Toxicity of Shiga Toxin 1 in the Central Nervous System of Rabbits

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The action of Shiga toxin (Stx) on the central nervous system was examined in rabbits. Intravenous Stx1 was 44 times more lethal than Stx2 and acted more rapidly than Stx2. However, Stx1 accumulated more slowly in the cerebrospinal fluid than did Stx2. Magnetic resonance imaging demonstrated a predominance of Stx1-dependent lesions in the spinal cord. Pretreatment of the animals with anti-Stx1 antiserum intravenously completely protected against both development of brain lesions and mortality.

In 1996, there were a total of 16 outbreaks of Shiga toxin (Stx)-producing *Escherichia coli* (STEC) infections in Japan. In Sakai City, the number of patients increased to 9,492, of which 7,889 were schoolchildren. Of these patients, 758 were hospitalized, 121 suffered from hemolytic uremic syndrome (HUS), and 11 children plus one adult died as a result of the infection (6a). Four of the 11 deaths were due to central nervous system (CNS) impairment. STEC produce one or more Stxs, such as Stx1, Stx2, and Stx2c, that are toxic to humans (4). Stx1 and Stx2 bind to a specific glycosphingolipid receptor, globotriaosylceramide (Gb3) (6), and are associated with hemorrhagic colitis (10), HUS (5), and neurological complications (11, 14). We previously reported that in mice Stx2c was toxic to both capillary endothelial cells and nerve fibers in the brain cortex and spinal cord following oral infection with STEC (3). Furthermore, using magnetic resonance imaging (MRI), we detected brain lesions of rabbits given purified Stx2 intravenously (1) and determined that the cause of death was a dysfunction of baroreflex (15). Impairment of the carotid sinus baroreflex control system in the brainstem leads to poor maintenance of blood pressure. In the present study, we have compared the action of Stx1 and Stx2 in the brain of rabbits exposed to the purified toxins and tested anti-Stx1 antibody as a protective agent in this model.

Determination of LD_{50} of intravenous Stx1 and Stx2 in rabbits. Stx1 and Stx2 used in this study were purified to homogeneity as described previously (17) and determined to be free of detectable lipopolysaccharide (LPS) by toxicolor test and sodium dodecyl sulfate-polyacrylamide gel electrophoresis with silver staining. The cytotoxic potency of Stx1 and Stx2 was 10^5 and 10^6 50% cytotoxic doses (CD_{50})/μg of protein, respectively, as tested in Vero cells using the WST-1 cell proliferation assay (Chemicon International, Inc.) as described Yoh et al. (16). The titers of anti-Stx1 and anti-Stx2 antisera were examined by the Ouchterlony double gel diffusion test and in the cytotoxicity neutralization assay. In both assay systems, the

Stx2 antiserum exhibited twice the potency of the Stx1 antiserum, 1:64 versus 1:32 and 1:6,400 versus 1:3,200, respectively.

The 50% lethal doses (LD_{50}) of intravenous Stx1 and Stx2 were measured using male Japanese white rabbits weighing 2 kg each (Shizuoka Experimental Animals, Hamamatsu, Japan). To determine the LD_{50} of intravenous Stx1, 10 groups of five rabbits each were challenged intravenously with 2.4, 4.9, 9.8, 20, 39, 78, 156, or 313 ng of Stx1 per kg. Using the same protocol, rabbits were challenged with intravenous Stx2 at 313, 625, 1,250, or 2,500 ng/kg. These animals were monitored for 1 month. The LD_{50} of intravenous Stx1 and Stx2 for rabbits was calculated to be 20 and 884 ng/kg, respectively (Table 1). Thus, the LD_{50} of Stx2 was 43.8-fold higher than that of Stx1 ($P = 0.0001$ by analysis of variance).

Survival curves of rabbits injected intravenously with either 1.9 LD_{50} (39 ng/kg) of Stx1 or 2.8 LD_{50} (2,500 ng/kg) of Stx2 are indicated in Fig. 1. The survival of the rabbits injected with Stx1 was significantly shorter than that of animals which received Stx2 ($P = 0.023$, log rank test) even though the LD_{50} of Stx1 administered was lower than that of Stx2. The mean survival days for Stx1 and Stx2 were 2.4 and 7.0, respectively.

To better understand why Stx1 is more toxic to rabbits than Stx2, we further examined the action of these toxins in vivo. The following data presented for our rabbit model help explain why Stx1 causes more CNS damage than Stx2 in rabbits. These results may also relate to the action of these toxins in humans as Stx2-producing STEC present a greater risk for development of acute renal failure and CNS abnormalities (8, 12, 13).

Measurement of Stx1 concentration in CSF of rabbits. Ten rabbits were given Stx1 (2 μg/kg) intravenously. At 0, 3, 8, and 24 h after injection of Stx1, cerebrospinal fluid (CSF) was obtained from the cisterna magna. Stx1 concentration of the fluid was determined by enzyme immunoassay (EIA) using the Novapath Verotoxin EIA kit (Japan Bio-Rad Laboratories, Tokyo) with values derived from a linear standard curve prepared using purified Stx1. The concentrations of Stx1 in CSF of rabbits after an intravenous injection of Stx1 are shown in Fig. 2 and compared to our previous results obtained with Stx2 (2). The results show that Stx1 accumulates more slowly in the CSF than does Stx2, suggesting that Stx2 crossed the blood-brain barrier more readily than did Stx1 (3, 8). However, sig-

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TABLE 1. Determination of LD_{50} of Stx1 and Stx2 for
rabbits after an intravenous injection

Stx	Dose (ng/kg)	No. of rabbits treated	No. of deaths	LD_{50} (ng/kg)
Stx1	313	5	5	20
	156	5	4	
	78	5	4	
	39	5	3	
	19	5	2	
	9.8	5	0	
	4.9	5	2	
	2.4	5	0	
Stx2	2,500	5	5	884[a]
	1,250	5	3	
	625	5	2	
	313	5	0	

[a] Probit method. Potency ratio, 43.8.

nificantly higher levels of Stx1 were detected in CSF at 24 h
compared with the time zero sample ($P < 0.05$ by t test).
Whether this was due to more rapid endothelial transport of
Stx2 or to selective destruction of the endothelial layer by Stx2
has not been determined. Stx2 not only entered the CSF more
readily, it also appeared to be more potent than Stx1 within
this site. Indeed, intrathecally injected Stx2 was more potent
than intrathecal Stx1 based on the LD_{50}s of intravenous versus
intrathecal Stx2, 3.4 versus 0.36 (μg/kg) (2). In support of this
concept, lethality data from the present study also revealed
some important properties of Stx1, as it was clearly demon-
strated that intravenously presented Stx1 was more than five
times more potent than intrathecally administered Stx1 based
on LD_{50} values of 20 and >100 ng/kg, respectively. Together,
the data suggest that the primary damage caused by Stx1 takes
place outside of the CNS, such as large intestine, because the
Stx1 toxemic rabbits showed hemorrhagic diarrhea. Stx2 tox-
emic rabbits also developed hemorrhagic diarrhea, but in the
Stx2 toxemic rabbits, Stx2 in CSF increased rapidly and
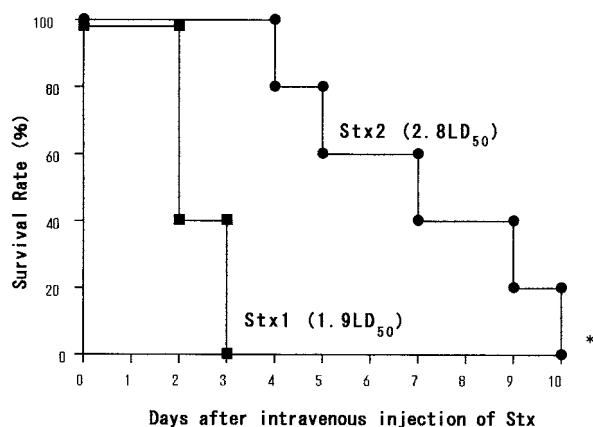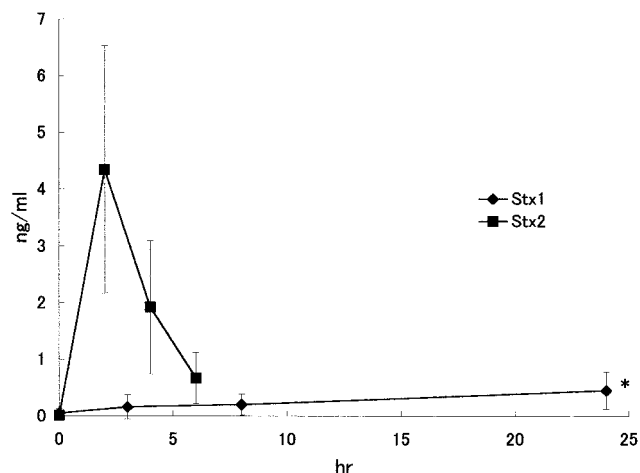reached a maximum 2 h after Stx2 injection (Fig. 2). This

FIG. 2. Time course of Stx1 and Stx2 concentrations in CSF after
intravenous injection of Stx1. Stx1 concentrations in CSF 8 and 24 h
after intravenous Stx1 injection were significantly higher than those in
the nontreated control ($*$, $P < 0.01$ by t test).

suggests that Stx2 may accumulate in rabbit CNS earlier than
in large intestine because the hemorrhagic diarrhea was rec-
ognized 24 h after the injection of Stx2 (1).

MRI of brain lesions induced by Stx1. Analysis of Stx1-
induced brain lesions in rabbits in the present study (Fig. 3)
was performed using previously reported MRI methodology
(1). The results revealed damage that was less severe than we
had described for Stx2 (1). Rabbits given a 1.95 LD_{50} dose of
Stx1 (39 ng/kg) developed a major spinal cord lesion, appear-
ing as lighter areas indicated by arrows in Fig. 3B (48 h) and
with an accompanying hematoma at 24 h (Fig. 3C). At 48 h,
additional lesions also developed in the brain stem (Fig. 3B,
large arrowhead) and midbrain (Fig. 3B, small arrowhead). In
control untreated animals, all of these areas appeared nor-
mally dark (Fig. 3A). It is likely that the observed flaccid
paresis in these Stx1-treated animals was a result of such lo-
calized damage. We could not find lesions in the cerebellum
and cerebrum of Stx1-treated rabbits.

In contrast, previous studies with intravenously Stx2-treated
animals revealed lesions of the brain stem but not of the spinal
cord (1). Lesions were first detected in the hypothalamic area
of all experimental animals at 24 h. After 57 h, lesions ap-
peared in the white matter of the cerebral hemisphere or the
hippocampus (1). It is important to note that both Stx1 and
Stx2 caused brain stem lesions that were the apparent cause of
death.

Aside from the different mobilities of Stx1 and Stx2 in cross-
ing the blood-brain barrier, receptor specificity for these toxins
may contribute to their different lethal potencies in the rabbit
model. Stx2 binds to the same receptor as Stx1, Gb3 (6).
However, it has become increasing clear that Gb3 represents a
heterogeneous group of glycolipids that differ in fatty acid
carbon chain length and degree of fatty acid saturation and
hydroxylation (9). It is well accepted that these structural dif-
ferences in toxin receptor may determine toxin binding affin-
ities. We propose that the structure of Gb3 may differ in the
spinal cord and the cerebrum or cerebellum. In humans and
mice, we guess that Stx2 may bind to the kidney and the target

FIG. 1. Survival curves for rabbits injected with Stx1 or Stx2 intra-
venously. Two groups of rabbits were injected intravenously with 1.9
LD_{50} (39 ng/kg) of Stx1 or with 2.8 LD_{50} (2,500 ng/kg) of Stx2. The
survival of the rabbits injected with Stx1 was significantly shorter than
that of rabbits that received Stx2 ($P = 0.023$ by log rank test). The
mean survival with Stx1 and Stx2 was 2.4 and 7.0 days, respectively.
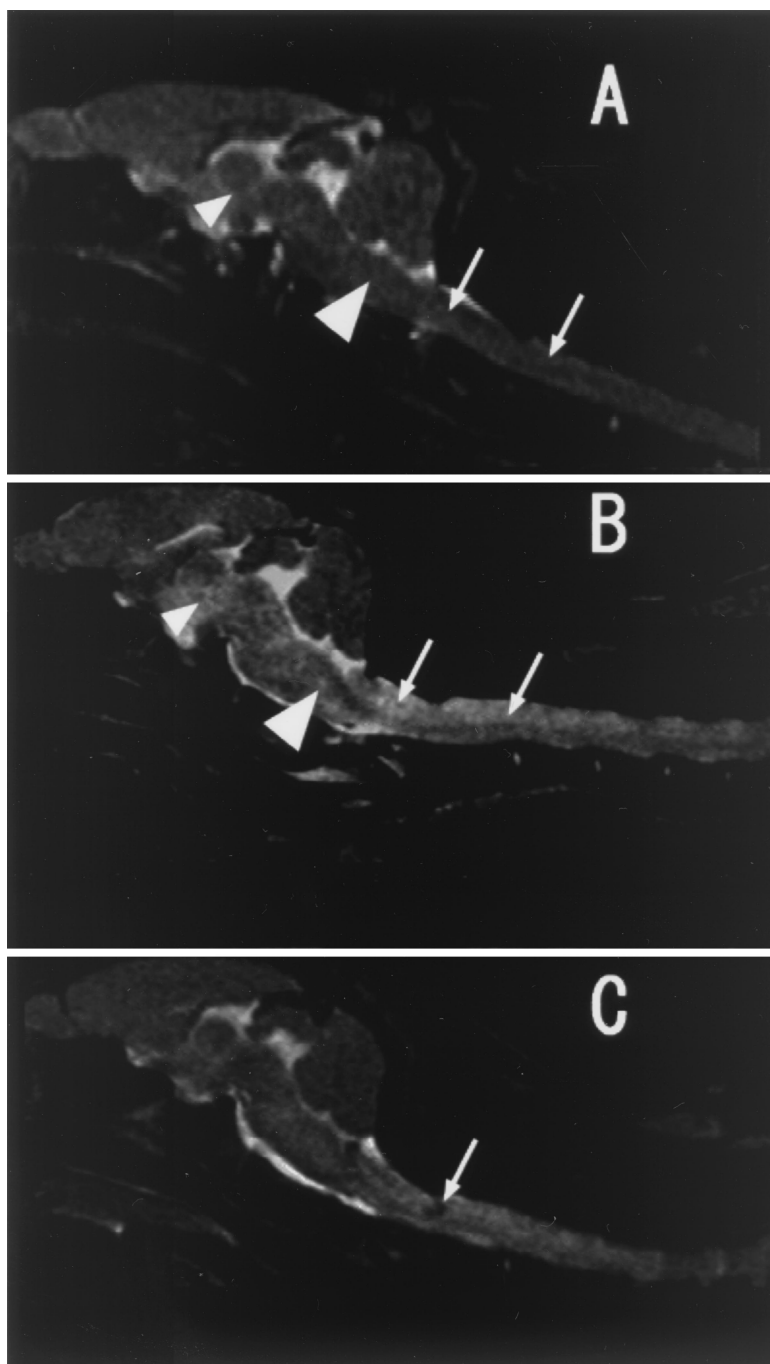
FIG. 3. MRI of brains of normal and Stx1-challenged rabbits. (A) Midsagittal T2-weighted image of a nontreated control rabbit's brain. Two arrows show spinal cord areas, a small arrowhead shows midbrain, and a large arrowhead shows brain stem. (B) T2-weighted image at 48 h after Stx1 administration (39 ng/kg, 1.95 LD_{50}). A high-intensity area of brain stem (large arrowhead) and midbrain (small arrowhead) with spinal cord lesion (two arrows) is seen. (C) T2-weighted image at 24 h after Stx1 administration (39 ng/kg, 1.95 LD_{50}). A low-intensity area of spinal cord is seen as a hematoma (arrow).

sites of brain rather than Stx1 because of the different structure of Gb3.

Effect of intrathecal versus intravenous injection of anti-Stx1 antiserum on brain damage induced by intravenous Stx1. To determine the protective effects of an intrathecal or intravenous injection of rabbit anti-Stx1 antiserum, 15 rabbits were examined in three different treatment groups of five rabbits

each. An intrathecal injection was used as described by Mizuguchi et al. (7). One group of rabbits were injected intrathecally with 0.5 ml of rabbit serum (the titer of anti-Stx1 antibody was 32) 1 h before an intravenous injection of Stx1 (78 ng/kg; 3.86 LD_{50}). Another group of rabbits was injected with the same dose of rabbit serum intravenously 1 h before the intravenous injection of Stx1. A control group of rabbits were in-

jected with Stx1 only. The lethality results recorded at day 10 showed 100% survival (five of five) of the rabbits injected intravenously with the rabbit serum containing anti-Stx1 antibody (32 titer) 1 h before the intravenous injection of Stx1. All five rabbits in the control group died. However, only 60% (three of five) survival was observed when the anti-Stx1 antiserum was injected directly into the CSF space 1 h before the intravenous injection of Stx1. This result differs from our previous success in therapy of an intrathecal injection of rabbit serum containing anti-Stx2 antibody for the brain lesion induced by intravenous Stx2 injection (2). It is concluded the lethal toxic effects of Stx1 in rabbits involve damage within as well as outside of the brain, whereas Stx2 acts primarily within the brain.

REFERENCES

1. **Fujii, J., Y. Kinoshita, T. Kita, A. Higure, T. Takeda, N. Tanaka, and S. Yoshida.** 1996. Magnetic resonance imaging and histopathological study of brain lesions in rabbits given intravenous verotoxin 2. Infect. Immun. **64:** 5053–5060.
2. **Fujii, J., Y. Kinoshita, Y. Yamada, T. Yutsudo, T. Kita, T. Takeda, and S. Yoshida.** 1998. Neurotoxicity of intrathecal Shiga toxin 2 and protection by intrathecal injection of anti-Shiga toxin 2 antiserum in rabbits. Microb. Pathog. **25:**139–146.
3. **Fujii, J., T. Kita, S. Yoshida, T. Takeda, H. Kobayashi, N. Tanaka, K. Ohsato, and Y. Mizuguchi.** 1994. Direct evidence of neuron impairment by oral infection with verotoxin-producing *Escherichia coli* O157:H− in mitomycin-treated mice. Infect. Immun. **62:**3447–3453.
4. **Karmali, M. A.** 1989. Infection by verocytotoxin-producing *Escherichia coli.* Clin. Microbiol. Rev. **2:**15–38.
5. **Karmali, M. A., B. T. Steele, M. Petric, and C. Lim.** 1983. Sporadic cases of haemolytic-uraemic syndrome associated with faecal cytotoxin and cytotoxin-producing *Escherichia coli* in stools. Lancet **i:**619–620.
6. **Lingwood, C. A., H. Law, S. Richardson, M. Petric, J. L. Brunton, G. S. De, and M. Karmali.** 1987. Glycolipid binding of purified and recombinant *Escherichia coli* produced verotoxin in vitro. J. Biol. Chem. **262:**8834–8839.
6a.**Michino, H., K. Araki, S. Minami, S. Takaya, N. Sakai, M. Miyazaki, A. Ono, and H. Yanagawa.** 1999. Massive outbreak of *Escherichia coli* O157:H7 infection in schoolchildren in Sakai City, Japan, associated with consumption of white radish sprouts. Am. J. Epidemiol. **150:**787–796.
7. **Mizuguchi, M., S. Tanaka, I. Fujii, H. Tanizawa, Y. Suzuki, T. Igarashi, T. Yamanaka, T. Takeda, and M. Miwa.** 1996. Neuronal and vascular pathology produced by verocytotoxin 2 in the rabbit central nervous system. Acta Neuropathol. Berl. **91:**254–262.
8. **Ostroff, S. M., J. M. Kobayashi, and J. H. Lewis.** 1989. Infections with *Escherichia coli* O157:H7 in Washington State. The first year of statewide disease surveillance. JAMA **262:**355–359.
9. **Pellizzari, A., H. Pang, and C. A. Lingwood.** 1992. Binding of verocytotoxin 1 to its receptor is influenced by differences in receptor fatty acid content. Biochemistry **31:**1363–1370.
10. **Riley, L. W., R. S. Remis, S. D. Helgerson, H. B. McGee, J. G. Wells, B. R. Davis, R. J. Hebert, E. S. Olcott, L. M. Johnson, N. T. Hargrett, P. A. Blake, and M. L. Cohen.** 1983. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. N. Engl. J. Med. **308:**681–685.
11. **Rooney, J. C., R. M. Anderson, and I. J. Hopkins.** 1971. Clinical and pathological aspects of central nervous system involvement in the haemolytic uraemic syndrome. Aust. Paediatr. J. **7:**28–33.
12. **Scotland, S. M., G. A. Willshaw, H. R. Smith, and B. Rowe.** 1987. Properties of strains of *Escherichia coli* belonging to serogroup O157 with special reference to production of Vero cytotoxins VT1 and VT2. Epidemiol. Infect. **99:**613–624.
13. **Thomas, A., H. Chart, T. Cheasty, H. R. Smith, J. A. Frost, and B. Rowe.** 1993. Vero cytotoxin-producing *Escherichia coli,* particularly serogroup O 157, associated with human infections in the United Kingdom: 1989–91. Epidemiol. Infect. **110:**591–600.
14. **Upadhyaya, K., K. Barwick, M. Fishaut, M. Kashgarian, and N. J. Siegel.** 1980. The importance of nonrenal involvement in hemolytic-uremic syndrome. Pediatrics **65:**115–120.
15. **Yamada, Y., J. Fujii, Y. Murasato, T. Nakamura, Y. Hayashida, Y. Kinoshita, T. Yutsudo, T. Matsumoto, and S. Yoshida.** 1999. Brainstem mechanisms of autonomic dysfunction in encephalopathy-associated Shiga toxin 2 intoxication. Ann. Neurol. **45:**716–723.
16. **Yoh, M., E. K. Frimpong, and T. Honda.** 1997. Effect of antimicrobial agents, especially fosfomycin, on the production and release of Vero toxin by enterohaemorrhagic *Escherichia coli* O157:H7. FEMS Immunol. Med. Microbiol. **19:**57–64.
17. **Yutsudo, T., N. Nakabayashi, T. Hirayama, and Y. Takeda.** 1987. Purification and some properties of a Vero toxin from *Escherichia coli* O157:H7 that is immunologically unrelated to Shiga toxin. Microb. Pathog. **3:**21–30.