JOURNAL OF VIROLOGY, June 1968, p. 553-557
Vol. 2, No. 6

Printed in U.S.A.

Mechanism of Resistance to Enteroviruses of Some Primate Cells in Tissue Culture

V. D. SOLOVIEV, T. I. KRISPIN, V. G. ZASLAVSKY, AND V. I. AGOL

N. F. Gamaleya Institute for Epidemiology and Microbiology, Academy of Medical Sciences; Institute of Poliomyelitis and Viral Encephalitides, Academy of Medical Sciences; and Department of Virology, Moscow State University, Moscow, USSR

Received for publication 6 March 1968

Two cell lines, M10-45-2 and L-41, were studied, each of which possessed specific resistance either to poliovirus or to coxsackievirus. Infection of M10-45-2 cells with poliovirus ribonucleic acid (RNA) and L-41 cells with infectious coxsackievirus RNA was accompanied by production of complete viruses in each of the resistant cell lines. During incubation of the cells with the virus to which they were resistant, the amount of infectious virus did not decrease. Treatment with glycine-HCl buffer solution (*p*H 2.5) of resistant M10-45-2 cells after incubation with poliovirus at 0 C did not result in recovery of infectious virus, although such release did take place after treatment of sensitive M10 cells. Infection of resistant cells with virus containing poliovirus RNA and coxsackievirus proteins resulted in production of poliovirus in M10-45-2 cells but not in L-41 cells. The resistant cells are apparently unable to adsorb the virus to which they are resistant.

Enterovirus-resistant cell lines can be obtained from sensitive tissue cultures by clonal selection (6, 11, 15, 20). Such lines can also be obtained by propagation of surviving cells which have maintained viability after massive infection of a sensitive population with the corresponding virus (16, 21). In cases where the mechanism of resistance has been investigated, it was found to be connected with inability of the cells to uncoat the adsorbed virus (7, 8).

The present communication describes the results of an investigation of two cell lines, M10-45-2 and L-41, which are highly resistant, respectively, to type I poliovirus and coxsackie virus B3. The evidence obtained is consistent with the conclusion that the investigated cells are unable to adsorb the corresponding virus. A preliminary report of this work has been published (18).

MATERIALS AND METHODS

Cell cultures. Human leukemic cells of the J-96 line (13) and monkey adenoid cells of the M10 line (17) were used for this study. Each of these cell cultures is sensitive to both poliovirus and coxsackievirus B3. By repeated infection of the J-96 line with coxsackievirus B3, a line resistant to this virus (L-34-2 line) was isolated (19). Also, by repeated infection of the M10 cell strain with type I poliovirus, a line resistant to this virus (M10-45 line) was isolated (17). Cell lines used in this study were L-41 (derived from the L-34-2 line after additional infection with coxsackievirus B3) and M10-45-2 (obtained from M10-45 cells after additional infection with type I poliovirus). Both resistant cell lines were obtained by N. E. Gulevitch. The sensitive and resistant cells, as well as SKE cells (continuous line of swine embryo kidney cells), were cultured in medium 199 with 10% normal bovine serum (NBS). HeLa cells were cultured in a nutrient mixture, 100 ml of which contained 56 ml of Eagle's medium, 24 ml of Earle's salt solution with 0.5% lactalbumin hydrolysate, and 20 ml of NBS.

Viruses. The following derivates of type I poliovirus, Mahoney strain, were used: M-I-2p line (1) and its guanidine-dependent variant M-g^d-180 (2). The coxsackievirus B3 was passaged in J-96 cells prior to investigation. A "ribonucleic acid (RNA)-protein hybrid" virus (3, 9, 22) was used which contained poliovirus RNA and coxsackievirus capsid (5). To prepare this virus (for brevity, henceforth referred to as PC-hybrid), HeLa cells were infected with a mixture of guanidine-dependent poliovirus (M-g^d-180) and guanidine-sensitive coxsackievirus. The yield was harvested after 8 hr of incubation at 37 C in maintenance medium without guanidine. All viruses were stored at −20 C.

Virus titrations. Viruses were assayed by the plaque method in cultures of kidney cells of *Macaca mulatta*. In some cases, HeLa and M10 cells were used for titrations of poliovirus, and SKE cells were used to assay coxsackievirus. Poliovirus was titrated under an agar overlay, the composition of which was reported earlier (1). For coxsackievirus assays, the concentration of sodium bicarbonate in the overlay was increased to 0.6%, and in some determinations protamine-sulfate was added in final concentrations of 600 μg/ml.

Infectious RNA. Infectious RNA was isolated from poliovirus preparations by phenol extraction (4). The infectious RNA of coxsackievirus was extracted from infected cells of the J-96 or M10 line by the method of Pagano and Vaheri (14); infection with such preparations was carried out after 15-min pretreatment of cells with diethylaminoethyl (DEAE)-dextran at a final concentration of 500 μg/ml.

Antiviral immune sera. The antiserum to poliovirus (APS) diluted 1:10 was capable of neutralizing 10^5 plaque-forming units (PFU) of type I poliovirus and had no effect on the infectious titer of the coxsackievirus. The antiserum to coxsackievirus B3 (ACS) diluted 1:4 produced practically complete neutralization of 4×10^4 PFU cf coxsackievirus B3. In experiments in which the antisera were incorporated into the overlay, the final concentration of APS was 1:500, whereas that of ACS was 1:80. In these dilutions, the antisera completely prevented development of plaques after application of at least 100 PFU of the corresponding virus. The immune sera were kindly provided by N. A. Graevskaya.

RESULTS

Replication of poliovirus and coxsackievirus in sensitive and resistant cell cultures. As can be seen from Table 1, in cultures of M10-45-2 and L-41 cells there was no detectable replication of poliovirus and coxsackie-virus, respectively. At the same time, the parent cells (M10 and J-96) from which the resistant lines had been isolated were highly sensitive to these viruses. Moreover, M10-45-2 cells supported replication of coxsackievirus, and L-41 cells supported replication of poliovirus.

Tests for possible persistence of infectious virus. L-41 or M10-45-2 cells (3×10^7 cells/ml) were homogenized and the nuclei-free homogenates, equivalent to 2.5×10^6 cells, were applied to monolayers of sensitive cells and covered either with agar overlay (to detect plaques) or with liquid medium (for cytopathic effect). An extract of coxsackievirus-infected L-41 cells, corresponding to 5×10^6 cells, was injected also into the brains of newborn mice. In none of these experiments could any infectious virus be detected in the extracts of resistant cells.

TABLE 1. *Multiplication of poliovirus and coxsackievirus in cell cultures*[a]

Cell line	Yield of virus (PFU/sample)	
	Polio	Coxsackie
M10	700×10^5	270×10^5
M10-45-2	$<1 \times 10^5$	230×10^5
J-96	500×10^5	320×10^5
L-41	250×10^5	$<1 \times 10^5$

[a] Monolayer cell cultures (7×10^5 cells per bottle) were infected at a multiplicity of infection for each virus of 140 PFU per cell. After adsorption for 60 min at 37 C, cells were thoroughly washed, Earle's salt solution was added, and the bottles were incubated for 8 hr at 37 C. After incubation, the cells were disrupted by three successive cycles of freezing and thawing. The amount of residual (not washed off) infectious virus did not exceed 1% of the inoculated virus.

TABLE 2. *Adsorption of poliovirus and coxsackievirus on susceptible and resistant cells at 37 C*[a]

Virus	Cell line	Infectivity of unadsorbed virus (percentage of the inoculated virus)
Polio	M10	<15
	M10-45-2	>90
Coxsackie	J-96	<15
	L-41	>90

[a] Cells were detached from the glass with ethylenediaminetetraacetate solution, washed, and resuspended in Earle's salt solution (10^7 cells/ml), containing virus (multiplicity of infection, 0.3 to 0.5 PFU per cell). The bottles were incubated in a water bath at 37 C for 2 hr with continuous shaking. Adsorption was stopped by dilution in a 50-fold volume of cold saline, and the material was preserved at −20 C until titration. Special experiments showed that the residual infectious virus remained in the supernatant fluid after centrifugation ($600 \times g$) and that disruption of cells by freezing and thawing had no influence on the level of infectious virus in the preparation.

Adsorption of poliovirus and coxsackievirus on cells of resistant tissue cultures. Adsorption of viruses on tissue culture cells was investigated by two methods. The first approach consisted in detecting unadsorbed virus. The amount of unadsorbed (infectious) virus was determined after incubation with cells at 0 and 37 C. In the first case, the reduction in infectious virus is due exclusively to reversible eclipse (adsorption), whereas in the second it is due to irreversible eclipse (8). At both incubation temperatures, results were qualitatively similar, but adsorption in the cold was less effective. Table 2 presents data on infectivity of the unadsorbed viruses after incubation with sensitive and resistant tissue cultures at 37 C. As can be seen, incubation of the viruses with cells of sensitive tissue cultures was accompanied by considerable reduction in infectivity of the viral preparations; after incuba-

tion with resistant cells, such reduction was absent. Since all the infectious virus was not associated with cells, it was concluded that the ability of resistant cells to adsorb the corresponding viruses was greatly impaired.

The second approach was based on the observation that it is possible to recover in infectious form virus which has been adsorbed by cells in the cold (12). Using this method, we studied the adsorption of poliovirus on M10-45-2 cells. Glycine-HCl buffer solution (0.1 M, *p*H 2.5) was used for recovery of the adsorbed virus. As can be seen in Table 3, after incubation with M10 cells about 25% of the inoculated virus could be recovered in infectious form after acid treatment (more than 90% of this virus was noninfectious prior to treatment with *p*H 2.5 buffer). In contrast, after incubation of poliovirus with M10-45-2 resistant cells, treatment with glycine buffer solution had no appreciable effect on the infectious titer of the preparation.

Production of poliovirus and coxsackievirus in cells of resistant tissue cultures after inoculation with infectious RNA. As shown in Table 4, infection of M10-45-2 cells with poliovirus-infectious RNA and of L-41 cells with coxsackievirus-infectious RNA resulted in considerable production (although somewhat less than in controls) of the corresponding viruses in their respective resistant tissue cultures.

TABLE 3. *Recovery of infectious poliovirus after incubation in the cold (0 C) with susceptible and resistant cells*[a]

Material	Content of infectious virus after treatment		
	With glycine HCl buffer		With Earle's salt solution (PFU/sample)
	PFU/sample	Percentage of inoculum	
Poliovirus (control).....	1.9×10^8	100	1.3×10^8
Poliovirus + M10.....	4.5×10^7	23	4.0×10^6
Poliovirus + M10-45-2.....	1.2×10^6	<1	7.3×10^5

[a] Cells of monolayer cultures were detached from the glass with ethylenediaminetetraacetate solution, washed with Earle's salt solution, and resuspended to contain 2×10^8 cells/ml, in 0.7 ml of Earle's salt solution, to which 1.3×10^8 PFU of poliovirus was added. This suspension was incubated for 4 hr at 0 C with continuous shaking. Adsorption was stopped by adding a 100-fold volume of saline; the cells were thoroughly washed and disrupted by repeated freezing and thawing. Cell debris was resuspended in 0.5 ml of Earle's salt solution. A 0.9-ml amount of glycine · HCl buffer (0.1 M, *p*H 2.5) of Earle's salt solution was added to 0.1 ml of each sample. After incubation for 2 min at 20 C, the material was diluted 100-fold, and the infectious virus was assayed.

TABLE 4. *Multiplication of poliovirus and coxsackievirus after infection of susceptible and resistant cells with infectious RNA*[a]

Infectious RNA	Cell line	Yield of virus (PFU/sample)
Poliovirus	M10	22×10^3
	M10-45-2	9×10^3
Coxsackievirus	J-96	15×10^3
	L-41	5×10^3

[a] Cells of corresponding monolayer cultures were infected with 50 PFU of infectious RNA of poliovirus or coxsackievirus. After adsorption (15 min, 20 C), the cells were washed, Earle's salt solution was added, and the cells were incubated for 8 hr at 37 C. The infectious virus progeny was titrated after three cycles of freezing and thawing. Treatment with ribonuclease (25 μg/ml, 15 min, 37 C) completely inactivated the infectivity of RNA preparations.

Interaction of tissue culture cells with the PC-hybrid. Table 5 presents results of a serological study of the PC-hybrid. Since titrations were made in the presence of guanidine, only those viral particles could be detected which possessed the RNA of guanidine-dependent poliovirus. It is shown in Table 5 that about 75% of such particles were neutralized with ACS; i.e., their capsids were composed of coxsackievirus proteins. Besides the PC-hybrid the preparation contained also 1.5×10^5 PFU/ml of guanidine-dependent poliovirus (not neutralized with ACS) and 5×10^7 PFU/ml of guanidine-sensitive coxsackievirus (the latter was measured in a separate assay without guanidine in the agar overlay).

Table 5 also shows that progeny of the PC-hybrid were neutralized with APS and, consequently, represented complete poliovirus.

Table 6 shows results of experiments in which the interaction of the PC-hybrid with resistant cell lines was studied. Since M10-45-2 cells are resistant to poliovirus but sensitive to coxsackievirus (Table 1) and to poliovirus RNA (Table 4), it was expected that infection of these cells with the PC-hybrid would be accompanied by production of poliovirus. Also, it was expected that infection of L-41 cells with the PC-hybrid would not be accompanied by production of poliovirus, although they are sensitive to it (Table 1), because these cells could not adsorb coxsackie-

virus (Table 2). To provide for replication of poliovirus RNA and to prevent replication of contaminating coxsackievirus, the experiments were carried out in medium with guanidine.

To study the role of viral capsid, additional experiments were done in which the PC-hybrid was treated before cells were infected with corresponding antiviral sera. It is clearly shown in Table 6 that infection of M10-45-2 cells resulted in production of poliovirus. This production was due exclusively to the hybrid virus, since it was blocked by ACS. In contrast, the production of poliovirus in L-41 cells was greatly reduced (compared with parent J-96 culture). The low virus production in these cells was caused by poliovirus present in the preparation, since pretreatment with APS blocked this reproduction. Thus, viral particle capsid composed of coxsackievirus proteins did not allow the reproduction of poliovirus in cells sensitive to poliovirus (but resistant to coxsackievirus). On the other hand, reproduction was achieved in cells resistant to poliovirus but sensitive to coxsackievirus.

TABLE 5. *Characterization of the PC-hybrid preparation*

Antiserum	PFU/ml	
	A[a]	B[b]
Normal bovine	6.0×10^5	4.5×10^5
Antipoliovirus	3.5×10^5	$<0.2 \times 10^5$
Anticoxsackievirus	1.5×10^5	4.5×10^5

[a] Equal volumes of the PC-hybrid (for preparation, see the Materials and Methods), and normal bovine serum, antipoliovirus serum or anticoxsackievirus serum were mixed and incubated for 60 min at 37 C. Samples (0.2 ml) of these mixtures were inoculated into bottles of monolayer cell cultures for assay. After 20 min, the inoculum was removed, the cells were washed once (to remove the antiserum), and agar overlay, containing 100 μg/ml of guanidine · HCl was added.

[b] A 0.2-ml amount of the PC-hybrid diluted 10^{-3} was inoculated into bottles with monolayer cell cultures, and after 20 min of adsorption the agar overlay containing 100 μg/ml of guanidine · HCl and corresponding antiserum was added.

DISCUSSION

In publications which reported isolation of cells resistant to poliovirus, such resistance was of a relative character and could be easily overcome with increased multiplicity of infection (7, 8). Tissue cultures at our disposal are different from those cells in three respects. First, their degree of resistance to corresponding viruses is extremely high (Table 1). Second, the mechanism of their resistance is due to inability of the cells to adsorb the virus (Tables 2 and 3). Third, each of the resistant cultures is highly resistant to one virus only (Table 1). All these properties of the cells are genetically determined, since the properties are maintained in progeny on prolonged cultivation.

It is known that adsorption of enteroviruses is dependent on the presence of special cellular lipoprotein structures (10) and that such structures are different for poliovirus and coxsackievirus (23). It is quite possible that the selective impairment of adsorption of the viruses studied was due to absence (or functional inactivity) of the virus-specific receptors on resistant cells.

In studying genetic interactions between poliovirus and coxsackievirus, the resistant cells may be used to select recombinants between these two viruses. If such recombinations are, in principle, possible, M10-45-2 cells (as well as nonprimate cells) may be used to select the recombinants in which the poliovirus RNA component is represented by the fragment from the virus which induces the formation of active RNA-polymerase only in the presence of guanidine. The coxsackievirus component in such a recombinant would be represented by the RNA fragment responsible for the synthesis of structural proteins. Obviously, L-41 cells may also be used to

TABLE 6. *Formation of poliovirus in cell cultures infected with the PC-hybrid preparation*[a]

PC-hybrid pretreated with	Virus yield (PFU sample)			
	M10-45-2	L-41	M10	J-96
Normal bovine serum	34×10^4	9×10^4	140×10^4	950×10^4
Antipolio serum	20×10^4	$<1 \times 10^4$	100×10^4	360×10^4
Anticoxsackie serum	$<0.01 \times 10^4$	45×10^4	9×10^4	35×10^4

[a] The PC-hybrid preparation (after treatment with respective antiserum) was inoculated into bottles with monolayer cell cultures. After incubation for 1 hr at 37 C, the cells were washed (to remove the antiserum) and incubated for 8 hr at 37 C in Earle's salt solution containing 100 μg/ml of guanidine·HCl. The virus yield was titrated with the same concentration of guanidine·HCl.

select recombinants, but of different type (the RNA fragment responsible for structural protein synthesis should be provided by the poliovirus component). It should be noted in this respect that formation of recombinants, if possible, would be accompanied by formation of multiple phenotypic RNA-protein hybrids. To exclude such hybrids it is sufficient to provide for one cycle of replication in a sensitive population.

LITERATURE CITED

1. Agol, V. I., S. V. Maslova, and M. Y. Chumakova. 1962. On the correlation between chromatographic behaviour and some other properties of poliovirus variants. Biokhimiya **27**:1071–1078.
2. Agol, V. I., and G. A. Shirman. 1964. Interaction of guanidine-sensitive and guanidine-dependent variants of poliovirus in mixedly infected cells. Biochem. Biophys. Res. Commun. **17**:28–33.
3. Agol, V. I., and G. A. Shirman. 1965. Formation of viral particles with participation of enzyme system and structural proteins, induced by another "helper"-virus. Vopr. Virusol. **10**:8–13.
4. Alexander, H. E., G. Koch, I. M. Mountain, and O. Van Damme. 1958. Infectivity of ribonucleic acid from poliovirus in human cell monolayers. J. Exptl. Med. **108**:493–506.
5. Cords, C. E., and J. J. Holland. 1964. Alteration of the species and tissue specificity of poliovirus by enclosure of its RNA with protein capsid of Coxsackie B1 virus. Virology **24**:492–495.
6. Darnell, J. E., and T. K. Sawyer. 1959. Variation of plaque-forming ability among parental and clonal strains of HeLa cells. Virology **8**:223–229.
7. Darnell, J. E., and T. K. Sawyer. 1960. The basis for variation in susceptibility to poliovirus in HeLa cells. Virology **11**:665–675.
8. Holland, J. J. 1962. Irreversible eclipse of poliovirus by HeLa cells. Virology **16**:163–176.
9. Holland, J. J., and C. E. Cords. 1964. Maturation of poliovirus RNA with capsid protein coded by heterologous enteroviruses. Proc. Natl. Acad. Sci. U.S. **51**:1082–1085.
10. Holland, J. J., and I. C. McLaren. 1961. The location and nature of enterovirus receptors in susceptible cells. J. Exptl. Med. **114**:161–172.
11. Leidy, G., K. Sprunt, W. Redman, and H. Alexander. 1959. Sensitivity of populations of clonal lines of HeLa cells to polioviruses. Proc. Soc. Exptl. Biol. Med. **102**:81–85.
12. McLaren, L. C., J. J. Holland, and J. T. Syverton. 1960. The mammalian cell-virus relationship. V. Susceptibility and resistance of cells in vitro to infection by Coxsackie A 9 virus. J. Exptl. Med. **112**:581–594.
13. Osgood, E. E., and J. H. Brooke. 1955. Continuous tissue culture of leucocytes from human leucemic bloods by application of "gradient" principles. Blood **10**:1010–1022.
14. Pagano, J. S., and A. Vaheri. 1965. Enhancement of infectivity of poliovirus RNA with diethylaminoethyl-dextran (DEAE-D). Arch. Ges. Virusforsch. **17**:456–464.
15. Soloviev, V. D., I. I. Georgadze, and N. B. Varshaver. 1965. A study of natural variations in susceptibility to viruses of the continuous cell culture J-96. Vopr. Virusol. **10**:699–703.
16. Soloviev, V. D., and N. E. Gulevich. 1960. Investigation of cell immunity to poliovirus by tissue culture method. Derivation of cells, resistant to poliovirus. Acta Virol. **4**:220–226.
17. Soloviev, V. D., N. E. Gulevich, and N. B. Varshaver. 1963. Virologic and karyologic investigation of a cell line, resistant to poliovirus. Vopr. Virusol. **8**:580–583.
18. Soloviev, V. D., T. I. Krispin, V. G. Zaslavsky, and V. I. Agol. 1967. Study on the mechanism of cellular resistance to some enteroviruses. Acta Virol. **11**:67.
19. Varshaver, N. B., and N. E. Gulevich. 1964. A study of genetic bases of cell immunity. II. Karyologic study of resistant leukemic cells. Vopr. Virusol. **9**:482–489.
20. Vogt, M. 1958. A genetic change in a tissue culture line of neoplastic cells. J. Cellular Comp. Physiol. Suppl. **1**:271–284.
21. Vogt, M., and R. Dulbecco. 1958. Properties of a HeLa cell culture with increased resistance to a poliomyelitis virus. Virology **5**:425–434.
22. Wecker, E., and G. Lederhilger. 1964. Genomic masking produced by double-infection of HeLa cells with heterotypic polioviruses. Proc. Natl. Acad. Sci. U.S. **52**:705–709.
32. Zajac, I., and R. I. Crowell. 1965. Effect of enzyme on the interaction of enteroviruses with living HeLa cells J. Bacteriol. **89**:574–582.