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Enhanced Efficiency of Echovirus 6 by a Transferable Factor in Cultured Human Cells.* (31978)

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In previous studies, stable mutant pairs of echovirus 6 strains were isolated in monkey kidney cell cultures(1). The plaque mutants, *m*+ and *m*, could be distinguished by several biological properties. In addition to differences in plaque size, cytopathogenicity, sensitivity to polyanions and neutralizability by antisera, the mutants differed in their host susceptibility(2). Most human cells were shown to have 100- to 1,000-fold greater sensitivity than monkey cells to infection by the large plaque mutant, *m*+. In contrast, human and monkey cells were approximately equally susceptible to the minute plaque mutant, *m*.

The greater sensitivity of human cells for *m*+ mutant was investigated further. The present paper describes a transferable factor in human cells which enhances specifically the efficiency of *m*+ virus for monkey cells. The intracellular location and some of the properties of the enhancing factor are reported.

Materials and methods. Cell cultures. Primary rhesus monkey kidney cells were grown and maintained in Eagle's basal medium (Hanks' salts) containing 2% calf serum and 0.2% SV_5 antiserum. Green monkey kidney cells (GM) were grown serially in Eagle's minimal essential medium (MEM) with 10% calf serum, and maintained in MEM containing 5% calf serum. BSC-1 cells were kindly provided by Dr. H. E. Hopps, Division of Biologics Standards, National Institutes of Health(3), and by Dr. F. Rapp, Baylor University, Houston, Texas. The latter lines were maintained in medium 199 with 2% fetal calf serum, and in MEM with 5% calf serum, respectively. Serially progagated human amnion cells, AV3(4), were grown in 0.45% lactalbumin hydrolyzate supplemented with Hanks' salts and 15% calf serum, and

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were maintained in MEM with 5% calf serum.

Virus preparations. The paired mutants, *m*+ and *m*, of Charles strain of echovirus 6, isolated from primary rhesus monkey kidney cultures, were plaque purified (3X) and subsequently passaged 11 times in rhesus monkey kidney cultures and 2 times in GM cells (1).

Virus assay. Monolayers prepared in 30 ml Falcon flasks were used for assay of plaque forming units (pfu). Four monolayers were each inoculated with 0.25 ml and incubated 1 hour at 36°C before agar overlay (2.5 ml). A second agar overlay (2.5 ml) containing 0.005% neutral red was applied on the 3rd or 4th day after inoculation. Overlay medium for monkey kidney cells consisted of a mixture of 2X MEM and 3% Ionagar (1:1). For AV3 monolayers, a mixture of bovine amniotic fluid (90%) and calf serum (10%) was combined with 3% Ionagar (1:1). TCD_{50} titers were determined in tube cultures after inoculation of serial 10-fold dilutions into each of three monolayers.

Enzymes. Sterile preparations of pancreatic ribonuclease, α-chymotrypsin and trypsin were obtained from Worthington Biochemical Corp., Freehold, N. J. Pronase (45,000 PUK/g) was obtained from Calbiochem, Los Angeles, Calif. Enzymes were diluted in 0.15 M saline containing 0.001 M calcium, 0.001 M magnesium and buffered at pH 7.0 with 0.001 M phosphate.

Results. Detection of enhancer in human cells. AV3 cells have been shown to be more sensitive to *m*+ mutant than monkey cells. An effort was made to determine whether this greater susceptibility could be imparted to monkey cells by the transfer of a subcellular component from AV3 cells. Therefore, the effect of homogenates of AV3 cells on efficiency of *m*+ infection in monkey cells was investigated. AV3 cell homogenates were prepared in the following manner: AV3 monolayers were washed 3 times with PBS (0.15 M saline buffered with 0.001 M phosphate at pH 7.0). Cells were harvested by scraping into buffer and concentrated to $10^{7.0}$ cells/ml by centrifugation (800 × *g*, 10 min). Cells were disrupted with a motor-driven Ten Broeck homogenizer; efficiency of the procedure was followed microscopically. Diluted virus (0.1 ml) was combined with 0.9 ml debris obtained from AV3 cells (AV_d), and the mixture was agitated frequently during an incubation period of 20 minutes at room temperature (23°C). Two virus dilutions containing initially 100 and 1,000 pfu/ml were used for each assay.

TABLE I. Effect of Debris Prepared from Human and Monkey Cells on Echovirus 6 Titers in Monkey Cells.

Debris	Virus	
	m+	*m*
None	1.2*	59.0
AV_d	51.3	24.0
GM_d	1.3	10.0

* pfu/ml × 10^5

As indicated in Table I, addition of AV_d to *m*+ virus increased the titer 43-fold in monkey cells. The activity of AV_d was specific for *m*+ mutant. AV_d did not increase the *m* titer in monkey cells. Debris from green monkey cells (GM_d) was prepared in the same manner and assayed in parallel. In contrast to the enhancing effect of AV_d for *m*+ virus, GM_d did not increase efficiency of either *m*+ or *m* for monkey cells. Thus, it appeared that AV_d contained a transferable factor, designated enhancer, which imparted greater sensitivity to monkey cells for *m*+ virus. The decreased *m* titer after treatment with either AV_d or GM_d may reflect the presence of viral receptor material in debris preparations.

Enhancer activity of various cell types. Human, monkey and other cultured cells were examined for presence of enhancer for *m*+ virus. Debris from each cell type was combined with *m*+ virus, and mixtures were assayed by plaque technique in monkey cells. Results, which are the average of 3 experiments, are presented in Table II. Increased *m*+ titer is expressed as fold-enhancement. The presence of enhancer was not limited to debris from AV3 cells. Debris prepared from all human cells tested, except erythrocytes, increased *m*+ titer 2- to 17-fold. However, debris obtained from monkey or turtle cells had no enhancing activity. None of the cells examined contained enhancer for *m* virus.

TABLE II. Presence of Enhancer for $m+$ Virus in Cultured Cells.

Cell type	Fold-enhancement
Human	
Primary fetal kidney	3.4
Primary amnion	3.7
AV3	17.3
WISH	13.0
HeLa	4.8
HEp-2	2.4
WI-38	2.6
RBC stroma	1.4
Monkey	
Primary rhesus kidney	1.2
GM	1.2
RBC stroma	1.2
Poikilothermic	
TH-1*	1.2

* Turtle heart cell line provided by Dr. H. Fred Clark.

With certain exceptions, which will be discussed later, presence of enhancer in cells coincided generally with the relatively greater sensitivity of human, as opposed to monkey, cells for $m+$ virus(2).

Effect of virus source on efficiency of infection. Attempts were made to increase efficiency of $m+$ virus for monkey cells by passage of virus in human cells. Undiluted $m+$ virus, harvested from GM cells, was passaged serially (3 to 5 times) in AV3 cells and subsequently passaged back into GM cells. Progeny of each passage was assayed in both AV3 and GM cells. Typical results are presented in Table III. One passage in AV3 cells rendered the virus 200-fold more sensitive for monkey cells. This increased $m+$ titer in monkey cells was maintained by virus obtained from all passages in AV3 cells. Virus harvested from AV3 cells continued to produce large plaques, and no alterations in virus characteristics were observed. Subsequent passage of $m+$ virus from AV3 back into GM cells resulted in a decreased titer in monkey cells.

These results indicated that host susceptibility for $m+$ virus was influenced by the cells in which the virus was grown. Furthermore, as indicated by the behavior of the virus returned to GM cells in passage 4 (AV3/GM titer ratio), it appeared that selection of a more efficient $m+$ variant for GM cells did not occur during passage of the virus in AV3 cells. The data rather suggested that the elevated $m+$ titers in GM cell observed in passages 1 through 3 may have reflected the enhancer activity of AV3 cells.

Location of enhancer in cells. Enhancing activity of various preparations of whole and fractionated AV3 cells was studied. Cells were harvested by scraping monolayers into PBS and were washed 3 times by centrifugation ($800 \times g$, 10 min). The cells were resuspended to a concentration of $10^{6.5}$ cells/ml. One portion of the cell suspension was stored at 4°C during further processing of cells. The remainder was quick-frozen and thawed 3 times employing a Dowanol-dry ice bath. Although most cells were not disrupted by this treatment, they were no longer viable as indicated by their inability to exclude eosin. Debris was prepared from a portion of the frozen cells by homogenization. Aliquots of the above 3 preparations (viable, frozen and disrupted frozen cells) containing equivalent concentration of cells were tested for presence of enhancer. As seen in Table IV, all preparations had enhancing activity. However, viable cells had 4.6-fold greater activity than frozen or disrupted cells.

AV_d prepared from another cell preparation ($10^{7.0}$ cells/ml) was fractionated further by

TABLE III. Influence of Virus Source on Efficiency of $m+$ Virus in Monkey and Human Cells.

Host cells	Passage No.	Assay cells GM	Assay cells AV3	Ratio AV3/GM
GM	0	2.3*	380	165.0
AV3	1	410.0	1880	4.6
	2	320.0	2380	7.4
	3	356.0	2540	7.1
GM	4	12.0	1095	91.0
	5	14.0	1585	113.0
	6	8.0	1080	135.0

* pfu/ml $\times 10^5$

TABLE IV. Enhancer Activity of AV3 Cellular Preparations.

	Titer*	Fold-enhancement
Virus alone	2.3	1.0
+ viable AV3 cells	220.0	96.0
+ frozen AV3 cells	47.5	21.0
+ disrupted AV3 cells (AV_d)	48.0	21.0

* pfu/ml $\times 10^5$

TABLE V. Location of Enhancer in Subcellular Fractions of AV_d.

	Titer*	Fold-enhancement	% Enhancement
Virus alone	0.8	1.0	—
Virus + fraction:			
Whole AV_d	31.2	39.0	100
Nuclear (800 × *g*, 10 min)	6.6	8.1	21
Mitochondrial (5,700 × *g*, 10 min)	14.4	18.0	46
Microsomal (54,000 × *g*, 1 hr)	43.2	53.0	138
Post-microsomal (78,000 × *g*, 3 hr)	13.2	16.0	41
Cell sap	2.1	2.6	7

* pfu/ml × 10^5

differential centrifugation in 0.25 M sucrose by the method of Hogeboom(5). The preparative Spinco L ultracentrifuge (rotor 30S) was used for sedimentation of fractions which were resuspended to original volume in 0.25 M sucrose and then titered for enhancer activity. As indicated in Table V, most of the enhancer activity sedimented with the microsomal fraction.

Additional evidence for the subcellular location of enhancer was obtained. Medium in contact with AV3 monolayers for 24 hours was decanted, clarified by centrifugation (800 × *g*, 20 min), and substituted for AV_d in the plaque system. AV_d was prepared from the same monolayers and assayed in parallel. Comparable enhancement of *m*+ plaque titer resulted after treatment with AV medium (AV_m) or AV_d resuspended to the same volume as AV_m. Therefore, it was concluded that an appreciable amount of enhancer appeared in medium which had been in contact with AV3 monolayers. In contrast, unconditioned medium had no enhancing effect.

Enhancement occurred not only under agar, as demonstrated above, but also in cell cultures maintained under fluid medium. Comparable dilutions of *m*+ virus were prepared in fresh maintenance medium and AV_m. The TCD_{50} titers of these preparations in different types of monkey cells were determined and compared (Table VI). AV_m enhanced virus titers by 1 to 3 logs in primary and serially propagated monkey cell lines. Furthermore, the relative insusceptibility of BSC-1 cell cultures for *m*+ virus was repaired by the presence of AV_m.

Properties of AV_d enhancement. AV_d was used as a model for investigation of some of the properties of enhancer in human cells. Enhancement of *m*+ efficiency by AV_d was expressed not only by increased titer, but also by rapidity of cell destruction. The earlier appearance of plaques in the presence of AV_d is illustrated in Fig. 1. Approximately 10% of the total plaques formed by untreated virus and 35% of the plaques formed by debris-treated was visible by day 4. The maximum pfu titer was reached 2 days earlier with debris-treated virus than with untreated virus.

The relationship between concentration and enhancer activity of a preparation of cell debris was studied. Dilutions of AV_d processed from $10^{7.0}$ cells/ml were combined with 40 pfu of virus and incubated for 20 minutes at room temperature. The mixtures were titered in rhesus monkey cells by the plaque assay method. Enhancer activity was detectable in debris prepared from 3 × 10^4 or more cells/ml (Fig. 2). Enhancement increased linearly with a slope of approximately 1.1. A 10-fold variation of virus concentration in the initial inoculum (10 to 100 pfu) did not affect enhancer activity of AV_d dilutions. A plateau in activity was reached with debris prepared from 3 × 10^6 cells/ml. Thus, within

TABLE VI. Enhancement of *m*+ Virus in Different Types of Monkey Cells by Clarified Media (AV_m) Obtained from AV3 Monolayers.

Cell culture	Titer*	
	Untreated virus	AV_m-treated virus
Primary rhesus	5.0	6.3
GM	4.8	6.3
BSC-1 (Rapp)	1.8	4.3
BSC-1 (Hopps)	≤1.5	4.3

* log TCD_{50}/ml

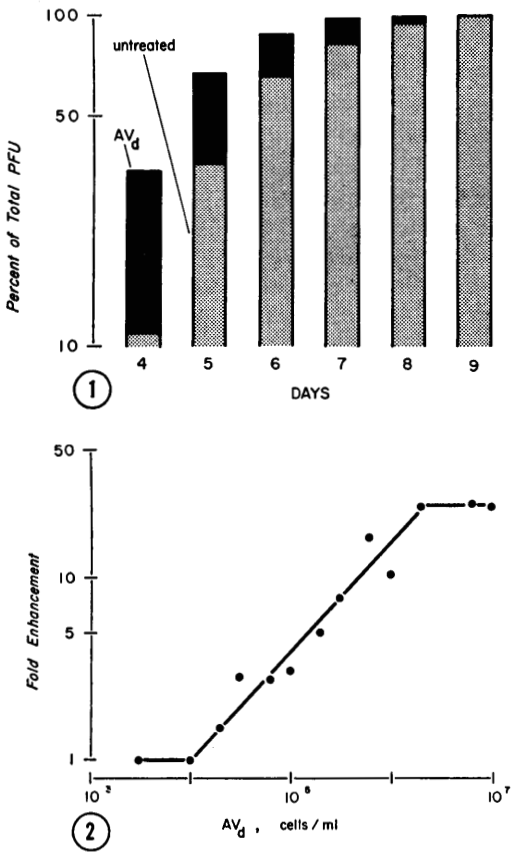

FIG. 1. Rate of early appearance of echovirus 6 plaques in rhesus monkey cells with and without addition of debris prepared from AV3 cells (AV_d). Final plaque titer (100% pfu): untreated virus = 2.4×10^5 pfu/ml; AV_d treated virus = 28.8×10^5 pfu/ml.

FIG. 2. Relationship between concentration and enhancer activity of a preparation of AV3 cell debris.

defined limits, there was a direct relationship between enhancer activity and concentration of cells used for preparation of debris.

The effect of time of addition of AV_d enhancer to the virus-cell system was studied. No significant difference in enhancer activity was observed when a mixture of AV_d and 25 pfu virus was incubated *in vitro* at 23°C for periods ranging from 0 to 120 minutes before inoculation on monolayers. When monolayers were pretreated with AV_d for 1 hour at 36°C and washed 3 times prior to addition of virus, full enhancement was observed. Undiminished enhancement was also evident when AV_d was added to monolayers at 5, 15 or 45 minutes after virus inoculation and overlaid at 60 minutes. Addition of AV_d to infected monolayers at 5 minutes before or at the time of agar overlay resulted in decreased enhancement (35% and 90%, respectively). However, when AV_d was combined directly with agar overlay medium, no enhancement occurred.

Further evidence for rapidity of action by enhancer was obtained. Cell monolayers were exposed to a mixture of AV_d and virus for 5 minutes. Monolayers were then washed 3 times and overlaid with agar. The observed enhancement was similar to that obtained after 60 minutes' incubation of virus-AV_d mixture on monolayers before agar overlay.

Physical and chemical properties of enhancer. Enhancer activity of AV_d suspensions did not penetrate cellophane membranes after dialysis with constant agitation (4°C, 24 hours) against 1 liter of PBS. AV_d retained its full enhancer activity after storage at —20°C for 3 months. Enhancer was stable for 1 hour at 45°C, but lost 50% of its activity after incubation at 56°C for 30 minutes. It was completely destroyed at 60°C in 1 minute.

The effect of acid and alkali on enhancer was determined by treatment of AV_d for 1 hour at 23°C. In each case, suspensions were neutralized to pH 7.0 before assay for enhancer activity. AV_d enhancer activity was destroyed by M NaOH and M HCl, but was not inactivated by M NaCl treatment. Partial destruction of enhancer occurred when the pH of AV_d (7.0) was lowered with 0.1 M HCl. A 70% and 35% decrease in enhancer activity occurred at pH 2.0 and pH 3.0, respectively.

Some of the enhancer activity was removed by treatment with ether. AV_d was extracted 3 times with ethyl ether (1:1) by agitation at room temperature, and residual ether was displaced by nitrogen. The resultant clear aqueous and cloudy interphase portions were assayed for enhancer activity. Each of these phases contained 20% of the initial enhancer activity.

AV_d was treated with 5% phenol for 1 hour at room temperature. The phenol was removed by centrifugation (110,000 × *g*, 2 hr) and the pellet was resuspended to the original volume in PBS. In contrast to untreated con-

trols, resuspended pellets obtained after phenol treatment of AV_d failed to enhance $m+$ infection in monkey cells. Complete inactivation of enhancer also resulted from treatment with trypsin (100 μg/ml, chymotrypsin (100 μg/ml) and pronase (1.0 mg/ml) for 1 hour at 37°C. The enzymes were removed by centrifugation as above, and resuspended pellets were assayed for enhancer activity. Residual phenol or enzymes in the pellet did not interfere with the plaque assay. Agents which did not destroy enhancer after treatment of AV_d (37°C, 1 hr) included 1% ethylenediaminetetraacetate (EDTA) and ribonuclease (10 μg/ml). These preliminary results suggested that enhancer activity may be dependent upon the integrity of a protein moiety.

Discussion. The results indicate that subcellular components of uninfected human cells contain an enhancer which imparts greater efficiency for echovirus 6 infection in monkey cells. The enhancer described herein was specific for the $m+$ mutant of echovirus 6 and detectable in all cultured human cells examined. Thus, the generally observed preferential susceptibility of human compared to monkey cells for $m+$ may be related to presence of enhancer. The results obtained previously (2) indicating that WI-38 and primary human amnion were only slightly more sensitive than monkey cell cultures for $m+$ virus may be explained by the low enhancer content in these human cells. A direct correlation between susceptibility of a specific cell type and enhancer content was not obtained with HEp-2 cells. It has been reported(6) and confirmed in this laboratory that HEp-2 cells do not support growth of echovirus 6. Therefore, it appears that the enhancer demonstrated in HEp-2 cells does not repair the relative inefficiency for $m+$ infection. Furthermore, sensitivity of HEp-2 cells to $m+$ virus was not imparted by addition of exogenous enhancer (AV_d). It is not unexpected that other mechanisms may block viral replication. The enhancement phenomenon described may not be limited to that observed with the $m+$ mutant of echovirus 6. Simon(7) reported that products of human cultured cells (but not monkey cells) increased the titer of variants of echoviruses 3,6,7 and 12 in monkey cells. Furthermore, Tsilinsky(8) obtained enhancement of cytopathic effect of echovirus 7 in monkey cells upon addition of media decanted from human cell cultures.

The mechanism of enhancement is unclear. Despite the greater enhancement by viable cells (Table IV), the data suggest that enhancement by AV_d cannot be explained by the presence of infectious centers in the reaction systems. Disrupted cells, clarified conditioned medium (AV_m) and microsomal fractions of AV3 cells increased $m+$ titer for monkey cells in fluid medium as well as under agar. The effect of enhancer was discernible after 5 minutes' treatment of infected monolayers. Enhancer was equally efficient when AV_d was added to monolayers before, during and after addition of virus. These results suggest that enhancer probably does not aid initial adsorption of virus to cells. However, the present studies do not permit interpretation of whether enhancer may affect events involving a later stage in the virus replication cycle. Since full enhancement occurred even after washing of AV_d treated monolayers before virus infection, it appears that enhancer attached irreversibly to the cell surface. The increased $m+$ plaque titer obtained after AV_d treatment indicates an increased efficiency of initial infection. Furthermore, the earlier appearance of plaques in the presence of AV_d suggests that enhancer continues to be effective during repeated cycles of virus infection under agar. Studies of the effect of enhancer at different stages of the virus growth cycle are in progress.

Certain physical and chemical properties of enhancer were examined. Enhancer was nondialyzable and sedimented with the microsomal fraction. Although the particulate nature of AV_d prevented direct chemical analysis, the relative thermolability and destruction of enhancer by phenol and proteolytic enzymes suggested that enhancer activity was dependent upon the integrity of protein. The possibility that other substances may be essential for enhancer activity has not been excluded.

The intracellular location of enhancer as well as some of its physical-chemical properties and relationship to cell susceptibility

resemble properties of enterovirus type receptors. Therefore, an analogy between receptor material and enhancer for $m+$ virus must be considered. Studies of cell receptors for $m+$ and m mutants and comparison of receptor and enhancer activity are in progress. Preliminary studies indicated that receptor concentration for $m+$ virus was similar in AV3 and monkey cells. Therefore, the low efficiency of $m+$ infection in monkey cells did not appear to be related to a deficiency of receptor material. In contrast to enhancer, receptor material was not inactivated by phenol, chymotrypsin, or trypsin, but was destroyed by ether treatment. These results suggest that enhanced and receptor activity may be different.

Summary. A transferable enhancer has been demonstrated in cultured human cells which increases the efficiency of infection of the $m+$ mutant of echovirus 6 in monkey cells. Enhancer was present in a variety of human cells but not in cells of other species. In general, presence of enhancer coincided with the greater susceptibility of human cells for $m+$ infection. Enhancer in AV3 cells was detected in viable and dead cells, cellular debris and subcellular fractions. Most of the enhancer activity sedimented with the microsomal fraction. Enhancer was thermolabile and destroyed by phenol, proteolytic enzymes, acid and alkali. Debris (AV_d) obtained by homogenization of AV3 cells enhanced the $m+$ titer (pfu and TCD_{50}) in monkey cells by 5- to 100-fold. Plaques appeared 2 days earlier in the presence of AV_d. There was a linear relationship between enhancer activity and concentration of cells ($10^{4.5}$ to $10^{6.5}$ cells/ml) used for preparation of AV_d. Prior *in vitro* contact of virus and AV_d was not required for enhancement. Full enhancement was evident when monolayers were treated before, during, or after addition of virus. The data suggest that enhancer binds rapidly and irreversibly to the cell surface and acts beyond the virus adsorption step.

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Ventricular Augmentor Fibers in the Cervical Vagosympathetic Trunk.* (31979)

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Conventional concepts of vagal control of the heart are limited to influences upon atrial excitability, conductivity and contractility and upon heart rate as determined by the sinoatrial node. In a survey of pertinent literature, DeGeest *et al* found that investigators were about equally divided between those who concluded that vagus had little or no influence on contractility of the ventricles and those who deduced that it elicits a negative inotropic effect(1). In their own experi-

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