APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1993, p. 1981–1983 Vol. 59, No. 6
0099-2240/93/061981-03$02.00/0

Neutral Red Assay for Measurement of Quantitative Vero Cell Cytotoxicity

A. VALDIVIESO-GARCIA,[1]†* R. C. CLARKE,[1] K. RAHN,[1] A. DURETTE,[1]
D. L. MACLEOD,[2] AND C. L. GYLES[2]

Health of Animals Laboratory, Agriculture Canada, 110 Stone Road West, Guelph, Ontario N1G 3W4,[1] and Department of Veterinary Microbiology and Immunology, Ontario Veterinary College, University of Guelph, Guelph, Ontario N1G 2W1,[2] Canada

Received 24 November 1992/Accepted 31 March 1993

A neutral red assay involving Vero cells was used to quantitate the cytotoxic activity of verotoxins (VT) produced by *Escherichia coli* and to investigate changes in titer caused by altering the composition of the cell culture medium. Three variations on medium 199 were investigated: one involved supplementing the medium with 5% fetal bovine serum (FBS), a second was the use of serum-free (SF) medium that contained 5% bovine serum albumin and 5 μg of fibronectin per ml, and the third involved the use of 4% Ultroser G, a commercial serum replacement. The level of cytotoxicity varied markedly with the type of VT and with the medium that was used. For VT1, there was no difference in cytotoxicity between medium with 5% FBS and SF medium, but cytotoxicity was reduced more than 100-fold in medium containing Ultroser G compared with cytotoxicity in the other media. For VT2, VT2v, and VTe, there was a slight reduction in cytotoxicity in medium containing 4% Ultroser G and a more marked reduction in SF medium compared with cytotoxicity in medium containing 5% FBS.

The Vero cell assay (VCA) has been widely used to assess the cytotoxicity of verotoxin-producing *Escherichia coli* (VTEC) strains (7). One of the major components of the tissue culture medium is fetal bovine serum (FBS). The efficacy of the VCA may be affected by the presence of contaminating bovine antibodies in FBS, thus creating variability between lots which could interfere with the quantification of cytotoxicity. In addition, variations in the glycolipid content of different lots of FBS (9) may result in various degrees of competition with verotoxins (VT) for binding to specific receptor sites on Vero cells.

The neutral red assay (NRA) has been used extensively for in vitro assessment of cytotoxicity of infectious agents (5, 16), food additives, and pharmaceuticals (1). In this study, we evaluated the use of the NRA for quantification of cytotoxicity in Vero cells and assessed the influence of the serum component of tissue culture medium on the VCA.

The VTEC strains used in this study are listed in Table 1. *E. coli* strains were grown overnight at 37°C in 2 ml of brain heart infusion broth (Difco, Detroit, Mich.); then 100 μl of culture was transferred into 1 ml of brain heart infusion, incubated for 6 h, and centrifuged. The culture supernatant was filtered through a 0.45-μm-pore-size membrane filter and tested for cytotoxic activity. Purified VT1 was provided by C. A. Lingwood, Hospital for Sick Children, Toronto, Ontario, Canada. The extraction and purification of VTe have been described elsewhere (10). A nonverotoxigenic *E. coli* K-12 strain was used as a negative control and failed to induce a response in Vero cells grown in any of the three media.

Growth medium consisted of medium 199 with Hanks salts (GIBCO, Burlington, Ontario, Canada) containing 1% basal medium Eagle (BME)–vitamins (GIBCO), 1% minimum essential medium–amino acids (GIBCO), 2 mM L-glutamine (GIBCO), 0.015 M sodium bicarbonate (Baxter), and 1% antibiotic antimycotic solution (GIBCO) and supplemented with a combination of 10% bovine calf serum (catalog no. A-2151-D; HyClone Laboratories, Logan, Utah) and 10% FBS (catalog no. A-111-D; HyClone Laboratories). A similar composition was used in three different combinations in which serum was replaced with one of the following: (i) 5% FBS, (ii) 4% Ultroser G (UG) (catalog no. 091-259504, batch numbers 22G3190 and 14F7398; GIBCO), or (iii) a combination of 5% bovine serum albumin (BSA) (Sigma, St. Louis, Mo.) and 5 μg of fibronectin (FN) per ml from bovine plasma (Sigma), which was designated serum-free (SF) medium. The osmolality of the three media (FBS, UG, and SF) ranged from 309 to 330 mmol/kg (advanced osmometer model 3MO; Advanced Instruments, Inc., Needham Heights, Mass.).

Vero cells (ATCC CC1 81) were detached by using a 0.25% solution of trypsin, and harvested cells were passed through a pipette tip 30 times and filtered through 25-μm sterile nylon mesh. The viability was determined by the dye exclusion technique (3), and the cell concentration was adjusted to 3.4×10^5 cells per ml. One hundred microliters of the cell suspension was added to each well of a 96-well tissue culture microtiter plate (Nunc; GIBCO) containing 200 μl of medium and 5-fold dilutions of VTEC culture supernatant (4). The plate was incubated at 37°C in a CO_2 incubator for 2 days.

The neutral red stock and working solutions were prepared as follows. A 1% (wt/vol) neutral red stock solution was prepared by dissolving neutral red powder (catalog no. N-129; Fisher Scientific Co.) in distilled water and standardized to a reading of 1.9 at A_{540} in an automated spectrophotometer (Titertek Multiskan; Flow Laboratories, Mississauga, Ontario, Canada). The neutral red working solution was prepared by diluting 1 volume of the neutral red stock solution into 40 volumes of chilled cell culture medium

* Corresponding author.
† Present address: Animal Diseases Research Institute, Agriculture Canada, 3851 Fallowfield Road, Nepean, Ontario, Canada K2H 8P9.

TABLE 1. $TCCD_{50}$s of culture supernatants of VTEC strains in FBS, SF, and UG cell culture media in the VCA

Strain	Toxin	$TCCD_{50}$ for the indicated cell culture[a]		
		5% FBS	SF	4% UG
H30	VT1	3.52 ± 0.31a	3.41 ± 0.18a	1.02 ± 0.13b
H19	VT1	3.70 ± 0.13a	3.65 ± 0.06a	1.27 ± 0.34b
933W	VT2	3.85 ± 0.31a	2.23 ± 0.08c	3.39 ± 0.20b
E32511	VT2v	3.67 ± 0.11a	1.70 ± 0.12c	2.92 ± 0.32b
K12B	VTe	2.91 ± 0.41a	1.76 ± 0.05b	2.43 ± 0.33a

[a] Numbers represent the mean and the standard deviation of three replicates of the reciprocal of the dilution (log_{10}) required to kill 50% of the cells as assessed by the modified NRA. $TCCD_{50}$s with different postscript letters within rows are statistically different at the 5% level.

containing 0.06% EDTA. All solutions were kept at 4°C, and a fresh neutral red working solution was made up each day.

The plate was stained as follows. The plate was washed twice with chilled cell culture medium containing 0.06% EDTA. The wells were then filled with 100 μl of neutral red working solution and incubated for 20 min at 37°C in 5% CO_2. The plate was washed three times with cell culture medium containing 0.06% EDTA. The wells were then filled with 200 μl of a mixture of 0.05 M glacial acetic acid and 0.5% sodium dodecyl sulfate and incubated overnight at room temperature. The optical density was measured at A_{540} in an automated spectrophotometer (Titertek Multiskan), blanked with the first column which was stained with neutral red but contained no cells.

In order to visualize the interaction between the toxins and UG, purified VT1 and VTe were immobilized on a 0.2-μm-pore-size nitrocellulose membrane (NCM) by the Easy-Titer enzyme-linked immunofiltration assay system (Pierce, Rockford, Ill.). The membrane was then cut into strips and blocked with phosphate-buffered saline (PBS) containing 0.3% Tween 20 (PBSTW) at 37°C for 1 h. The NCM strips were rocked continuously throughout this step and subsequent steps of the assay. UG (4%), ammonium sulfate-treated UG, and polyclonal antibodies to purified VT1 and VTe (1:100), raised in pigs, were added, and the mixture was incubated at 4°C overnight. The NCM strips were washed in PBSTW five times (4 min each), and alkaline phosphatase-conjugated antibody (1:1,000) was added and allowed to react for 2 h at room temperature. The NCM strips were again washed with PBSTW five times (4 min each) and then twice with PBS (1 min each) and once with substrate buffer for 4 min. Substrate (5-chromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium [BCIP-NBT]) was added, and the reaction was stopped after 10 min or when strong color developed.

To determine binding of VT1 and VTe to FN and BSA, the following procedure was applied. BSA and FN were immobilized on 0.2-μm-pore-size NCM by the ELIFA system. Strips were cut, blocked with PBSTW at 37°C for 1 h, and exposed to purified VT1 and VTe overnight at 4°C. Porcine anti-VT1 and anti-VTe (1:100) were used as primary antibodies with the corresponding alkaline phosphatase conjugate by the protocol outlined in the previous paragraph.

The 50% tissue culture cytotoxic doses ($TCCD_{50}$s) for three replicates were calculated (5, 16) by taking the percent cytotoxicity for each dilution of culture supernatant as follows: % cytotoxicity = [(A−B)/A] × 100, where A is the optical density of control wells (cells with no VTEC culture supernatant) and B is the optical density of the wells containing cells challenged with VT. The 50% cutoff point

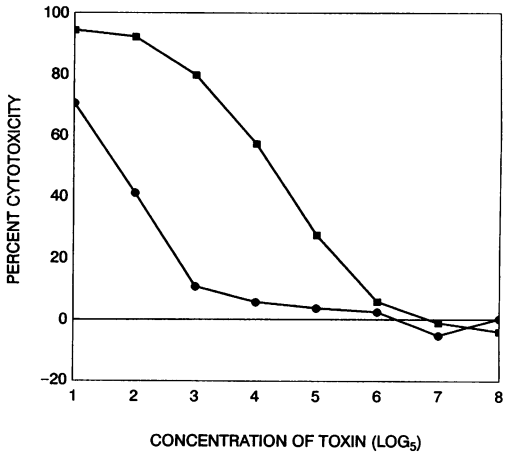

FIG. 1. VCA of strain H19 in culture medium supplemented with 4% UG (circles) and ammonium sulfate-treated UG (squares) as assessed by the modified NRA. Abscissa, reciprocal of log_5 of the toxin concentration.

was established by the method of Rovozzo and Burke (11), adjusting the proportionate distance to the log base 5 and converting to log base 10. Analysis of variance was performed, and Duncan's multiple range test (13) was used to determine whether significant differences existed among the three medium formulations ($P = 0.05$).

When the medium containing UG was used, the supernatants from VT1-producing strains of VTEC had a reduced $TCCD_{50}$ compared with titers obtained with cells grown in SF and FBS media (Table 1). Precipitation of UG with ammonium sulfate increased cytotoxicity from $10^{1.19}$ to $10^{2.97}$, as demonstrated in the cytotoxicity curve for strain H19 (Fig. 1). Immobilization of purified VT1 on NCM and subsequent application of UG as a primary antibody gave a positive signal with alkaline phosphatase-conjugated goat anti-bovine immunoglobulin G (Fig. 2). For VT1, there were no significant differences in the $TCCD_{50}$ levels measured for SF and FBS media. In contrast, for the VT2 subgroup of toxins (VT2, VT2v, and VTe), there was significant inhibition of cytotoxicity ($P = 0.05$) with SF medium compared with that for FBS medium (Table 1). Binding of these toxins to medium components such as FN or BSA could have explained the reduced cytotoxicity. However, dot immuno-

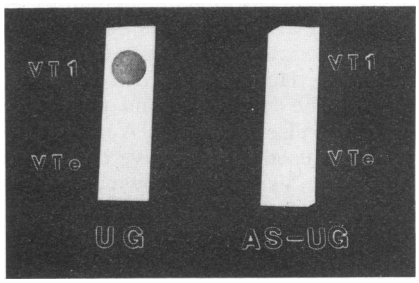

FIG. 2. Dot blot immunobinding. Purified VT1 and VTe (3 μg each) were immobilized on an NCM by the Easy-Titer ELIFA system and incubated with 4% UG and ammonium sulfate-treated UG (AS-UG) followed by alkaline phosphatase-conjugated anti-bovine immunoglobulin G (heavy- and light-chain specific) and BCIP-NBT substrate.

blot analysis demonstrated no binding of purified VTe and VT1 to BSA or FN.

Previous studies have established that VT1-neutralizing antibodies are prevalent in the cattle population (2), and since UG is an undefined product of bovine origin, it is possible that the serum content in it is high. When UG was subjected to ammonium sulfate treatment, there was a 1.78 log increase of cytotoxicity for VT1, suggesting that the low cytotoxicity level might have been due to specific antibodies. The dot immunoblot analysis confirmed the inhibiting factor in UG as bovine immunoglobulins, since the conjugate is an enzyme-labelled affinity-purified antibody that specifically recognizes bovine immunoglobulin G.

Naturally occurring antibodies against glycolipids containing the Galα1-4Galβ sequence (Forssmann and Tja antibodies) have been described previously (8, 9). The functional glycolipid receptor for both VT1 and VT2 (9, 17) is globotriaosylceramide. It is therefore unlikely that antiglycolipid antibodies were mediating this effect, since there was no corresponding decrease in VT2 cytotoxicity.

There were no significant differences in the $TCCD_{50}$ levels measured for SF and FBS media for VT1. However, in the VT2 subgroup of toxins (VT2, VT2v, and VTe), significant inhibition of cytotoxicity was observed ($P = 0.05$) with SF medium (Table 1). This could be attributed to the BSA and/or FN present in the medium. FN was included in the SF medium to promote cell attachment, but it is known to bind proteins (12). However, the fact that neither BSA nor FN bound to purified VT1 or VTe suggests that binding of toxin to these components was not the factor responsible for the decrease in titer. Furthermore, since the VT2 subgroup is a heterogeneous cluster with different binding specificities (6, 14), it is unlikely that specific binding was a factor.

When used with appropriate control strains, the NRA allowed quantitative assessment of cytotoxicity and detection of interfering substances among different medium formulations. In this study, neither UG nor SF proved to be a satisfactory replacement for FBS in the VCA. The NRA has also been successfully used with endothelial cells and LS180 human adenocarcinoma cells to assess cytotoxicity of VT (15).

The NRA proved to be a valuable quantitative tool and provides a useful adjunct to the routine VCA. The VCA using the NRA modification will allow factors that play a role in diseases caused by VTEC to be dissected in a more systematic manner.

We are grateful to Deborah A. McCabe, Brian W. Brooks, Nazar H. Shakarchi, and Ed Riche for valuable suggestions.

This work was supported by the Visiting Fellowships in Canadian Government Laboratories program.

REFERENCES

1. **Babich, H., and E. Borenfreund.** 1990. Cytotoxic effects of food additives and pharmaceuticals on cells in culture as determined with the neutral red assay. J. Pharm. Sci. **79**:592–594.
2. **Clarke, R. C., S. A. McEwen, V. P. Gannon, V. E. O. Valli, H. Lior, and C. L. Gyles.** 1987. Isolation of verotoxin-producing *Escherichia coli* from milk filters and calves in Ontario, abstr. LFE-15. Int. Symp. Workshop Verocytotoxin-(Shiga-like toxin) Producing *Escherichia coli* (VTEC) Infections, Toronto.
3. **Freshney, R. I.** 1983. Culture of animal cells, p. 199–215. Alan R. Liss, Inc., New York.
4. **Gannon, V. P., C. L. Gyles, and R. W. Friendship.** 1988. Characteristics of verotoxigenic *Escherichia coli* from pigs. Can. J. Vet. Res. **52**:331–337.
5. **Greer, C. N., and P. E. Shewen.** 1986. Automated colorimetric assay for the detection of *Pasteurella haemolytica* leucotoxin. Vet. Microbiol. **12**:33–42.
6. **Head, S. C., M. A. Karmali, and C. A. Lingwood.** 1991. Preparation of VT1 and VT2 hybrid toxins from their purified dissociated subunits. J. Biol. Chem. **266**:3617–3621.
7. **Konowalchuk, J., J. I. Speirs, and S. Stavric.** 1977. Vero response to a cytotoxin of *Escherichia coli*. Infect. Immun. **18**:775–779.
8. **Linderberg, A. A., E. Schultz, M. Westling, J. E. Brown, S. W. Rothman, K.-A. Karlsson, and N. Stromberg.** 1986. Identification of the receptor glycolipid for Shiga toxin produced by *Shigella dysenteriae* type 1, p. 439–446. *In* D. Lark (ed.), Protein-carbohydrate interactions in biological systems. Academic Press Ltd., London.
9. **Lingwood, C. A., H. Law, S. Richardson, M. Petric, J. L. Brunton, S. De Grandis, and M. Karmali.** 1987. Glycolipid binding of purified and recombinant *Escherichia coli* produced verotoxin *in vitro*. J. Biol. Chem. **262**:8834–8839.
10. **MacLeod, D. L., and C. L. Gyles.** 1990. Purification and characterization of an *Escherichia coli* shiga-like toxin II variant. Infect. Immun. **58**:1232–1239.
11. **Rovozzo, G. C., and C. Burke.** 1973. A manual of basic virological techniques, p. 87–93. Prentice-Hall, Inc., Englewood Cliffs, N.J.
12. **Ruoslahti, E.** 1988. Fibronectin and its receptors. Annu. Rev. Biochem. **57**:375–413.
13. **Snedecor, G. W., and W. G. Cochran.** 1980. Statistical methods, 7th ed. The Iowa State University Press, Ames.
14. **Tyrrell, G. J., K. Ramotar, B. Toye, B. Boyd, C. A. Lingwood, and J. L. Brunton.** 1992. Alteration of the carbohydrate binding specificity of verotoxins from Galα1-4Gal to GalNAcβ1-3Galα1-4Gal and vice versa by site-directed mutagenesis of the binding subunit. Proc. Natl. Acad. Sci. USA **89**:524–528.
15. **Valdivieso-Garcia, A., D. L. MacLeod, R. C. Clarke, and C. L. Gyles.** 1990. Comparative cytotoxicity of shiga-like toxin-IIv on bovine and porcine endothelial and human colonic adenocarcinoma cells, abstr. B-201, p. 60. Abstr. 90th. Annu. Meet. Am. Soc. Microbiol. 1990.
16. **Valdivieso-Garcia, A., S. Rosendal, B. Allen, C. M. Thompson, and S. Watson.** 1989. Cytotoxicity of *Mycoplasma mycoides* subspecies *mycoides* for cultured endothelial cells. Zentralbl. Bakteriol. **272**:202–209.
17. **Waddell, T., S. Head, M. Petric, A. Cohen, and C. Lingwood.** 1988. Globotriosylceramide is specifically recognized by the *Escherichia coli* verocytotoxin 2. Biochem. Biophys. Res. Commun. **152**:674–679.