THE MAMMALIAN CELL-VIRUS RELATIONSHIP

II. Adsorption, Reception, and Eclipse of Poliovirus by HeLa Cells*

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Experiments (1) with primate and non-primate cells, susceptible or insusceptible to poliovirus infection, showed that the ability of some mammalian cells to adsorb the virus extensively was correlated with capacity to reproduce virus and to suffer cytopathogenic effect. Both susceptible and insusceptible cells adsorbed non-productively a small amount of virus which, although retained despite repeated rinsing of cells following adsorption, was neutralized in part by antiserum and eluted continuously from cells with continued incubation. Since it was not apparent how this behavior differed in mechanism from retention culminating in cellular infection, the process of poliovirus infection of susceptible cells *in vitro* was studied further. This paper is concerned with phases of adsorption and reception of poliovirus by susceptible primate cells of the HeLa strain.

Materials and Methods

Cell Cultures and Procedures.—Origin and preparation of primary and established strain cell cultures were described in the preceding paper (1). The primary cultures were from calf kidney, guinea pig kidney, monkey kidney, and human amnion; the continuous cultures included the strains known as Minn. Hu-EE, Harris, and Detroit-6 of human origin, and Minn. DRF, Minn. CRE, Minn. CRP, Westwood's Porton ERK-1, and ERK-2 of rabbit origin, and strain L mouse fibroblasts.

Viruses.—Types 1 (Mahoney) and 2 (MEF-1) polioviruses were prepared from uniformly infected HeLa monolayers. A pool of Type 1 virus was dialyzed for 2 days against running tap water and for 1 day against distilled water frequently changed.

Virus Assays.—For poliovirus plaque titration, 2-day monolayer cultures of HeLa cells grown in calf serum were rinsed and drained thoroughly. These monolayers were exposed to virus suspended in 0.1 ml. Hanks' solution (BSS), either for: (*a*) 2 hours at 37°C., or (*b*) 2 minutes at room temperature. In 2 minutes about 10 per cent of the total input virus was adsorbed when bottles were rocked continuously. Unattached virus was removed with four BSS rinses. The brief attachment period was favored for measurement of relative reduction in concentration of plaque-forming units (PFU) because it: (*a*) facilitated timing of the infectious cycle,

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(*b*) permitted early treatment of adsorbed virus, and (*c*) represented a sensitive measure of virus attachment rate.

Cell Debris.—To produce debris of disrupted cells, 5×10^7 cells or more from bottle monolayer cultures were washed *in situ* five times with glucose-potassium-sodium solution (GKN) (1), dispersed by trypsinization, rewashed three times, and subjected to 10 cycles of rapid freezing and thawing at −70° and 37°C. As seen microscopically, the cells were almost all disrupted. Resultant debris was pipetted vigorously and diluted in BSS to final concentration equivalent to about 5×10^6 cells per ml. The pH was adjusted to 7.2 (the dispersed debris flocculated rapidly in acid solution).

Antisera.—Monkeys hyperimmunized over a period of years by injections of infectious polioviruses yielded antiviral serum. For production of anticellular serum, HeLa cells were disrupted by three cycles of freezing and thawing; weekly, cell debris equivalent to about 5×10^6 cells was mixed with Bayol-arlacel A (4:1) adjuvant and injected intraperitoneally and subcutaneously into each of 6 guinea pigs; injections were continued for 6 to 8 months until, 10 days prior to final bleeding, HeLa cell antigens without adjuvant were given intraperitoneally to the animals. Serum was removed aseptically from blood collected by cardiac puncture and stored at −20°C. until used, when it was inactivated at 56°C. for 30 minutes. Diluted 1:50 and mixed with added fresh guinea pig serum, the anti-HeLa serum was strongly cytotoxic to HeLa cell cultures.

EXPERIMENTAL

Poliovirus Attachment and Penetration

Influence of Ionic Environment on Rate and Reversibility of Poliovirus Attachment to HeLa Cells.—Results shown in Table I indicate that Type 1 poliovirus in contact with HeLa cell monolayers for 2 minutes at room temperature could not be eluted by repeated washing of cells with 0.25 M sucrose solution or with 0.1 M calcium chloride solution. The data also illustrate the reproducibility of the assay procedure. Dialyzed poliovirus was diluted to final required concentration in media of various ionic strengths, HeLa cell monolayers were exposed to 1.0 ml. of respective virus suspensions for 30 minutes at 37°C., monolayers were washed four times with the medium used to dilute the virus, and overlaid with agar medium for plaque counts. Table II shows that cation balance was not necessary for attachment of poliovirus to HeLa cells; although reduced, adsorption was not prevented either by absence of ions or by high cation concentrations. The next experiment was modified so that dialyzed poliovirus diluted in distilled water was added to suspensions of HeLa cells that had been trypsinized, washed three times, and suspended in the attachment media previously employed. After incubation at 37°C. for 1 hour, cells were sedimented by centrifugation for assay of unadsorbed supernate virus. It can be seen from Table III that, with HeLa cells in suspension, in the presence of balanced salt solution about 70 per cent of input virus was adsorbed in 1 hour, while in the absence of salts or presence of divalent cations in high concentration, barely measurable amounts of virus were adsorbed.

Influence of Temperature on Attachment.—Monolayer bottle cultures of HeLa cells were equilibrated at 37° or 1°C., exposed to Type 1 poliovirus in 0.1 ml.

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of BSS, washed 5 times with cold BSS to remove unattached or lightly attached virus, and overlaid with agar medium for plaque counts. A similar experiment was carried out with HeLa cells suspended as described for the experiment with various ionic media; BSS was used as suspending medium. Adsorption by cell

TABLE I

Eluting Effect of Media of High and Low Ionic Strength on Type 1 Poliovirus Adsorbed to HeLa Cells in Monolayer

Washing medium	No. of plaques on individual monolayers
BSS (control)	73, 77, 68, 83, 76
0.25 M sucrose	67, 75, 80, 66, 79
0.1 M $CaCl_2$	81, 69, 69, 72, 74
1.0 M NaCl	80, 82, 76, 72, 69

Each monolayer was (*a*) exposed to approximately 70 PFU for 2 minutes in 0.1 ml BSS at room temperature, (*b*) washed 4 times with, and finally bathed 5 minutes in, the indicated medium, and (*c*) overlaid with agar medium for plaque counting.

TABLE II

Influence of Ionic Strength of Attachment Medium on Adsorption of Type 1 Poliovirus to HeLa Cell Monolayers

Attachment medium	Average No. of plaques after 30 min. attachment	Virus attached as compared to control
		per cent
BSS (control)	203	100
0.25 M sucrose	43	21
Sucrose + $CaCl_2$ (0.001 M)	210	About 100
Sucrose + $CaCl_2$ (0.1 M)	9	4
Sucrose + NaCl (0.14 M)	196	About 100
Sucrose + NaCl (1.0 M)	224	About 100

Dialyzed poliovirus was diluted 10^{-4} in distilled water. The final dilution was made into the indicated attachment medium and 1.0 ml. of each virus suspension was added to the cell monolayer for 30 minutes at 37°C. After attachment the monolayers were washed 4 times with medium used for virus attachment, then overlaid with agar medium for plaque counting.

monolayers was about 10-fold less at 1° than at 37°C. (Table IV); only a 2-fold difference was found with suspended cells. It was observed further that poliovirus adsorbed by monolayers at 1°C. was not eluted by washing with 0.25 M sucrose.

Rate and Temperature Dependence of Poliovirus Penetration into HeLa Cells.—Poliovirus adsorption kinetics for HeLa cells, as determined in this laboratory

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(1), showed that the proportion of input virus adsorbed in 2 minutes at 37°C. was reproducible. Use of the 2 minute attachment period permitted measurement of the time required for adsorbed virus to become resistant to neutralization by externally applied antibody; that is, time required for virus to enter the cell. After exposure to virus, HeLa monolayers were rinsed free of unattached virus four times with BSS and, after further intervals of incubation at 37°C., exposed to 0.5 ml. of 20 per cent Type 1 poliovirus antiserum in BSS for 15

TABLE III

Influence of Ionic Strength of Attachment Medium on Attachment of Type 1 Poliovirus to HeLa Cells in Suspension

Attachment medium	Virus not attached to suspended cells after 1 hr. at 37°C.
	per cent
BSS (control)	33
0.25 M sucrose	95
0.25 M sucrose + $CaCl_2$ (0.001 M)	26
0.25 M sucrose + $CaCl_2$ (0.1 M)	97
0.25 M sucrose + NaCl (0.14 M)	30

Trypsinized HeLa cells were washed 3 times and suspended in the indicated attachment medium. Poliovirus (dialyzed and diluted in distilled water) was added to each suspension and left at 37°C. for 1 hour. The cells were then centrifuged off and residual unattached virus in the supernate was determined.

TABLE IV

Influence of Temperature on Attachment of Type 1 Poliovirus to HeLa Monolayers

Adsorption temperature	No. of PFU adsorbed by individual monolayers
°C.	
37	128, 133, 119, 124, 130
1	8, 9, 11, 12, 14

Each plaque bottle was equilibrated to temperature, exposed to 0.1 ml. of poliovirus in BSS for 15 minutes, washed 5 times with cold BSS, and overlaid with agar medium for plaque counting.

minutes at room temperature. After removal of antiserum, monolayers were washed three times with BSS and overlaid with agar medium.

Results and variation in plaque counts for test and control systems are shown in Table V. The experiment was expanded to include additional intervals of time between virus adsorption and antiserum application, and to study the course of the reaction at 1° as well as 37°C., with results shown in Fig. 1. Table V and Fig. 1 reveal that poliovirus in contact with HeLa monolayers became progressively unavailable for neutralization by antibody, at a temperature-dependent rate. At 37°C., 50 per cent of the virus became antiserum-resistant

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after about 30 minutes; the remaining 50 per cent of input virus became resistant in an additional 10 minutes. An insignificant amount of virus became resistant when cells were held at 1°C. for 2 hours following virus adsorption; after 20

TABLE V

Influence of Antiserum on Type 1 Poliovirus Adsorbed by HeLa Monolayers

Treatment of cell monolayers after virus attachment	Plaques per monolayer
No treatment	84, 82, 91, 83, 76
Normal serum immediately after attachment	83, 80, 78, 78, 84
Antiviral serum immediately after attachment	0, 3, 0, 1, 1
Antiviral serum 1 hr. after attachment	86, 69, 84, 77, 83

Monolayers were exposed to virus for 2 minutes at room temperature; rinsed 4 times with BSS; exposed to 0.5 ml. of 20 per cent normal or immune monkey serum for 15 minutes at room temperature, immediately following exposure to virus or after incubation with BSS or 1 hour; and overlaid with agar medium after removal of serum by further rinsing.

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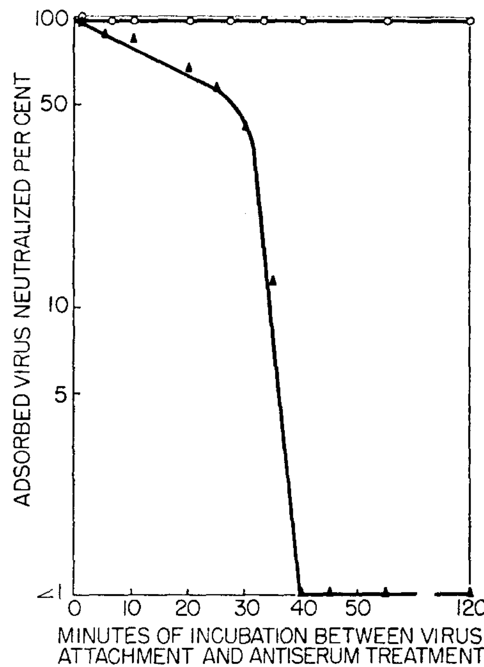

FIG. 1. Neutralization of Type 1 poliovirus adsorbed to HeLa cells in monolayer by antiserum applied after varying periods of incubation at 37° C. (▲) or 1°C. (O).

hours at this temperature all of the virus became antiserum-resistant. These results indicate that adsorbed virus is held initially at the cell surface where it is susceptible to antiserum, but at 37°C. penetrates beyond the sphere of anti-

serum influence within 40 minutes. The idea of "penetration" is employed primarily for convenience, since these experiments do not indicate what physical interpretation should be applied to the course of reception of virus by cells thinly extended in monolayer.

Effect of Anticellular Agents on Virus Penetration Rate.—Various anticellular agents were employed to see whether the capacity of HeLa monolayers to protect adsorbed poliovirus from neutralization by antiserum resulted from active cellular participation. Metabolic inhibitors (10^{-2} M sodium fluoride, 10^{-2} M sodium azide, and 5×10^{-6} M 2,4-dinitrophenol), cytotoxic antiserum plus complement, and a surface-active agent (tween 80) were used to reduce cellular activity. The doses employed had been found to alter the cells morphologically and resulted in rounding, crenation, granularity, pyknosis, *etc.*, without reducing the capacity for virus production or causing gross detachment

TABLE VI

Influence of Ribonuclease on Type 1 Poliovirus Adsorbed by HeLa Monolayers

Treatment of monolayers	Plaques per monolayer
Untreated controls	37, 35, 40, 36, 38
Ribonuclease before virus adsorption	32, 29, 27, 30, 33
Ribonuclease after virus adsorption	29, 34, 30, 35, 28

Washed HeLa monolayers were bathed with 0.1 per cent ribonuclease in BSS without Mg^{++} at 37°C. for 1 hour before or after exposure to virus for 2 minutes.

of cells from glass. All reagents were dissolved or diluted in BSS. A cytotoxic antiserum was diluted 1:50 in BSS containing 20 per cent fresh pooled guinea pig serum as a source of complement. Tween 80 was employed in 0.01 per cent concentration. Rinsed HeLa monolayers were: (*a*) exposed to 2.0 ml. of metabolic inhibitor at 37°C. for 1 hour, or cytotoxic antiserum or tween 80 for 15 minutes; (*b*) after removal of agents, exposed to sufficient poliovirus to allow adsorption of about 60 PFU in 2 minutes at room temperature, then rinsed four times with BSS; (*c*) exposed to anticellular agent again for 45 minutes at 37°C.; (*d*) after removal of agent, exposed to 0.5 ml. of homotypic poliovirus antiserum for 15 minutes at room temperature; and (*e*) rinsed and overlaid with agar medium for plaque counts. Time of treatment of cells with cytotoxic serum or tween 80 was restricted because prolonged exposure produced extensive rounding and detachment of cells. Both untreated control monolayers and treated cultures rendered adsorbed poliovirus immune to neutralization in 45 minutes. Despite obvious damage to cells, virus penetration was insignificantly affected. Since measurement depended upon the ability of infected cells to generate plaques, greater concentrations of damaging agents could not be employed. Active cell participation did not appear essential to virus penetration.

Effect of Ribonuclease on Adsorbed Poliovirus.—Since infectivity of poliovirus

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ribonucleic acid (RNA) is not sensitive to antiserum but is destroyed by ribonuclease (2), enzymatic treatment was used to determine whether virus penetration involved intact virus or dissociated RNA. Ribonuclease was used in relatively high concentration (0.1 per cent) and dissolved in BSS without added magnesium ions. Washed HeLa monolayers were exposed to ribonuclease solution at 37°C. for 1 hour preceding or succeeding standard exposure to virus for 2 minutes. Table VI shows that, in comparison with control untreated monolayers, monolayers treated with enzyme before or after adsorption of virus produced slightly fewer plaques. These results suggested only a minor effect of ribonuclease on cells similar to that already reported (3), but no indication that poliovirus RNA at the cell surface was affected by the enzyme.

Eclipse of Infecting Poliovirus

Effect of Physiological State on Capacity of HeLa Cells to Destroy Infectivity of Adsorbed Poliovirus.—It was verified (1) with HeLa and monkey kidney cultures prepared in this laboratory that adsorption of Type 1 poliovirus was followed by loss of infectivity (eclipse) of intracellular virus. To determine the role of cellular physiological state in initiation of the eclipse phase, HeLa cells were: (*a*) heat-killed by exposure in suspension to 56°C. for 2 minutes; (*b*) "starved" by storage in BSS at room temperature for 5 days; (*c*) poisoned by exposure to 10^{-2} M sodium azide in BSS at 37°C. for 8 hours; or (*d*) left untreated as controls. In comparison with controls, damage to treated cells was such that their efficiency of colonial plating was less than 0.1 per cent. Two million cells of each type were mixed with 10^5 PFU of poliovirus in 1.0 ml. of BSS, incubated for 2 hours at 37°C., and sedimented by centrifugation. Supernates were assayed for residual unadsorbed virus. Sedimented cells were twice washed with BSS, disrupted by three cycles of freezing and thawing and assayed for infective cell-associated virus (CAV). In test as in control systems, more than 95 per cent of adsorbed virus (total input virus minus residual supernatant virus) was inactivated (total adsorbed virus minus CAV) indicating that unimpaired cell vitality was not essential to virus eclipse.

To determine whether intact cellular organization was required for virus adsorption and inactivation, HeLa cells were disrupted by 10 cycles of rapid freezing and thawing. Resultant debris of 5×10^6 HeLa cells was incubated for 2 hours at 37°C. with about 10^5 PFU of poliovirus in 1.0 ml. BSS, diluted 1:100, and plated in 0.1 ml. amounts on 5 HeLa monolayers for plaque counts. Control virus was incubated with BSS in place of HeLa debris. HeLa cell debris rendered over 90 per cent of input Type 1 or 2 poliovirus non-infectious (Table VII). It was observed that Type 3 poliovirus was inactivated similarly by cell debris. The per cent of virus inactivated by debris was independent of virus input; that is, an inoculum of 10^8 PFU was reduced to about 10^6 PFU under the same conditions.

Nature of Virus-Inactivating Mechanism.—Antiviral activity of HeLa cell

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debris could have been mediated by action of: (*a*) specific cellular receptor material, (*b*) cellular enzyme, or (*c*) cell-associated antibody accumulated from human serum growth medium. Table VIII shows that debris inactivating Type

TABLE VII

Inactivation of Poliovirus by Debris from Disrupted HeLa Cells

Reaction mixture	Plaques per monolayer
Type 1 poliovirus + BSS (control)	117, 124, 109, 116, 122
Type 1 poliovirus + HeLa debris	3, 1, 2, 4, 2
Type 2 poliovirus + BSS (control)	87, 78, 91, 83, 87
Type 2 poliovirus + HeLa debris	0, 1, 3, 1, 2

Virus was incubated at 37°C. for 2 hours in 1.0 ml. BSS (control) or in 1.0 ml. BSS containing debris from 5×10^6 disrupted HeLa cells. After 2 hours, mixtures were diluted 1:100 and 0.1 ml. aliquots were plaque-assayed for remaining virus.

TABLE VIII

Inactivation of Type 1 Poliovirus by Debris from Susceptible and Insusceptible Mammalian Cells

Cell and species source of debris	Poliovirus inactivated by cell debris*
	per cent
HeLa (human)	98
Harris (human)	88
HeLa grown in calf serum	92
Esophageal epithelium (human)	>99
Detroit 6 (human)	92
Primary human amnion	96
Primary monkey kidney	>99
ERK-1 (transformed rabbit cell)	98
ERK-2 (untransformed rabbit cell)	0
Domestic rabbit fibroblasts	0
Cottontail rabbit epithelium	0
Cottontail rabbit papilloma	0
L strain mouse fibroblasts	0
Primary calf kidney	0
Primary rabbit kidney	0
Primary guinea pig kidney	0
HeLa + domestic rabbit fibroblast	95
HeLa + primary calf kidney	97

* Per cent poliovirus losing infectivity after 2 hours' incubation at 37°C. with debris from 5×10^6 cells of the type indicated.

1 poliovirus was obtained only from cells found previously to adsorb and replicate poliovirus (1). Addition of inactive debris (domestic rabbit fibroblast or primary calf kidney) to active debris (HeLa) did not diminish the antiviral activity of debris from the susceptible primate cells. Since antiviral activity

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was exhibited by debris from both ERK-1 and HeLa cells propagated for many months in antibody-free calf serum medium, action of antibody or other accumulated virucidal substance was unlikely. The association of debris activity with susceptibility of source cells, and the complete inactivity of debris from insusceptible non-primate cells, suggested that the active material was specific cellular receptor for virus. Harvests of debris from different susceptible cells (Table VIII) differed in antiviral potency; similar variation in potency was observed even with different lots of debris prepared from a single cell strain.

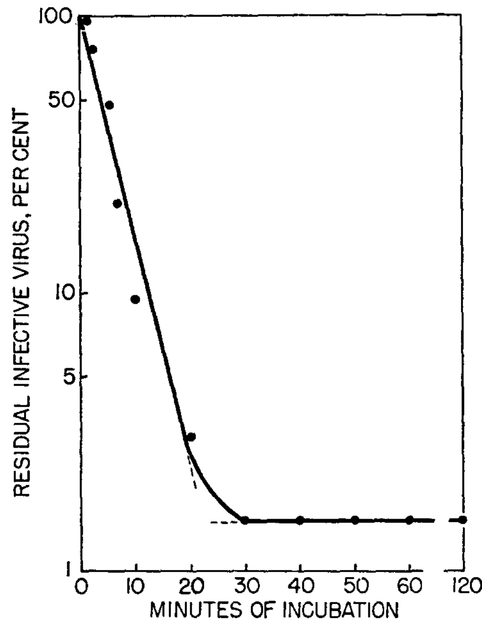

FIG. 2. Kinetics of poliovirus neutralization by HeLa cell debris at 37°C.

To determine the kinetics of poliovirus inactivation by debris, 1.8 ml. of HeLa debris suspended in BSS was equilibrated in a water bath at 37°C., then mixed with 0.2 ml. of poliovirus containing 4×10^5 PFU. Samples were withdrawn at intervals, immediately diluted 1:100, pipetted vigorously and assayed for residual infective virus. Results (Fig. 2) showed that: (*a*) the course of debris inactivation of virus was strikingly similar to the course of virus attachment to intact cells (1); and (*b*) an amount of virus representing about 1 per cent of the inoculum escaped inactivation. This proportion was similar to that retaining infectivity as CAV after adsorption by intact HeLa cells (1, 4).

It was of interest to determine whether the receptor material in the active debris could interfere with penetration of intact HeLa cells by adsorbed poliovirus. HeLa monolayers were exposed for 2 minutes to virus calculated to yield a countable number of PFU, immediately washed four times with BSS, and:

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(*a*) exposed to 1.0 ml. of suspension of HeLa cell debris for 30 minutes at room temperature, then rinsed free of debris with BSS and overlaid with agar medium; or (*b*) treated with cell debris at 15 minutes at room temperature, washed once, and treated with poliovirus antiserum for 15 minutes at room temperature before being overlaid with agar medium. Table IX shows that

TABLE IX

Effect of HeLa Cell Debris on Penetration of Intact HeLa Cells by Attached Type 1 Poliovirus and Inactivation of Adsorbed Virus by Antiserum

Treatment of monolayers after adsorption of virus	Plaques per monolayer
No treatment	69, 65, 68, 72, 64
HeLa cell debris	73, 70, 64, 67, 69
HeLa cell debris followed by antiserum	0, 0, 1, 0, 1

HeLa cell monolayers were exposed to sufficient virus to produce a countable number of plaques, rinsed 4 times with BSS, and (*a*) exposed to HeLa cell debris for 30 minutes at room temperature, or (*b*) exposed to HeLa debris for 15 minutes, rinsed, and exposed to antiviral serum for 15 minutes at room temperature.

TABLE X

Effect of Centrifugation on Antiviral Activity of HeLa Cell Debris

Gravitational force, *g*	Poliovirus inactivated by:	
	Supernate	Sediment
	per cent	*per cent*
0 (untreated cell debris)	97	—
1000	98	0
10,000	18	96
20,000	0	94

One ml. aliquots of HeLa cell debris in BSS at pH 7.2 were centrifuged for 20 minutes under the indicated forces. Centrifugation at 1000 *g* was performed at room temperature, sedimentations at 10,000 and 20,000 *g* at 4°C. Before testing for antiviral activity the sediment was washed once and resuspended to 1.0 ml. volume in BSS. The supernate was tested without further treatment.

cell debris neither hindered penetration of attached virus, nor reduced the ability of antiserum to prevent penetration.

Properties of the Antiviral Component of HeLa Cell Debris.—To learn something of the size and density of the active material, HeLa cell debris was sedimented in BSS at 1000 *g* for 15 minutes. Virus-inactivating activity was associated wholly with the supernate (Table X) rather than the washed and resuspended sediment, which consisted mainly of intact cell nuclei. The antiviral component was sedimented at 10,000 or 20,000 *g*, and retained activity after several BSS washings and resedimentation at 30,000 *g*.

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Insolubility of the receptor substance suggested association with insoluble cellular lipoprotein (5). A large pool of HeLa cell debris was prepared and 1.0 ml. aliquots treated variously. Samples of cell debris were mixed thoroughly with equal volumes of ether or chloroform for 5 minutes. After separation by centrifugation the aqueous phase was tested immediately for antiviral activity, and the ether or chloroform phase tested after evaporation to dryness and resuspension of residue in 1.0 ml. of BSS. These fat solvents either inactivated the antiviral substance or rendered it undispersible in BSS (Table XI). Antiviral activity was not destroyed by treatment similar to that recommended (6) for destruction of cellular receptor for influenza virus. Potassium periodate (10^{-2} M) in 0.85 per cent NaCl solution was added to 10^7 HeLa cells; after 15 minutes

TABLE XI

Effect of Chemical and Physical Treatment on Antiviral Activity of HeLa Cell Debris

Treatment	Poliovirus inactivated by treated debris at 37°C. in 2 hrs.
	per cent
None	>99
Heated 56°C. 15 min.	0
Incubation at 37°C. for 24 hrs.	0
Extracted with ether	0
Extracted with chloroform	0
Periodate	>99
Trypsin	0
Lipase	>99
RDE	>99
Sonic disruption for 15 min.	0

at room temperature, cells were washed 4 times with 10^{-2} M glycerol in saline solution, washed 3 times with BSS, frozen and thawed, and resuspended in 2.0 ml. of BSS. The resultant debris and cell debris prepared prior to periodate treatment and similarly handled were fully active. The activity of debris was destroyed by trypsin. Debris sedimented at 50,000 *g* for 15 minutes was suspended in 2.0 ml. of 1 per cent trypsin, dispersed by pipetting, incubated for 3 hours at 37°C., resedimented at 50,000 *g* for 15 minutes, and resuspended in 1.0 ml. BSS for test. Control debris treated similarly but not exposed to trypsin retained activity. Activity also was destroyed by: (*a*) 15 minutes in a Raytheon magnetostrictor operating at maximum rate of 9,000 cycles per second, (*b*) heating for 30 minutes at 56°C., and (*c*) incubation for 24 hours at 37°C. Contrariwise, 1 per cent lipase and 160 units receptor-destroying enzyme (RDE) were ineffective.

Other Properties of Antiviral Activity by HeLa Cells or Debris.—Results of physical and chemical treatments reinforced the suggestion that cellular antiviral material was associated with lipoprotein structures of cell membrane.

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Since non-primate cells insusceptible to poliovirus apparently lacked such a receptor, the material might be antigenic in non-primate animals. Pooled, inactivated hyperimmune anti-HeLa guinea pig serum was diluted 1:5 in BSS. Monolayers of HeLa, ERK-1, monkey kidney, and EE were exposed for 15 minutes at 37°C. to 0.5 ml. of anticellular serum before or after 2-minute adsorption of virus, washed twice with BSS, and overlaid for plaque count. Control monolayers were treated similarly with 20 per cent normal guinea pig serum. Under test conditions the antiserum was not cytotoxic. Results (Table

TABLE XII

Effect of Anticellular Serum on Specific Attachment of Type 1 Poliovirus to Susceptible Cells

Cell type	Treatment of cell monloayer	Plaques per monolayer
HeLa	Normal serum before virus attachment	90, 78, 74, 88
	Anticellular serum before virus attachment	21, 17, 23, 19
	Anticellular serum after poliovirus	87, 94, 83, 87
ERK-1	Normal serum before virus attachment	31, 25, 29, 31
	Anticellular serum before virus attachment	6, 8, 5, 4
	Anticellular serum after virus attachment	29, 33, 28, 35
Minn. EE	Normal serum before virus attachment	63, 67, 63, 60
	Anticellular serum before virus attachment	18, 21, 17, 18
	Anticellular serum after virus attachment	61, 58, 66, 65
Monkey kidney	Normal serum before virus attachment	103, 111, 98, 106
	Anticellular serum before virus attachment	25, 22, 22, 27
	Anticellular serum after virus attachment	104, 107, 114, 95

Cell monolayers were exposed to Type 1 poliovirus for 2 minutes, immediately before or after treatment of cells with normal guinea pig serum or anti-HeLa serum, then washed and overlaid with agar medium.

XII) showed reduction of virus adsorption rate by antiserum treatment of cells preceding, but not following, virus attachment. Influence of antiserum did not result from general cellular inhibition (7), since antiserum was inactive when applied immediately after virus adsorption. Tests showed that HeLa cells treated or not treated with anticellular serum before exposure to Type 1 poliovirus both returned about 1 per cent of input virus as CAV (adsorbed virus retaining infectivity). At the dilution employed, the anti-HeLa serum showed little evidence of variation in activity toward the different cells, since virus adsorption by each type of cell was reduced in about the same proportion.

Temperature sensitivity of the poliovirus-inactivating capacity of the cell material was tested with two 0.9 ml. samples of HeLa cell debris equilibrated at 37° and 1°C., respectively. Virus suspension containing more that 10^5 PFU in 0.1 ml. was incubated with each sample for 1 hour at the indicated tempera-

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ture. Mixtures then were diluted 1:100, pipetted vigorously, and assayed in 0.1 ml. amounts for residual infectivity. Plaque counts showed that more than 99 per cent of input Type 1 poliovirus had been inactivated by HeLa cell debris in 1 hour at 37°C., while only 21 per cent of input virus had been similarly inactivated at 1°C.

Reversibility of Poliovirus Inactivation by HeLa Cell Debris.—Debris-inactivated Type 1 poliovirus was treated in several ways, to determine whether loss of infectivity was reversible. After incubation at 37°C. for 2 hours, mixtures of Type 1 poliovirus and HeLa cell debris were diluted 1:100 and subjected to: (*a*) 3 cycles of freezing and thawing, (*b*) sonic disruption at 9000 cycles per second, or (*c*) extraction with ether for 1 minute. None of these treatments had

TABLE XIII

Effect of Brief Ether Extraction, Sonic Oscillation, or Freeze-Thawing on Infectivity of Type 1 Poliovirus Inactivated by HeLa Cell Debris

Test system	Plaques per monolayer
Untreated poliovirus (control)	124, 118, 131, 127
Poliovirus + HeLa debris	2, 1, 1, 2
Poliovirus + ether treated HeLa debris	128, 134, 121, 123
Poliovirus + sonicated HeLa debris	117, 112, 120, 109
Ether treatment after virus-debris reaction	12, 10, 8, 14
Sonic disruption after virus-debris reaction	29, 22, 28, 33
Freezing-thawing after virus-debris reaction	3, 1, 2, 2

Poliovirus and HeLa cell debris were allowed to react for 2 hours at 37°C. The debris was treated as indicated either before or after combination with virus.

been found sufficiently drastic to reduce the titer of virus incubated without debris. For control purposes, treatments were applied to other samples of HeLa debris before mixture with Type 1 virus. It can be seen from Table XIII that treatment of debris with ether or sonic oscillation before mixture with virus, as expected, destroyed its inactivating capacity. Treatment with ether or sonic oscillation restored a small portion of the infectivity of the debris-inactivated original virus, but much less than the full amount. The amount of infectivity restored could not be increased by varying length of treatments. These results suggested that the reaction between virus and receptor did not represent reversible attachment, but physical or chemical alteration with permanent impairment of infectivity.

DISCUSSION

Poliovirus in contact with HeLa cells in culture is adsorbed, somehow received into the cell, deprived of infectivity, reproduced, and finally released in infectious form with accompanying cell destruction. The experiments reported

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in this paper deal with phases of attachment, reception or penetration, and eclipse of Type 1 poliovirus infecting HeLa cells in monolayer. Firm attachment was but little dependent on salt concentration, and fairly sensitive to temperature. Like attachment, penetration (progressive immunity of adsorbed virus to inactivation by externally applied antibody) was temperature-sensitive. Penetration was shown to be independent of physiologic integrity of cells. Virus in process of penetration was not affected by ribonuclease. Eclipse of adsorbed virus was not dependent on metabolic activity of HeLa cells or on physical integrity. Debris from poliovirus-susceptible cells inactivated the virus in a manner similar to the kinetics of virus adsorption by intact cells, and released CAV (cell-associated virus) in similar amounts. All cells insusceptible to poliovirus infection failed to yield active debris. Virus inactivation by debris, like virus reception by intact cells, was temperature-sensitive. Debris could not neutralize virus adsorbed to cells, nor alter the progressive incapacity of antibody to neutralize penetrating virus. The active debris factor was not associated with cell nuclei, was inactivated by fat solvents and trypsin treatment, and was destroyed by heat inactivation or sonic disruption. The material apparently was specifically antigenic, since anti-HeLa serum applied to cells before exposure to virus reduced the rate of virus adsorption, while antiserum treatment following virus adsorption was ineffective. These findings suggested that the capacity of HeLa and other susceptible cells to adsorb, receive, and eclipse poliovirus was associated with organized cytoplasmic lipoprotein structures not possessed by insusceptible cells. The reaction of virus with this receptor substance contained in debris was not readily reversed by treatment shown not to affect virus and to destroy activity of uncombined debris. Studies are underway to learn whether this process is reversible. Since productive adsorption of virus was associated with specific structures possessed only by susceptible cells, while unproductive adsorption occurred with insusceptible as well as susceptible cells, it appeared that the two types of adsorption were not closely related.

It has been established that influenza and some other myxoviruses attach electrostatically to specific receptor sites on the surface of host cells (8, 9). Attachment of poliovirus to monkey kidney cells has been reported to be electrostatic, salt-dependent, and temperature-independent (10). The results reported here are consistent with knowledge of coliphage infection, and with findings of other workers for mammalian cells, when biological and technical differences are considered. Attachment of poliovirus by HeLa cells in monolayer was not absolutely sensitive to salt concentration, in contrast to attachment assessed by the less sensitive method of measuring residual unattached virus (10). Cation leakage from cells might explain attachment in the absence of salt, but not in the presence of excess salt. Some bacterial viruses, such as T2 coliphage, are not prevented from attaching by high salt concentration (11). In agreement with Bachtold *et al.* (10), measurement of residual virus unattached

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by HeLa cells suspended at 37° or 1°C. showed that the effect of temperature on virus attachment to cells was little more than could be expected on the basis of viscosity change. The much greater effect demonstrable by direct measurement of attached virus, to monolayer cultures which were then thoroughly washed, is in agreement with Youngner's findings for monkey kidney monolayers (12) and may have been obtained because only firmly adsorbed virus was measured. If so, firm attachment of poliovirus is temperature-sensitive like irreversible binding of T1 coliphage (13). It was observed that poliovirus antiserum applied immediately after virus attachment to host cells inactivated most of the virus. Although Delbrück (14) originally showed that attached phage was not neutralized by antiserum, such neutralization has since been demonstrated when virus penetration was retarded by cold (15) or normally delayed (16).

The concept of Fazekas de St. Groth (17) that attached virus is engulfed or pinocytosed by the host cell (viropexis), although accepted (18, 19) in explanation of virus penetration, must be reconsidered in the light of recent findings (20, 21). Little is known of the stages immediately following poliovirus attachment to cells, except that most of the attached virus enters an eclipse period (4, 22).

Preliminary physical and chemical characterization of the antiviral substance in mammalian cell debris indicated a specific cellular receptor residing in the lipoprotein of the cell membrane. No role for carbohydrate, as found in myxovirus receptors (6), was suggested. Bacterial cell walls, and purified lipopolysaccharides and lipoprotein from cell walls, are known similarly to inactivate homologous phage (23–25). By use of a brief virus attachment period in the experiments here reported, it was demonstrated that anticellular serum reduced the rate of poliovirus attachment to susceptible cells. Such a specific action on poliovirus attachment was not mentioned by Habel *et al.* (7) in their report of inhibition of poliovirus and other viruses by anticellular sera but it was suggested by the findings of Quersin-Thiry (26). Inhibition by antiserum of phage attachment to bacterial cells appears mediated by antigenically related receptor sites possessed by different cells, and/or steric hindrance to virus adsorption exerted by antibody combined with antigen elsewhere on the cell surface than at receptor sites (27). Apparently a similar effect is exerted on the poliovirus receptor sites of HeLa cells by anti-cellular serum. The observation that attached poliovirus was neutralized by antiviral antibody agrees with views of Rubin (20) and Rubin and Franklin (28) and Mandel (29) and with the general conclusion for bacterial viruses that penetration even more than reversible attachment, is vulnerable to antibody action (27). In contrast to the course of virus penetration of HeLa cells here observed, Baluda (30) found that Newcastle disease virus was immune to antiserum only 7½ minutes after adsorption by chick embryo lung cells.

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It can be concluded that the capacity of cell debris to inactivate poliovirus, and the capacity of intact cells to receive and eclipse poliovirus, involve similar though not identical mechanisms since both: (*a*) operate following virus attachment to specific cellular receptor, (*b*) are temperature-sensitive, and (*c*) appear independent of cell physiological integrity. Inactivation of Newcastle disease virus may also be similar, since it can result from attachment to erythrocytes, which are unable to replicate virus (20). Unimpeded penetration of damaged HeLa cells by poliovirus, here observed, does not appear to support the concept of viropexis. The mechanism common to debris inactivation and cell eclipse and penetration of poliovirus may involve dismantling of virus into protein coat and ribonucleic acid (RNA). If so, since externally applied ribonuclease was harmless during penetration, it must be assumed that the protein coat releases its RNA content into or through the cell membrane without exposing it to external fluid. It is interesting that Hoyle and Finter (21) have reported that influenza virus infection is initiated when viral nucleoprotein enters the cell, leaving protein envelope and hemagglutinin on the cell surface. These findings suggest a similarity in the role of nucleic acid in penetration by mammalian and bacterial viruses.

SUMMARY

Phases of attachment, reception or penetration, and eclipse of Type 1 poliovirus infecting HeLa cells in monolayer were studied. Firm attachment was not completely dependent on salt concentration, and was sensitive to temperature change. Like attachment, progressive resistance of adsorbed virus to inactivation by externally applied antibody was temperature-sensitive. Penetration was shown to be independent of physiologic integrity of cells. Virus in process of penetration was not affected by ribonuclease. Eclipse of adsorbed virus was not dependent on metabolic activity or physical integrity of HeLa cells. Debris from poliovirus-susceptible cells inactivated the virus in a manner similar to the kinetic course of virus adsorption by intact cells, and released cell-associated infective virus in similar amounts. All cells insusceptible to poliovirus infection failed to yield active debris. Virus inactivation by debris, like virus reception by intact cells, was temperature-sensitive. Debris could not inactivate virus adsorbed to cells, or alter the progressive incapacity of antibody to neutralize penetrating virus. The active debris factor was insoluble, was not associated with cell nuclei, was inactivated by fat solvents and trypsin treatment, and was destroyed by heat inactivation or sonic disruption. Anti-HeLa serum applied to cells before exposure to virus reduced the rate of virus adsorption, while antiserum treatment immediately following virus adsorption was ineffective. These findings suggested that the capacity of HeLa and other susceptible cells to adsorb, receive, and eclipse poliovirus was associated with organized cytoplasmic lipoprotein structures not possessed by insusceptible cells. The reaction

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of virus with receptor substance contained in debris was not readily reversed by treatment shown not to affect virus and to destroy activity of uncombined debris. Sensitivity of poliovirus adsorption by HeLa cells to change in environmental salt concentration or temperature was dependent on the method of measurement.

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