Exocytotic Secretion of Toxins from Macrophages Infected with *Escherichia coli* O157

Osamu Shimada^{1,2,*}, Harunori Ishikawa², Hisami Tosaka-Shimada², and Saoko Atsumi¹

Department of Anatomy, ¹Yamanashi Medical University, Tamaho, Yamanashi 409-3821 and ²Gunma University School of Medicine, Maebashi, Gunma 371-8511, Japan

ABSTRACT. This study examined whether macrophages are involved in the development of pathogenicity in Shiga-like toxin (SLT)-producing enterohemorrhagic *Escherichia coli* (EHEC) O157:H7. Macrophages were infected with the bacteria, after which the macrophage culture medium showed a clear increase in toxicity in rats *in vivo* as well as in rat aortic endothelial cells *in vitro*. The increased toxicity resulted mainly from a rapid increase in the concentrations of SLT type I (SLT-I) and type II (SLT-II) and partly from an increase in concentrations of the proinflammatory cytokines, tumor necrosis factor α (TNF α) and interleukin-1 (IL-1), in the culture medium. Most of the EHEC O157 added to the macrophage culture were quickly incorporated to form phagosomes, which then fused with lysosomes to become phagolysosomes. During this intracellular digestion process, the EHEC O157 remained alive for about 15 min, and continued synthesizing and secreting the toxins SLT-I and SLT-II. The bacteria were then killed and digested in the phagolysosomes with significant amounts of the toxins retained. Subsequently, the contents of the phagolysosomes were exocytotically secreted from the macrophage cell membrane into the surrounding culture medium. Such a sequence of events in macrophages may occur *in vivo*, suggesting the active involvement of macrophages in the rapid increase in pathogenicity, such as seen in the onset of hemolytic-uremic syndrome (HUS) in patients infected with EHEC O157. The exocytotic secretion is considered to be one of the most basic cellular functions in macrophages.

Key words: Shiga-like toxins/*Escherichia coli* O157/macrophages/secretion/exocytosis

There have been several large outbreaks of food poisoning caused by enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 in the world (2, 33). The patients are at increased risk of developing life-threatening complications, particularly hemolytic-uremic syndrome (HUS) (2, 16, 26, 28, 29, 32). HUS is a clinical syndrome consisting of the triad of hemolytic anemia, thrombocytopenia and acute renal failure, the epidemic form of which starts with an acute, often bloody, gastroenteritis (2, 14, 16, 26, 28, 29, 32). Karmali *et al.* (15) were the first to associate this form of HUS with EHEC O157 infection. Several other investigators around the world have subsequently shown that EHEC O157 infection is responsible for most cases of HUS (2, 14, 16, 26, 28, 29, 32), though the mecha-

nism causing such sudden HUS is still unclear.

Although the mechanism by which EHEC O157 causes tissue or cell damage is not clear, it has been assumed that the damage occurs as a result of the spread of the bacterial products, Shiga-like toxins type I (SLT-I) and type II (SLT-II) (3), from the intestine to target organs (10). It has been reported that administration of SLT-I and/or SLT-II causes systemic symptoms in animals (1, 8, 18), and their cytotoxicity for several cultured cells has been confirmed (7, 20, 21, 22). After binding to cells, the toxin is endocytosed (13, 25, 30), binds to 60S ribosomes, and inhibits peptide chain elongation and protein synthesis (10, 24), thereby leading to cell death.

Macrophages are a very important part of the host defense system (11, 18, 19), and the lamina propria of the large intestine is rich in macrophages. When the intestinal epithelial cells are damaged and microorganisms penetrate the basement membrane, the macrophages may be the first line of the host defense system in the microbial infection such as EHEC O157. Hence, we

* To whom correspondence should be addressed: Department of Anatomy, Yamanashi-Medical University School of Medicine, Shimokawahigashi 1110, Tamaho, Yamanashi 409-3821, Japan.

Tel: +81-55-273-9474, Fax: +81-55-274-3552

E-mail: oshimada@swallow.res.yamanashi-med.ac.jp

examined the possibility that macrophages are involved in the development of pathogenicity of EHEC O157.

Materials and Methods

Isolation of EHEC O157:H7

EHEC O157:H7 used in the present experiment was isolated from feces of patients. Sorbitol-MacConkey agar plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) were used to isolate the bacterium (16, 28). The bacteria were grown aerobically at 37°C in tryptic soy broth (TSB) (Difco, Detroit, Mich., USA). Culture samples were examined for SLTs as described by Karmali *et al.* (16, 17). The bacterial serotype was determined using specific antibodies against EHEC antigens (Denka Seiken, Tokyo, Japan). Two different strains of *Escherichia coli*, DH1 and O126:H27, which are non-pathogenic and non-SLTs-secreting bacteria, respectively, were used as controls.

Macrophage, rAE and MDCK cell culture

Female Wistar rats weighing about 200 g were injected intraperitoneally with 2 ml of 3% thioglycollate medium (Wako Pure Chem., Osaka, Japan) that had been stored for two months, and four days later, the peritoneal exudate was harvested, along with the cells according to previous methods (34). The cells were suspended in Dulbecco's modified Eagle's medium (DMEM) (Flow Laboratories, Rickmansworth, UK) supplemented with 10% fetal bovine serum (FBS) (Gibco, Madison, WI, USA) but no antibiotics. The number of cells in the suspension was counted in a hemacytometer, adjusted to a density of 5×10^6 cells per ml, and then 1.0 ml of the suspension was placed on glass slides in a 9-cm-diameter Petri dish (Flow Laboratories). After 3 hours of incubation at 37°C under a 5% CO₂ atmosphere, each glass slide was washed three times with fresh DMEM to remove non-adherent cells. The adherent cell population consisted of more than 95% macrophages, as shown by specific staining with anti-rat macrophage antiserum (Inter-Cell Technologies, Hopewell, NJ, USA). The macrophages showed about 80% confluence after 2 days of incubation, at which point the culture medium was exchanged for the infection medium (DMEM lacking serum and antibiotics). After a further 24 hour-incubation, the macrophage culture was used for the following experiments.

The rat aortic endothelial (rAE) cells were isolated by outgrowth from rat aortic rings exactly as described (23). Finally, the cells were placed on glass slides in RPMI 1640 medium (Sigma Aldrich Japan, Tokyo, Japan) with 10% FBS, 1 µg/ml of endothelial cell growth factor (Sigma Aldrich) and 10 µg/ml of endothelial cell growth supplement (Sigma Aldrich). The cells were incubated at 37°C in a 5% CO₂ atmosphere and fed every 48 hours by replacing the existing medi-

um with fresh medium. MDCK cells were cultured using DMEM with 10% FBS. When both types of cell had almost reached a confluent population, they were used for the following experiments. Cell viability was measured by the procedure of Riddell *et al.* (27).

Infection with EHEC O157

The wild bacteria isolated from the fecal samples were grown in TSB, then washed three times in order to remove SLTs. After the final wash, the pellet was resuspended in DMEM to give a final concentration of 5×10^8 bacteria per ml. To synchronize the infection process, 1 ml of the bacterial suspension was placed on each glass slide; then the slide was centrifuged at 700 g for one min. After washing well, the fresh culture medium was added and the macrophage cell culture was returned to the incubator under a 5% CO₂ atmosphere. We defined the moment when the medium was finally washed as time 0 medium for assay. Giemsa staining of macrophages showed that about 85% of cells contained bacteria 15 min after infection. In the present experiment, all macrophages remained alive for at least 24 hours.

Antibodies

Synthetic peptides composed of the 20-amino acid sequences corresponding to the N- and C-terminal residues of the A subunits of SLT-I and SLT-II were separately prepared (3). According to previously described methods (5), monoclonal antibodies against SLT-I and SLT-II were obtained from mice immunized with each antigen. Commercially available monoclonal antibodies against SLT-I and SLT-II were also used (Toxin Tech., Sarasota, FL, USA). Polyclonal antibodies against mouse TNFα and mouse IL-1α were purchased commercially (Genzyme, Cambridge, MA, USA), and their cross-reactivities with rat TNFα and rat IL-1α, respectively, were confirmed to be almost 100% in our laboratory. Polyclonal antibody against acid phosphatase was purchased (Chemicon Int. Inc., Temecula, CA, USA), as was a monoclonal antibody against rat IL-1β (Genzyme). Preneutralization of the macrophage culture medium was performed by incubation for one hour with an adequate amount of each antibody: 20 µg/ml immunoglobulin for SLT-I and -II, 5 µg/ml immunoglobulin for IL-1β, and 100 µl/ml antisera for TNFα and IL-1α.

SLTs and cytokines assay

Macrophage culture supernatants which had been filter-sterilized and diluted 1:10³ to 1:10⁵ with PBS were assayed by individual enzyme-linked immunosorbent assays (ELISA) for SLT-I (5), SLT-II (5), TNFα, IL-1α and IL-1β. For SLT-I and SLT-II assays, a previously reported bioassay (16, 20) was also performed to confirm that the toxins released were active, and these results were compared with those of the ELISA. Briefly, the macrophage culture supernatants were

preneutralized with anti-SLT-I or -SLT-II antibody, filter-sterilized, added to 96-well plates containing about 75% confluent Vero cells (at a density of 2×10^4 cells per well), and then incubated overnight in a 5% CO₂ atmosphere. The cells were examined for cytopathologic effects and detachment during the next two days.

Electron microscopy

For electron microscopic analysis, macrophage cell cultures on glass slides were rapidly fixed with 2.5% acrolein in 0.01 M PBS, pH 7.2, at 37°C for 10 min and then at 4°C for 2 hours, and post-fixed with 1% osmium tetroxide for 1 hour at 4°C. After fixation, the cells were dehydrated with a graded ethanol series, embedded in Epon 812, and then cured for 3 days. Thin sections were cut and treated with 3% hydrogen peroxide for 10 min, according to the method reported previously (31). In order to block aldehyde formation, thin sections on grids were floated in a blocking solution, Tris-glycine buffer (0.1 M, pH 7.2) containing 1% freshly prepared borohydride (Wako Pure Chem.) and 1% bovine serum albumin (BSA), for 10 min. Thin sections were treated with 1% normal goat serum for 1 hour, and then incubated with the required anti-SLT-I or -SLT-II immunoglobulin (1 ng/ml) in PBS, pH 7.4, containing 1% BSA. After rinsing with PBS, the sections were incubated for 1 hour with 10-nm colloidal gold-labelled goat anti-mouse IgG immunoglobulin (1:200 dilution) (British Biocell Int., Cardiff, UK), followed by rinsing with PBS and then with distilled water. The sections were stained with uranyl acetate and lead citrate, and then examined with an electron microscope (Hitachi H-800, Hitachi, Japan). The specificity of each immunoreaction was confirmed by a preadsorption test involving the addition of excess SLTs to the first antibody.

Results and Discussion

The supernatant of culture medium of the infected macrophages was tested for its toxicity in rats. As a result, a clear increase in toxicity was recognized in rats *in vivo* as well as in rat aortic endothelial (RAE) cells *in vitro* (Fig. 1). After intraperitoneal injection (1.5 ml) of the infected macrophage culture medium (sterilized supernatant), all rats showed gastrointestinal, neurologic and systemic symptoms, which were consistent with previous reports (8, 12, 17, 18), and almost all the rats died within 5 days. This toxicity was reduced to basal levels by preneutralization of the culture medium with a mixture of antibodies against SLT-I and SLT-II (Fig. 1A). In contrast to macrophages, the culture medium of MDCK cells, which had receptors for SLTs on the cell surface (25), had no significant effect on the rats. These results provide evidence for the active participation of macrophages in SLT-induced lethality in rats. The toxicity appeared to be slightly reduced when the macro-

phage culture medium was pretreated with a mixture of antibodies against TNF α and IL-1 α , β , but not when treated with normal animal immunoglobulin. Concerning the effects of cytokines, the present data agree with the results of previous studies (1, 9, 19, 31), which found that cytokines showed synergistic toxic effects of SLTs and increased expression of SLT receptors on

Fig. 1. Toxic effects of the culture medium of macrophages infected with EHEC O157 in live rats (A) and isolated rat aortic endothelial cells (B). (A) After 3 hours of infection 1.5 ml of the culture medium was injected intraperitoneally into male Wistar rats weighing about 200 g. Before injection, the supernatant of the culture medium was sterilized by double filtration (0.45 and 0.10 μ m filters). Preneutralization using a mixture of monoclonal antibodies against SLT-I and SLT-II causes complete diminution of toxic activity in rats. Preneutralization using a mixture of antibodies against the cytokines TNF α , IL-1 α and IL-1 β also decreases lethality. As a result, the culture medium of MDCK cells infected with the bacteria produces no lethal effect. The denominator shows the number of injected animals and the numerator that of surviving animals for 5 days after the infection. Significant difference was confirmed by χ^2 analysis ($P < 0.05$). (B) Cytotoxic effect of the macrophage incubation medium which was treated with EHEC O157 for 3 hours on the viability of rat aortic endothelial cells *in vitro*. The addition of macrophage culture medium produces a dose-dependent cytotoxic effect on the endothelial cells. Three different amounts of the culture medium were added to 1.5 ml medium in total per dish. Preneutralization by adding a mixture of SLT-I and SLT-II antibodies resulted in no significant cytotoxic activity against the endothelial cells. Data are shown as means $\pm$ SEM of the results of 10–12 experiments. MDCK/O157; culture medium of MDCK cells infected with EHEC O157; Mac/NPEC; culture medium of macrophages infected with non-pathogenic *Escherichia coli*; Mac/O157; culture medium of macrophages infected with EHEC O157; +anti-cytokines; preneutralization with a mixture of anti-cytokines antibodies; +anti-SLTs; preneutralization with a mixture of anti-SLT-I and SLT-II antibodies.

some types of cells.

The cytotoxic activity of macrophage culture medium (sterilized supernatant) was measured using isolated rAE cells (23) (Fig. 1B). Cell viability was dramatically decreased after the addition of the culture medium. This cytotoxic activity was minimized by preneutralization of the supernatants with a mixture of antibodies against SLT-I and SLT-II. In cases where higher concentrations of macrophage culture medium (0.05 ml and 0.25 ml) were added, preneutralization with a mixture of antibodies against TNF α and IL-1 α , β resulted in a weak but significant lowering of the SLTs-induced cytotoxic effect on the viability of rAE cells ($P < 0.01$ by Welch's t-test).

Figure 2 shows the kinetics of the concentrations of SLT-I (A), SLT-II (B), TNF α (C) and IL-1 α , β (D) in the culture medium of macrophages infected with EHEC O157. Interestingly, a rapid and clear increase in SLT concentrations was observed between 15 and 60 min after infection, although the number of free bacteria fell due to phagocytosis of macrophages. When cultured without macrophages, EHEC O157 secreted both SLT-I and SLT-II into the culture medium, but the rate of increase was about 10 times less than that observed in the presence of macrophages from 15 to 60 min after the beginning of incubation. Such a clear increase in toxin concentrations was ever found in the culture medium of MDCK cells (25) infected with EHEC O157 (Fig. 2A, B). Two possibilities are conceivable: 1) macrophages may stimulate the secretion of SLTs from the bacteria, or 2) macrophages may directly secrete SLTs (produced by the bacteria). Compared with the kinetics of SLT increase, the increase in TNF α and IL-1 α , β in the macrophage culture medium was slower, and their release from macrophages continued for at least 24 hours after infection.

Electron microscopy showed that macrophages infected with EHEC O157 developed characteristic morphological features (Fig. 3). The digestion process consisted of three steps: 1) engulfment of bacteria and phagosome formation, 2) phagolysosome formation (35) and bacterial killing/digestion, and 3) secretion of incompletely digested materials from phagolysosomes into the surrounding culture medium. On immunoelectron microscopy to detect SLT-I or SLT-II, no significant numbers of immunogold particles were observed in macrophages fixed at the onset of EHEC O157 infection, but a clear increase of gold particles in the lumen of phagosomes was apparent later (Fig. 3A, C). The immunolabelling was confined within the phagosomes and phagolysosomes. In phagosomes, the EHEC O157 continued synthesizing and secreting SLT-I and SLT-II for about 15 min (Fig. 3A, C). No gold particles were significantly localized in the cytoplasm outside phagosomes and phagolysosomes at the bacteri-

Fig. 2. The kinetics of SLT-I (A), SLT-II (B), TNF α (C), IL-1 α (D) and IL-1 β (D) concentrations in macrophage culture medium after the infection with EHEC O157 (closed circles in A, B and C). The culture media of MDCK cells infected with the bacteria were used as controls (open circles in A and B). All results were obtained by ELISA using specific antibodies, though a bioassay was also used for determination of SLT concentrations, showing results similar to those obtained by ELISA. Data are shown as means $\pm$ SEM of the results of 5–12 experiments. Note a rapid and clear increase in SLTs concentration between 15 and 60 min after infection. The patterns of increase in the SLT contents are quite different from those of cytokines, TNF α , IL-1 α and IL-1 β .

al density used, and all macrophages remained alive for at least 24 hours (12, 27). In the present experiment, the phagolysosomes were identified by the content of acid phosphatase. Interestingly, the macrophages then exocytotically secreted incompletely digested substances in the phagolysosomes, including a significant amount of both SLT-I and SLT-II (Fig. 3B, D).

It has generally been believed that macrophages utilize digested materials in lysosomes for protein synthesis *en route* to the Golgi apparatus and rough endoplasmic reticulum in cells, or that macrophages become "foreign-body giant cells" and sometimes die when the digested materials are very large and/or toxic (6). However, in the case of EHEC O157 incorporated by macrophages, SLT-I and SLT-II synthesized by the bacteria are released into phagosomes and phagolysosomes, and then exocytotically secreted from the macrophage plasma membrane; thus the macrophages remain alive. In accordance with the morphological features of the toxin secretion from macrophages (Fig. 3), SLT concentrations in the culture medium were clearly and sharply increased from 15 min to 60 min after the bacterial infection (Fig. 2A, B).

To examine effect of phagocytosis on the production of the toxins by EHEC O157, macrophages were pre-treated with cytochalasin D (5 μ g/ml), which inhibits actin polymerization (4), for 20 min. The phagocytosis of bacteria was significantly blocked and the increase in

Fig. 3. Immunoelectron micrographs of SLT-II localization in macrophages 10 min (A and C) and 30 min (B and D) after the infection with EHEC O157. (A) Immunogold particles are mainly confined within the macrophage phagosomes, suggesting that the incorporated EHEC O157 continues secreting SLT-II. 10,000 $\times$. (B) Incorporated bacteria were killed and digested in phagolysosomes (L). Significant amounts of SLT-II are seen in all phagolysosomes. Some of the remaining contents in phagolysosomes are exocytotically secreted. 10,000 $\times$. (C) Higher magnification of part of the cell shown in A (arrowhead in A). Note that immunogold particles of SLT-II are found around the bacterial bodies in phagosomes. 23,000 $\times$. (D) Higher magnification of part of the cell shown in B (arrowhead in B). A number of immunogold particles are found in exocytotic materials. 23,000 $\times$. The same results were obtained using the antibody against SLT-I. *, incorporated EHEC O157; L, phagolysosome.

concentrations of SLT-I and SLT-II in the infected macrophage culture medium was markedly inhibited (Fig. 4), as compared with the controls without the drug.

The present study shows that EHEC O157 remains alive for about 15 min in the phagosomes in a macrophage, since they are able to synthesize SLTs. Moreover, the digestion and killing functions of macrophages cause a rapid secretion of high dose toxins, together with certain cytokines. The sequence of events observed in macrophages *in vitro* — phagocytosis, digestion and secretion of SLTs — may be applicable to what occurs in the human colon in patients infected with EHEC O157. As a result, during the bacterial digestion process in macrophages, bacteria can produce considerable amounts of SLTs, and the toxin content increases in the tissue. It is likely that the tissue damage caused by the SLTs released by macrophages may allow certain inflammatory mediators to gain access to the circulation (22) and attract more macrophages, thus forming a vicious circle. As a result, the functions of macrophages may cause development of acute clinical conditions such as the onset of HUS.

During the present experiment, all infected macrophages could survive, probably because toxins were confined within the phagosomes and phagolysosomes. If the contents leaked, the toxins released by the bacteria would diffuse throughout the cytoplasm, suppressing protein synthesis in the macrophage and causing macrophage cell death (7, 21). Although the internal-

Fig. 4. Effects of cytochalasin D on SLT-I and SLT-II concentrations in the cell culture medium of macrophages and MDCK cells infected with EHEC O157 for 60 min. Compared with the controls without cytochalasin D (—), the increase in concentrations of SLT-I and SLT-II in the infected macrophage culture medium is clearly inhibited by the treatment with the drug (+). Concentrations of the toxins in the culture medium of MDCK cells are very low and not significantly different (n.s.) with or without cytochalasin D. Data are shown as means $\pm$ SEM of the results of 5–12 experiments. Statistical significance was evaluated with Welch's t-test.

ized O157 remained alive for about 15 min and could secrete toxin, the toxin distribution was restricted to the lumen of lysosomes or outside the cell membrane. Before being damaged seriously, the macrophage may secrete the toxins from the cell, and simultaneously gather other immune cells by the secreted cytokines. Thus, the sequence of events observed in macrophages are considered to be one of the basic cellular functions for macrophage survival.

Although EHEC O157 were not invasive, the possibility that intestinal epithelial cells are damaged and that microorganisms penetrate the basement membrane cannot be neglected. In the case of bacterial invasion, macrophages may be the first line of the host defense system. Additional experiments are required in order to clarify whether the same events happen in the intestinal mucous membrane *in vivo*.

Acknowledgments. We thank Drs. S. Fujimoto and Y. Ike, Dept. of Microbiology, Gunma University School of Medicine, Gunma, Japan, and Dr. S. Kurashige, Department Laboratory Science, Gunma University School of Health Science, for their valuable suggestions and comments.

References

1. BARRETT, T.J., POTTER, M.E., and STROCKBINE, N.A. 1990. Evidence for participation of the macrophage in Shiga-like toxin II-induced lethality in mice. *Microbiol. Pathogenesis*, **9**: 95–103.
2. BOYCE, T.G., SWERDLOW, D.L., and GRIFFIN, P.M. 1995. *Escherichia coli* O157:H7 and the hemolytic-uremic syndrome. *N. Engl. J. Med.*, **333**: 364–368.
3. CALDERWOOD, S.B., AUCLAIR, F., DONOHUE-ROLFE, A., KEUSCH, G.T., and MEKALANOS, J. 1987. Nucleotide sequence of the Shiga-like toxin genes of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA*, **84**: 4364–4368.
4. COOPER, J.A. 1987. Effects of cytochalasin and phalloidin on actin. *J. Cell Biol.*, **105**: 1473–1478.
5. DONOHUE-ROLFE, A., KELLEY, M.A., BENNISH, M., and KEUSCH, G.T. 1986. Enzyme-linked immunosorbent assay for Shiga toxin. *J. Clin. Microbiol.*, **24**: 65–68.
6. FAWCETT, D.W. 1986. *A Textbook of Histology*, 11th ed. W.B. Saunders Company, Philadelphia, PA.
7. FERNANDEZ-PRADA, C., TALL, B.D., ELLIOTT, S.E., HOOVER, D.L., NATARO, J.P., and VENKATESAN, M.M. 1998. Hemolysin-positive enteroaggregative and cell-detaching *Escherichia coli* strains cause oncosis of human monocyte-derived macrophages and apoptosis of murine J774 cells. *Infect. Immun.*, **66**: 3918–3924.
8. FUJII, J., KINOSHITA, Y., KITA, T., HIGURE, A., TAKEDA, T., TANAKA, N., and YOSHIDA, S. 1996. Magnetic resonance imaging and histopathological study of brain lesions in rabbits given intravenous verotoxin 2. *Infect. Immun.*, **64**: 5053–5060.
9. GOOSNEY, D.L., CELLI, J., KENNY, B., and FINLAY, B.B. 1999. Enteropathogenic *Escherichia coli* inhibits phagocytosis. *Infect. Immun.*, **67**: 490–495.
10. GRIFFIN, P.M. 1995. *Escherichia coli* O157:H7 and other enterohemorrhagic *Escherichia coli*. In *Infections of the Gastrointestinal Tract* (M.J. Blaser, P.D. Smith, J.I. Ravdin, H.B. Greenberg, and R.L. Cuerrant, eds.). Raven Press Ltd. New York, NY, pp.739–761.
11. HUME, D.A. and DOE, W.F. 1988. Role of macrophages in cellular defense. In *Immunology of the Gastrointestinal Tract and the Liver* (M.F. Heyworth and A.L. Jones, eds.). Raven Press, New York, NY, pp.23–45.
12. ISOGAI, E., ISOGAI, H., KIMURA, K., HAYASHI, S., KUBOTA, T., FUJII, N., and TAKESHI, K. 1998. Role of tumor necrosis factor alpha in gnotobiotic mice infected with an *Escherichia coli* O157:H7 strain. *Infect. Immun.*, **66**: 197–202.
13. JOHANNES, L., TENZA, D., ANTONY, C., and GOUD, B. 1997. Retrograde transport of KDEL-bearing B-fragment of Shiga toxin. *J. Biol. Chem.*, **272**: 19554–19561.
14. KAPLAN, B.S., CLEARY, T.G., and OBRIG, T.G. 1990. Recent advances in understanding the pathogenesis of the hemolytic uremic syndromes. *Pediatr. Nephrol.*, **4**: 276–283.
15. KARMALI, M.A., STEELE, B.T., PETRIC, M., and LIM, C. 1983. Sporadic cases of haemolytic-uraemic syndrome associated with faecal cytotoxin and cytotoxin-producing *Escherichia coli* in stools. *Lancet*, **1**: 619–620.
16. KARMALI, M.A., PETRIC, M., LIM, C., FLEMING, P.C., ARBUS, G.S., and LIOR, H. 1985. The association between idiopathic hemolytic uremic syndrome and infection by verotoxin-producing *Escherichia coli*. *J. Infect. Dis.*, **151**: 775–782.
17. KARMALI, M.A. 1989. Infection by verocytotoxin-producing *Escherichia coli*. *Clin. Microbiol. Rev.*, **2**: 15–38.
18. KARPAN, D., CONNELL, H., SVENSSON, M., SCHEUTZ, F., ALM, P., and SVANBORN, C. 1997. The role of lipopolysaccharide and Shiga-like toxin in a mouse model of *Escherichia coli* O157:H7 infection. *J. Infect. Dis.*, **175**: 611–620.
19. KESHAV, S. and GORDON, S. 1990. Macrophage function in the gastrointestinal tract. In *Inflammation in the Gastrointestinal Tract*. (T. Peters, ed.). Comers Publications, Hull.
20. KONOWALCHUK, J., SPEIRS, J.I., and STAVRIC, S. 1977. Vero response to a cytotoxin of *Escherichia coli*. *Infect. Immun.*, **18**: 775–779.
21. LAI, X.H., XU, J.G., MELGAR, S., and UHLIN, B.E. 1999. An apoptotic response by J774 macrophage cells is common upon infection with diarrheagenic *Escherichia coli*. *FEMS Microbiol. Lett.*, **172**: 29–34.
22. LOUISE, C.B. and OBRIG, T.G. 1991. Shiga toxin-associated hemolytic-uremic syndrome: combined cytotoxic effects of Shiga toxin, interleukin-1 β , and tumor necrosis factor alpha on human vascular endothelial cells in vitro. *Infect. Immun.*, **59**: 4173–4179.
23. MCGUIRE, P.G. and ORKIN, R.W. 1987. Isolation of rat aortic endothelial cells by primary explant techniques and their phenotypic modulation by defined substrata. *Lab. Invest.*, **57**: 94–105.
24. OBRIG, T.G., MORAN, T.P., and BROWN, J.E. 1987. The mode of action of Shiga toxin on peptide elongation of eukaryotic protein synthesis. *Biochem. J.*, **244**: 287–294.
25. SANDVIG, K., PRYDZ, K., RYD, M., and VAN DEURS, B. 1991. Endocytosis and intracellular transport of the glycolipid-binding ligand Shiga toxin in polarized MDCK cells. *J. Cell Biol.*, **113**: 553–526.
26. PICKERING, L.K., OBRIG, T.G., and STAPLETON, F.B. 1994. Hemolytic-uremic syndrome and enterohemorrhagic *Escherichia coli*. *Pediatr. Infect. Dis.*, **13**: 459–476.
27. RIDDELL, R., CLOTHER, R., and BALLS, M. 1986. An evaluation of three in vitro cytotoxicity assays. *Food Chem. Toxicol.*, **24**: 469–471.
28. RILEY, L.W., REMIS, R.S., HELGERSON, S.D., MCGEE, H.B., WELLS, J.G., DAVIS, B.R., HEBERT, R.J., OLCOTT, E.S.,

- JOHNSON, L.M., HARGRETT, N.T., BLAKE, P.A., and COHEN, M.L. 1983. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *N. Engl. J. Med.*, **308**: 681–685.
29. ROWE, P.C., ORRBIN, E., LIOR, H., WELLS, G.A., and MCLANE, P.N. 1993. A prospective study of exposure to verotoxin-producing *Escherichia coli* among Canadian children with haemolytic uremic syndrome. *Epidemiol. Infect.*, **110**: 1–7.
30. SANDVIG, K., PRYDZ, K., RYD, M., and DEURS, B.V. 1992. Retrograde transport of endocytosed Shiga toxin to the endoplasmic reticulum. *Nature*, **358**: 510–512.
31. SHIMADA, O., ISHIKAWA, H., TOSAKA-SHIMADA, H., YASUDA, T., KISHI, K., and SUZUKI, S. 1998. Detection of deoxyribonuclease I along secretory pathway in Paneth cells of human small intestine. *J. Histochem. Cytochem.*, **46**: 833–840.
32. SIEGLER, R.L., PAVIA, A.T., CHRISTOFFERSON, R.D., and MILLIGAN, M.K. 1994. A 20-year population-based study of post-diarrheal hemolytic uremic syndrome in Utah. *Pediatrics*, **94**: 35–40.
33. TAKEDA, Y. 1997. Enterohaemorrhagic *Escherichia coli*. *World Health Stat. Q.*, **50**: 74–80.
34. TESH, V.L., RAMEGOWDA, B., and SAMUEL, J.E. 1994. Purified Shiga-like toxins induce expression of proinflammatory cytokines from murine peritoneal macrophages. *Infect. Immun.*, **62**: 5085–5094.
35. ZIMMERLI, S., MAJEED, M., GUSTAVSSON, M., STENDAHL, O., SANAN, D.A., and ERNST, J.D. 1996. Phagosome-lysosome fusion is a calcium-independent event in macrophages. *J. Cell Biol.*, **132**: 49–61.

(Received for publication, July 27, 1999

and in revised form, August 24, 1999)