J. Med. Microbiol. — Vol. 49 (2000), 905–910
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ISSN 0022-2615

MICROBIAL PATHOGENICITY

Effects of interaction between *Escherichia coli* verotoxin and lipopolysaccharide on cytokine induction and lethality in mice

KURI SUZUKI, KAZUHIRO TATEDA*, TETSUYA MATSUMOTO*, FUMIO GONDAIRA†, SHIROU TSUJIMOTO‡ and KEIZO YAMAGUCHI*

*First Department of Urology and Departments of *Microbiology and ‡Pathology, Toho University School of Medicine, 5–21–16 Ohmorinishi, Ohtaku, Tokyo 143–8540 and †Denkaseiken Co. Ltd. Gosenshi, Minami-motomachi 1–2–2, Niigata 959–16, Japan*

In *Escherichia coli* O157 infections, verotoxins (VT) play a critical role in causing the disease, although other factors such as lipopolysaccharide (LPS) and inflammatory cytokines may affect the progression and course of the disease. The present study examined the roles of VT and LPS in induction of serum cytokines and lethality in mice. LD50 of VT2 (13 ng) was *c.* 10^4-fold smaller than that of LPS (400 μg). Although the lethal toxicity of these toxins was examined in several experimental conditions, such as VT2 (5, 10, 20, 40 ng/mouse) alone or in combination with LPS (100 μg/mouse) at various times (-2 days to $+2$ days), no evidence of synergy was observed. VT2 did not augment LPS-induced tumour necrosis factor-α (TNF-α) or interleukin-6 production, and conversely suppressed TNF-α production when it was injected 2 days before LPS challenge. The data failed to indicate either synergic or additive effects of VT and LPS on cytokine production or lethality in mice. In contrast, antagonistic interactions were clearly observed in cytokine production in certain conditions. The results suggested that these toxins may be co-operatively involved in the pathology of VT-related diseases, but not through synergic interactions.

Introduction

Escherichia coli O157 is associated with outbreaks of diarrhoea, haemorrhagic colitis and haemolytic uraemic syndrome (HUS) [1, 2]. This serotype belongs to the family of enterohaemorrhagic *E. coli*, which produces several virulence factors, such as intimin [3], haemolysins [4], lipopolysaccharide (LPS) and verotoxins (VT) [5]. In particular, the last two factors are crucial in the pathogenesis of HUS. The pro-inflammatory cytokines, tumour necrosis factor (TNF) and interleukin-1 (IL-1), have been shown to affect the severity and pathophysiology of VT-associated disease [6, 7].

It is well known that LPS is capable of stimulating the induction of cytokines *in vitro* and *in vivo*, whereas the activity of VT is controversial. Tesh *et al*. [8] reported that VT induced expression of pro-inflammatory cytokines from murine peritoneal macrophages, whereas Harel *et al*. [9] showed that intraperitoneal injection of VT in mice did not induce the production of serum TNF. Several investigators have shown that TNF and IL-1 induce expression of the VT receptor (globotriacylceramide, Gb3) on human endothelial cells *in vitro* [6, 7], which may sensitise hosts to VT, although the exact interactions and co-operative roles of VT, LPS and cytokines *in vitro* remain to be clarified. CD-18-mediated leucocyte accumulation and adherence to the endothelium was suggested to be a critical event in the development of HUS [10, 11].

The present study examined the in-vivo effects of VT and LPS on the induction of pro-inflammatory cytokines, such as TNF-α, IL-1 and IL-6, and lethality in mice.

Materials and methods

Animals

BALB/c mice (5–6 weeks old, male, specific pathogen-free) were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan). Mice were housed in

Received 14 Sept. 1999; revised version accepted 8 March 2000.
Corresponding author: Dr K. Tateda (e-mail: kazu@med.toho-u.ac.jp).

groups of 10 and were provided with food and water *ad libitum*. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Toho University Medical School.

Toxin preparation

Verotoxins 1 and 2 were purified from cell lysates of *E. coli* strain 87–27 and *E. coli* strain Tp8, respectively, as described previously with minor modifications [12]: ammonium sulphate 60% precipitation followed by DEAE-sepharose column chromatography with a gradient elution of 0–0.4 M NaCl in 10 mM Tris-HCl (pH 8.5); Q-sepharose High Performance column (Amersham Pharmacia Biotech, Uppsala, Sweden) chromatography with a gradient elution of 0–0.4 M NaCl in 10 mM Tris-HCl (pH 8.5); gel filtration on TSK-gel G30000SW_{XL} (Tosoh, Tokyo, Japan). Endotoxin content in VT2 was determined by a commercial *Limulus* test (Endospecy ES-6, Seikagaku, Tokyo, Japan) as 90 pg of LPS/1 μg of VT2. *E. coli* O55:B5 LPS was purchased from Difco Laboratories (Detroit, MI, USA) and was diluted before use to the desired concentration with pyrogen-free saline.

Determination of LD50 of VT2 and LPS

Mice were inoculated intraperitoneally (i.p.) or intravenously (i.v.) with two-fold dilutions of purified VT2 (0.16–400 ng/mouse) or LPS (100–800 μg/mouse) and cumulative mortality was then monitored over the next 7 days.

Effect of LPS and VT2 on mortality rate

A protocol involving the i.p. administration of various concentrations of VT2 (5, 10, 20 and 40 ng) to mice in combination with sub-lethal LPS (100 μg), which was injected i.p. 48 h earlier, simultaneously, 24 or 48 h after VT2 administration was followed. In some experiments, different doses of LPS (100–800 μg) were administered simultaneously with 0.8 ng of VT2/mouse and changes in the mortality rate were examined.

Serum pro-inflammatory cytokines

Mice (n = 5) were inoculated i.p. with 100 μg of LPS or 20 ng of VT2 alone or in combination with LPS. Blood samples were collected by cardiac puncture at 0, 1.5, 3, 6, 24, 48 h after inoculation. Blood was allowed to clot at 4°C for 2 h and centrifuged to obtain serum samples that were stored in divided volumes at −80°C before being used for cytokine assays. The concentrations of TNF-α, IL-6 and IL-1β in sera were determined with commercially available ELISA kits (Biosource International, Camarillo, CA, USA).

Histological studies

Mice were inoculated i.p. with 100 ng (*c.* 10 × LD50) of purified VT2 or 100 μg of LPS alone or in a combination of the two. They were then killed at 1, 2, or 3 days after injection. The colon, liver and kidneys were surgically removed and examined for pathological changes. Tissues were then fixed in formalin 10% and routinely processed. Sections of paraffin-embedded tissues were stained with haematoxylineosin and examined by light microscopy.

Statistical analysis

Student's *t* test was used to compare the mean values of different groups and the χ^2 test was used to compare survival rates; $p < 5\%$ was accepted as statistically significant.

Results

LD50 of VT_2 and LPS

Groups of five mice were inoculated i.p. or i.v. with two-fold dilutions of purified VT_1, VT_2 or LPS. The LD50s of VT2 (i.v. and i.p.) were *c.* 1.5 ng and 13 ng/mouse, respectively (Fig. 1). The LD50 of LPS administered i.p. was estimated to be *c.* 400 μg. These data indicated that VT2 was *c.* 10^4-fold more lethal than LPS.

Changes in lethality following injection with VT2 in combination with sub-lethal LPS at different time intervals

Treatment of mice with 20 or 40 ng of VT2 was associated with a >90% mortality rate even in the absence of LPS administration (Fig. 2). Although higher mortality rates were demonstrated in groups of mice treated with 10 ng of VT2 in combination with LPS than that with VT2 alone, these differences were not significant.

Histological examination

In mice treated with VT2 (100 ng), nuclear vesiculation and cytoplasmic vacuolation in kidney tubules were noted after 3 days, whereas no apparent changes were observed earlier (Fig. 3a and b). No pathological changes were seen in the colon, small intestine or liver.

Serum cytokine levels

Although the peak concentrations of TNF-α and IL-6 were noted, at 1.5 and 3 h, respectively, after administration of a sub-lethal dose of LPS, injection of the lethal dose of VT2 did not induce detectable levels of these cytokines (Fig. 4). Moreover, the concentration of these cytokines was not different in mice treated with LPS and LPS + VT2. Only small amounts of IL-1β

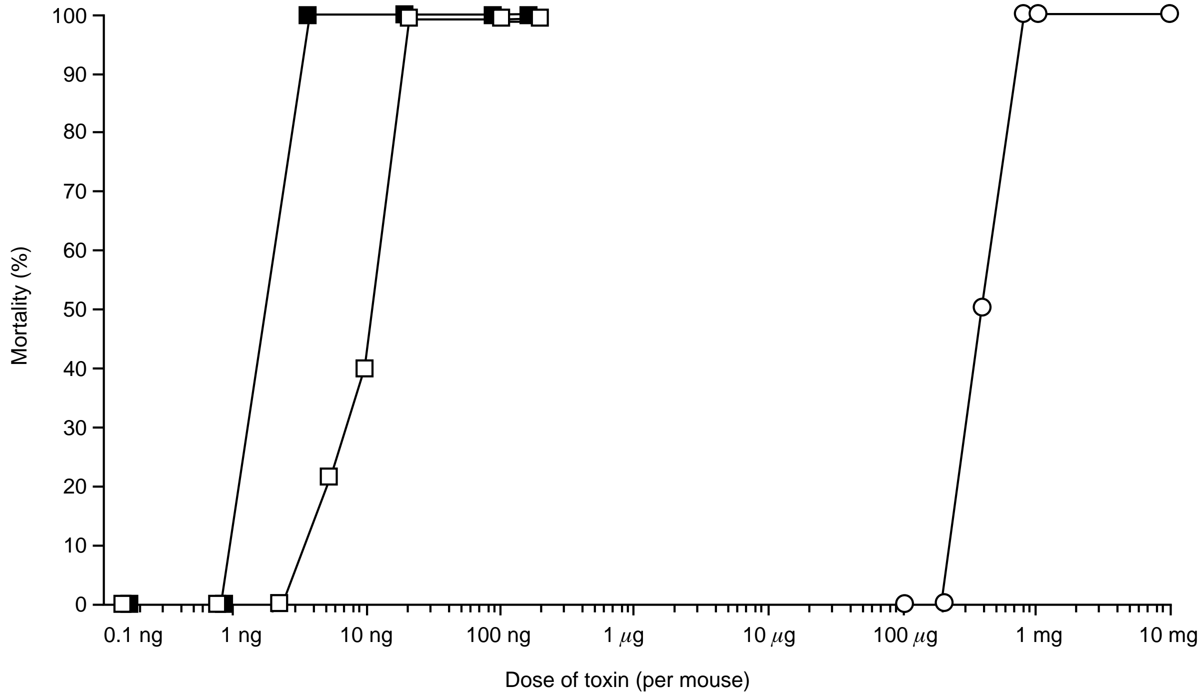

Fig. 1. Comparison of lethal toxicity of VT2 (■, i.v.; □, i.p.;) and LPS (○, i.p.) for mice.

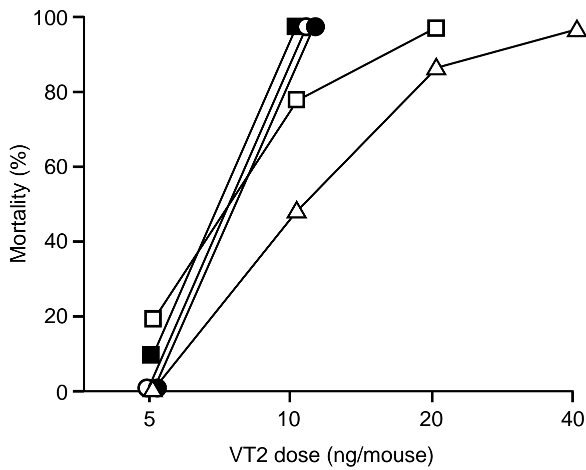

Fig. 2. Effect of a sub-lethal dose of LPS (100 μg) administered at different times on mouse lethality induced by VT2: ○—○, LPS 48 h before VT2; ●—●, LPS simultaneously with VT2; □—□, LPS 24 h after VT2; ■–■, LPS 48 h after VT2; △—△, VT2 alone.

were detected between 3 and 6 h, and significant differences were not demonstrated among the three groups. The study also investigated the effects of VT2 on LPS-induced TNF-α production. When a higher dose of VT2 (100 ng) was administered simultaneously with LPS, a slight suppression of TNF-α production was observed (Fig. 5). Interestingly, a marked inhibition of LPS-induced TNF-α production was noted in mice treated with VT2 48 h before induction of LPS.

Discussion

The data showed that VT was highly toxic for mice. The LD50 of VT2 was *c*. 10^4-fold higher than that of LPS. Such toxicity was similar to the results described in previous reports [13]. In the present study, LPS contamination in VT2 was minimal (only 90 pg/1 μg of VT2). However, great care must be taken when examining cytokine induction *in vivo* by bacterial products or components, because LPS is one of the most potent inducers of inflammatory cytokines. Interestingly, no induction of IL-1, IL-6 or TNF-α was observed with 20 ng of VT2, which was 10 times higher than the LD50 for this toxin. These data strongly suggest that VT2 does not induce these inflammatory cytokines.

Several clinical studies have demonstrated a low frequency of patients with HUS who have high serum levels of TNF-α. Inward *et al*. [14] reported that two of 19 children with VT-producing *E. coli* infection complicated with HUS were positive for serum TNF-α. Furthermore, van de Kar *et al*. [15] showed no increase in plasma TNF-α in HUS patients compared to healthy controls, and Fitzpatrick *et al*. [16] found TNF-α present in the plasma of only one of 16 patients with HUS. The data on serum cytokines in mice from the present study were largely consistent with these clinical data. Recently, the molecular basis for induction and localisation of TNF-α by VT was investigated in an elegant experiment in mice bearing a chloramphenicol acetyltransferase (CAT) reporter gene that indicates TNF-α synthesis [9]. After injection of VT in

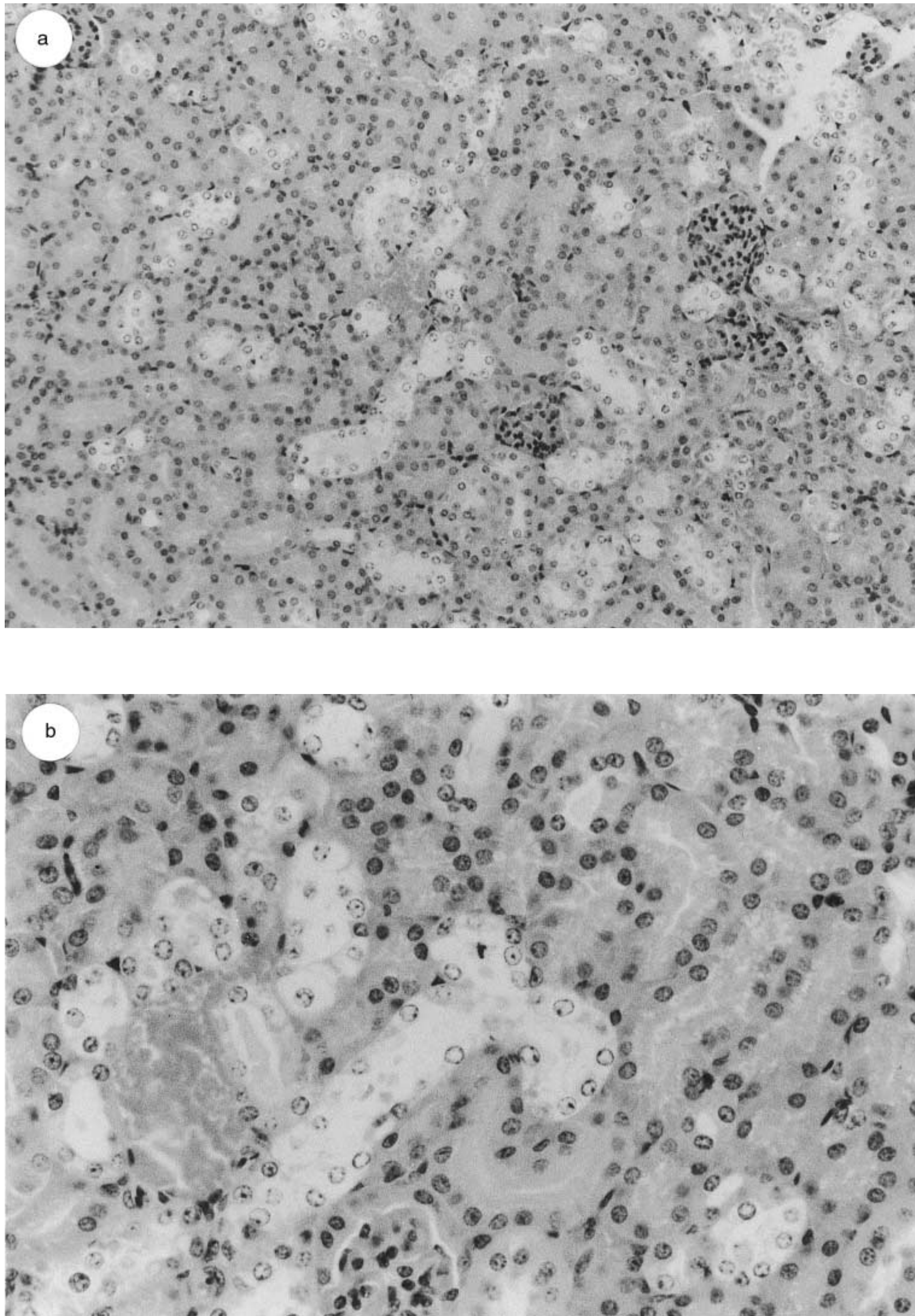

Fig. 3. Pathological characteristics in kidneys of mice given VT2 (100 ng). Nuclear vesiculation and cytoplasmic vacuolation in kidney tubules were noted at 3 days. (**a**) $\times$ 100; (**b**) $\times$ 400.

these mice, CAT activity was induced selectively in the kidney. These data demonstrated the distinct potential of VT to upregulate TNF-α gene expression in the kidney, which fits well with the unique sensitivity of the kidney to microvascular damage in HUS, although specific expression of the TNF-α gene in the human kidney has not been investigated.

Simultaneous administration of VT2 and LPS did not result in a synergic effect with regard to serum cytokine levels. In contrast, a marked inhibition of LPS-induced TNF-α production occurred when mice were treated with VT2 48 h before LPS challenge. The molecular mode of action of VT has been studied extensively [5]. VT inhibits protein synthesis in eukaryotic cells by inactivating 60S ribosomal subunits, resulting in the inhibition of elongation factor-1-dependent aminoacyl-tRNA binding to 60S ribosomal subunits. It is most likely that the lethal dose of VT inhibits the synthesis of several proteins, which may

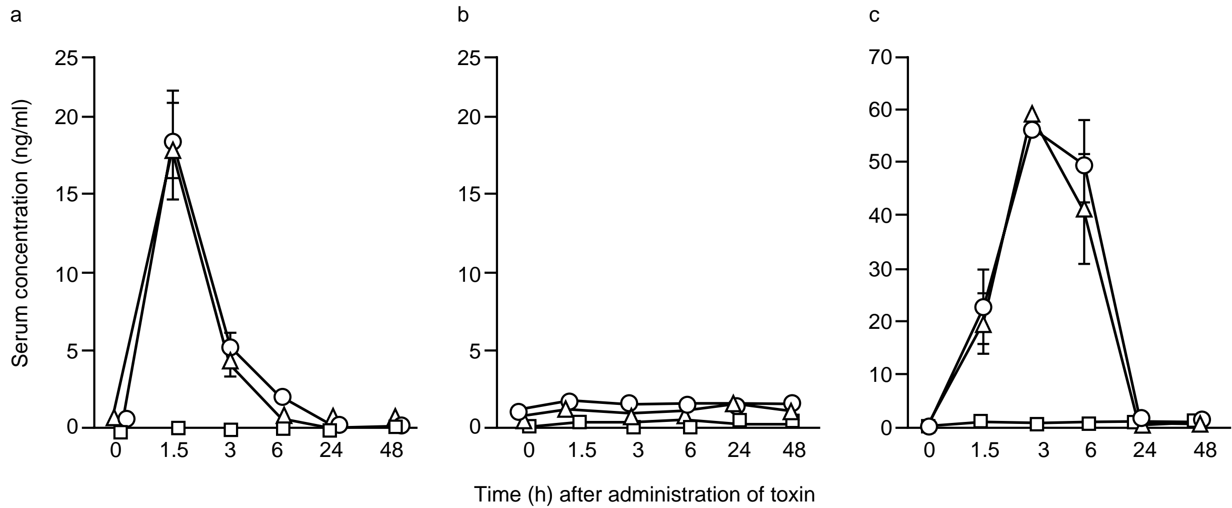

Fig. 4. Serum TNF-α (**a**), IL-1β (**b**) and IL-6 (**c**) levels after administration of VT2 (20 ng/mouse, □), LPS (100 μg/mouse, ○) or VT2 plus LPS (△).

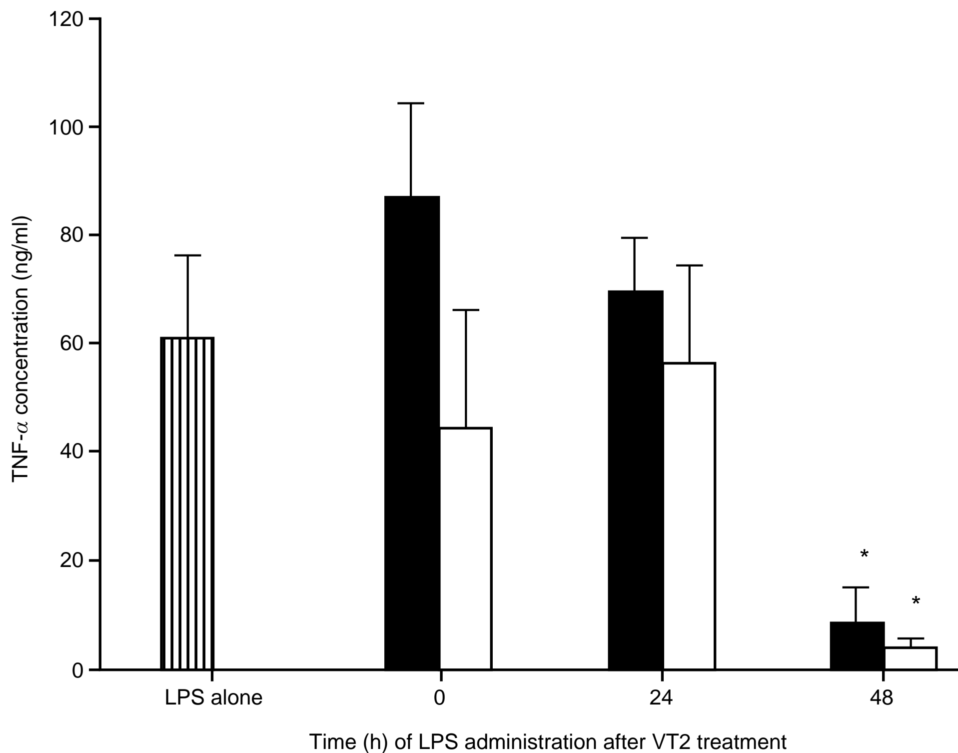

Fig. 5. Serum TNF-α concentrations after administration of VT2 and LPS at various times: ▥, LPS (100 μg/mouse); ■, VT2 (20 ng/mouse) + LPS; □, VT2 (100 mg/mouse) + LPS. Serum TNF-α levels were measured 1.5 h after administration of LPS. *$p < 0.01$, compared with LPS alone.

include factors responsible for the expression of TNF-α.

The present study examined changes in the mortality rates of mice under several experimental conditions, in which various concentrations of VT were administered in combination with sub-lethal doses of LPS at various times, but significant effects of the combination were not observed. In LPS-responder (C3H/HeN) and LPS-non-respondent mice (C3H/HeJ), Karpman *et al.* [17] reported different courses of disease with intragastrically inoculated VT-producing *E. coli*. In this regard, Harel *et al.* [9] reported that VT sensitised C3H/HeN mice to the lethal effect of LPS and TNF, whereas pre-treatment with either LPS or TNF did not noticeably sensitise the mice to the lethal effect of VT. Barrett *et al.* [18] also reported that pre-treatment of C3H/HeN mice with LPS protected the animals from challenge with an LD50 but not an LD100 of VT. On the other hand, LPS enhanced the lethal effect of VT when it was administered at 8 or 24 h but not 0 or 72 h after VT challenge. However, these effects were relatively minor

compared with in-vitro results, where the cytotoxic activity of VT was markedly enhanced by up to 6 $\log_{10}$ orders by pro-inflammatory cytokines [6, 8]. It is difficult to explain the discrepancy between previous and present data, but possible reasons may include differences in the strain of mice used, preparation of toxins and experimental conditions.

The characteristic features of renal histology in human patients with HUS include thrombotic micro-angiopathy in the glomeruli and cortical tubular necrosis [19]. Glomerular damage is thought to be a consequence of the cytotoxic effect of VT on capillary endothelial cells. In the present study, the pathological changes in the kidneys of mice treated with lethal doses of VT were observed mainly in tubules (nuclear vesiculation and cytoplasmic vacuolation), but not in glomeruli. These observations were quite similar to those described by other investigators [20]. The lack of glomerular damage may be due to the absence of a functional receptor specific for VT. These results indicated that the pathological states induced by VT in mice are clearly different from those in man. Accordingly, care must be taken when considering the relevance of results obtained from a mouse model of enterohaemorrhagic *E. coli* infection to the human disease.

We thank Nobuhisa Ishii for critically reading the manuscript and helpful suggestions.

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