THE MAMMALIAN CELL-VIRUS RELATIONSHIP

I. Attachment of Poliovirus to Cultivated Cells of Primate and Non-Primate Origin*

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The relationship of viruses with mammalian host cells involves two types of processes: those concerned with intake and output of infectious virus by cells, and those concerned with intracellular interaction of virus and cell components. At present the connection between virus intake and virus replication has not been delineated, nor is it known whether the ability of a mammalian cell to respond to virus infection depends on processes facilitating virus entry and egress, or on processes of viral synthesis. The following study was concerned with primary attachment of poliovirus to fully susceptible or resistant cells in cultures of primate and non-primate origin.

Materials and Methods

Cell Origin and Cultivation.—Established strain cells were propagated in 20 per cent homologous serum diluted in yeast extract medium YEM[1] (1). Human strains included Minnesota HeLa (2) of malignant origin, Minn. Harris[2] (3) and Detroit-6 (4) possibly of malignant origin, and Minn. Hu-EE (Minn. 55-12-1) esophageal epithelium of normal origin (1). Rabbit strains originated in this laboratory (5) included DRF[3] (Minn. 56-8-6) fibroblastic cells from skin of a normal domestic 12 to 15 day old rabbit foetus, CRE[4] (Minn. 57-8-19) epithelial cells from normal cottontail rabbit skin, and CRP[5] (Minn. 56-8-8) epithelial cells from cottontail rabbit papilloma. The ERK-1 and ERK-2 embryonic rabbit kidney lines of Westwood *et al.* (6) also were used.

For virus assays, to avoid use of antibody-containing human serum and fix the cells to the

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[1] YEM, yeast extract medium.

[2] The Harris cell strain was derived by Professor R. J. V. Pulvertaft from a lymph node of an adult patient with Hodgkin's disease.

[3] DRF, domestic rabbit fibroblast.

[4] CRE, epithelial cells from normal cottontail rabbit skin.

[5] CRP, epithelial cells from cottontail rabbit papilloma.

glass surface, HeLa cells were grown in 20 per cent calf serum (CaS-20),[6] 80 per cent YEM (YEM-80), containing 100 units penicillin and 100 micrograms streptomycin per ml. The HeLa cells were dispersed with 0.05 per cent trypsin as described (7), and dispensed in the same CaS medium to form monolayers of about 2×10^6 cells per 30×60 mm. bottle (8). Dispersed cell suspensions from monkey, dog, cat, rabbit, guinea pig, calf, and pig kidney, and from mouse and chick embryo, were prepared by standard technic (9) and established in primary culture in 5 per cent calf serum with 0.5 per cent lactalbumin hydrolysate in Hanks' solution (BSS). Strain L (10) was propagated in 20 per cent horse serum (HoS-20),[7] and YEM-80. For culture of chick cells, chick embryo extract was added in final 5 per cent concentration. Primary cultures of human amnion were established in HuS-20, BSS-80 containing 0.5 per cent lactalbumin hydrolysate.

Virus.—A single virus pool containing 2×10^9 plaque-forming units (PFU)[8] per ml. was prepared from Type 1 (Mahoney) poliovirus representing fluid of the eighth HeLa-culture passage following reception from Connaught Laboratories.[9]

Virus Assay.—Replicate HeLa monolayers, prepared in bottles as described, were inoculated with virus and incubated for a given time at 37°C. with periodic rocking. Adsorption was halted by addition to bottles of sufficient cold BSS to dilute the inoculum 1:100. This fluid was removed for assay of residual virus and replaced completely with fresh cold BSS four times. Rinsed monolayers thus freed of unadsorbed virus were, (*a*) overlaid with 5 ml. of CaS-20, YEM-80 containing 0.5 per cent Difco agar (8), (*b*) incubated at 37°C. for 40 to 48 hours to allow plaque development, and (*c*) overlaid again with 2 ml. of 2 per cent agar in BSS containing 1:10,000 neutral red. Virus concentrations were recorded as plaque-forming units (PFU) per milliliter. To hasten virus adsorption, an inoculum volume of 0.1 ml. was adopted as standard. Suspension of virus by employing as diluent GKN[10] (glucose, 1.0 gm.; KCl, 0.4 gm.; NaCl, 8.0 gm.; H_2O containing 0.002 per cent phenol red, *q. s. ad* 1000 ml.), BSS or CaS-5, YEM-95, did not affect titers of stock virus, nor did use of different lots of pooled and inactivated calf serum.

Cell Disruption.—Infected cell monolayers rinsed with BSS were overlaid with 1.0 ml. of BSS. Prepared cultures were frozen by immersion of bottles in alcohol–dry ice mixture, thawed rapidly in a 37°C water bath, pipetted vigorously, refrozen, and rethawed. This procedure destroys cells without perceptible effect on virus infectivity (11).

EXPERIMENTAL

Virus Adsorption by HeLa Cultures.—For comparison, it was necessary to establish two essential properties of the Type 1 poliovirus-HeLa assay system employed. First, to learn whether virus released from plaques could penetrate overlying agar freely, HeLa monolayers were exposed to virus: (*a*) added in fluid and removed after 90 minutes at 37°C. preceding overlay; (*b*) mixed directly with soft agar overlay; or (*c*) mixed in soft agar layered over previously applied overlay. The results (Table I) indicated that the soft agar overlay retarded penetration of suspended virus to underlying cells. Second, the time course of virus adsorption from 1.0 ml. and from the standard 0.1 ml. inoculum

[6] CaS-20, 20 per cent calf serum.

[7] HoS-20, 20 per cent horse serum.

[8] PFU, plaque-forming units.

[9] Standard control poliovirus was provided by Connaught Medical Research Laboratories of Toronto for the Poliomyelitis Vaccine Evaluation Program.

[10] GKN, glucose-potassium-sodium solution.

volume was determined. Fig. 1 shows that 50 per cent of virus was adsorbed from the small volume in 10 minutes, while 90 minutes was required for adsorption of this proportion from the larger volume. The rate constant is the same for both reactions; *i.e.*, 2.7 to 4.2 × 10^{-9} cm.^{3} min.$^{-1}$ cell^{-1}. Use of the 0.1 ml.

TABLE I

Diffusion of Poliovirus through Agar Overlay Medium to HeLa Cell Monolayers

Treatment of virus inoculum	Proportion of virus adsorbed
0.1 ml. virus adsorbed for 90 min., then overlaid with 0.7 per cent agar medium, 5 ml.	1.0*
0.1 ml. virus mixed directly with 5 ml. overlay medium	0.38
0.1 ml. virus mixed with 0.4 ml. agar overlay medium and pipetted onto monolayers already overlaid with 4.5 ml. agar medium	0.01

* Number of plaques recorded for this treatment 40 to 48 hours after overlay of cultures was considered as base value; plaque counts for other treatments were recorded as proportions relative to this count.

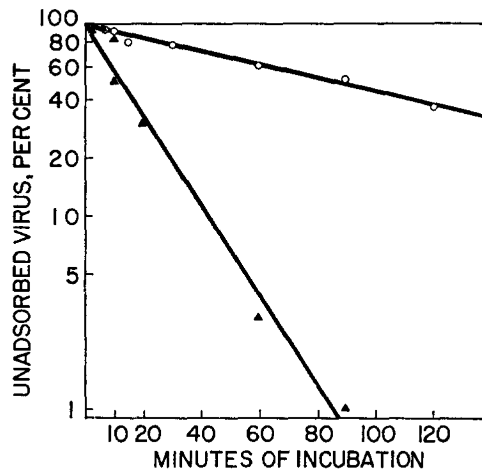

FIG. 1. Rate of adsorption of Type 1 poliovirus by 1 to 3 × 10^6 HeLa cells in monolayer from 0.1 ml. (▲) and 1.0 ml. (○) inoculum volume.

inoculum not only expedited assay, but permitted brief exposure of cells to reduced medium level.

Virus Adsorption by Primate and Non-Primate Cells.—The course of poliovirus adsorption by normally susceptible primate cells was determined with monolayers of HeLa, human amnion, and monkey kidney cells. Rates of attachment for the 3 cell types (Fig. 2) were similar; *i.e.*, *circa* 5.0 × 10^{-9} cm.^{3} min.$^{-1}$ cell^{-1}.

Ability of monolayer cell cultures differing in type and species origin to adsorb virus was measured by the technic specified earlier. The results are presented in Table II. Except for ERK-1, non-primate cells neither adsorbed poliovirus to any marked extent, nor responded pathologically to virus exposure. Fluids from unaffected cultures were passed serially through homologous cultures, without subsequent appearance of cytopathogenic effect or evidence of viral multiplication apparent on plaque assay with HeLa cells.

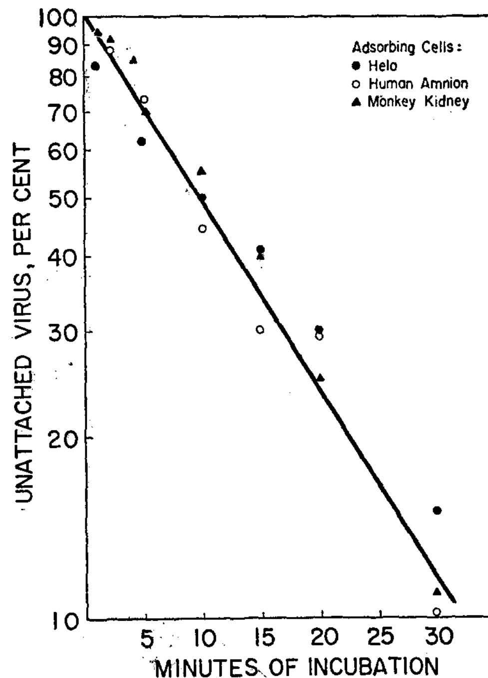

FIG. 2. Adsorption of Type 1 poliovirus by HeLa, human amnion, and monkey kidney monolayers as measured by numbers of plaques formed on replicate monolayers.

Fate of Virus Adsorbed by Susceptible and Insusceptible Cells.—After exposure for 2 hours at 37°C. and thorough rinsing, non-primate cell monolayers consistently retained about 1 per cent of the input virus. To determine whether this virus was adsorbed by a small number of susceptible cells, monolayers varying in origin were: (*a*) exposed to poliovirus at a multiplicity of about 10 PFU per cell; (*b*) rinsed; and (*c*) trypsinized to yield essentially singly dispersed cells. Dispersed cells were counted in a hemocytometer, diluted, mixed with an equal volume (0.1 ml.) of overlay medium, and distributed over HeLa monolayers. Finally, 5 ml. of overlay medium was layered carefully over each of the thin gelled, agar cell layers. In this manner, infected cells were held close to the

susceptible monolayer, as suggested by Kaplan (12). To reveal unproductively adsorbed virus within or on cells, samples of virus-treated cell suspensions were disrupted by freezing and thawing after trypsinization. It is evident from Table III that HeLa and monkey kidney cells were able to adsorb virus readily and on exposure to virus in high concentration became infected in high pro-

TABLE II

Adsorption of Poliovirus to Cell Cultures Derived from Several Animal Sources

Cells*	Type culture	Source	Cyto-pathic effect observed	Virus adsorbed from 0.1 ml. inoculum in 2 hrs. at 37°C.
				per cent
HeLa	Continuous culture	Human	+	>90
Detroit-6	“ “	“	+	>90
Harris	“ “	“	+	>90
Esophageal (EE)	“ “	“	+	>90
Amnion	Primary “	“	+	>90
Kidney	“ “	“	+	>90
“	“ “	Monkey	+	>90
Kidney (ERK-1)	Continuous “	Rabbit	+	>90
Kidney (ERK-2)	“ “	“	0	<10‡
Kidney	Primary “	“	0	<10
“	“ “	Dog	0	<10
“	“ “	Cat	0	<10
“	“ “	Swine	0	<10
“	“ “	Calf	0	<10
“	“ “	Guinea pig	0	<10
Embryonic, mixed	“ “	Mouse	0	<10
“ “	“ “	Chick	0	<10
Papilloma (CRP)	Continuous “	Rabbit	0	<10
Skin (DRF)	“ “	“	0	<10
Skin (CRE)	“ “	“	0	<10
Strain L	“ “	Mouse	0	<10

* Approximately 2×10^6 cells per monolayer.
‡ No adsorption within limits of method.

portion, yet they yielded little infective virus when disrupted soon after exposure. In contrast, the calf and rabbit cells that were unable to adsorb much virus yielded some infective virus whether disrupted or left intact. Some virus thus was received by the insusceptible cells but did not enter an eclipse phase, as did the majority of virus received by susceptible cells. The variation in yield of "infective centers" from intact HeLa and monkey kidney cultures may indicate that some cells were destroyed during dispersal and plating.

Unproductive Association of Virus with Primate and Non-Primate Cells.—It was of interest to learn whether unproductive association with poliovirus was

typical only of the non-primate cells studied. Monolayer cultures of various cell species and for control purposes bottles without cells were exposed to about 10 PFU of virus in 0.1 ml. for 2 hours at 37°C. Adsorbed virus was determined by the technic described earlier. The experimental procedure was repeated with HeLa cultures and infectious virus released from disrupted cells (cell-associated virus = CAV[11] (11)). As shown in Table IV, 1 to 3 per cent of virus adsorbed by susceptible primate cells did not enter an eclipse phase; a similar amount of CAV was recovered from insusceptible non-primate cells.

TABLE III

Proportion of Cells Giving Rise to Plaques after Exposure to Poliovirus at High Multiplicity

Cell culture	Source	Treatment	Cells producing plaques
			per cent
HeLa	Human	None	70–95
		Disrupted*	0.1–1.0
Kidney	Monkey	None	50–90
"	"	Disrupted	<0.1
"	Calf	None	<0.1
"	"	Disrupted	<0.1
"	Rabbit	None	<0.1
"	"	Disrupted	<0.1
Papilloma (CRP)	"	None	<0.1
" "	"	Disrupted	<0.1
Skin (DRF)	"	None	<0.1
" "	"	Disrupted	<0.1

* After cells were enumerated, a sample was disrupted by freezing and thawing during the eclipse phase; *i.e.*, 2.5 hours after exposure.

The control bottles despite thorough washing retained less than 0.1 per cent of virus, an amount without appreciable influence on CAV. CAV recovered from disrupted cells behaved as original virus: 95 per cent was infectious for HeLa cells after adsorption, and 1 to 3 per cent again was recovered as CAV. To determine whether CAV adsorbed by susceptible primate cells differed qualitatively from that adsorbed by non-primate cells, the experiments were repeated except that monolayers exposed to virus were treated or not treated for 15 minutes at 37°C. with homotypic antiserum before rinsing and disruption. Findings (Table IV) for test systems compared to control systems indicated that 5 to 16 per cent of CAV was serum-resistant regardless of cell source.

Insusceptible DRF monolayers were selected for study of the stability of the CAV-cell relationship because eluted CAV would not be lost by infective read-

[11] CAV, cell-associated virus.

sorption. Cells were rinsed 10 times with BSS after exposure to poliovirus, 10 PFU per cell, and incubated at 37°C. under CaS-5, YEM-95 maintenance medium. At various times, cells and fluid were separated for assay of cell-associated and eluted virus. CAV eluted continuously from cells; after 40 hours only 10 per cent of CAV remained with cells.

TABLE IV

Proportion of Input Poliovirus Associating Unproductively with Primate and Non-Primate Cells and Proportion of Cell-Associated Virus (CAV) Unaffected by Homotypic Antiserum

Cell	Strain	Per cent CAV	Per cent of total CAV resistant to antiserum
HeLa	Minn.-HeLa	0.4–2.0	6–11
Esophagus	Minn. EE (55-12-1)	1	8
Amnion	Primary	0.8–2.0	13
Monkey kidney	Primary	1–3	7
Embryonic rabbit kidney	Westwood's Porton ERK-1	1	5
Dog kidney	Primary	2–3	16
Rabbit kidney	Primary	3	N.D.*
Calf kidney	Primary	3	11
Chick embryo	Primary	2	N.D.
Papilloma	Minn. CRP (56-8-8)	2–4	10
Rabbit skin fibroblast	Minn. DRF (56-8-6)	1–4	12

Cell monolayers were exposed to approximately 10 PFU per cell. After a 2 hour adsorption period, monolayers were rinsed 4 times with BSS and disrupted by freezing and thawing three times in 1 ml. BSS to determine CAV. Serum-resistant CAV was measured by treating rinsed monolayers with 1:20 antiserum for 15 minutes at 37°C., rinsing treated monolayers, and disrupting cells for plaque assay of unneutralized virus.

* Not done.

DISCUSSION

Resistance of insensitive strains of *Escherichia coli* B to bacteriophage is correlated with failure of the cells to adsorb virus irreversibly (13). Although experiments have shown that susceptible cells of primate origin adsorb poliovirus *in vitro*, the adsorptive capacity of completely resistant mammalian cells has not previously been documented (14). Capuchin monkey kidney cultures whose partial susceptibility to poliovirus infection was explained by action of a small proportion of fully susceptible cells, did not adsorb measurable amounts of virus (15). The studies described in this paper reveal that Type 1 poliovirus was adsorbed by a variety of primate cell cultures susceptible to cytopathic infection, but that non-primate cultures, not so affected, did not adsorb virus extensively. The ERK-1 cell line, exceptional in its sensitivity to poliovirus infection, resembled primate lines in capacity to adsorb virus.

Susceptible or insusceptible, primate or non-primate, all cell cultures adsorbed a small amount of input virus. This cell-associated virus (CAV) retained infectivity after attachment and did not enter an eclipse phase. Whether associated with susceptible or insusceptible cells, about the same amount of CAV was not neutralized by specific antiserum. CAV attached to the insusceptible non-primate cells was about 90 per cent neutralized by homotypic antiserum; on continued incubation, CAV dissociated continuously from cells, so that about 90 per cent of initially associated virus separated from the cells after 40 hours. This relatively loose association of poliovirus with non-primate cells, differs from the observations of Darnell (16), who found that 2 to 6 per cent of poliovirus attached to HeLa cells did not elute but could be recovered in infectious form by disruption of cells during the latent period. The experiments with insusceptible non-primate cells as well as susceptible cells, showing no apparent difference between CAV and original virus on influence of host cell type, suggest that the alternatives proposed by Darnell are inapplicable to our system. Since 95 per cent of eluted CAV could infect susceptible cells normally, and since CAV was recovered equally from susceptible and insusceptible cells, the mechanism responsible for existence of CAV apparently involves inactive cellular sites rather than viral sites.

Association of virus with cells unproductive of destructive infection is not peculiar to poliovirus, for Ackermann *et al.* (17) have reported that a proportion of influenza virus, representing less than 1 per cent of inoculum, also attaches unproductively. The authors designated such virus as BIV[12] ("bound infectious virus"). Rubin *et al.* (11) observed that approximately 50 per cent of Newcastle disease virus unproductively associated with chick embryo lung cells could not be neutralized by antiserum after cell disruption. This finding, when compared to ours, may indicate that poliovirus CAV is more loosely associated with cell material than Newcastle disease virus.

Characterization of adsorptive capacity of susceptible primate cells for poliovirus, necessary for comparison with behavior of insusceptible non-primate cells, produced findings not entirely in agreement with those of other workers. HeLa, human amnion, monkey kidney, and ERK-1 lines, under our experimental conditions, adsorbed more than 90 per cent of input Type 1 poliovirus at similar rates. Our cultures of calf kidney cells, however, did not adsorb poliovirus significantly, contrary to the findings of Warren *et al.* (18). The rate of virus attachment to HeLa cell monolayers was not greatly affected by variation in virus diluent, as has been reported for foot-and-mouth disease virus (19). A rate of attachment of poliovirus to susceptible cells greater than expected from previous reports (16, 20) may be attributed to use of smaller inoculum volume, 0.1 ml., for adsorption. This reduction in reaction volume could be expected to increase the rate but not alter the rate constant for adsorption.

[12] BIV, bound infectious virus.

The observation that agar overlay hindered penetration of poliovirus to susceptible monolayers agrees with the report of Hsiung *et al.* (21) but not that of Youngner (20); such differences may relate largely to viscosity of the agar employed. Comparison of the interactions of primate and non-primate cells with poliovirus is of interest because: (*a*) susceptible cells in continuous culture from non-primate sources are desirable for preparation of poliovaccine for human use, and (*b*) the role of adsorptive capacity on resistance of cells to destructive infection is significant to an understanding of the relation between phases of cell-virus association relating to virus reception and synthesis. In these studies of a variety of primate and non-primate cells, capacity to adsorb virus and to respond pathologically to reception of virus was characteristic of primate cells. The ability of the ERK-1 cell line to adsorb and reproduce poliovirus thus is peculiar. Since only "transformed" cells in culture of non-primate origin have been found susceptible to poliovirus (6, 22, 23), the characteristic of poliovirus susceptibility of the ERK-1 line observed here suggests that "transformation" involves fundamental cellular properties not seen in other cells of non-primate origin.

The finding that the capacity to adsorb virus was associated with the capacity to reproduce virus and suffer destruction supports the reasonable conclusion that poliovirus must be specifically taken up and penetrate the mammalian cell for reproduction of infectious virus. Lack of specific adsorption of poliovirus by non-primate cells might be reason for insusceptibility rather than metabolic incompetency of these cells to synthesize poliovirus. The accompanying paper (24) deals with the nature of the cell-poliovirus interaction.

SUMMARY

Primary or established strain cultures of a variety of primate cells that were susceptible to cytopathic infection strongly adsorbed poliovirus. Insusceptible non-primate cells in primary or established-strain culture did not so adsorb virus (or propagate it), with exception of the ERK-1 embryo rabbit kidney strain. All tested cells, regardless of type or susceptibility, adsorbed about 1 per cent of input virus, which became cell-associated (CAV) without loss of infectivity. In combination with susceptible or insusceptible cells, CAV was only about 90 per cent neutralized by homotypic antiserum. CAV eluted continuously from non-susceptible cells with continued incubation; eluted virus gave rise to infection and new CAV to the same degree in susceptible cells as did original virus.

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