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Direct Cytotoxic Action of Shiga Toxin on Human Vascular Endothelial Cells

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To help explain a role of the Shiga toxin family in hemorrhagic colitis and hemolytic-uremic syndrome in humans, it has been hypothesized that these toxins cause direct damage to the vascular endothelium. We now report that Shiga toxin purified from *Shigella dysenteriae* 1 does indeed have a direct cytotoxic effec on vascular endothelial cells in cultures. Human umbilical vein endothelial cells (HUVEC) in confluent monolayers were reduced 50% by 10^{-8} M Shiga toxin after a lag period of 48 to 96 h. In comparison, nonconfluent HUVEC were reduced 50% by 10^{-10} M Shiga toxin within a 24-h period. These data suggest that dividing endothelial cells are more sensitive to Shiga toxin than are quiescent cells in confluent monolayers. Both confluent and nonconfluent HUVEC specifically bound ^{125}I-Shiga toxin. However, in response to the toxin, rates of incorporation of [^{3}H]leucine into protein were more severely reduced in nonconfluent cells than in confluent cells. Toxin inhibition of protein synthesis preceded detachment of cells from the substratum. The specific binding of ^{125}I-Shiga toxin to human endothelial cells and the cytotoxic response were both toxin dose dependent and neutralized by anti-Shiga toxin antibody. Heat-denatured Shiga toxin was without the cytotoxic effect. In addition, the complete culture system contained less than 0.1 ng of bacterial endotoxin per ml, as measured by the *Limulus* amoebocyte lysate test.

Shiga toxin is the multiple-subunit proteinaceous toxin produced by *Shigella dysenteriae* 1. It has been the subject of several recent reviews (7, 24, 31). During the past decade, studies from different laboratories have led to a more complete knowledge of the toxin molecule, its interaction with cells, and its biochemical mode of action at the subcellular level (1, 3–5, 10, 13, 15, 29, 32–35, 37, 39). While such studies have advanced our current understanding of how Shiga toxin may actually exert its effect at the cellular level, the exact role of Shiga toxin in pathogenesis remains to be established. Recently, attention has turned to resolving the role of Shiga toxin in human disease by using isolated target cell types, such as intestinal epithelia, which are purported to be damaged during the natural progression of the infectious disease process (23, 30).

In addition to the typical diarrhea and dysentery syndromes resulting from *S. dysenteriae* 1 infection (7, 24, 25), a small but significant number of cases have been reported to progress towards the development of disseminated vascular complications and hemolytic-uremic syndrome (HUS) (6, 16, 19, 26, 36). The pathology of HUS includes microangiopathic nephrotoxicity, disseminated microvascular thrombosis, anemia, and thrombocytopenia (14). Several reports point to the vascular endothelial lining as being a primary cell type affected in HUS (2, 8, 14, 22). A role for bacterial endotoxin in HUS has been suggested but has not been established (27). In contrast, parenteral presentation of Shiga toxin to animals suggested that systemic Shiga toxin may have a direct or indirect cytotoxic effect on endothelial cells (2, 22). Thus, the purpose of the present study was to determine if highly purified Shiga toxin from *S. dysenteriae* 1 exerts direct cytotoxic activity on human umbilical vein endothelial cells (HUVEC) in cultures.

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MATERIALS AND METHODS

Reagents and chemicals. Cell culture materials and their sources were as follows: Dulbecco modified Eagle medium, GIBCO Laboratories, Grand Island, N.Y.; amphotericin B, Flow Laboratories, Inc., McLean, Va.; gentamicin sulfate, M.A. Bioproducts, Walkersville, Md.; povidone-iodine, Sherwood Pharmaceutical Co., Mahwah, N.J.; CLS II collagenase, Organon Teknika, Malvern, Pa.; RPMI 1640 medium and fetal bovine serum, Hyclone Labs, Logan, Utah; bovine serum albumin and culture plasticware, Corning Glass Works, Corning, N.Y.; and heparin, trypsin, polyvinylpyrrolidone, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetracetic acid (EGTA), and endotoxin test kit, Sigma Chemical Co., St. Louis, Mo. [4,5-^{3}H]leucine (60 Ci/mmol), was obtained from Dupont, NEN Research Products, Boston, Mass. Type 1 water of 18 megaohm resistance was utilized for the preparation of solutions.

Toxin. Shiga toxin was purified from cell lysates of *S. dysenteriae* 1 3818-0 as described previously (3). The toxin was stored at −70°C in a solution of 20 mM Tris hydrochloride (pH 8.0)–0.1 M NaCl. In some cases, the toxin was incubated sequentially with the following: trypsin, 10 μg/ml; phenylmethylsulfonyl fluoride, 0.02 μg/ml; urea, 10 M; and dithiothreitol, 10 mM. The toxin was then dialyzed against water, lyophilyzed, and stored at −70°C. Upon reconstitution in water, this toxin preparation retained full biological activity in cell-free protein synthesis systems but was without cytotoxic activity in whole cells (5, 33).

Antitoxin. Polyclonal antibody was produced in rabbits

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treated with purified Shiga toxin. The immunoglobulin G fraction was partially purified from rabbit serum by protein A affinity chromatography and preincubated (37°C for 1 h) with Shiga toxin when needed before addition to cell cultures. Monoclonal antibody against the β-subunit of Shiga toxin was prepared as described elsewhere (18).

Cells. HUVEC were isolated by a method described previously (21). At the time of collection, the umbilical cord was cut from the placenta, the blood was drained, and the cord was placed in a sterile container with 25 ml of Dulbecco modified Eagle medium supplemented with 2.5 μg of amphotericin B and 100 μg of gentamicin sulfate per ml. Under sterile conditions, the outside of the cord was wiped with a 10% povidone-iodine solution, and the ends were cut clean with a no. 10 scalpel blade. Cannulas were inserted into each end and secured with braided umbilical tape. The cord was perfused with calcium- and magnesium-free phosphate-buffered saline supplemented with 2.5 μg of amphotericin B and 100 μg of gentamicin sulfate per ml. The cord was then perfused with 500 U of CLS II collagenase per ml in Dulbecco modified Eagle medium-RPMI 1640 medium supplemented with 2.5% bovine serum albumin containing 10% fetal bovine serum and was incubated at 37°C for 10 min. The umbilical cord was gently massaged and then perfused with RPMI 1640 medium supplemented with 20% fetal bovine serum. The resultant effluents were placed in a 15-ml tube and centrifuged at 500 × *g* for 10 min at 10°C. The supernatant was removed, and the pellet was suspended in complete medium which consisted of PRMI 1640 supplemented with 20% fetal bovine serum, 75 μg of heparin per ml, and 6.7 μg of retina-derived growth factor per ml (17). This cell suspension was placed in a 25-cm^2 tissue culture flask and incubated in a 5% CO_2 atmosphere at 37°C. Cells were fed every third day by exchanging existing media with new complete media. When confluent, cells were removed from the plate with a solution containing, in final concentrations, 0.05% trypsin, 0.02% EGTA, and 1% polyvinylpyrrolidone–0.9% NaCl in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (28). Characteristic of endothelial cells, virtually all cells contained factor VIII-related protein, revealed by a fluorescent-double-antibody test.

For each experiment, cells were seeded into 24-well (16-mm-diameter) plates at a cell density of 2.5 × 10^4 cells per well (nonconfluent) or 1 × 10^5 cells per well (confluent) and incubated at 37°C for 12 to 24 h prior to use. Each well contained 1 ml of complete medium. All experimental components were prepared in RPMI 1640 medium, sterilized by filtration, and added in a total volume of ≤100 μl. Control wells received an equal volume of RPMI 1640 medium only. Following incubation at 37°C in a 5% CO_2 atmosphere, medium was removed carefully from each well, and the remaining substratum-attached cells were released from the plate with 250 μl of a trypsin-EGTA solution containing 0.04% (wt/vol) trypan blue. The cells were enumerated with a hemacytometer or a Coulter Counter. All experiments were performed with triplicate or quadruplicate wells for each treatment mode.

Binding of ^{125}I-Shiga toxin to HUVEC. Purified Shiga toxin was iodinated to a specific activity of approximately 9.2 μCi/μg of protein by the immobilized-lactoperoxidase method (9). The biological activity of mock-iodinated Shiga toxin was unaltered. The binding assay was carried out as follows. To each well of a 24-well culture plate was added ^{125}I-Shiga toxin (5 μl, 10^6 cpm) in 0.5 ml of HUVEC complete medium, yielding a final concentration of approximately, 10^{-9} M toxin. The cells were incubated at 4 or 37°C for 60 min in a 5% CO_2 atmosphere. To terminate the reaction, we removed medium from the wells and washed each well three times with 1 ml of complete medium. Total cell protein was solubilized by incubation in 0.5 ml of 0.5 M sodium hydroxide at 37°C for 20 min. Radioactivity present in this solution was measured in a gamma counter. In some cases, before addition to cell cultures, ^{125}I-Shiga toxin was preincubated (37°C for 2 h) with either a 100-fold excess of unlabeled Shiga toxin or a 1:50 dilution of Shiga toxin anti-β-subunit monoclonal antibody.

Protein synthesis. To measure total cellular protein synthesis, we added [^{3}H]leucine (specific activity, 60 Ci/mmol) to complete medium in each well of either 24- or 96-well culture plates. After incubation for various times, each reaction was terminated with the addition of NaOH to 0.2 M. A 0.10 volume of 100% trichloroacetic acid solution was then added to each well. The resultant precipitate was collected on glass fiber filters and washed with 5% trichloroacetic acid. These filters were dried at 60°C for 30 min, placed in a scintillation vial with 5 ml of nonaqueous scintillant, and monitored for radioactivity.

Endotoxin. A *Limulus* amoebocyte lysate assay was used to determine bacterial endotoxin levels in cell culture components. The lower level of endotoxin detection was 0.01 ng/ml with this technique. A final estimate of endotoxin concentrations in the complete cell culture assays was based on the dilution of each component in the assays.

RESULTS

Effect of Shiga toxin on nonconfluent HUVEC. To test for a direct cytotoxic effect of Shiga toxin on HUVEC, we added the toxin to wells containing confluent monolayers or nonconfluent substratum-attached cells. The number of remain-

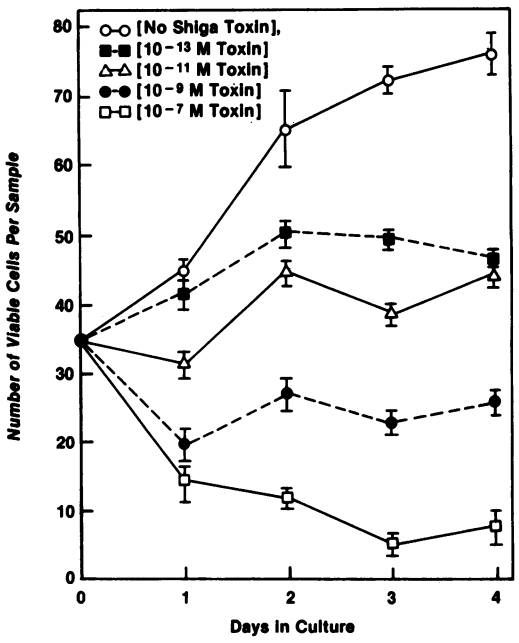

FIG. 1. Effect of Shiga toxin on nonconfluent HUVEC. Cells were incubated in complete medium in the presence or absence of Shiga toxin for the times indicated. The number of remaining viable substratum-attached cells in each well was then determined as described in Materials and Methods. Vertical bars represent the standard deviations of triplicate samples.

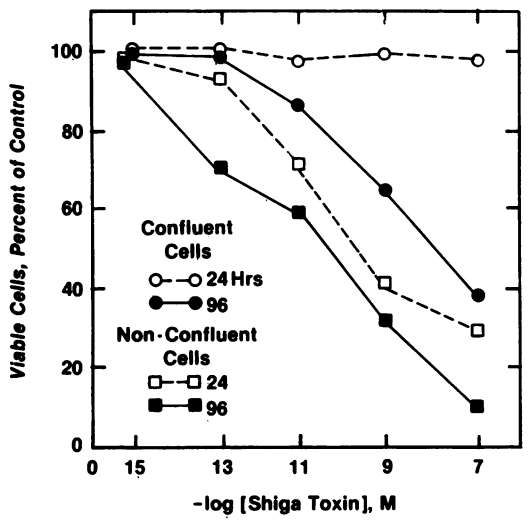

FIG. 2. Comparison of the effect of Shiga toxin on confluent and nonconfluent HUVEC. Different concentrations of Shiga toxin were added to cell cultures at time zero, and the number of viable attached cells was measured at either 24 or 96 h. The respective 100% values for 24- and 96-h samples of confluent cells were 150,000 and 291,200 cells per well, while the 100% values for nonconfluent cells were 29,000 and 76,500 cells per well.

ing viable attached cells was then monitored between 24 and 96 h. The results demonstrated that Shiga toxin added to actively dividing, nonconfluent HUVEC caused a time- and dose-dependent reduction in the number of viable attached HUVEC (Fig. 1 and 2). Cells continued to grow and divide at a reduced rate for 48 h in the presence of 0.1 pM or 0.01 nM Shiga toxin (Fig. 1). Thereafter, a constant cell number was maintained. Higher concentrations of Shiga toxin (1 and 100 nM) resulted in an abrupt reduction in viable cell number within 24 h (Fig. 1). Only in the absence of toxin did the nonconfluent HUVEC continue to increase in number over the 4-day period. All concentrations of Shiga toxin examined yielded a steady-state number of HUVEC beyond 48 h of incubation with the toxin (Fig. 1).

Differential toxin sensitivity of confluent and nonconfluent HUVEC. A clear distinction was observed between the effect of Shiga toxin on nonconfluent and confluent vascular endothelial cells. Typically, a 24-h incubation of toxin (1 nM) with nonconfluent HUVEC resulted in a 60% reduction in viable attached cells, while a similar incubation of toxin with confluent HUVEC was without effect (Fig. 2). Shiga toxin was a less potent inhibitor of confluent than of nonconfluent HUVEC at all incubation times examined. For example, the concentrations of toxin yielding 50% inhibition of the test system for a 96-h incubation with confluent HUVEC and a 24-h incubation with nonconfluent HUVEC were approximately 10 and 0.2 nM, respectively. This 50-fold difference in toxin sensitivity suggests that specific growth phases of HUVEC may recognize, process, or respond to Shiga toxin in a different manner.

TABLE 2. Binding of ^{125}I-Shiga toxin to confluent HUVEC at 37°C[a]

Addition(s) (nM)	cpm (%) of Shiga toxin bound
^{125}I-Shiga toxin (1)	238,175 (100)
^{125}I-Shiga toxin (1) plus unlabeled Shiga toxin (100)	30,187 (13)
^{125}I-Shiga toxin (1) plus 1:50-diluted antitoxin	4,009 (2)

[a] Assay conditions were as described in Table 1, footnote *a*, and Materials and Methods.

Binding of ^{125}I-Shiga toxin to HUVEC. To determine if the recognition of Shiga toxin by human vascular endothelial cells was specific and perhaps receptor mediated, we measured the binding of ^{125}I-toxin to HUVEC. ^{125}I-Shiga toxin (1 nM) bound efficiently at 4°C to both confluent and nonconfluent cells (Table 1). Toxin binding was saturable, and specificity was indicated by the decrease in binding in the presence of excess unlabeled Shiga toxin. On a per-cell basis, nonconfluent HUVEC bound twice as much toxin as did confluent HUVEC at 4°C (Table 1). Our results indicate that toxin bound at 4°C was not internalized by antitoxin antibody. These data suggest that the toxin sensitivity of nonconfluent and confluent HUVEC may not be due to the differential binding of toxin to these two cell types. This concept was further supported by additional data indicating that Shiga toxin could be internalized by confluent HUVEC. Incubation of confluent HUVEC with ^{125}I-Shiga toxin at 37°C resulted in a 72% increase in the amount of cell-associated toxin (Table 2) as compared with that observed during a similar incubation performed at 4°C (Table 1). Preliminary studies have shown that Shiga toxin bound to confluent HUVEC during incubation at 37°C for 1 h becomes refractory to neutralization by anti-Shiga toxin antibody protein. Such results have raised the possibility that protein synthesis is inhibited by Shiga toxin in nonconfluent HUVEC but not in confluent HUVEC.

Effect of Shiga toxin on protein synthesis in HUVEC. The rate of incorporation of [^{3}H]leucine into total cellular protein was measured in nonconfluent and confluent HUVEC. In the absence of Shiga toxin, the rate of protein synthesis in nonconfluent cells was twice that in confluent cells (Fig. 3). After 1 h in the presence of 10 nM Shiga toxin, protein synthesis was rapidly shut off in nonconfluent cells. In contrast, confluent HUVEC monolayers incubated with 10 nM Shiga toxin continued to synthesize protein at a rate 60% of the control rate observed in the absence of toxin (Fig. 3). These results indicate that protein synthesis in confluent HUVEC is partially refractory to Shiga toxin.

Another possible explanation for these results is that Shiga toxin preferentially inhibits the uptake of [^{3}H]leucine by nonconfluent HUVEC. That the latter is not responsible for our results was indicated by similar protein synthesis exper-

TABLE 1. Binding of ^{125}I-Shiga toxin to HUVEC at 4°C[a]

Addition(s) (nM)	cpm (%) of Shiga toxin bound to:	
	Confluent cells	Nonconfluent cells
^{125}I-Shiga toxin (1)	138,674 (100)	139,418 (100)
^{125}I-Shiga toxin (1) plus unlabeled Shiga toxin (100)	35,289 (25)	25,371 (18)

[a] Toxin was incubated for 1 h with 100,000 confluent or 50,000 nonconfluent cells per well. Cell-associated radioactivity was measured as described in Materials and Methods.

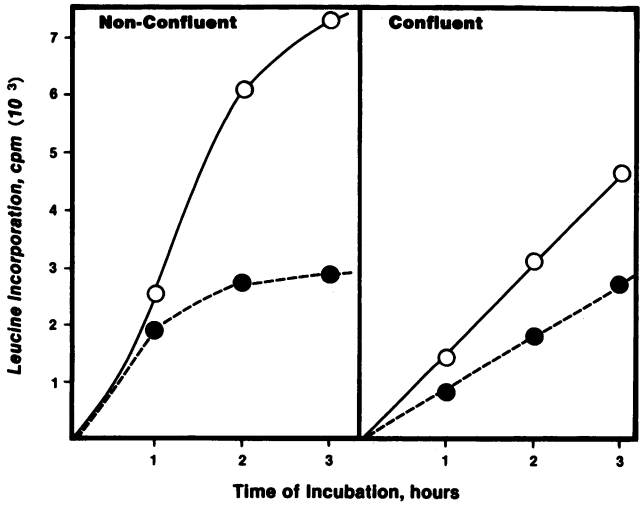

FIG. 3. Shiga toxin inhibition of human endothelial cell protein synthesis. HUVEC were seeded into 96-well culture plates at 4,000 cells (nonconfluent) or 16,000 cells (confluent) per well and incubated overnight at 37°C in a 5% CO_2 atmosphere. Shiga toxin was added to 10^{-8} M (●), while control wells received an equal volume of medium (○). Following a 1-h preincubation of cells with toxin, each well received 1 μCi of [^{3}H]leucine at time zero. At the times indicated, each reaction was terminated, and acid-insoluble radioactivity was collected mechanically onto filters for the measurement of [^{3}H]leucine incorporation into total cellular protein by liquid scintillation (see Materials and Methods). A background radioactivity of 400 cpm has been subtracted from all values. To assist in a direct comparison of ^{3}H incorporation by the two cell growth phases, we have normalized data for nonconfluent cells to represent radioactivity per 16,000 cells.

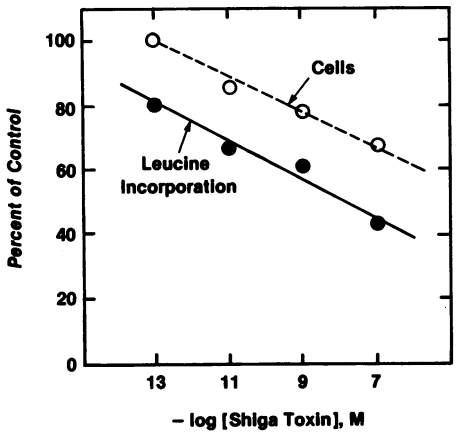

FIG. 4. Shiga toxin dose-response effect on protein synthesis in confluent HUVEC. Shiga toxin was added to confluent HUVEC in 24-well culture plates at time zero. At 60 h the cells in each well were pulsed with 25 μCi of [^{3}H]leucine for 2 h, and the incorporation of radioactivity into acid-insoluble protein was measured as described in Materials and Methods. The 100% values were 260,600 cells per well and 28,061 cpm per well.

iments conducted with HUVEC preincubated for 4 h with [^{3}H]leucine prior to the addition of Shiga toxin. Again, protein synthesis was completely inhibited 2 h after the addition of toxin to nonconfluent cells which had been equilibrated with [^{3}H]leucine (data not shown). Under the same conditions of preincubation with [^{3}H]leucine, confluent HUVEC incubated with toxin exhibited a steady rate of protein synthesis equal to 60 to 70% that of control (minus toxin) cells.

It should be noted that a partial inhibition of protein synthesis was also a characteristic of confluent HUVEC which had been incubated for a prolonged period (60 h) with Shiga toxin (Fig. 4). While a portion of the reduced rates of protein synthesis in this case was due to an overall decrease in the number of viable cells, there also appears to have been a toxin dose-dependent inhibition of protein synthesis in the remaining viable confluent cells. Thus, accumulated data indicate that Shiga toxin quickly elicits a slower rate of protein synthesis in confluent HUVEC (Fig. 3) under conditions in which cells remain fully viable for at least 24 h (Fig. 2), and the slower rate persists in these cultures through 60 h (Fig. 4).

Responsibility of Shiga toxin for the cytotoxic response. It was possible that the effect observed on the endothelial cell cultures was caused by some unknown minor contaminant in the toxin preparation. To test this possibility, we examined whether specific treatments known to destroy cytotoxic activity would eliminate the effect on endothelial cells. Firstly, heat-treated toxin was without cytotoxic activity. Preincubation of the purified toxin for 15 min at 90°C completely eliminated the cytotoxic response (Table 3). A similar preincubation at 37°C did not alter the activity of Shiga toxin in this assay. Secondly, pretreatment of toxin with trypsin, dithiothreitol, and urea reduced its cytotoxicity to zero (Table 3). This procedure has been used previously to "activate" or increase the cell-free protein synthesis inhibitory activity of Shiga toxin (4, 33). The activation procedure eliminates all biological activity of Shiga holotoxin on intact HeLa cells (37). Thirdly, antitoxin antibody neutralized the cytotoxic effects of Shiga toxin on HUVEC (Table 3). Preincubation of rabbit polyclonal anti-Shiga toxin immunoglobulin G protein with purified toxin completely eliminated the cytotoxic activity in cultures of HUVEC. Antibody alone was without effect in the assay system.

We also examined the possibility that bacterial endotoxin was responsible, partially or totally, for the observed cytotoxic effects. All components of the culture system, including Shiga toxin preparations, were measured for endotoxin content by the *Limulus* amoebocyte lysate assay. In all cases, our results indicated that final endotoxin concentrations in the HUVEC assay system were less than 0.1 ng/ml, taking into account the dilution of individual components in the assays. Studies from other laboratories indicate that this concentration of endotoxin has no effect on vascular endothelial cells in vitro (38).

In summary, as mentioned above, Shiga toxin was an active inhibitor of whole-cell protein synthesis in HUVEC cultures (Fig. 3 and 4). These data suggest that damage to

TABLE 3. Effect of toxin pretreatments on cytotoxic activity[a]

Pretreatment (nM)	Viable cells (% of control)
None	100
Shiga toxin (10) not treated	32
Shiga toxin (10) treated at 90°C for 15 min	141
Shiga toxin (10) plus 1:20-diluted antitoxin	101
Shiga toxin (10) plus trypsin, urea, and dithiothreitol	108

[a] Shiga toxin was pretreated as described and added to nonconfluent HUVEC at a 10 nM final concentration. Following incubation of the toxin with cells at 37°C for 24 h, HUVEC were processed as described in Materials and Methods.

human nonconfluent and confluent vascular endothelial cells is due primarily to Shiga toxin inhibition of protein synthesis.

DISCUSSION

The purpose of this study was to determine if Shiga toxin exerts direct cytotoxic activity on human vascular endothelial cells in cultures. The rationale for this study was based on observations by others that Shiga toxins may be responsible for the endothelial damage observed in hematologic complications following bacillary dysentery in humans (2, 6, 14, 22). The data presented here indicate that purified Shiga toxin does indeed have a direct cytotoxic effect on human vascular endothelial cells and that this activity is both toxin dose and time dependent. In addition, we have observed similar results with purified Shiga-like toxin 1 (verotoxin 1) from *Escherichia coli* (T. G. Obrig, P. J. Del Vecchio, M. A. Karmali, M. Petric, T. P. Moran, and T. K. Judge, Letter, Lancet ii:687, 1987). The cytotoxic effect is not due to endotoxin contaminants and is eliminated by treatments which specifically inactivate Shiga toxins.

It is generally accepted that the toxic actions of Shiga toxin are based on its ability to inhibit eucaryotic protein synthesis (11, 20, 33). A toxin dose-dependent reduction in protein synthesis was also a characteristic response of endothelial cells to Shiga toxin. In such cultures, the inhibition of protein synthesis appeared to be a more sensitive measure of cytotoxic effects than did counts of total viable substratum-attached cells. These data also suggest that pertubation of human endothelial protein synthesis may be responsible for the early developmental stages and onset of HUS.

Nonconfluent HUVEC are much more sensitive to both Shiga toxin and Shiga-like toxin than are confluent HUVEC. A delayed cytotoxic response by confluent cells was observed following their incubation with Shiga toxin for ≥48 h. There exist several possible explanations for the preferential toxin sensitivity of actively growing and dividing endothelial cells. An intriguing similarity exists between the results obtained with HUVEC cultures and those obtained with Shiga toxin-sensitive and Shiga toxin-resistant nonendothelial cell lines (10, 20). Many toxin-resistant nonendothelial cell lines remained capable of binding Shiga toxin with high affinity. In our case, both confluent and nonconfluent HUVEC efficiently bound Shiga toxin. Thus, it could be that confluent HUVEC are more resistant to the toxin than are nonconfluent HUVEC because of slow internalization of the toxin, inefficient intracellular processing of the toxin, or lower sensitivity of the protein synthesis mechanism. It also seems plausible that active cell division is a prerequisite for the expression of the maximum toxin sensitivity of target cells.

In nonconfluent HUVEC, protein synthesis is shut off completely within 2 h after the addition of toxin to these metabolically active cells. Therefore, it seems most likely that the cytotoxic activity of Shiga toxin on nonconfluent HUVEC is due to its effect on protein synthesis.

Confluent HUVEC respond to Shiga toxin with a steady but partially reduced rate of protein synthesis which appears to continue for 3 days or longer. Again, it is possible that Shiga toxin may be cytotoxic for confluent HUVEC because cell viability simply cannot be sustained under the pressure of reduced protein synthesis for prolonged periods. The data presented here indicate that cells in a confluent monolayer of human vascular endothelial cells remain fully viable for approximately 48 h in the presence of 10^{-7} M Shiga toxin, although overall protein synthesis rates are suppressed.

In humans, HUS commonly develops 3 to 4 days following the peak of bacterial dysentery. In relating our in vitro vascular endothelial cell data to the clinical situation, it seems logical to suggest that the onset of HUS could be a result of Shiga toxin-dependent changes in the synthesis of vascular endothelial cell components necessary to maintain the normal antithrombotic physiological state. If so, confluent endothelial cells may be involved primarily for the reasons discussed above. Confluent cells are the predominant endothelial cell type in human vasculature. However, one cannot also rule out a role for actively dividing nonconfluent endothelial cells in the development of HUS, as these cells are more sensitive to Shiga toxins than are confluent endothelial cells.

A perplexing result of the present study was the shallow Shiga toxin dose-response curves observed with endothelial cells. A similar but less exaggerated situation was described for the interaction of Shiga toxin with HeLa cells (12, 37). It was discovered that HeLa cell cultures consisted of a heterogeneous cell population with a ≥5-log toxin concentration response. Subsequent cloning of individual HeLa cells from the total population resulted in a series of cell lines, each with a defined sensitivity to Shiga toxin which was retained through further propagation (37). HUVEC may also consist of individual cells with different but defined toxin sensitivities.

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