Cytochalasin D Enhances Infectivity of Poliovirus (37527)

ARLINE D. DEITCH, GABRIEL C. GODMAN, AND STUART W. TANENBAUM
(Introduced by H. M. Rose)

Departments of Pathology and Microbiology, Columbia University, New York, New York 10032

The cytochalasins, congeneric fungal metabolites, are known to inhibit cell motility and cytokinesis (1–3), and to depress both cell permeability to some smaller molecules (4, 5), and some kinds of endocytosis (6–9). On the other hand, exocytosis of the lysosomal enzymes of leukocytes is augmented (10, 11) and cytoplasmic contracture is induced by these compounds (12). Although the common basis of these effects on cells is not agreed on, cytochalasin appears to act, directly or indirectly, on the cell surface (4, 7), and on microfilaments in the cortical cytoplasm which are presumed to have a contractile function (3, 13, 14). In this communication, we report the results of experiments which show that treatment of cells with cytochalasin D increases both their susceptibility to limiting doses of poliovirus and the productivity of the infection. The potentiation of infection would not appear to be an obvious result of cytochalasin-induced interference with microfilament function or impairment of endocytosis.

Methods. Cytochalasin D (CD), a less familiar but biologically more active congener of the more frequently used cytochalasin B (1, 15), was applied in this study in conjunction with the Sabin strain of type I poliovirus to three cell types: Vero, an established line of African green monkey kidney cells (16, 17); HeLa, both maintained and infected in monolayer culture; and WIL_2 (18), a diploid human lymphoblastic line grown in suspension culture. A high titer stock of type I poliovirus, purified by sucrose gradient centrifugation, was kindly made available by D. F. Summers. The extent of poliovirus infection was scored by direct plaque count or by infectious center assay according to standard procedures. Cytochalasin D, obtained from filtrates of a strain of *Zygosporium masonii* and purified by preparative dry column chromatography, was dissolved (at 1 mg/ml) in dimethyl sulfoxide (DMSO). Stock solutions of 100 μg/ml CD in 10% DMSO were kept at −20°. Just before use the drug was further diluted to the concentrations used in these experiments (0.25–4.0 μg/ml) in Eagle's medium supplemented with 10% fetal calf serum and 75 units of penicillin–streptomycin/ml. Comparable concentrations of DMSO (0.025–0.4%) were used in treatment of control cultures.

Results and Discussion. When Vero or HeLa cells, which are permissive hosts for poliovirus, were infected at low infectious virus-to-cell ratios in the presence of CD, a two- to threefold enhancement in the number of infected cells was found as compared with drug-free cultures. The extent of this enhancement was a function of concentration of CD. Figure 1 indicates that for Vero cells after infection with approximately one infectious viral particle for every ten thousand cells, the ratio of plaques formed in drug-treated as compared with drug-free or DMSO-treated cultures rose sharply until a concentration of 1 μg/ml CD was reached, and less sharply thereafter; maximal enhancement was found at 4 μg/ml, the highest dose of CD used. Although CD concentrations of more than 1 μg/ml were somewhat toxic to these cells if applied for several hours, Vero cells treated with 1 μg/ml for 1–2 hr remained viable, and the morphological effects of drug treatment (chiefly contraction of the peripheral cytoplasm) were readily and to-

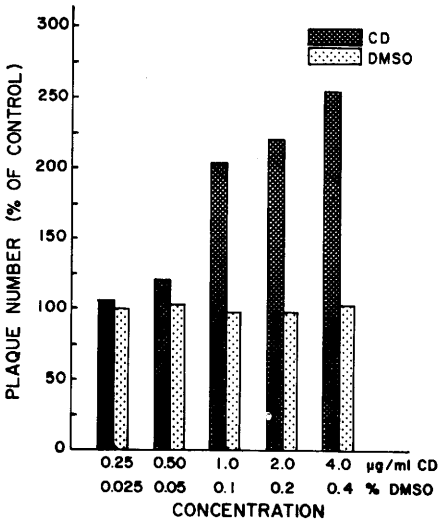

FIG. 1. Effect of CD on infectivity of poliovirus in Vero cells. Monolayers (2×10^6/mm dish) were passaged 18–24 hr before being treated with CD or DMSO-supplemented medium. The cultures were treated for 30 min at 37° with medium containing CD or DMSO and were then exposed to 8×10^{-5} plaque-forming units (PFU) per cell of poliovirus in CD- or DMSO-medium for 1 hr at 37°. The medium during infection was Eagle's Minimal Essential Medium (MEM) supplemented with 25 m*M* Mg Cl_2 (19) and 2% fetal calf serum and with 20 m*M* HEPES (20) replacing bicarbonate. After rinsing, the cultures were treated with rabbit type-specific serum (kindly supplied by B. Mandel) and overlayed with bicarbonate-buffered MEM and agar. The number of plaques formed was counted at 48 hr. The data are expressed as percent of the plaque number found in control cultures without added CD or DMSO. The CD-cultures also contained DMSO at the concentrations indicated.

tally reversed within 2–3 hr after refeeding with drug-free medium. HeLa cells were rather more susceptible to the toxic effects of CD at 1 µg/ml or higher, however, HeLa cultures treated with 0.5 µg/ml CD and infected with low input multiplicities of poliovirus had twice as many productively infected cells as were found in comparable drug-free, virus-infected cultures.

The WIL_2 lymphoblastic line is a semipermissive host for poliovirus; when these cells were infected in control medium at an infectious virus-to-cell ratio of 1, less than one in a thousand cells was detectable as an infectious center on a Vero monolayer. After pretreatment with 0.5 µg/ml CD and infection in the presence of the same concentration of the drug, more than three times as many WIL_2 cells became detectable as infectious centers (Table I). Although this dose of CD was not toxic to WIL_2 cells, higher concentrations of CD were lethal to a part of the population and this is apparently the reason for the reduction in enhancement of poliovirus infection that was found after infection in the presence of 1 µg/ml of CD.

Treatment with cytochalasin D, therefore, facilitates infection by poliovirus as reflected in the increased number of cells that can be productively infected using either permissive or inefficiently infected cell populations.

Infection of Vero cultures with poliovirus in the presence of CD brought about an increased yield of infectious virus particles per culture, but only after inoculation with low multiplicities of virus. Table II shows that on infection with less than one plaque-forming unit per cell, CD-treated Vero cultures produced two to four times as much virus as was found in the corresponding drug-free infected cultures. However at infectious virus-to-cell ratios sufficient to insure the infection of every cell (*e.g.*, at 8 PFU/cell) there was little or no augmentation in the yield of virus per culture. It is notable, however, even at high multiplicities of infection, that Vero cultures pretreated and infected in CD-medi-

TABLE I. Effect of CD on Infection of WIL_2 Cells with Poliovirus.[a]

Treatment	Ratio infected:uninfected cells	Percent of control ratio
0.5 µg/ml CD[b]	1.9×10^{-3}	316%
1 µg/ml CD	1.0×10^{-3}	166%
0.075% DMSO	6×10^{-4}	100%

[a] Aliquots of 2×10^7 WIL_2 cells in suspension culture were treated with 10 ml of CD- or DMSO-medium at 37°. Thereafter 0.1 ml of a polioviral concentrate was added per sample at an input multiplicity of 1.2 PFU per cell. After 3 hr at 37° the cells were rinsed and treated with rabbit type-specific serum before performing infectious center assays on Vero cell monolayers.

[b] 0.5 µg/ml = 10^{-6} *M* CD.

TABLE II. Yield of Infective Virus from Vero Cells Exposed to CD and Different Doses of Poliovirus.[a]

Viral inoculum (PFU)	Supernatant after adsorption (PFU)	Multiplicity of infection[b]	Virus produced (PFU/culture)		Virus production (percent of DMSO control)
			DMSO	CD	
8.0×10^5	4.0×10^5	0.2	3.2×10^7	7.8×10^7	244%
3.6×10^6	2.5×10^6	0.6	8.2×10^7	3.0×10^8	366%
6.8×10^6	4.5×10^6	1.2	1.1×10^8	1.5×10^8	136%
2.2×10^7	1.0×10^7	8.1	1.4×10^8	1.5×10^8	116%

[a] Vero monolayers were treated with 1 μg/ml CD, or 0.1% DMSO as detailed in the description of Fig. 1 and then exposed to different amounts of poliovirus in CD- or DMSO-medium for 1 hr at 37° before refeeding with drug-free medium. At 10 hr after introduction of virus, the combined cells and medium were frozen and thawed to liberate virus and the supernatant clarified by centrifugation and assayed by plaquing on Vero cells.

[b] Multiplicity of infection = number of infectious particles adsorbed/number of cells.

um showed the characteristic cytopathic effects (CPE) of enteroviral infection more rapidly than they did in drug-free infected cultures, and that the onset of these cytopathic changes was more rapid in CD-treated cultures at all multiplicities of infection tested (Table III). There is a direct relationship between the rapidity of onset of cytopathic changes after exposure to poliovirus and the number of copies of the viral genome initially present in the cells (21). It is, therefore, probable that the more rapid cytopathic changes in cultures infected in the presence of CD, as well as the increased numbers of cells found to be productively infected at low viral multiplicities, reflects the more efficient infectivity of the entering viral particles under the influence of CD.

Infection with poliovirus is a notoriously inefficient process; after having been initially attached to cell surface receptors, more than half of the viral particles are rejected into the medium in an altered noninfectious state (22, 23), while an additional 7–12% never penetrate but remain attached to the cell surface in infectious form throughout the viral growth cycle (24). Furthermore, as much as 90% of the virus that does succeed in penetrating the cell is degraded to acid-soluble form (22). Thus only a small fraction of the poliovirus originally received at the cell surface survives in the host cell with an intact genome that is capable of initiating the replicative cycle. The efficiency of both translation and replication of viral RNA may be influenced by many factors intrinsic to the host cell (25, 26), or its phase in the cell cycle (24), as well as by agents extrinsically applied (28, 29). The increase in efficiency of infection in the presence of CD might reflect drug-induced cellular alterations affecting attachment and penetration, viral elution, uncoating, or biosynthetic events occurring during the viral growth cycle. We, therefore, attempted to ascertain the point or event in the infective sequence that was influenced by CD by exposing cells to this agent at progressively later stages in the infectious cycle, using lower temperatures

TABLE III. Effect of CD on the Proportion of Vero Cells Showing Advanced Cytopathic Changes (CPE) at Different Multiplicities of Infection with Poliovirus.[a]

Viral input (PFU/cell)	Percentage of cells showing CPE		Percent of control
	0.1% DMSO	1 μg/ml CD	
1	21.2	43.0	203%
2	28.5	50.3	176%
20	68.4	96.2	141%

[a] Vero monolayers treated and infected as in Fig. 1 were fixed and stained at 6 hr after initiating infection. Cells were scored as showing advanced cytopathic changes if they exhibited characteristic viral-induced cytoplasmic retraction and marked nuclear shrinkage.

to separate adsorption and penetration from events occurring after penetration.

It was found that CD promoted infectivity even when it was first added after removal of the viral inoculum. In these experiments, Vero cells were exposed to poliovirus in control medium at 4°: conditions under which the virus attaches to surface receptors, but does not penetrate (30). When medium containing 1 μg/ml CD was added for 1 hr at 37° immediately after removing the viral inoculum (following thorough rinsing to remove nonadsorbed virus), more than twofold enhancement in plaque number occurred. If, however, addition of CD was delayed for an hour, only a 1.5-fold enhancement occurred, and when the delay before adding CD was prolonged to 90 min, the number of plaques formed was the same in CD- and DMSO-treated cultures. The results of these experiments suggest that adsorption of virus is not the step potentiated by CD, and they indicate also that the sensitive period lasts for only 1.5 hr after infection begins.

At 22°, viral penetration occurs but viral particles are not uncoated (30, 31). Table IV shows that the augmentation of infectivity by CD as judged by infectious center assay was the same whether Vero cells were first exposed to CD during the penetration or in the post-penetration periods, and the enhancement of infectivity was comparable to that which occurred when cells were both preconditioned by CD and infected in the presence of CD. Cytochalasin caused the same morphological changes in cells at 22° and at 37° and these changes were not reversed for 2 hr or more after withdrawal of the drug. It is probable therefore that the potentiating effects of CD applied during viral penetration actually persist through the post-penetration period.

TABLE IV. Effects of Applying CD During or After Penetration on the Infectivity of Poliovirus for Vero.[a]

Medium during			
Viral penetration (2 hr, 22°)	Post-penetration (1 hr, 37°)	Infectious centers per 10^4 cells	Percent of control
1 μg/ml CD	0.1% DMSO	8.6	263%
0.1% DMSO	1 μg/ml CD	8.9	270%
0.1% DMSO	0.1% DMSO	3.3	100%

[a] Vero cultures were infected with 1.6×10^{-3} PFU/cell of poliovirus at 4° for 1 hr to permit viral attachment. After rinsing to remove nonadsorbed virus, CD- or DMSO-medium was added at 22° for 2 hr to permit viral penetration. Subsequently this medium was replaced with CD- or DMSO-medium at 37° for 1 hr (post-penetration period). An infectious center assay was performed on Vero monolayers 3 hr after initiating the infection.

The data suggest that CD acts to augment infection during or after cell penetration by the virus. The time course of the enhancement of infectivity would seem to point to events concerned with either the uncoating of the viral genome or the subsequent earliest stages in the beginning of viral translation, such as, possibly, the attachment of viral RNA to host ribosomes, as the CD-sensitive stages. Subsequent events, such as the replication of viral RNA, probably occur too late [*i.e.*, about 1–1.5 hr after viral penetration (32)] to be affected by cytochalasin.

The manner in which CD acts on cells to augment infectivity with poliovirus is not immediately apparent. Most of the known effects of this agent are inhibitions of normal cellular functions, which result from changes in the cortical cytoplasm or plasma membrane and are accompanied by disturbances in the disposition of microfilaments. The present experiments suggest that the step potentiated by cytochalasin occurs during or shortly after viral penetration. Whether the drug prevents the usual elution of virus, diminishes the degradation of parental polioviral RNA, or causes more efficient virus-directed use of cellular machinery, remains to be determined. In preliminary observations we have found that not all classes of viruses are similarly affected (33), and that pretreatment of Vero cells with dibutyryl cyclic adenosine 3′:5′-monophosphate can prevent the cytochalasin-induced potentiation of poliovirus infectivity.

Summary. Treatment of permissive or semirestrictive cell lines with cytochalasin D (CD) enhances their susceptibility to infec-

tion with poliovirus. A two-to-threefold increase in the number of productively infected cells, as well as an augmented viral yield and a more rapid onset of cytopathic effects occur in drug-treated cultures. CD exerts its potentiating effect early after adsorption either during or, more probably, after viral penetration.

This work was supported by grant VC-76 from the American Cancer Society and grants CA-13835 and AI-06801-07 from the National Institutes of Health. We are grateful to Stanley G. Sawicki for many fruitful discussions and helpful suggestions.

1. Carter, S. B., Nature (London) **213**, 261 (1967).

2. Estensen, R. D., Proc. Soc. Exp. Biol. Med. **136**, 1256 (1971).

3. Spooner, B. S., Yamada, K. M., and Wessells, N. K., J. Cell Biol. **49**, 595 (1971).

4. Estensen, R. D., and Plagemann, P. G. W., Proc. Nat. Acad. Sci. USA **69**, 1430 (1972).

5. Kletzein, R. F., Perdue, J. F., and Springer, A., J. Biol. Chem. **247**, 2964 (1972).

6. Malawista, S. E., Gee, J. B. L., and Bensch, K. G., Yale J. Biol. Med. **44**, 286 (1971).

7. Davis, A. T., Estensen, R., and Quie, P. G., Proc. Soc. Exp. Biol. Med. **137**, 161 (1972).

8. Wagner, R., Rosenberg, M., and Estensen, R., J. Cell Biol. **50**, 804 (1971).

9. Wills, E. J., Davies, P., Allison, A. C., and Haswell, A. D., Nature New Biol. **240**, 59 (1972).

10. Hawkins, D., J. Immunol. **110**, 294 (1973).

11. Henson, P. M., and Oades, Z. G., J. Immunol. **110**, 290 (1973).

12. Miranda, A. F., and Godman, G. C., Tissue Cell **5**, 1 (1973).

13. Schroeder, T. E., Biol. Bull. **137**, 413 (1969).

14. Goldman, R. D., J. Cell Biol. **52**, 246 (1972).

15. Tanenbaum, S. W., Amer. Chem. Soc. 162nd meeting, Wash., D.C., B 128 (1971).

16. Yasamura, Y., and Kawakita, Y., Nippon Rinsho **21**, 1201 (1963).

17. MacFarlane, D. E., and Sommerville, R. G., Arch Ges. Virusforsch. **27**, 379 (1969).

18. Levy, J. A., Virolainen, M., and Defendi, V., Cancer **22**, 517 (1968).

19. Wallis, C., Melnick, J. L., and Bianchi, M., Tex. Rep. Biol. Med. **20**, 693 (1962).

20. Shipman, C., Proc. Soc. Exp. Biol. Med. **130**, 305 (1969).

21. Howes, D. W., and Melnick, J. L., Virology **4**, 97 (1957).

22. Joklik, W. K., and Darnell, J. E., Virology **13**, 439 (1961).

23. Fenwick, M. L., and Cooper, P. D., Virology **18**, 212 (1962).

24. Mandel, B., Virology **25**, 152 (1965).

25. Dunnebacke, T. H., and Reaume, M. B., Virology **6**, 8 (1958).

26. Buck, C. A., Granger, G. A., Taylor, M. W., and Holland, J. J., Virology **33**, 36 (1967).

27. Eremenko, T., Benedetto, A., and Volpe, P., Nature New Biol. **237**, 116 (1972).

28. Tamm, I., and Eggers, A. J., Science **142**, 24 (1963).

29. Cooper, P. D., Virology **28**, 663 (1966).

30. Mandel, B., Cold Spring Harbor Symp. Quant. Biol. **27**, 123 (1962).

31. Mandel, B., Virology **31**, 702 (1967).

32. Baltimore, D., Girard, M., and Darnell, J. E., Virology **29**, 179 (1966).

33. Deitch, A. D., and Godman, G. C., J. Cell Biol. **55**, 58a (1972).

Received April 13, 1973. P.S.E.B.M., 1973, Vol. 144.