THE MAMMALIAN CELL VIRUS RELATIONSHIP

IV. Infection of Naturally Insusceptible Cells with Enterovirus Ribonucleic Acid*

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Primate cells naturally susceptible to poliovirus infection possess specific surface receptors that permit poliovirus to be adsorbed quantitatively, received within the cell, eclipsed with loss of infectivity, replicated, and released with accompanying cytopathic effect. Conversely, non-primate cells are naturally insusceptible to poliovirus infection, do not possess active receptors, adsorb only a small fraction of supplied poliovirus, fail to eclipse or replicate measurable amounts of the cell-associated virus, and undergo no cytopathic change (1, 2). It was of interest to learn whether the specific capacity of primate cells to receive, replicate, and release poliovirus was conditioned wholly by presence of specific receptors. Preliminary findings indicated lack of receptors required for cell infection by poliovirus could be surmounted by use of viral RNA (3).

The present communication describes production of infectious poliovirus and other enteroviruses by non-primate cells exposed to viral ribonucleic acid, comparative efficiency of non-primate and primate cells for such virus production, and properties of the produced virus.

Materials and Methods

Cell Culture Methods.—Cell sources, cultural methods, solutions, and media have been described previously (1, 4).

Viruses.—HeLa-grown Type 1 poliovirus (Mahoney) was obtained originally from Connaught Medical Laboratories, Toronto, and employed as virus pools representing fluid of the ninth HeLa culture passage. This virus stock was used for all experiments unless it is stated that Type 1 Mahoney virus grown in FL cultures and partly purified by Cutter Laboratories by application of the first step of the Schwerdt-Schaffer procedure (5) was employed. This latter virus pool was obtained by courtesy of Dr. Ralph B. Houlihan as the first celite eluate (El fraction) in phosphate-buffered 1 M sodium chloride, with a titer of 8.9 log $TCID_{50}$ per

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0.5 ml. Coxsackie A-9 (Wiederhold strain) was supplied by Dr. J. L. Melnick. A pool representing fluid of the fifteenth human amnion primary culture passage was prepared by Dr. F. Lehmann-Grube. This pool of Coxsackie A-9 virus gave final virus yields in tube cultures exceeding 10^9 plaque-forming units (PFU) per ml. Coxsackie B-1 virus (Conn. 5 strain), purchased from American Type Culture Collection, was employed as a fourth HELa passage pool. ECHO 8 (Bryson strain) virus, obtained by courtesy of Dr. A. B. Sabin, was used as fluid from a third human amnion passage pool.

All virus pools were prepared by a method designed to give very high concentrations of infectious virus; the method depended on the high peak of intracellular poliovirus production preceding extracellular release (6, 7). HeLa or amnion monolayers were rinsed, exposed for 30 minutes at room temperature to virus at multiplicities greater than 20, overlaid with 5 per cent calf serum in yeast extract medium (YEM) (1), and incubated at 37°C. for 10.5 hours. Maintenance medium was removed, infected monolayers were washed gently with isotonic (0.14 M) NaCl solution, cells were scraped from glass and were suspended in 0.2 M phosphate-buffered salt solution to a concentration of 3 to 4 $\times$ 10^7 cells per ml., and suspended cells were frozen and thawed 3 times to release intracellular virus. Initially, virus pools were centrifuged to remove cellular debris which could inactivate virus (2), but this precaution appeared unnecessary so long as pools were frozen immediately and stored at −20°C. The procedure yielded poliovirus at 0.4 to 1.0 $\times$ 10^{10} PFU per ml, Coxsackie A-9 virus at 0.8 to 3.0 $\times$ 10^{10}, Coxsackie B-1 at 0.9–2.0 $\times$ 10^{10}, and ECHO 8 virus at 7 $\times$ 10^9 PFU per ml.

Virus Assay.—Virus potency was measured by plaque count, as described previously (1), by use of HeLa cells adapted to growth in calf serum for assay of poliovirus and Coxsackie B-1 virus, and primary human amnion monolayers for Coxsackie A-9 and ECHO 8 virus assays.

Preparation of Viral Ribonucleic Acid.—Viral RNA was prepared by methods modified from those of Gierer and Schramm (8), Colter *et al.* (9), and Alexander *et al.* (10). Reagents included (*a*) Mallinckrodt analytical reagent liquefied phenol saturated with distilled water and stored at 4°C.; (*b*) fresh Merck ether repeatedly washed with distilled water and stored at 4°C. (used within a week of opening); and (*c*) ethylenediamine sodium tetraacetate (versene) for addition to the primary virus suspension in final concentration of 5 $\times$ 10^{-4} M to prevent inactivation of nucleic acid by metal ions (11). Three to six ml. quantities of virus were mixed with equal amounts of phenol, shaken in screw-capped pyrex test tubes vigorously by hand for 5 minutes at room temperature, chilled in an ice bath, and centrifuged to separate the aqueous layer. The separated aqueous layer was treated similarly, and the next aqueous layer treated likewise. The final aqueous layer was chilled in an ice bath and extracted 5 times with equal volumes of ether. Nitrogen was bubbled through the final aqueous preparation for 10 minutes at 0°C. to remove residual ether. This nucleic acid preparation was used without further modification for animal inoculation; for treatment of cells in culture, the extract was adjusted to pH 7.2 with sodium bicarbonate solution and phenol red indicator, and made 1 M with sterile 5 M NaCl solution (10).

Preparation of Ribonuclease—Crystalline ribonuclease (Nutritional Biochemicals Corp., Cleveland) was dissolved in 0.14 M NaCl solution to a concentration of 1 mg. per ml. to give a 10 times stock solution which was sterilized by filtration and stored at 4°C.

Use of Ribonucleic Acid.—Guinea pigs, hamsters, rabbits, and chicks were injected intracerebrally with 0.05 ml. of nucleic acid solution, and mice with 0.33 ml. Monolayer cell cultures containing about 2 $\times$ 10^6 cells in rectangular screw-cap bottles were triply rinsed with 0.5 M NaCl solution and thoroughly drained; cultures were treated with 0.1 ml. of RNA solution at room temperature, with frequent rocking to distribute the inoculum over the cell sheet during a 15 minute period. For counting of plaques, exposed monolayers were

overlaid with 5.0 ml. of semisolid agar medium, incubated 2 days at 37°C., given a second overlay of neutral-red agar, and incubated further for another day at 37°C. for development of plaques. For harvest of infectious virus, exposed monolayers were incubated at 37°C. for varying periods in 5.0 ml. of 10 per cent calf serum in YEM. After incubation, cultures in liquid medium were stored at −20°C. until assayed for virus.

Antisera.—Monkeys were inoculated repeatedly with infectious virus to yield specific antisera to Type 1 poliovirus, Coxsackie A-9 and Coxsackie B-1 viruses. Monkey antiserum to ECHO 8 (Bryson) virus prepared by Dr. H. A. Wenner was received from The National Foundation.

EXPERIMENTAL

Infection of Naturally Susceptible Cell Monolayers with Poliovirus RNA

Infectivity of the ribonucleic acid fraction of poliovirus for naturally susceptible cells in culture, as described by Alexander *et al.* (10), was confirmed.

TABLE I

*Influence of Salt Concentration of Exposure Medium on Production of Infectious Type 1 Poliovirus by HeLa and Monkey Kidney Cells Treated with Poliovirus RNA**

Cell type	Plaque count per monolayer when RNA was suspended in:	
	0.1 M NaCl	1.0 M NaCl
HeLa‡	0, 0, 1, 0, 1, 2	17, 10, 5, 18, 6, 9
Monkey kidney	0, 3, 1, 0, 2, 0	13, 12, 9, 10, 3, 24

* RNA was prepared from partially purified and concentrated poliovirus kindly supplied by Dr. Ralph Houlihan, Cutter Laboratories, Berkeley, California.

‡ Before RNA inoculation, cell monolayers were washed with NaCl solution corresponding in molarity to the RNA solution. RNA was freshly prepared from poliovirus suspended in 1.0 M NaCl solution, diluted by adding 1 part to 9 parts of NaCl solution of molarity as stated, and added in 0.1 ml. amount to each bottle culture.

As observed by these workers, infectivity of RNA was enhanced by use of hypertonic saline. Table I shows the response of HeLa and monkey kidney monolayers to poliovirus RNA extracted in 1.0 M NaCl solution and diluted 1:10 with 0.1 M or 1.0 M NaCl solution immediately before addition to monolayer cultures washed with NaCl solution of corresponding molarity. Table II illustrates the comparative response of HeLa cells, human amnion, and monkey kidney cells in primary culture, to four lots of Type 1 poliovirus RNA prepared at different times from the same pool of partially purified poliovirus (Cutter Laboratories). The results show variation in infectivity of each RNA lot, and variation in receptivity of the different cell types. The results for human amnion cells are representative; such membranes infrequently were found to yield cells as receptive to RNA infection as HeLa cells. Treatment of RNA with ribonuclease for 1 minute before addition to monolayers destroyed infectivity. Through many experiments, not so much as a single

plaque-forming unit of enterovirus RNA was observed to survive ribonuclease treatment. Although variable, these results provided a base determination of RNA infectivity for experiments with naturally insusceptible cells.

Infection of Naturally Insusceptible Cells in Vitro and in Vivo with Enterovirus RNA

Response of Insusceptible Cells to Poliovirus RNA.—Type 1 poliovirus RNA was added in 0.1 ml. amounts to monolayer cultures of naturally insusceptible (1) rabbit cells and to naturally susceptible cell monolayers as controls, or injected intracerebrally in 0.03 ml. amounts into suckling mice. Inoculated

TABLE II

Production of Type 1 Poliovirus by HeLa, Primary Human Amnion and Monkey Kidney Cells Treated with Different Lots of Viral RNA

Cell type*	Plaque count per monolayer for RNA lot No.‡			
	1	2	3	4
HeLa.....	78, 112, 84, 110	4, 0, 7, 14	84, 117, 110, 124	39, 42, 67, 67
Monkey kidney..	89, 85, 70, 97	0, 3, 0, 0	>150§	73, 91, 62, 71
Human amnion...	0, 0, 0, 0	0, 0, 0, 0	0, 1, 0, 0‖	47, 52, 28, 40

* For each experiment, cells in primary culture were obtained from different monkeys and amniotic membranes.

‡ For each experiment, control cultures inoculated with RNA pretreated for 1 minute with ribonuclease (100 μg. per ml.) showed no plaques.

§ Confluent cytopathic effect (>150 plaques present) on each of 4 cultures.

‖ Amnion Lot 3 despite refractoriness to RNA infection was proved fully susceptible to intact poliovirus by titration as shown by its yielding the expected number of plaques upon exposure to dilutions of stock poliovirus pool of known titer (110 plaques at 10^{-4} dilution).

control HeLa cultures were overlaid with agar medium for plaque counting. Inoculated rabbit cell cultures and mouse brains were harvested after various periods of incubation and stored at −20°C. for virus assay. Table III shows that infectious poliovirus was not present in inoculated cultures or animals from 20 minutes to 3 hours after inoculation, that poliovirus plaque-forming units had appeared by 11 hours, and that the numbers of plaque-forming units produced within 11 hours by insusceptible cells did not increase much with further incubation. As usual, pretreatment of RNA with ribonuclease completely prevented production of plaque-forming units. RNA-inoculated cell cultures did not exhibit visible cytopathic effect on virus production, and mice did not respond with apparent disease. The latent period of 3 hours or more preceding appearance of infectious virus nonetheless suggested an infection rather than a reactivation of viral RNA.

Nature of Virus Produced by Insusceptible Cells from Poliovirus RNA.—Numbers of plaque-forming units produced by non-primate cells *in vitro* or

TABLE III

*Production of Type 1 Poliovirus by Insusceptible Rabbit Cell Cultures and Mouse Brain Inoculated with Poliovirus RNA**

Cell type‡	Total PFU of poliovirus at indicated times after inoculation of poliovirus RNA§			
	20 mins.	3 hrs.	11 hrs.	24 hrs.
Naturally susceptible				
HeLa	0	0	8×10^3	6×10^3
Human amnion	0	0	0	0
Naturally insusceptible:				
Domestic rabbit fibroblast (DRF, Minn. 56-8-6)	0	0	5×10^3	6×10^3
Cottontail rabbit papilloma (CRP, Minn. 56-8-8)	0	0	7×10^1	2×10^2
ERK-2 (Westwood's Porton normal rabbit kidney)	0	0	7×10^3	8×10^2
Mouse brain	0	0	7×10^2	8×10^2

* RNA was prepared from partially purified and concentrated poliovirus kindly supplied by Cutter Laboratories.

‡ Monolayers (about 2×10^6 cells) were inoculated with 0.1 ml. RNA solution; mice received 0.03 ml. intracerebrally.

§ Ribonuclease pretreatment of RNA prevented production of infectious virus.

TABLE IV

Effect of Homotypic Antiserum or Ribonuclease on Type 1 Poliovirus Produced from Viral RNA Material by Rabbit Cells and Mouse Brain

Treatment of virus produced from RNA*	Plaque counts per HeLa monolayer for virus from:	
	Rabbit cells	Mouse brain
None	84, 75, 81, 87	31, 26, 33
Antiserum (1 per cent)	0, 0, 0, 0	0, 0, 0
Ribonuclease (0.1 per cent)	90, 79, 80, 83	30, 30, 37

* Virus pools were derived from freeze-thaw–disrupted domestic rabbit fibroblasts (DRF, Minn. 56-8-6) or mouse brain 12 hours after RNA inoculation. Antiserum or ribonuclease and virus suspension was incubated for 1 hour at 37°C., diluted 1:100 in BSS, and plated over HeLa monolayers for plaque counts.

in vivo were of the same order as numbers produced by control primate cells. Infectious virus produced by non-primate cells inoculated with poliovirus RNA was indistinguishable from the virus donating the RNA; the virus produced

was neutralized by homotypic antiserum and unaffected by ribonuclease treatment (Table IV).

For measurement of virus release rate, 0.1 ml. of poliovirus RNA was added to washed (*a*) ERK-2 monolayers, and (*b*) monolayers of rabbit skin cultures in primary culture, each containing about 2×10^6 cells. After 15 minutes, 10 ml. of maintenance medium was added and cultures were incubated at 37°C. At intervals, 0.3 to 1.5 ml. amounts of medium were withdrawn from cultures and frozen at −20°C. for virus assay; culture medium was restored to volume after each withdrawal. Fig. 1 shows the kinetics of virus release: a

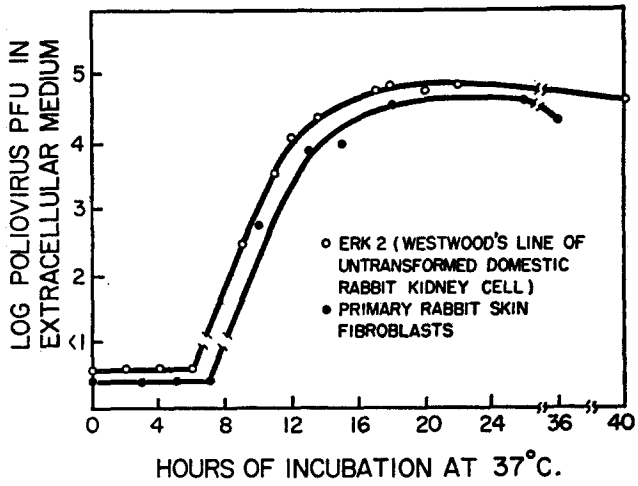

FIG. 1. Production of Type 1 poliovirus by insusceptible rabbit cells infected with poliovirus RNA.

one-step growth curve with no indication of significant readsorption of released virus.

To test the ability of poliovirus produced from RNA received by nonprimate cells to attach to cells of homologous and heterologous relationship, poliovirus released from RNA-infected domestic rabbit fibroblasts (DRF, Minn. 56-8-6) was diluted in balanced salt solution to a concentration of about 70,000 PFU per ml. Four rinsed and drained monolayer cultures of HeLa cells and domestic rabbit fibroblasts each containing about 2×10^6 cells were inoculated with 0.1 ml. of this virus. Bottles were incubated at 37°C. with occasional rocking to distribute the inoculum. At intervals, 0.9 ml. of balanced salt solution was added to a culture, mixed with the inoculum, and removed for virus assay. As shown in Fig. 2, poliovirus produced by RNA-infected domestic rabbit fibroblasts was rapidly adsorbed by HeLa cells but not adsorbed by cells of the donor strain. In this respect also, the poliovirus from RNA infection was

indistinguishable from virus produced by susceptible cells infected with intact virus (1). These results explained why virus production by non-primate cells was not accompanied by overt cytopathogenic effect or disease, and why non-primate cell populations yielded limited amounts of infectious virus after infection with RNA. Virus produced from RNA material again was dependent on specific cell-virus interaction.

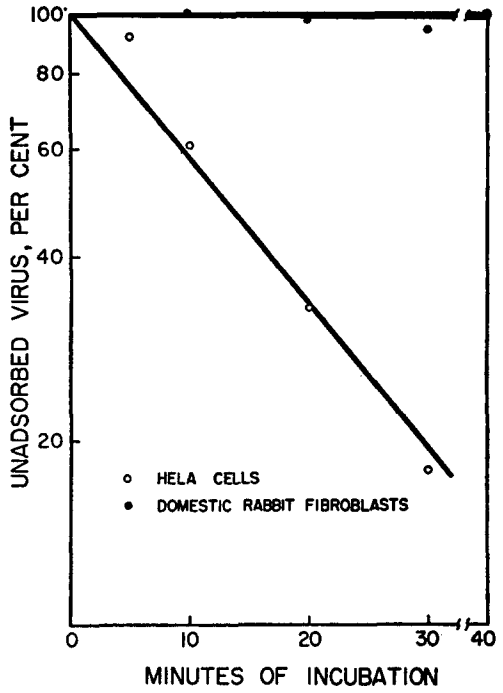

FIG. 2. Attachment of Type 1 poliovirus produced by RNA-infected rabbit cells to HeLa cells and to cells of the donor domestic rabbit fibroblast line.

As previously observed (3), a variety of naturally insusceptible cells or animals inoculated with poliovirus RNA could produce infectious poliovirus (Table V). Varied cell capability for production of poliovirus from RNA possibly indicated variation in cell competency and variation in potency of RNA lots as previously noted with susceptible cells. Variation in competency was suggested because some cultures produced larger yields of poliovirus from most RNA preparations than did other cultures of the same strain. Without regard to natural susceptibility to poliovirus, some cell types (human amnion in primary culture, cottontail rabbit papilloma and cottontail rabbit epithelium) consistently produced only small amounts of virus from RNA, while other cell

types (ERK-2, domestic rabbit fibroblasts, HeLa, rabbit skin cells in primary culture) usually but not always produced larger amounts of virus.

TABLE V

Production of Type 1 Poliovirus by Insusceptible Cells and Animals Inoculated with Viral RNA

Test system*	Total PFU per culture vessel 18 hrs. after inoculation of poliovirus RNA‡	
	Experiment A	Experiment B
Susceptible cell control:		
HeLa	5×10^4	4×10^3
Established lines of non-primate cells:		
Domestic rabbit fibroblast (Minn. 56-8-6)	7×10^4	9×10^2
Cottontail rabbit epithelium (Minn. 57-8-19)	2×10^3	4×10^2
Cottontail rabbit papilloma (Minn. 56-8-8)	0	3×10^2
ERK-2 (Westwood's normal rabbit kidney cell)	2×10^5	4×10^3
Strain L mouse fibroblasts	5×10^2	8×10^4
Primary monolayer cultures of non-primate cells:		
Rabbit skin	4×10^3	9×10^4
Rabbit kidney	6×10^4	5×10^2
Guinea pig kidney	0	1×10^4
Swine lung	2×10^3	5×10^4
Embryonic mouse carcass	8×10^3	—
Maitland-type cultures		
Rabbit liver	3×10^3	—
Guinea pig liver	4×10^5	1×10^4
Hamster liver	9×10^2	—
Mouse liver	2×10^2	5×10^3
Chick liver	0	7×10^3
Minced chick embryo	1×10^3	5×10^2
Living animals inoculated intracerebrally:		
Rabbit	4×10^3	—
Guinea pig	3×10^4	8×10^2
Hamster	6×10^3	—
Chick	2×10^3	0

* Monolayer cultures of about 2×10^6 cells were inoculated with 0.1 ml. of RNA; 0.5 ml. was added to washed tissue fragments by Maitland-type cultures; 0.03 ml. was injected intracerebrally into mice, and 0.05 ml. into the larger animals.

‡ No poliovirus was produced when RNA was treated with ribonuclease before inoculation, or when cultures were harvested 3 hours after inoculation.

Response of Insusceptible Cells or Animals to RNA from Other Enteroviruses. —RNA lots were prepared from Coxsackie A-9 and B-1, and ECHO-8 viruses, as representative enteroviruses that are produced to high titer in cell cultures and that can be assayed accurately by plaque count. Natural susceptibility of

human amnion cells in primary culture and insusceptibility of cells of rabbit strains was correlated with ability to adsorb each of the selected enteroviruses quantitatively. Fig. 3 shows that the enteroviruses were adsorbed rapidly from a 0.1 ml. inoculum by naturally susceptible human amnion monolayers (about

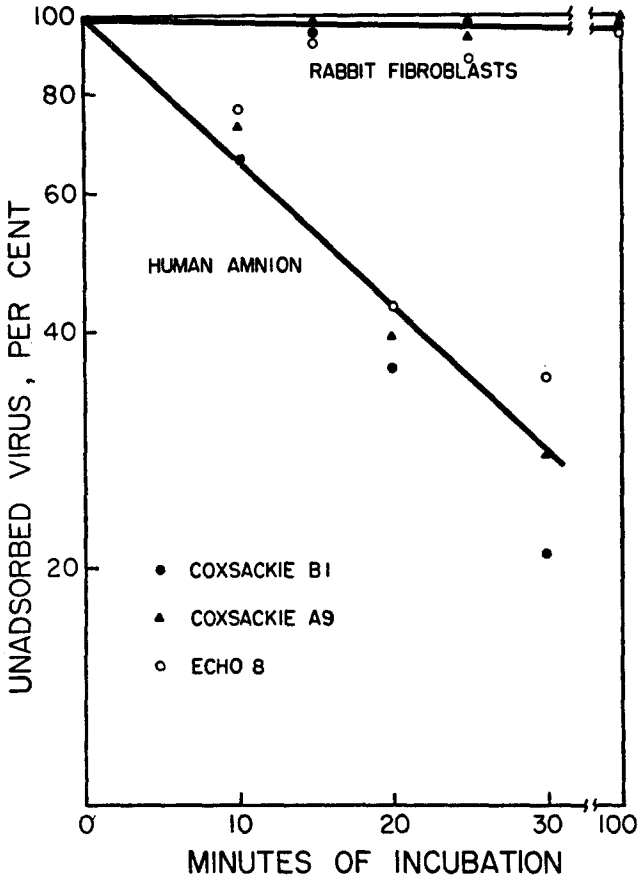

FIG. 3. Adsorption of Coxsackie A-9 and B-1 and ECHO 8 viruses by susceptible human amnion cells and by insusceptible domestic rabbit fibroblasts (Minn. 56-8-6).

2×10^6 cells), but insignificantly by insusceptible domestic rabbit fibroblast monolayers of similar cell density. Host range of these enteroviruses, like poliovirus, was determined by specific adsorption.

RNA from each of the three enteroviruses was extracted with phenol by the procedure used for poliovirus, except that more infective preparations resulted when virus suspensions were kept chilled during the entire extraction procedure by periodic immersion of extraction tubes in an ice bath. Various insusceptible

cell types *in vivo* or *in vitro* were able to produce infectious enterovirus from RNA (Tables VI to VIII). Human amnion cells treated with viral RNA were variably able to produce infectious Coxsackie or ECHO virus.

Virus produced from RNA, whether from poliovirus, Coxsackie or ECHO viruses, reacted like the original virus donating RNA; it was neutralized by homotypic antiserum, was ribonuclease-resistant, and was neither strongly adsorbed nor replicated by cells of the non-primate strain giving rise to the virus. Serial blind passage of poliovirus produced from RNA in non-primate cell cultures able to produce such virus resulted in eventual loss of all infectivity. No evidence of adaptation to insusceptible cells was found by alternating pas-

TABLE VI

Production of Coxsackie A-9 Virus from Viral RNA by Insusceptible Cells

Test system*	Total PFU per culture vessel 18 hrs. after inoculation of Coxsackie A-9 RNA‡
Human amnion in primary culture as susceptible cell control	9×10^2
HeLa	2×10^5
Domestic rabbit fibroblasts (Minn. 56-8-6)	3×10^4
Rabbit skin in primary culture	3×10^5
Cottontail rabbit epithelium (Minn. 57-8-19)	3×10^4
Hamster liver Maitland culture	4×10^2
Chick liver Maitland culture	5×10^4
Guinea pig liver Maitland culture	9×10^4

* Monolayer cultures of about 2×10^6 cells were inoculated with 0.1 ml. of Coxsackie A-9 viral RNA; washed tissue-fragment cultures, with 0.5 ml. of RNA.

‡ No virus was produced when RNA was treated with ribonuclease prior to inoculation, or when cultures were harvested 3 hours after inoculation.

sage of virus produced by RNA infection between the non-primate (parent) cell line and HeLa cells. In addition, poliovirus produced by RNA-infected domestic rabbit fibroblasts was inactivated by receptor material present in HeLa debris (2), as was the original poliovirus.

Infection of Naturally Insusceptible Rabbit Cells with Intact Poliovirus

Infectivity of enterovirus RNA indicated that natural insusceptibility of non-primate cells was determined primarily by specific cell receptors rather than specific ability of cells to respond to information conveyed by RNA material. It was of interest to learn whether the role of cell receptors possessed by naturally susceptible cells involved only facilitation of viral entry, or included separation of RNA from protein coat by virus attachment mechanisms. If specific dismantling of virus by receptors was unnecessary for cell infection, non-primate cells lacking specific receptors should have been able to reproduce

TABLE VII

Production of Coxsackie B-1 Virus from Viral RNA by Insusceptible Cells and Animals

Test system*	Total PFU obtained 18 hrs. after inoculation of Coxsackie B-1 RNA‡
Cells in culture:	
HeLa as susceptible cell control	9×10^4
Human amnion in primary culture	0
Domestic rabbit fibroblast (Minn. 56-8-6)	5×10^4
Cottontail rabbit papilloma (Minn. 56-8-8)	7×10^1
Cottontail rabbit epithelium (Minn. 57-8-19)	5×10^3
Rabbit skin in primary culture	8×10^4
ERK-2 (Westwood's Porton strain of normal rabbit kidney)	8×10^4
Guinea pig liver Maitland culture	2×10^4
Chick liver Maitland culture	6×10^3
Living animals inoculated intracerebrally:	
Hamster	5×10^3
Guinea pig	0
Chick	4×10^2

* Monolayer cultures of about 2×10^6 cells were inoculated with 0.1 ml. of Coxsackie B-1 viral RNA; washed tissue-fragment cultures were inoculated with 0.5 ml., mice intracerebrally with 0.03 ml. and other animals with 0.05 ml.

‡ No virus was produced when RNA was treated with ribonuclease prior to inoculation, or when cultures were harvested 3 hours after inoculation.

TABLE VIII

Production of ECHO 8 Virus from Viral RNA by Insusceptible Cells and Animals

Test system*	Total PFU obtained 18 hrs. after inoculation of ECHO 8 RNA‡
Cells in culture:	
Human amnion in primary culture as susceptible cell control	2×10^3
Domestic rabbit fibroblast (Minn. 56-8-6)	2×10^3
Cottontail rabbit papilloma (Minn. 56-8-8)	8×10^2
Cottontail rabbit epithelium (Minn. 57-8-19)	1×10^2
Rabbit skin in primary culture	1×10^3
ERK-2 (Westwood's Porton strain of normal rabbit kidney)	1×10^4
Guinea pig liver Maitland culture	3×10^4
Living animals inoculated intracerebrally:	
Mouse	2×10^4
Chick	4×10^3

* Monolayer cultures of about 2×10^6 cells were inoculated with 0.1 ml. of ECHO 8 viral RNA; washed tissue-fragment cultures were inoculated with 0.5 ml., mice intracerebrally with 0.03 ml. and chicks with 0.05 ml.

‡ No virus was produced when RNA was treated with ribonuclease prior to inoculation, or when cultures were harvested 3 hours after inoculation.

intact virus successfully introduced into the cells. To achieve and to detect replication of small amounts of virus non-specifically received by non-primate cells, it was necessary to inoculate massive amounts of virus and to remove cell-associated virus (1) from inoculated cell cultures after allowing sufficient time for reception of some virus into the cells.

Heavy (about 4×10^6 cells) washed monolayers of domestic rabbit fibroblasts were incubated at 37°C. with 1.0 ml. of balanced salt solution containing 10^{10} Type 1 poliovirus PFU. After incubation, unattached virus was removed by 5 rinses with balanced salt solution.

TABLE IX

*Multiplication of Type 1 Poliovirus in Insusceptible Domestic Rabbit Fibroblast Cultures Massively Inoculated with Intact Virus and Exhaustively Eluted**

	Experiment 1	Experiment 2
Viral inoculum, PFU in 1.0 ml.	1×10^{10}	1×10^{10}
Infective viral PFU still attached to cells after washing 5 times with BSS	2×10^7	2×10^7
Infective viral PFU associated with cells disrupted after 3 hrs. of elution treatments and incubated at 37° an additional 12 hrs.	2×10^3	1×10^3
Infective viral PFU in cultures following an additional 12 hrs. incubation to allow virus multiplication	7×10^4	2×10^4
Increase in virus PFU between 3 hrs. and 15 hrs. after removal of inoculum	6.8×10^4	1.9×10^4
Estimated‡ number of cells successfully infected (*i.e.*, number of PFU replicated), average of Experiments 1 and 2	About 100	
Estimated fraction of input virus replicated	About 1/10,000,000th	

* Dense monolayer cultures were inoculated with 10^{10} PFU in 1.0 ml. of BSS, incubated for 1 hr. at 37°C., and washed and treated exhaustively to elute cell-associated virus.

‡ An average yield of Type 1 poliovirus (Mahoney strain) per cell of about 400 PFU was assumed, as reported by other workers (7, 12, 13).

After this rinsing, cells were scraped from some bottles, disrupted by three freeze-thaw cycles, and frozen for assay of cell-associated virus (CAV). Remaining monolayers were washed 5 more times with balanced salt solution, 3 times with 1 M NaCl solution, 3 times with 0.25 M sucrose, and twice again with balanced salt solution. This treatment was possible when domestic rabbit fibroblast monolayers or other cell strains which adhere firmly to glass were employed. Thoroughly drained monolayers were incubated for 10 minutes at 37°C. with 0.1 ml. of homotypic antiserum able to neutralize 100 $TCID_{50}$ when diluted 1:25,000. After incubation with antiserum, monolayers were washed 3 times with balanced salt solution and incubated at 37°C. under 12 ml. of 5 per cent calf serum in YEM containing 1 per cent homotypic antiserum. This entire procedure was repeated at 30 minute intervals for 3 hours, when cells were dispersed with trypsin, washed twice with balanced salt solution, resuspended in 2 ml. of balanced salt solution per monolayer, and divided into two 1 ml. samples containing about 2×10^6 cells each. One sample of cells was disrupted by 5 freeze-thaw cycles, and the suspension of cell debris incubated at 37°C. in 5 ml. of maintenance medium for

12 hours. Cells of the other sample, kept intact, were incubated similarly. After 12 hours, the second sample was disrupted and both samples assayed for intracellular virus.

Table IX shows that the exhaustive elution reduced CAV to a level sufficiently low for unequivocal detection of virus replication by the naturally insusceptible rabbit cells. On the basis of an assumed yield of Mahoney-strain poliovirus of about 400 PFU per cell (7, 12, 13) it was calculated that not more than 10 of a billion inoculated PFU were productively received by the naturally insusceptible cells. The virus produced was indistinguishable from the parental stock virus in that it was not adsorbed or replicated by rabbit cells, indicating that this replication of intact virus did not result by selection of virus mutants.

DISCUSSION

Studies reported herein reveal that naturally insusceptible as well as susceptible animal cells, *in vivo* or *in vitro*, can produce typical poliovirus and other representative enteroviruses from phenol-extracted viral ribonucleic acid. The term "viral RNA" is here used to designate the ribonuclease-sensitive infective fraction of phenol-extracted virus. It is not germane to the present consideration whether some amount of viral protein must remain associated with ribonucleic acid to retain infectivity (14). Whether phenol-extracted virus yielded ribonucleic acid or ribonucleoprotein, the RNA material bypassed an initial stage of infection which limited host range of intact poliovirus to primate cells. Previous findings (1, 2) indicated that naturally susceptible primate cells possessed receptor sites necessary for quantitative poliovirus adsorption, and that naturally insusceptible non-primate cells lacked such receptors. The present observation that many non-primate cells indeed can produce infectious poliovirus when inoculated with incomplete virus material leads to the conclusion that initiation of interaction between poliovirus and animal cells is primarily limited by a reaction of virus and cell surfaces. The conclusion seems applicable to enteroviruses in general, and possibly to other animal viruses as well. Observed ability of cells of every examined non-primate species (including avian cells) to produce poliovirus from RNA furthers revision of the original belief that polioviruses were able to infect only neural tissue of certain primates; revision was initiated by the work of Enders *et al.* (15) showing that polioviruses could infect and destroy a variety of non-nervous primate tissues *in vitro*.

The reported data support the contention that RNA infectivity was not mediated by intact virus particles. RNA preparations were ribonuclease-sensitive, and equally capable of infecting primate and non-primate cells. Non-primate cells have been shown unable to adsorb similar quantities of intact virus (1). Such adsorption as occurs to yield cell-associated virus is not sufficient to cause infection. When naturally insusceptible rabbit cells were infected successfully with intact virus, although cell-associated virus represented an average of more than 5 PFU attached per rabbit cell, at most only 1 in 10,000

of these attached PFU was able to infect. This low efficiency suggests that specific cell receptors not only increase frequency of contact between cell and virus, but facilitate penetration of essential virus components into cells. Unaltered host range and serologic specificity of enteroviruses produced by insusceptible non-primate cells indicate that specific composition of enterovirus protein coat was uninfluenced by host cells, but determined by information presumably contained in the viral ribonucleic acid. RNA-infected insusceptible cell monolayers did not show even microscopic plaques. This absence of cytopathic effect and the course of virus production characteristic of a one-step growth curve, showed that enteroviruses did not spread from cell to cell through protoplasmic bridges or in any other manner that might circumvent operation of cell receptors.

Ability of RNA-infected cells from every tested warm-blooded animal infected to produce poliovirus suggests that most if not all of the cells of the primate body may possess the metabolic machinery needed for poliovirus production. However, the conclusion that natural susceptibility of primate and non-primate cells to enteroviruses is dependent mainly on cell receptors may need modification for application to more complex *in vivo* states. *In vivo* susceptibility may be affected also by hormonal, immunological, and physiological factors influencing entrance of virus into cells and subsequent intracellular activities. Ability of non-primate cells to produce enteroviruses is interesting in view of the desirability of employing domestic animal cells for production of human virus vaccine. Use of non-primate cells will demand considerable increase in efficiency of RNA infection or efficiency of interaction of intact virus with receptorless cells. Attempts to increase interaction efficiency by coating HeLa receptor substance onto rabbit cells have been unsuccessful.

Species limitation may be operative if examined far enough. Unadapted Newcastle disease virus has been reported to attach and enter Ehrlich ascites tumor cells but not to multiply (16). Coxsackie A-9 and ECHO 1 viruses were found (17) to attach to patas monkey cells but to induce viral replication and cytopathic effect only when heavy inocula were used. We have failed to infect frog cells and bacterial protoplasts with poliovirus RNA. It is not clear whether failure resulted from inherent inability of these cells to replicate poliovirus, or from technical deficiency in introduction of active RNA into cells.

Correlation of host-cell susceptibility to virus infection with adsorptive capacity, found here for animal cells and enteroviruses, generally agrees with findings for bacterial viruses. Garen and Puck (18) related resistance of *Escherichia coli* B/1, 5 to T1 coliphage to failure of cells to adsorb phage. A B/1 strain of the bacterium adsorbed the phage reversibly but not irreversibly. Spizizen (19) and Fraser *et al.* (20) infected protoplasts of both susceptible and resistant bacteria with osmotically disrupted and urea-extracted T2 phage respectively. Apparently both protein and deoxyribonucleic acid were required

for infectivity of the disrupted phage (19). Our findings with insusceptible animal cells do not preclude the necessity of some protein for RNA infectivity, but support the conclusion that infectivity does not result from surviving intact virus.

SUMMARY

Ribonucleic acid extracted with phenol from Type 1 poliovirus, Coxsackie A-9, Coxsackie B-1, and ECHO 8 viruses infected non-primate cells and animals insusceptible to whole virus as such. Viral RNA was proved infectious for insusceptible cells in test systems of established cell lines, primary monolayer cultures, Maitland type cultures, and living animals inoculated intracerebrally. Cells of rabbit, swine, mouse, guinea pig, chicken, and hamster were infected. Each virus produced was identical with the virus donating RNA, in (*a*) neutralization by homotypic antiserum, (*b*) resistance to ribonuclease treatment, and (*c*) failure to be adsorbed or replicated by nonprimate cells, even of the strain producing the virus from RNA. Produced virus was adsorbed and replicated by susceptible primate cells as usual. Virus in RNA-infected cell cultures was produced in a single cycle unaccompanied by overt cytopathic effect on non-primate cells or disease of intracerebrally inoculated animals.

By drastic elution of infective poliovirus associated with rabbit cells exposed to massive inocula of intact virus, intact poliovirus was shown to infect insusceptible non-primate cells to produce progeny indistinguishable from the parent virus population. Under these conditions, infection was accomplished by about 10 virus plaque-forming units per billion inoculated.

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