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Effects of Cycloheximide and Puromycin on Cytotoxic Activity of *Escherichia coli* Verocytotoxin (Shiga-Like Toxin)

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Verocytotoxin (VT)-producing *Escherichia coli* is closely associated with hemorrhagic colitis and hemolytic uremic syndrome. The diagnosis of this infection requires the demonstration of VT activity in fecal filtrates or the isolation of VT-producing *E. coli* from stools. To improve the sensitivity of the Vero cell assay for detecting VT, we investigated the interaction between this toxin and cycloheximide and puromycin, agents which, like VT and the related Shiga toxin, are protein synthesis inhibitors. Cycloheximide-treated cells were found to be about eightfold more sensitive to VT, this effect being most pronounced when the the drug was added before the toxin. In contrast, puromycin treatment had an antagonistic effect in that it decreased the sensitivity of the cells to VT. In assays of VT in fecal filtrates, the addition of cycloheximide (at 4 to 8 μg/ml) increased the sensitivity without affecting the specificity of the assays. Likewise, the use of cycloheximide led to an increase in the sensitivity of the serum VT-neutralizing antibody test by a factor of over eightfold.

Verocytotoxin (VT; Shiga-like toxin)-producing *Escherichia coli* (VTEC) have become established as the most likely causative agents of hemorrhagic colitis (2, 17) and hemolytic uremic syndrome (HUS) (4). The definitive diagnosis of VTEC infection involves the demonstration of VT, using either Vero cells or HeLa cells, in fecal filtrates, and the isolation of VTEC from stools (3, 4). It would be helpful to improve the sensitivity of the Vero cell assay because the titers of VT in fecal filtrates are generally low (3, 4).

VT, which has been shown to be closely related to the Shiga toxin of *Shigella dysenteriae* type 1, is thought to be composed of two covalently linked subunits referred to as the "A" or active subunit and "B" or binding subunit (9). Although the mechanism of action of VT at the cellular level has yet to be established, it is probably similar to that of Shiga toxin, to which VT is closely related (10). In the case of Shiga toxin, the A subunit is considered to be responsible for the cytopathology by interacting with the 60S ribosomal subunit of the mammalian cell and inhibiting protein synthesis (1, 13). It is thought to act by inhibiting the elongation of the nascent polypeptide chain, but the exact mechanism remains to be established (12, 14, 16).

The objective of this study was to investigate the interaction of VT with two other protein synthesis inhibitors, cycloheximide (11) and puromycin (15), to determine whether they could be used for enhancing the sensitivity of the Vero cell assay for detecting VT.

MATERIALS AND METHODS

Bacterial strains, media, and reagents. The VT-positive (VT^+) reference strain *E. coli* H.30 was provided by J. Konowalchuk (8) and described previously (3). This strain has been documented to be a high-level producer of Shiga-like toxin (10). The medium used for VT production was Penassay broth (antibiotic medium no. 3; Difco Laboratories, Detroit, Mich.) (3). Penassay broth cultures of *E. coli* H.30 or stool suspensions were filtered through 0.22-μm-pore-size filters (Millipore Corp., Bedford, Mass.), and the filtrates were tested for VT activity (3, 4). These filtrates were used as sources of toxin in our studies.

Cytotoxicity test. Volumes (50 μl) of serial twofold dilutions of the test sample were inoculated onto a confluent Vero cell monolayer overlaid by 200 μl of medium in an assay system described previously (3, 4). The microtiter trays were incubated at 37°C in a 5% CO_2 atmosphere and monitored on 3 consecutive days for the characteristic VT effect. The endpoint was taken as the highest dilution (expressed as titer) of the sample that caused a cytotoxic effect on at least 50% of the cell monolayer. The cytotoxic effect consisted of the rounding and detachment of cells from the monolayer and was monitored daily by an inverted microscope. Each sample was tested in duplicate, and the results were recorded as the mean titer of the two assays. Good agreement was found between duplicates, as reported previously (3, 4). Each experiment was repeated at least three times, and the titers did not vary by more than one twofold dilution. The specificity of VT activity was confirmed by neutralization with a specific H.30 VT antiserum (5).

Protein synthesis inhibitors. Cycloheximide and puromycin (Boehringer Mannheim Canada, Dorval, Quebec) were diluted to the appropriate stock concentration (250 to 1,000 μg/ml) in phosphate-buffered saline (Dulbecco "A"; Oxoid Ltd., Basingstoke, United Kingdom). A volume of 10 μl or less was added to the 200-μl cell cultures to give the desired final concentration. Unless otherwise stated, these agents were added immediately after the addition of the VT.

Interaction of VT with cycloheximide and puromycin. Samples of VT preparations were titrated in a Vero cell assay. To respective pairs of titrations was added a solution of cycloheximide or puromycin at serially increasing final concentrations from 0.5 to 16 μg/ml. The assay plates were monitored for cytotoxicity daily for 3 days. The specificity of VT activity was confirmed by neutralization with a specific H.30 VT antiserum (5).

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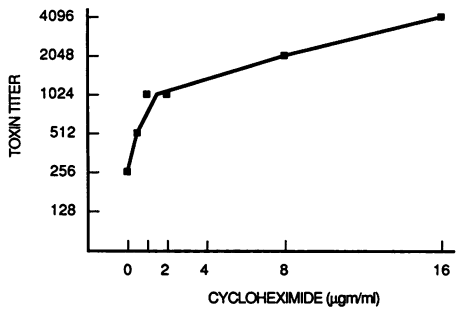

FIG. 1. Effects of cycloheximide on the toxicity of VT. Portions of a VT-positive broth culture filtrate were titrated on Vero cell monolayers exposed to the indicated concentrations of cycloheximide.

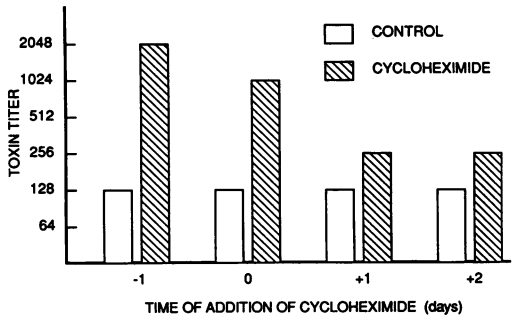

FIG. 2. Effects of duration of cycloheximide exposure of Vero cells on the VT titer. Vero cells treated with cycloheximide at a final concentration of 16 μg/ml were used to titrate samples of a VT preparation. Cycloheximide, as a concentrated stock solution, was added at the times indicated relative to the time of addition of the VT. Control preparations received an equivalent volume of phosphate-buffered saline. The assays were scored after 3 days of incubation at 37°C.

VT-neutralizing antibody titers in sera. Pre- and postimmune rabbit antisera were assayed for VT-neutralizing antibody titers as described (4, 5).

Detection of VT in fecal filtrates. The stool samples used in this study comprised 12 samples from an outbreak of VTEC serotype O157:H7 diarrhea among kindergarten children and 14 stools from a family outbreak of HUS associated with the same serotype (unpublished data). Samples from the latter outbreak included sequential specimens from four children. Control samples consisted of eight stools obtained from sporadic cases of diarrhea in our hospital that were shown to be negative for VT in fecal filtrates by our conventional assay (3, 4). All stool specimens were preserved at −70°C before testing. The specificity of the VT activity in fecal filtrates was confirmed by neutralization with a high-titered convalescent-phase VT antiserum from a patient with O157:H7 infection (3–5).

RESULTS

Effects of cycloheximide on Vero cells. Cycloheximide alone was cytotoxic to Vero cells at concentrations of 64 μg/ml and greater. No appreciable cytotoxic effect was noted at concentrations of 16 μg/ml or less for as long as 5 days. The cells of the treated monolayers ceased growing but did not show signs of detachment.

Effects of cycloheximide on the cytotoxicity of H.30 VT. The addition of cycloheximide at various concentrations to the assay system before the addition of H.30 VT (Fig. 1) led to a marked enhancement of VT titer. The VT titer increased with increasing cycloheximide concentration (Fig. 1), the effect being most pronounced when the cycloheximide concentration was varied from 0 to 2 μg/ml.

Effects of duration of exposure of cells to cycloheximide on H.30 VT titer. H.30 VT activity was titrated on Vero cell monolayers which had been exposed to cycloheximide (16 μg/ml) for different periods of time (Fig. 2), i.e., 1 day before, immediately after, or 1 and 2 days after the addition of VT. In all of the above assays the control titer of VT (in the absence of cycloheximide) was 128, which indicated the level of consistency of the VT titers. The highest VT titer was obtained when cycloheximide was added to the cells 1 day before VT. When cycloheximide was added at the same time as VT, or 1 or 2 days later, a significant fall in VT titer was noted (Fig. 2).

Effects of puromycin on VT activity. The interaction between H.30 VT and puromycin was investigated in a manner similar to that with cycloheximide. In contrast to cycloheximide, the addition of puromycin to the monolayer before the addition of VT decreased the VT titer with increasing drug concentrations from 2 to 8 μg/ml (Fig. 3). At higher concentrations, puromycin alone became toxic to the cells. The addition of puromycin to Vero cells at different times relative to the addition of VT did not significantly influence the VT titer.

Use of cycloheximide in the VT assay on stool specimens. The titers of VT activity in fecal filtrates were markedly enhanced in several samples in the presence of cycloheximide at 4 μg/ml (Tables 1 and 2). The VT activity in both the conventional assay and that with added cycloheximide was specifically neutralizable. It should be noted that two samples that were negative for VT by the conventional assay were positive in the presence of cycloheximide. All control samples were negative for VT by both assays.

Studies on sequential samples from patients from a known family VTEC outbreak showed that VT activity was detectable for a longer period by the cycloheximide-enhanced assay than by the conventional method (Table 2).

Effect of cycloheximide on VT-neutralizing antibody titers in sera. A series of serum neutralization assays were performed (4) on a rabbit VT antiserum. To each well of the assay was added cycloheximide at 8 μg/ml. Each assay was performed with a different amount of VT, ranging from 2 to 0.03 U of toxin and decreasing in twofold serial dilutions (Fig. 4). A unit of toxin was taken as the highest dilution of toxin that produced a 50% cytopathic effect. The concentration of antiserum at which toxin was neutralized was determined by using a conventional VT cytotoxicity assay as

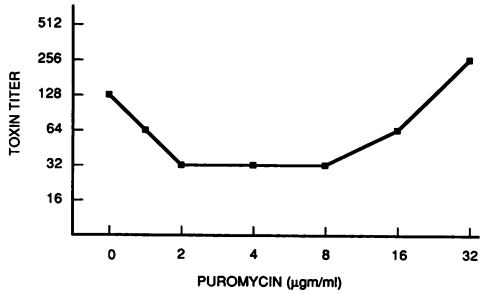

FIG. 3. Effects of puromycin on the activity of VT. Samples of a VT-positive broth culture were titrated on Vero cell monolayers exposed to the indicated concentrations of puromycin.

TABLE 1. Effects of cycloheximide on free VT titer in stool specimens

No. of stools[a]	Control titer[b]	Cycloheximide-enhanced titer[c] (median; GMT)
8[d]	Negative	Negative
2	Negative	−4, 16
6	2	4 to 16 (8; 6.3)
1	4	16
1	8	32
1	32	512
1	256	>2,048

[a] Stools were obtained from HUS contacts at a school outbreak of VTEC and from control hospitalized patients.
[b] Conventional Vero cell assay endpoint after 3 days of incubation of twofold dilutions at 37°C.
[c] Cycloheximide was added to a final concentration of 4 μg/ml at the time of the assay. GMT, Geometric mean titer.
[d] Control stool specimens from hospitalized children having no known contact with VTEC.

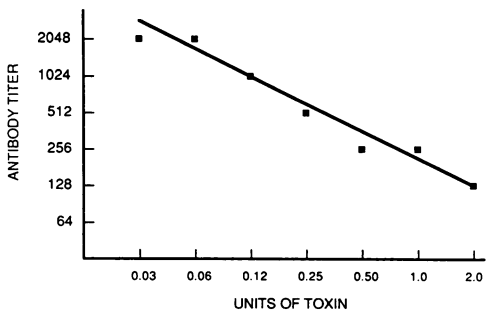

FIG. 4. Effects of cycloheximide on the neutralizing antibody titer to VT. Preparations of a rabbit serum immune to VT were titrated on Vero cells exposed to cycloheximide (8 μg/ml), using indicated quantities of VT. No VT-neutralizing activity was detected with the control nonimmune rabbit serum. A unit of toxin was defined as the highest dilution of VT in 50 μl of medium which resulted in the production of cytopathic effects in half of the cells of the monolayer.

described above. The titer of the antiserum, in the absence of cycloheximide, using 2 U of toxin, was 128. In the presence of cycloheximide, the neutralizing antibody titer, using 2 U of toxin, was 64. This increased to 2,048 when 0.06 U of toxin was employed. Hence, the incorporation of cycloheximide into the assay resulted in an increased sensitivity of VT neutralization assay by 16-fold. The addition of cycloheximide did not influence the negative VT-neutralizing antibody titer in the preimmune rabbit antiserum.

DISCUSSION

Our findings show that cycloheximide, at concentrations of less than 32 μg/ml, significantly enhances H.30 VT activity in Vero cells. The fact that this activity was specifically neutralizable by a VT antiserum indicates that it was due to VT rather than to cycloheximide. The greatest enhancement occurred when cycloheximide was added to the monolayer 1 day before the addition of VT and decreased when it was added at the same time or 1 or 2 days after VT. The reasons for this are not known. In contrast to cycloheximide, puromycin inhibited H.30 VT activity in Vero cells.

The enhancing effect of cycloheximide was successfully used for improving the sensitivity of the Vero cell assay for detecting VT activity in fecal filtrates, without affecting the specificity of this assay. The practical importance of the cycloheximide assay was illustrated in diagnosing VTEC infection in two outbreaks. In one (Table 1), the assay enabled a diagnosis to be made in two patients who would have been missed by the conventional assay. Moreover, in that outbreak, 6 of 12 samples had low levels of VT activity (titer of 2) by the conventional assay. Cycloheximide enhancement resulted in an increase of titer to levels of 4 to 16, allowing for an increased confidence in the diagnosis. VT titers of less than 2 are considered negative (4), precluding specific neutralization from being performed. In the second outbreak (Table 2), a substantial number of the stool specimens would thus fall in the negative catetory. However, in the presence of cycloheximide, only one stool specimen late in convalescence (day 47) proved negative. The detection of VT in late-convalescent-phase stools by cycloheximide enhancement (Table 2) has implications for infection control in that it identifies patients as being potentially infectious where, by the conventional assay, they would have been considered noninfectious. We have previously shown that in some patients with HUS, a diagnosis of VTEC infection could only be made by demonstrating rises in VT-neutralizing antibody titer; stools from these patients were negative for fecal VT and for VTEC presumably because they were taken during late convalescence. The use of a cycloheximide-enhanced assay could have permitted a direct demonstration of fecal VT in these cases.

TABLE 2. Comparison of VT titers in sequential stool specimens of patients in a family outbreak of HUS

Patient (age [yr])	VT titer (control/cycloheximide-enhanced)[a] at (days after onset of illness)[b]
Female (7.5)	−/8 (26); −/4 (27); −/8 (52)
Male (1.5)	512/>2,048 (13); 2/16 (16); 4/32 (18); −/− (47)
Female (4)	>2,048/>2,048 (8); 128/2,048 (15); 8/64 (16); −/2 (42)
Male (6)	>2,048/>2,048 (6); 2/16 (20); −/8 (22)

[a] See Table 1. −, Negative.
[b] Date of onset of acute gastrointestinal prodromal illness.

To date, no studies have been performed on the effects of cycloheximide on other VTs. The stool specimens tested were derived from defined outbreaks, and the toxic activity was neutralized by antibody to our reference strain of VT.

Since cycloheximide allows for the detection of lower amounts of toxin, it is logical that proportionally lower amounts of antitoxin will be required to neutralize this toxin. Consequently, the synergistic effects of cycloheximide and VT can be exploited for the enhancement of the sensitivity of the VT-neutralizing antibody assay. Using an immune rabbit serum, it was demonstrated that this sensitivity can be increased up to 16-fold over the conventional assay for measuring VT-neutralizing antibodies. This effect was specific in that the preimmune serum, negative for VT-neutralizing antibodies by the conventional assay, remained negative for antibody in the presence of cycloheximide.

The effects of cycloheximide on the cytotoxicity of Shiga toxin have been previously explored, with results inconsistent with our observations (6, 7). The difference is probably related to the concentration of cycloheximide employed which was 0.1 μg/ml, or at least 10-fold less than in our studies.

It has been proposed that Shiga toxin inhibits protein synthesis through an interaction with 60S ribosomes which leads to an inhibition of peptide chain elongation (12, 18).

The close similarity of VT to Shiga toxin (9) suggests that VT probably acts in a similar manner. Our observations on the modulation of the cytotoxicity of VT by cycloheximide and puromycin may provide further insight into the mechanism of action of this toxin.

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LITERATURE CITED

1. **Brown, J. E., S. W. Rothman, and B. P. Doctor.** 1980. Inhibition of protein synthesis in intact HeLa cells by *Shigella dysenteriae* 1 toxin. Infect. Immun. **29**:98–107.
2. **Johnson, W. M., H. Lior, and G. S. Bezanson.** 1983. Cytotoxic *Escherichia coli* O157:H7 associated with hemorrhagic colitis in Canada. Lancet **i**:76.
3. **Karmali, M. A., M. Petric, C. Lim, R. Cheung, and G. S. Arbus.** 1985. Sensitive method for detecting low numbers of verotoxin-producing *Escherichia coli* in mixed cultures by use of colony sweeps and polymyxin extraction of verotoxin. J. Clin. Microbiol. **22**:614–619.
4. **Karmali, M. A., M. Petric, C. Lim, P. C. Fleming, G. S. Arbus, and H. Lior.** 1985. The association between hemolytic uremic syndrome and infection by verotoxin-producing *Escherichia coli*. J. Infect. Dis. **151**:775–782.
5. **Karmali, M. A., M. Petric, S. Louie, and R. Cheung.** 1986. Antigenic heterogeneity of *Escherichia coli* verotoxins. Lancet **i**:164–165.
6. **Keusch, G. T.** 1973. Pathogenesis of *Shigella* diarrhea. III. Effects of *Shigella* enterotoxin in cell culture. Trans. N.Y. Acad. Sci. **35**:51–58.
7. **Keusch, G. T.** 1982. *Shigella* toxins: description and role in diarrhea and dysentery. Pharmacol. Ther. **15**:403–438.
8. **Konowalchuk, J., J. I. Speirs, and S. Stavric.** 1977. Vero cell response to a cytotoxin of *Escherichia coli*. Infect. Immun. **18**:775–779.
9. **O'Brien, A. D., and G. D. LaVeck.** 1983. Purification and characterization of a *Shigella dysenteriae* 1-like toxin produced by *Escherichia coli*. Infect. Immun. **40**:675–683.
10. **O'Brien, A. D., G. D. LaVeck, M. R. Thompson, and S. B. Formal.** 1982. Production of *Shigella dysenteriae* type 1-like cytotoxin by *Escherichia coli*. J. Infect. Dis. **146**:763–769.
11. **Obrig, T. G., W. J. Culp, W. C. McKeehan, and B. Hardesti.** 1971. The mechanism by which cycloheximide and other glutarimide antibiotics inhibit peptide synthesis on reticulocyte ribosomes. J. Biol. Chem. **246**:174–181.
12. **Obrig, T. G., T. P. Moran, and R. J. Collins.** 1985. Ribonuclease activity associated with the 60 S ribosome interacting proteins ricin A, phytolaccin and Shigatoxin. Biochem. Biophys. Res. Commun. **130**:879–884.
13. **Olsnes, S., R. Reisbig, and K. Eiklid.** 1981. Subunit structure of *Shigella* cytotoxin. J. Biol. Chem. **256**:8732–8738.
14. **Osato, M. S., T. A. Brawner, and D. J. Hentges.** 1979. In vitro inhibition of DNA, RNA and protein synthesis by *Shigella dysenteriae* type 1 enterotoxin. Am J. Clin. Nutr. **32**:268–275.
15. **Pestka, S.** 1972. Peptidyl puromycin synthesis on polyribosomes of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **69**:624–628.
16. **Reisbig, R., S. Olsnes, and K. Eiklid.** 1981. The cytotoxic activity of *Shigella* toxin. J. Biol. Chem. **256**:8739–8744.
17. **Riley, L. W., R. S. Remis, S. D. Helgerson, H. B. McGee, J. G. Wells, B. R. Davis, R. J. Hebert, E. S. Olcut, L. M. Johnson, N. T. Hargrett, P. A. Blake, and M. L. Cohen.** 1983. Hemmorhagic colitis associated with a rare *Escherichia coli* serotype. N. Engl. J. Med. **308**:681–685.
18. **Thompson, M. R., M. S. Steinberg, P. Gemski, S. B. Formal, and B. P. Doctor.** 1976. Inhibition of *in vitro* protein synthesis by *Shigella dysenteriae* 1 toxin. Biochem. Biophys. Res. Commun. **71**:783–788.