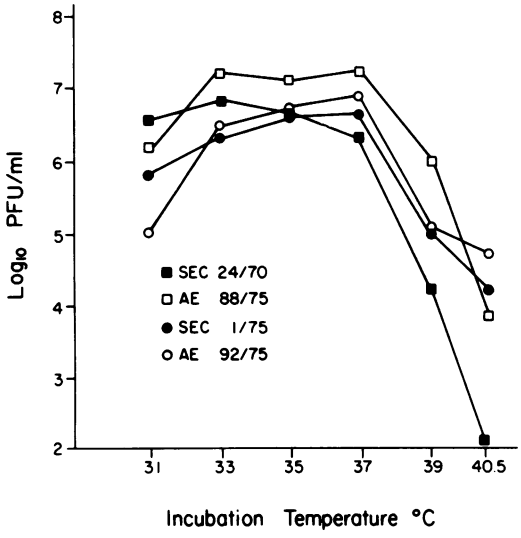

FIG. 2. *Replicative ability of four CA 24 isolates in human WISH cells at various incubation temperatures. Each point represents the mean of three assays, each performed in triplicate.*

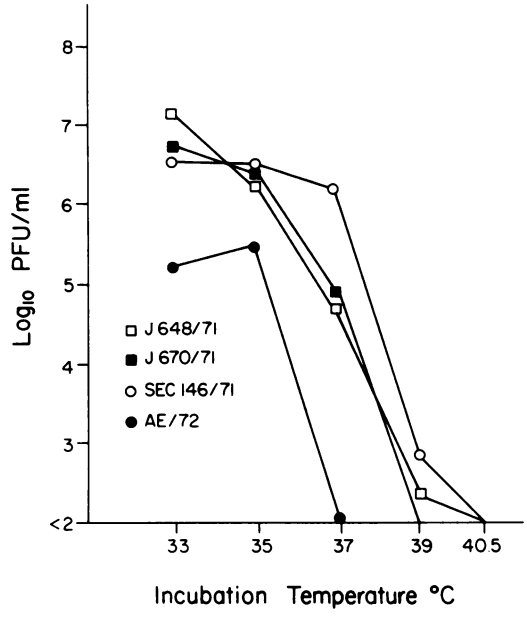

FIG. 3. *Replicative ability of four E 70 virus isolates in mouse L-cells at various incubation temperatures. Each point represents the mean of three assays, each performed in triplicate.*

TABLE 3. *Comparison of IF sensitivity of E 70 and CA 24 viruses to VSV*

Virus strain	Ratio of IF titers with AHC viruses to VSV					
	Yield reduction assay (5 PFU/cell) in WISH cells		Plaque reduction assay (0.0003 PFU/cell) in:			
			WISH cells		L-cells	
	33°C	37°C	33°C	37°C	33°C	37°C
E 70						
J 670/71	0.03[a]	0.01	2.0	6.3	1.2	1.2
J 648/71	0.01	0.31	2.5	10.0	1.3	1.1
AE /72	0.03	3.00	3.2	2.0	1.3	ND[b]
260/74	0.50	1.00	2.0	10.0	ND	ND
SEC 146/71	0.50	0.31	2.0	16.0	1.0	1.2
CA 24						
SEC 24/70		0.31	5.0[c]	10.0	ND	ND
SEC 1/75	0.10	0.20	5.0	12.6	ND	ND
SE 88/75	0.01			5.0	ND	ND
AE 92/75	0.10	1.00	4.0	6.3	ND	ND

[a] E 70 is 33 times less sensitive to IF than is VSV at an MOI of 5 at 33°C in a yield reduction asay in WISH cells.

[b] ND, Not done.

[c] CA 24 is five times more sensitive to IF than is VSV at an MOI of 0.0003 at 33°C in WISH cells.

activity was detected in the UV-treated controls. The data given in Table 4 indicate that in WISH cells the E 70 isolates induced IF, ranging from <2 to 110 U, whereas the CA 24 isolates did not induce detectable amounts (<2) of IF in WISH cells. In contrast, all of the L-cell-adapted E 70 isolates induced from 10 to 300 U of IF in L-cells. It is of interest that the AE/72 Thailand isolate (E 70) was the best inducer of IF in both WISH and L-cells, inducing 110 and 300 U, respectively. In early studies when only the E 70 isolate J 670/71 was available, it induced no detectable IF in human embryonic kidney cells (personal communication, Carol Uhlendorf, Laboratory of Cellular Virology, National Institutes of Health). Although the E 70 viruses induced varying amounts of IF, the amounts stimulated ranged from moderate to undetectable. In comparison, the E 70 and CA 24 isolates induced 20 to 50 times less IF than NDV. Thus, if endogenous IF levels during natural infection are equally low, it might be possible to exceed the endogenous level by application of highly concentrated exogenous IF or a potent inducer of IF.

DISCUSSION

E 70 and CA 24 are etiological agents of a newly recognized and highly contagious eye disease of humans known as AHC or epidemic picornavirus conjunctivitis, respectively (38). The infection is primarily localized in the superficial epithelial layers of the conjunctiva and cornea but may spread to the preauricular lymph nodes (4, 6–8, 16–18, 26, 30, 33, 37, 38) and, rarely, to the central nervous system (3, 4, 12, 21, 22, 32, 35, 36).

TABLE 4. *Induction of IF by E 70, CA 24, and NDV in human WISH cells and mouse L-cells at 33°C*

Virus isolate	U of IF/ml induced in WISH cells	U of IF/ml induced in L-cells
E 70		
J 670/71	5	30
J 648/71	10	10
SEC 146/71	<2	10
AE /72	110	300
260/74	30	ND[a]
CA 24		
SEC 24/70	<2	ND
SEC 1/75	<2	ND
AE 88/75	<2	ND
AE 92/75	<2	ND
NDV	5,000	8,000

[a] ND, Not done. Viruses were not adaptable to L-cells. UV-treated control IF were not significantly inactivated by UV.

In the present study, five E 70 and four CA 24 virus isolates were studied in cell culture to determine basic biological properties to aid in the understanding of the infectious process, development of animal models, and control of disease. The virus properties studied included their (i) relative adaptability to human and mouse cells, (ii) ability to replicate at various temperatures, (iii) sensitivity to IF, and (iv) ability to induce IF.

We found that all isolates were readily adaptable to human WISH cells (Table 2), which allowed easy preparation in a readily available cell type. These high-titered stock suspensions of virus are useful for adaptation studies and for induction of antibody and IF. Although others have been able to grow E 70 viruses in different human and primate cell types (17, 31, 38), this is the first report of adaptation of E 70 isolates to L-cells. The ability to grow in mouse cells to high titers may be a step toward developing a murine model system for studying this disease. Also, adaptation to cells of other species may help select avirulent variants.

All the E 70 and CA 24 isolates were found to replicate well between 33 and 37°C in human WISH cells. At temperatures above 37°C, there was a rapid decline of replication. These observations are comparable to the findings of Miyamura et al. (31), who used monkey kidney cells. Replication of the E 70 isolates was inhibited at slightly lower temperatures in mouse L-cells as compared with WISH cells. These results suggest that application of hot packs to the eye may help control the infection. One drawback to this therapy, however, may be the selection of more virulent forms of virus that grow better at higher temperatures. We have found in WISH cells two subpassages of some E 70 and CA 24 isolates grown at 37 or 40.5°C selected for virus populations that grew better at these temperatures and poorly at 33°C (data not given). An animal model would be useful for determining changes in virulence of these virus populations. The ability of the viruses to adapt to replication at higher temperatures may help explain their rare spread to the central nervous system (3, 12, 21, 22, 32, 35, 36). The ability to select variants that replicate optimally at different temperatures opens the possibility of developing attenuated vaccine strains (46) if this becomes necessary because of changes to increased neurotropism.

All E 70 and CA 24 viruses at low MOI were equally or more sensitive to IF in WISH cells at 33 or 37°C than the IF-sensitive VSV. These conditions would be expected in vivo during the early stages of infection. The ability of IF to protect cultures at high virus-to-cell ratios, as

might be expected to occur during the latter parts of the infectious process, was much less. Thus, application of IF or IF inducers during the latter phases of infection may not be particularly effective in controlling local infection. It will be important to determine whether IF in combination with other antiviral factors (e.g., elevated temperatures and antibody) could be effective during the later stages of infection in preventing spread of virus to other foci, including the nervous system. These experiments also await development of a suitable animal model. It is noteworthy that IF therapy and prophylaxis have already proven to be effective, in certain circumstances, in controlling eye infections caused by viruses of lower sensitivity to IF than the AHC viruses (11).

To help determine the effect of applied IF or IF inducers on an infection, knowledge concerning the relative ability of the infecting virus to induce IF is needed, since the concentrations of IF induced by the virus may be greater than those that can be applied. This may not be a major problem in evaluating IF therapy of AHC infections, since E 70 and CA 24 isolates were all found to induce moderate to low levels of IF in human cell cultures. It is noteworthy that different virus strains induced various amounts of IF; thus, they can be used to explore the natural role of IF during infection.

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