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In Vitro Interactions Between Virus and Enhancer

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Cell-free supernatant fluids of permissive host cells contained an enhancer for the *m*+ variant of echovirus 6. The enhancer activity remained in supernatant fractions after centrifugation at 110,000 × *g* for 4 hr. Under in vitro conditions, a complex between enhancer and virus formed rapidly. The complex was not dissociated by dilution and was sedimented by centrifugation. The effect of enhancer on the distribution of virus in CsCl density gradients was determined. In the absence of enhancer, virus was recovered in one peak at 1.33 g/ml. Incubation of enhancer with virus before centrifugation resulted in the formation of two peaks of infectivity at 1.33 g/ml and 1.24 g/ml. The heavier virus peak represented 43% of the recovered infectivity and could be enhanced. The light virus peak contained 26% of the infectivity and could not be enhanced. The density of enhancer was 1.20 g/ml and approximated the density of the light virus population.

It was reported previously that cell cultures derived from human tissues were more susceptible than simian cell cultures to infection by the large-plaque variant (*m*+) of echovirus 6 (7). Although comparable yields of virus from restrictive simian cells (542 plaque-forming units [PFU]/cell) and permissive human cells (337 PFU/cell) were obtained, less than 1% of the virus in the inoculum was detected by simian cells. Subsequently, it was observed that uninfected human cell cultures contained a subcellular component, designated enhancer, that increased the susceptibility of simian cells to *m*+ infection by 10- to 50-fold (4). Simultaneous addition of enhancer and virus accelerated the growth cycle of *m*+ in restrictive cells so that it resembled the *m*+ growth cycle in permissive cells (5). However, inoculation of enhancer after attachment of virus to restrictive cells did not increase the virus titer or rate of viral replication. These observations suggested that the enhancement process may be initiated before attachment of virus to restrictive host cells. To test this possibility, interactions between *m*+ virus and enhancer under in vitro conditions were studied. The results indicate that enhancer complexes with virus and alters the physical properties of the virus.

MATERIALS AND METHODS

Cell cultures. Primary and serially propagated rhesus monkey kidney cells (MK) were used as restrictive host cells. Primary cell cultures were grown and maintained in Eagle basal medium (Hanks salts) containing 2% calf serum. The LLC-MK_2 cell line, obtained from American Type Culture Collection (CCL 7), was grown in Hanks salts supplemented with 0.45% lactalbumin hydrolysate and 2% calf serum and maintained in Eagle minimal essential medium (MEM) containing 5% calf serum. Human amnion cell lines served as permissive host cells. AV_3 (CCL 21) and WISH (CCL 25) cells were propagated in Hanks salts supplemented with 0.45% lactalbumin hydrolysate and 15% calf serum and were maintained in MEM with 5% calf serum. All cells used for experiments were free of mycoplasma.

Cell homogenates. Cell monolayers were washed 3 times with Dulbecco phosphate-buffered saline (BSS) at *p*H 7.2. Cells (5×10^6 to 10^7 cells/ml) were harvested from washed monolayers by scraping into BSS and disrupted by either freezing and thawing or by a motor-driven Ten Broeck homogenizer. Disruption of cells was monitored microscopically.

Virus preparations and assay. Plaque-purified preparations of the *m*+ variant of echovirus 6 (1) were passaged serially three or more times in MK and AV_3 cell lines. Virus assays were performed by the plaque technique. The agar overlay medium for MK cells consisted of MEM, 5% calf serum, and 1.2% Ionagar no. 2 (Colab, Chicago Heights, Ill.). WISH cell monolayers were overlaid with medium containing 45% bovine amniotic fluid, 5% calf serum, and 1.2% Ionagar no. 2.

Enhancer preparations and assay. Homogenates and cell-free supernatant fluids of AV_3 or WISH cell suspensions were employed as sources of enhancer. Cell homogenates were prepared from 10^7 cells. Supernatant fluids were obtained after centrifugation of concentrated, washed cell suspensions (5×10^7 to 10×10^7 cells/ml) at 20,000 × *g* for 1 hr at 5 C. Enhancer activity was determined by assay of the above preparations with *m*+ virus on MK cells. One unit of enhancer activity was expressed as the minimal volume of enhancer preparations that increased the *m*+ plaque titer on MK cells by twofold. For standard assays, 0.1 ml of an appropriate dilution of virus was

incubated with 0.9 ml of enhancer preparations (5 to 30 units) for 20 min at 23 C before the mixture was inoculated on MK cell monolayers.

Isopycnic density centrifugation. Samples (1.0 ml) were mixed with 4.0 ml of 60% CsCl at a final density of 1.23 to 1.25 g/ml and centrifuged in a Spinco SW39 rotor at 5 C for 20 to 24 hr at 36,000 rev/min. Fractions (0.4 to 0.5 ml) were collected dropwise from the bottom of the tube. Densities were determined from the weight of filled 100-μliter micropipettes and expressed as grams per milliliter. Fractions were dialyzed for 24 hr at 5 C against BSS and stored at −20 C before assay.

RESULTS

Effect of dilution on virus-enhancer mixtures. It was shown previously that enhancer activity was lost completely when homogenates obtained from 10^7 permissive cells were diluted 1,000-fold before incubation with the $m+$ variant of echovirus 6 (4). The possibility that virus may complex with enhancer and prevent dilution of enhancer activity was examined. Undiluted virus (0.1 ml) was incubated with 0.9 ml of homogenates of AV_3 cells for 20 min at 23 C. The mixture was diluted (10^{-3}) before assay on MK cells. Mixtures containing 0.1 ml of diluted virus (10^{-3}) and 0.9 ml of undiluted cell homogenates were incubated as above and assayed directly without further dilution. Also, virus and enhancer preparations were each diluted separately (10^{-3}) and incubated together before assay. As shown in Table 1, mixtures containing diluted virus and undiluted cell homogenates increased the virus titer in MK cells by 31-fold. No enhancement of virus titer occurred when homogenates were diluted before combination with virus. However, dilution of a preincubated mixture of virus and cell homogenates to beyond the end point of enhancer activity (10^{-3}) resulted in a 53-fold increase of virus titer. The enhancer activity in supernatant fractions of AV_3 cell suspensions was also retained if the supernatant fluids were preincubated with virus before dilution. Varying the time (5 to 60 min) and temperature of the incubation period did not alter the enhancer effect. Maximum enhancement was obtained within 5 min after incubation of virus-enhancer mixtures at either 23 or 37 C.

TABLE 1. *Effect of dilution on titer of virus-enhancer mixtures*

Reacting mixture[a] dilutions		Dilution after incubation	MK titer (PFU/ml × 10^4)	Titer increase
Virus	Enhancer[b]			
10^{-5}		None	11.7	
10^{-4}	10^0	None	357.0	30.5
10^{-3}	10^{-3}	None	10.5	0
10^{-1}	10^0	10^{-3}	616.0	52.6

[a] Incubated for 20 min at 23 C.
[b] Homogenates of permissive AV_3 cells.

Sedimentation of virus-enhancer mixtures. Supernatant fluids (9.0 ml) of WISH cell suspensions, that contained 7 units of enhancer activity per ml, were incubated with 1.0 ml of $m+$ virus (3.8×10^6 PFU) for 20 min at 23 C. As controls, virus (1.0 ml) and enhancer preparations (9.0 ml) were each diluted to 10.0 ml with BSS and incubated separately. All preparations were centrifuged at 110,000 × *g* for 4 hr at 5 C. The supernatant and reconstituted pellet fractions of untreated and enhancer-treated virus were diluted and assayed on MK and WISH cells. The centrifuged enhancer fractions were incubated with diluted virus for 20 min at 23 C before inoculation on MK and WISH cells. Before centrifugation, enhancer preparations (1.0 ml) increased the $m+$ titer in MK cells from 2.5×10^4 to 35.0×10^4 PFU/ml, but did not alter the virus titer in WISH cells (3.8×10^6 PFU/ml).

As indicated in Table 2, 96% of untreated virus (WISH cell assay) sedimented after centrifugation. In the absence of virus, most of the enhancer activity (5 units/ml) remained in the supernatant fraction as determined by the increased virus titer (10-fold) in MK cells. The pellet fractions of enhancer preparations did not increase the virus titer appreciably in MK cells. Incubation of enhancer preparations with virus before centrifugation resulted in sedimentation

TABLE 2. *Sedimentation of virus-enhancer mixtures*

Samples[a]	Assay system (PFU/ml × 10^3)	
	MK	WISH
Untreated virus		
Supernatant	2.1	140
Pellet	22.0	3,600
Enhancer[b]		
Supernatant	252.0	3,800
Pellet	3.5	ND[c]
Virus + enhancer		
Supernatant	3.7	300
Pellet	120.0	3,300

[a] Centrifuged at 110,000 × *g* for 4 hr at 5 C. Input virus titer was 3.8×10^6 PFU/ml on WISH cells and 2.5×10^4 PFU/ml on MK cells. Enhancer preparations (supernatant fluids of WISH cell suspensions) contained 7 units of activity per ml.
[b] Fractions were incubated with virus for 20 min at 23 C.
[c] Not done.

of enhancer activity. The pellet fractions recovered from virus-enhancer mixtures titered fivefold higher in MK cells than pellets of untreated virus. However, less virus (92%) sedimented in the presence of enhancer. Supernatant fractions of virus-enhancer mixtures had twofold higher titers than supernatant fractions of untreated virus in both MK and WISH cells. In other experiments, it was observed that high concentrations of enhancer (17 to 30 units/ml) prevented sedimentation of 10 to 25% of the applied virus. Although most of the enhancer sedimented with virus, some enhancer activity (30 to 40%) was detected in the supernatant fractions of virus-enhancer mixtures.

Effect of host cell components on density of virus. The possibility that combination of enhancer with virus may alter the density of the virus was explored. Initially, the density of virus harvested from restrictive MK and permissive AV_3 cells was determined. Samples of the different virus preparations were subjected to equilibrium sedimentation in CsCl. Distribution of the infectivity in the linear gradients was determined by assay of the collected fractions on AV_3 cells. Approximately 60% (33 to 84%) of the applied virus was recovered from the gradients. The average results obtained from eight different gradients containing MK-harvested virus and five gradients containing AV-harvested virus are presented in Fig. 1.

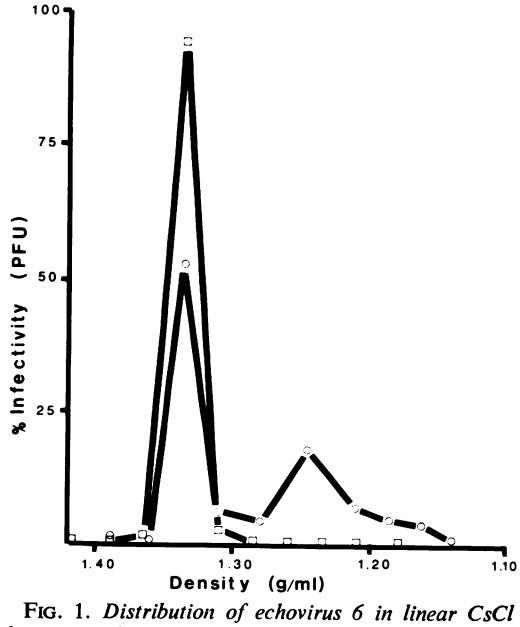

FIG. 1. *Distribution of echovirus 6 in linear CsCl density gradients. Squares, Virus harvested from restrictive MK cells. Circles, Virus harvested from permissive AV_3 cells.*

Most of the virus harvested from MK cells (95%) was recovered in one band at a density of 1.335 ± 0.002 g/ml. In contrast, two peaks of infectivity were obtained from gradients containing virus harvested from AV_3 cells. Approximately 55% of the recovered virus had a density of 1.335 ± 0.005 g/ml, whereas 18% of the infectivity banded at 1.245 ± 0.015 g/ml. Each of the two density populations was recentrifuged in a CsCl gradient, and 80% of the applied infectivity was recovered. Most of the heavier virus population (90%) resedimented at 1.331 ± 0.006 g/ml. The density of the lighter virus population remained at approximately 1.250 ± 0.01 g/ml.

The recentrifuged heavier virus population (1.331 g/ml) corresponded to the density reported for the *m*+ variant of echovirus 6 (2) and was used for subsequent experiments. Virus preparations were incubated with BSS or preparations obtained from either restrictive MK or permissive AV_3 and WISH cells for 20 min at 23 C before equilibrium centrifugation in CsCl. The average results of four experiments are shown in Fig. 2. Homogenates of MK cells did not alter the density or distribution of the recovered virus in CsCl gradients. Approximately 90% of

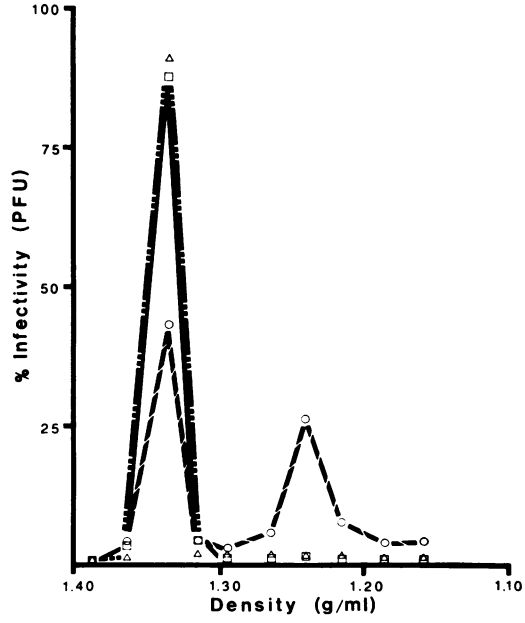

FIG. 2. *Effect of enhancer on distribution of echovirus 6 in CsCl density gradients. Squares, Untreated virus. Circles, Virus incubated with enhancer preparations (extracts of permissive host cells) before centrifugation. Triangles, Virus incubated with extracts of restrictive host cells before centrifugation.*

the detectable virus sedimented in one band at 1.332 ± 0.006 g/ml. Preincubation of virus with homogenates or cell-free supernatant fluids of permissive cells resulted in redistribution of virus in linear CsCl gradients. Approximately 26% of the virus banded at 1.238 ± 0.005 g/ml, whereas 43% of the infectivity remained at 1.333 ± 0.002 g/ml.

The titers of the two virus fractions obtained from gradients were determined on MK and AV_3 cells and compared with titers of the input virus samples (Table 3). Permissive cell extracts (AV_3) enhanced the input virus titer on MK cells by 18-fold, whereas pretreatment of virus with restrictive cell homogenates (MK) resulted in a 70% reduction in titer. As indicated by the AV_3/MK ratio, the titers of recovered heavier virus populations (1.33 g/ml) were increased on AV_3 cells by approximately 25-fold. Also, addition of enhancer preparations to these virus populations increased the virus titer in MK cells by 10-fold. The minor fraction (2 to 7%) of less dense virus populations (1.25 g/ml), recovered after centrifugation of untreated virus or virus pretreated with MK cell homogenates, had 12- to 24-fold higher titers on AV_3 than on MK cells. However, addition of enhancer preparations (AV_3 homogenates or cell-free supernatant fluids) to virus before centrifugation reduced the AV_3/MK ratio of the lighter virus population from approximately 20 to 4. The latter ratio was not altered by addition of permissive cell preparations (enhancer) to the less dense virus population before assay on MK and AV_3 cells.

TABLE 3. *Effect of host cell components on titers of heavy and light virus populations*[a] *in restrictive and permissive cells*

Samples	Virus treatment before centrifugation	Assay system (PFU/ml × 10^3)		
		MK	AV_3	AV_3/MK
Input virus				
1.33 g/ml	None	6.1	441.0	72
	MK cells[b]	2.3	121.0	53
	AV_3 cells[c]	107.1	461.3	4
Recovered virus				
1.33 g/ml	None	5.8	142.5	25
	MK cells	2.3	75.0	33
	AV_3 cells	5.3	103.7	20
1.25 g/ml	None	0.1	2.4	24
	MK cells	0.5	5.9	12
	AV_3 cells	9.3	37.5	4

[a] Recovered from linear CsCl density gradients.
[b] Homogenates of MK cells.
[c] Homogenates or cell-free supernatant fluids of AV_3 cells.

Distribution of enhancer in CsCl gradients. Cell-free supernatant fluids of WISH cell suspensions, that contained 35 units of enhancer activity, were subjected to isopycnic centrifugation in CsCl. The fractions were collected from linear gradients (1.16 to 1.40 g/ml) and were assayed for enhancer activity on MK cells. Enhancer (22.5 units) was recovered in two fractions at densities between 1.186 g/ml (12.0 units) and 1.214 g/ml (10.5 units). When the enhancer preparation was incubated with virus before centrifugation, a small but detectable shift (10%) in virus density from 1.336 to 1.252 g/ml was observed. In two other experiments, enhancer activity in particulate AV_3 cell homogenates was recovered from linear CsCl gradients at densities between 1.225 and 1.247 g/ml.

DISCUSSION

The effects of host cell components on infectivity and density of the *m*+ variant of echovirus 6 were examined. The results indicate that cell-free supernatant fluids, as well as homogenates, of permissive host cells contain an enhancer that complexes with virus and increases the virus titer in restrictive host cells. Under in vitro conditions, the complex between enhancer and virus forms rapidly (within 5 min) and is not dissociated by dilution.

Additional evidence for the formation of a complex between enhancer and virus was obtained from isopycnic centrifugation studies. Virus harvested from restrictive host cells bands in one peak at a density of 1.335 g/ml, whereas virus harvested from permissive cells forms two peaks of infectivity in CsCl gradients at densities of 1.335 g/ml and 1.250 g/ml. The density of the heavier virus population corresponds to the reported density (1.34 g/ml) of the *m*+ variant of echovirus 6 (2) as well as other enteroviruses (3, 6). The presence of a light-density virus population suggests that permissive cells either produce two virus populations or contain a component that alters the density of a portion of the virus population. To examine these possibilities, the effect of host cell components on the distribution of the heavier virus population in density gradients was investigated.

Preincubation of virus with cell-free supernatant fluids or homogenates of permissive cells increased the virus titer in restrictive (MK) host cells. Centrifugation of this mixture in CsCl gradients caused a shift of 26% of the infectivity from a high (1.33 g/ml) to lower density (1.24 g/ml). In contrast, treatment of virus with homogenates of restrictive cells reduced the virus

titer in MK cells and did not alter the density of virus after isopycnic centrifugation. The reduction in virus titer may be due to receptor material or substances in cell homogenates that combine with virus and render it noninfectious. Such complexes may have lighter densities that would not be detected by the methods employed for these studies. Thus, the light virus population is detected only after centrifugation of virus in the presence of host cell components that contain enhancer activity.

The two virus populations also differ in their ability to infect restrictive host cells. The titers of the heavier virus populations in restrictive cells can be increased by addition of enhancer preparations before assay. The less dense virus has approximately the same titer in restrictive and permissive cells, and these titers cannot be increased further by addition of enhancer. In an earlier study (5), it was reported that virus cannot be enhanced after it has attached to cells or cellular components. It was also observed that the density of enhancer (1.20 g/ml) approximated the density of the light virus population. These results suggest that enhancer attaches to $m+$ virus and confers a lighter density to the virions. Separation of enhancer activity from the less dense band of infectivity would provide further evidence for the formation of a complex between virus and enhancer. Development of procedures to inactivate virus without destruction of enhancer activity are in progress.

In the above experiments, enhancer redistributed only a portion (26%) of the virus population from a heavier to a lighter density. The results from centrifugation experiments with mixtures of crude virus preparations and enhancer suggest that the sedimentability of virus with enhancer is dependent upon the concentrations of virus and enhancer in the reacting mixtures. Thus, enhancer concentrations used in the isopycnic centrifugation studies may have been insufficient to bind all of the virus. However, other factors may be responsible for the observed results. The enhancer preparations employed in this study were not purified Therefore, these preparations may contain substances that prevent complexing between virus and enhancer or alter the stability of these complexes in CsCl. Use of purified enhancer preparations should aid further studies on the mechanism of enhancement. The results suggest that enhancer may facilitate virus infection by complexing with virus prior to attachment of virus to restrictive host cells.

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