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Shiga-like Toxin-Ⅱ-Producing *Escherichia coli* 0157 : H7 infection in gnotobiotic piglets : Protection against brain vascular lesions with SLT-Ⅱ antiserum

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Shiga-like Toxin Ⅱ 항독소에 의한 Shiga-like Toxin Ⅱ-Producing *Escherichia coli* 0157 : H7 감염돼지에서의 뇌혈관 병변의 방어

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초록 : Shiga-like toxin-Ⅱ(SLT-Ⅱ)-producing *Escherichia coli* 0157 : H7 strain B2387이 분비하는 SLT-Ⅱ가 gnotobiotic 자돈에서의 뇌혈관 병변을 일으키는 pathogenesis에 관해서 실험을 했다. 제왕절개 수술로 태어난 자돈들을 두 그룹으로 나누어서, 한 그룹에는 SLT-Ⅱ 중화항체를 포함한 혈청을 구강을 통해서 수동면역을 시키고, 또다른 한 그룹에는 SLT-Ⅱ 중화항체가 포함되어 있지 않은 혈청을 구강을 통해서 수동면역시켰다. 24시간후 두 그룹 모두에게 SLT-Ⅱ producing *Escherichia coli* 0157 : H7 strain B2387를 구강으로 접종했다. SLT-Ⅱ 중화항체가 포함되어 있지 않은 혈청으로 수동면역시킨 그룹의 자돈들은 설사와 맹결장염, 신경증상, 뇌혈관병변을 일으키고, plasma의 prostacyclin의 level이 증가했다. 하지만 SLT-Ⅱ 중화항체가 포함되어 있는 혈청으로 수동면역시킨 그룹의 자돈들은 설사와 맹결장염은 유발했지만, 신경증상과 뇌혈관병변은 관찰되지 않았고, prostacyclin의 level도 증가하지 않았다. 이런 실험결과는 SLT-Ⅱ 중화항체는 뇌혈관병변은 방어하지만 맹결장염은 방어하지 못한다는 의미를 나타내며, prostacyclin의 증가는 뇌혈관의 endothelium의 병변을 의미한다.

Introduction

Infection with Shiga-like toxin (SLT or verocytotoxin)-producing *Escherichia coli* 0157 : H7 is associated with several diseases or syndromes, particularly hemorrhagic colitis, hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura(TTP)[19,27,34]. Shiga-like toxin-Ⅱ-producing strains are the predominant genotype isolated from these patients[30] and arteriolar damage in the brain, colon, and kidney is central to the pathogenesis of organ dysfunction and symptoms.[22,33] Gnotobiotic piglets have been used as a model to study the pathogenesis of vascular lesions caused by infection with *E coli* 0157 : H7.[9,38] Experimental infection results in vascular lesions which are identical to edema disease, a natural disease of swine caused by *E coli* strains that produce SLT-Ⅱ variant (SLT-IIv).[24~26] Shiga-like toxin-IIv has >90% genetic homology with SLT-Ⅱ and is functionally identical to SLT-Ⅱ.[11~17]

A major question concerning the role of SLT in the pathogenesis of various diseases if whether or not the toxine induces vascular damage, and whether the en-

dothelial cell or some other cell is the target of the toxin. In our previous studies of the vascular lesions in the brains of gnotobiotic pigs infected with SLT-Ⅱ-producing *E coli* 0157 : H7, we found endothelial degeneration and necrosis in arterioles, capillaries, and venules, with elevated prostacyclin and IL-1 activity, neutropenia, and monocytopenia.[9] Although vascular lesions were consistently present, it could not be determined whether they were induced by SLT-Ⅱ or some other virulence factor. In addition, it was not clear whether the increase in prostacyclin and interleukin-1 (IL-1) activity, or the other clinical pathologic features of the infection were a manifestation of brain lesions, cecocolitis or both.

The objective of this experiment was to determine the effect(s) of SLT-Ⅱ by providing passive immunity to SLT-Ⅱ and challenging piglets by oral inoculation with live *E coli* 0157 : H7. As SLT-Ⅱ-related diseases such as idiopathic HUS and TTP are often sequelae to foodborne hemorrhagic colitis, we felt this would more closely mimic a natural SLT-Ⅱ toxemia than injection with purified SLT-Ⅱ. Also, a passive immunization study might provide practical information for clinicians. challenged piglets given control antiserum lacking SLT-Ⅱ-neutralizing antibodies were used as positive controls, and non-challenged piglets given SLT-Ⅱ antiserum were used as negative controls. Specific parameters addressed were whether or not SLT-Ⅱ is responsible for (1) brain endothelial lesions ; (2) elevated levels of prostacyclin activity ; and (3) decreased neutrophil and monocyte counts that have been associated with SLT-Ⅱ-producing *E coli* 0157 : H7 infection of the gnotobiotic piglet.

Materials and Methods

Bacterial strains : *E coli* C600 (933w), kindly provided by Dr. Helge Karch, University of Hamburg, Hamburg, Germany, was used for preparation of polyclonal porcine antiserum to SLT-Ⅱ. *E coli* C600 (933w) is a K12 C600 strain that was lysogenized with the SLT-Ⅱ toxin converting phage 933w. The non-lysogenized *E coli* C600 parent strain was used for preparation of the control antiserum. *E coli* 0157 : H7 strain B2387, which produces high levels of SLT-Ⅱ, was used for challenge inoculation and induction of cytotoxicity in the neutralization assay. Strain B2387 was isolated from an adult patient in Nebraska with bloody diarrhea, and was kindly provided by Dr. Kay Wachsmuth at the Centers for Disease Control, Atlanta, GA. The Inoculum consisted of 10㎖ of broth culture containing 5×10^9 CFU/㎖ of strain B2387. The SLT-Ⅱ titer of inoculum filtrate in the HeLa cell cytotoxicity assay[21] was 128.

Antiserum preparation : Antiserum was prepared by repeatedly inoculating pigs with culture supernatant from K12 *E coli* strain C600 (933w), a C600 strain that was lysogenized with the SLT-Ⅱ toxin-converting phage 933W. Bacteria were cultured for 48 hr with aeration in syncase broth prepared as described previously[28], except that the medium was not depleted of iron. Culture supernate was passed through an endotoxin removing gel column(Pierce, Rockford, IL), mixed in equal proportions with Freund's incomplete adjuvant and 2㎖ of the mixture was injected intramuscularly at 2 to 3 week intervals until the SLT-Ⅱ-neutralizing antibody titer rose above 16,000. Control antiserum was prepared by inoculating pigs with antigens from the culture supernatant of the C600 parent *E coli* strain. Pigs used in antiserum production were delivered by Cesarian section, deprived of colostrum, and reard to 3 weeks of age in germ-free isolators, then placed into isolation rooms for inoculation and remained there until antiserum was colleted. The SLT-Ⅱ-neutralizing antibody titer for the SLT-Ⅱ antiserum in HeLa cells was 64,000 and <2 for the control antiserum.

Cytotoxicity and neutralization assays : SLT-Ⅱ titers in sera were tested by a cytotoxicity assay[21], but using HeLa(American Type Culture Collection, Rockville, MD) instead of Vero cells since HeLa cells are more sensitive to SLT-Ⅱ.[10] HeLa cells were propagated in modified McCoy 5a medium containing L-glutamine(Gibco Laboratories, Grand Island, NY) and 10% fetal bovine serum(Sigma Chemical Co., St. Louus, MO). Serial 2-fold dilutions of sera were made and cytotoxicity was assessed at 20-24 hr incubation.

Serum-neutralizing SLT-Ⅱ antibody titers against extracts of strain B2387 on HeLa cells were assessed in serial 2-food-diluted sera, and tests were conducted in duplicate.[10,20]

Characterization of the SLT-Ⅱ-neutralizing and control antisera : The SLT-Ⅱ-neutralizing antibody titer of sera from pigs hyperimmunized with C600 (933w) culture

supernatant ranged from 32,000 to 64,000, while no antibody was detected in the control sera. The anti-C600 (933w) and control antisera contained < 0.007 ng/mℓ endotoxin as assessed by the limulus amebocyte lysate(L-AL) chromogenic assay(Whittaker, Walkersville, MD).

Purified SLT-Ⅱ (obtained from Dr. James E. Samuel, MicroCarb, Gaithersburg, MD), sonically disrupted C600 *E coli* and culture supernatant from C600(933w) were heated at 100℃ for 2 min in the presence of sample buffer(62.5mM Tris-HCl, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, pH 6.8) and applied at 0.2 μg, 93 μg, and 1.9 μg protein/lane, respectively, to 12% SDS-polyacrylamide gels and subjected to electrophoresis.[23] Proteins were electrophoretically transferred to nitrocellulose filters(0.45 μm) by the method of Towbin.[41] Nitrocellulose filters were incubated in phosphate buffered saline(PBS) containing 0.5% Tween-20 (0.5% PBST ; Sigma, St. Louis, MO) for 30min at 4℃, then incubated for 1 hr at 37℃ with swine serum diluted 1:500 in 0.05% PBST. They were then washed once with 0.5% PBST and twice with 0.05% PBST. Filters were incubated for 1 hr at room temperature with ^{125}I-labeled protein G(20mℓ of 0.0445 μCi/mℓ ; specific activity 11.6 μCi/mℓ ; ICN, Irvine, CA) diluted in 0.05% PBST, rinsed 3 times for 10min in 0.05% PBST, and once in PBS, then dried and exposed to autoradiography film for 24 hr at -70℃.

Immunoblots of C600 *E coli* incubated with the anti-C600 (933w) or control sera exhibited numerous heavily labeled bands, indicating that each contained antibodies to C600 *E coli*. Immunoblots of purified SLT-Ⅱ incubated with porcine anti-C600 (933w) antiserum exhibited a heavily labeled band of approximately 33 kDa, a moderately well labeled band of approximately 27 kDa and a weakly labeled band of approximately 10 kDa. These bands corresponded in M^r to the A, A^1 and B subunits, respectively, of SLT-Ⅱ[7], and indicate that the sera contained antibodies to SLT-Ⅱ. A blot of purified SLT-Ⅱ incubated with the control serum contained no bands, indicating the control serum contained no anti-SLT-Ⅱ antibodies. Immunoblots of culture supernatant of C600 (933w), when incubated with the anti-C600 (933w) serum contained 9 bands. However, when the same blot was incubated with the anti-C600 (933w) serum previously absorbed with sonically disrupted C600 *E coli*, only 2 bands (33 and 27 kDa), corresponding to SLT-Ⅱ subunits A and A^1, were exhibited. When incubated with the control serum, blots of C600 (933w) culture supernatant exhibited only weakly labeled bands which were not evident in blots incubated with control serum previously absorbed with C600 *E coli*. The results of the culture supernatant immunoblot tests indicated that culture supernatant from C600 (933w) contained both SLT-Ⅱ and *E coli* antigens to which hyperimmunized pigs responded. Further, they indicated that the control sera contained no anti-SLT-Ⅱ antibodies, and a lower concentration of antibodies to the *E coli* andigens found in the culture supernatant than did the anti-SLT-2-Ⅱ antisera.

Experimental animals : Twenty gnotobiotic piglets were derived by Caesarian section from 2 Yorkshire-Hampshire SPF sows, and maintained in germ-free isolator units.[3] The methods of procurement, animal facilities and experimental design were previously approved by the University of Nebraska-Lincoln Institutional Animal Care & Use Committee. Sterility was confirmed by pre-inoculation bacterial culture of the rectum.

Experimental design : Piglets were randomly divided into 3 groups : (1) group 1 those administered SLT-Ⅱ antiserum at birth and inoculated 24 hours later ; (2) group 2 those administered control antiserum at birth and inoculated 24 hours later ; and (3) group 3 those only administered SLT-Ⅱ antiserum at birth to test for antibody absorption and decay. Piglets were (A) fed 50mℓ antiserum mixed with 50mℓ sterile milk replacer (SPFLac, Borden, Hampshirs, IL) ; or (B) administered 50mℓ antiserum without milk replacer by stomach tube. The piglets were examined for diarrhea and neurologic signs, and rectal temperatures were taken 3 times daily post-inoculation(PI).

Blood sample collection : Pre-immunization blood was collected from the umbilicus of all piglets at birth, at 24 hours post-immunization from the orbital sinus[16] under sedation by intramuscular (IM) injection of tiletamine hydrochloride and zolazepam hydrochloride (Telazol® 3 mg/kg B.W., Animal Health Group, A. H. Robins Company, Richmond, VA) and xylazine hydrochloride(Rompun® 1 mg/kg B.W., Mobay Corporation, Shawnee, KA), and immediately before euthanasia. Serum samples at birth and 24 hr post-immunization were assayed for SLT-Ⅱ cytotoxicity titers and SLT-Ⅱ-

neutralizing antibody titers. Terminal blood samples were assayed for hematologic parameters. Sera from these samples were assayed for tumor necrosis factor (TNF) activity, endotoxin and SLT-Ⅱ levels, and SLT-Ⅱ-neutralizing antibody titers. Plasma from these samples was assayed for prostacyclin(6-keto-$PGF_{1\alpha}$) and fibrinogen levels.

Perfusion fixation : Upon the onset of neurologic sings of 8 days PI, gnotobiotic piglets were anesthetized with a combination of tiletamine hydrochloride and zolazepam hydrochloride(Telazol ®, 6mg/kg B.W., Animal Health Group, A. H. Robins Company, Richmond, VA) and xylazine hydrochloride (Rompun ®, 2 mg/kg B.W., Mobay Corporation, Shawnee, KS) given by IM injection. Terminal blood samples were taken from the right ventricle via percutaneous needle puncture, and a midsternal thorocotomy was performed. Whole body perfusion fixation was conducted on 7 of 10 piglets in Group 1, 4 of 6 piglets in group 2, and 3 of 4 piglets in group 3 ; tissues of the remaining piglets were fixed by immersion in the same fixative, following euthanasia by electrocution, and were examined only by light microscopy. For perfusion, the left ventricle was catheterized with a 14 gauge, 39.4mm catheter (Abbocath Hospitals, Inc., Chicago, IL), the right ventricle was incised, and using a peristaltic pump (Cole-Palmer Instrument Co., Chicago, IL), 1.0 liter of heparinized (0.5%) cacodylate buffer (0.1 M, pH 7.2, 4℃), immediately followed by 0.7 liter of heparinized, cacodylate-buffered paraformaldehyde(1.5%)-glutaraldehyde(1.5%) fixative was perfused over 7-minute period. Brains were removed 30min after perfusion to allow adequate penetration of the fixative.[5] Following removal, perfused brains were immersed in cacodylate-buffered paraformaldehyde(1.0%)-glutaraldehyde (3.0%) fixative for 2 hr at room temperature or overnight at 4℃, and transferred to cacodylate buffer until further processed.

Bacterial culture : After perfusion, a sample of cecal contents for each piglet was obtained at necropsy and later cultured aerobically on bovine and MacConkey agars at 37℃ for 24 hr. The cytotoxicity of the bacterial isolates from the ceca were tested in the HeLa cell assay.[21]

Tissue processing : Following perfusion or exsanguination without perfusion, the piglets were necropsied and examined for gross lesions, and samples of brain, intestines and other tissues were collected for histopathology. Five mm slices of brain were taken for histopathology from 3 areas at the following reference points:(1) frontal cerebrum at the genu of the corpus collasum ; (2) parietal cerebrum at the interthalamic adhesion ; and (3) cerebellum with brain stem at the fastigial recess of the fourth ventricle. All fixed tissues, including intestines and brain slices were processed by routine methods for light microscopy. Light microscopic sections were examined for lesions, and areas in the brain stem with perivascular and perineuronal hemorrhages were selected for transmission electron microscopy (TEM). The surface of the tissue remaining in cacodylate buffer that opposed the area with hemorrhages was located, and a block of tissue was removed with a 2mm circular skin biopsy punch (Baker Cummins Pharmaceuticals, INC., Miami, FL). punched brain samples were processed for TEM by standard methods. Theree arterioles, venules and capillaries in the brain stem of each perfused piglet were examined by TEM.

Endotoxin, prostacyclin and cytokine assays : Serum samples were diluted 1 : 10 in pyrogen-free water and heated to 100℃ for 10min. Endotoxin levels were determined by the LAL chromogenic assay with a sensitivity of 0.007 ng/mℓ.

Blood samples for the prostacyclin assay were collected in one-tenth volume of 3.8% trisodium citrate (Sigma Chemical Co., St. Louis, MO), and centrifuged twice at 3000×g for 15min. platelet-poor plasma samples were aliquoted and stored at -70℃.

The stable hydrolytic metabolite of prostacyclin, 6-keto-$PGF_{1\alpha}$, was measured by radioimmunoassy (RIA) with 6-keto-$PGF_{1\alpha}$ antibody and 6-keto-$PGF_{1\alpha}$-[^{125}I]-iodotrysine methylester tracer(Amersham, Arlington Height, IL).[36] The 6-keto-$PGF_{1\alpha}$ antibody had 100% specific reactivity for 6-keto-$PGF_{1\alpha}$, but less than 1% cross-reactivity for other prostaglandins, such as $PGF_{2\alpha}$, PGE_1, PGE_2 and thromboxane B_2. Tumor necrosis factor levels were assayed with the murine L-929 fibroblast cell line cytotoxicity assay.[1,11]

Statistical analyses : Since the piglets for this study were derived from 2 litters, and analysis of variance was conducted on all tests to assess for variation. An experiment-wise F test was conducted to determine whether perceived differences among groups for all analyzed parameters were statistically significant. The least significant difference test was conducted to determine the levels of

significance among groups.

Results

Clinical signs and gross pathology : All piglets developed diarrhea after inoculation, but those administered SLT-Ⅱ antiserum had a delay in the onset. Ten of 10 piglets in group 1 developed watery diarrhea at 27 to 82 hr PI, compared to 6 of 6 piglets in group 2, which had watery diarrhea at approximately 27 to 54 hr PI (Table 1). One of 4 piglets in group 3 developed mild diarrhea at 124 hr post-immunization.

Four (67%) of 6 piglets in group 2 developed neurologic sings at 144.8 ± 19.0 hr (mean ± standard deviation) PI (Table 1). Two piglets had muscle tremors and 2 had posterior paresis ; 1 piglet did not develop meurologic signs until 197 hr PI, and 1 piglet was found dead at 126 hr PI. None of the 10 piglets in group 1 developed neurologic signs over and 8-day(194.5 ± 2.6 hr) PI observation period. No neurologic signs were seen in the groups 3 piglets over a 9-day(219.8 hr ± 0.5) observation period following immunization. No significant differences in rectal temperature were detected among the 3 groups at necropsy. Gross lesions at necropsy were limited to edema in the mesentery of the spiral colon in 8 (80%) of 10 piglets in group 1 and 5 (83%) of 6 piglets in group 2, ranging from 1 to 3 mm thickness at the widest point between adjacent colonic coils.

Light microscopy : All group 1 and 2 piglets had suppurative inflammation of the cecum and colon associated with bacterial colonization of the epithelium. Only 1 piglet in group 1 had detectable brain lesions, namely, a focus of malacia in the parietal cerebrum ; however, all piglets in group 2 had brain lesions. Six of 6 piglets had hemorrhages in the cerebellar folia, cerebellar peduncles, and brain stem. Three of 6 piglets had multifocal malacia in the subcortical frontal and parietal cerebrum, and the

Table 1. Incidence of clinical signs and light microscopic lesions

Signs and lesions	Group 1 (n=10)	Group 2 (n=6)	Group 3 (n=4)
Neurologic signs	0*	4	0
Brain vascular lesions	1	6	0
Diarrhea	10	6	1
Colitis	10	6	0

Note. group 1, piglets administered SLT-Ⅱ-neutralizing antiserum at birth and inoculated 24 hours later ; Group 2, piglets administerd control antiserum at birth and inoculated 24 hours later ; Group 3 piglets not inoculated, but administerd SLT-Ⅱ-neutralizing antiserum at birth.

*Number of piglets affected.

Table 2. Geometric mean serum SLT-Ⅱ-neutralizing antibody titers.

Time groups	At birth	At 24 hr post-immunization	Terminal stage
Group 1A (n=7)	<2	5.38	5.38
Group 1B (n=3)	<2	64	64
Group 2A (n=3)	<2	<2	<2
Group 2B (n=3)	<2	<2	<2
Group 3A (n=2)	<2	2.82	2.82
Group 3B (n=2)	<2	256	256

Note. group 1, piglets administered anit-SLT-Ⅱ-neutralizing serum at birth and inoculated 24 hours later ; group 2, piglets administered control antiserum at birth and inoculated 24 hours later ; group 3 piglets administerd SLT-Ⅱ-neutralizing antiserum at birth and not inoculated. Within each group, piglets were (A) fed 50㎖ anfiserum mixed with 50 ㎖ sterile milk replacer or(B) administered 50㎖ antiserum withot milk replacer by stomach tube.

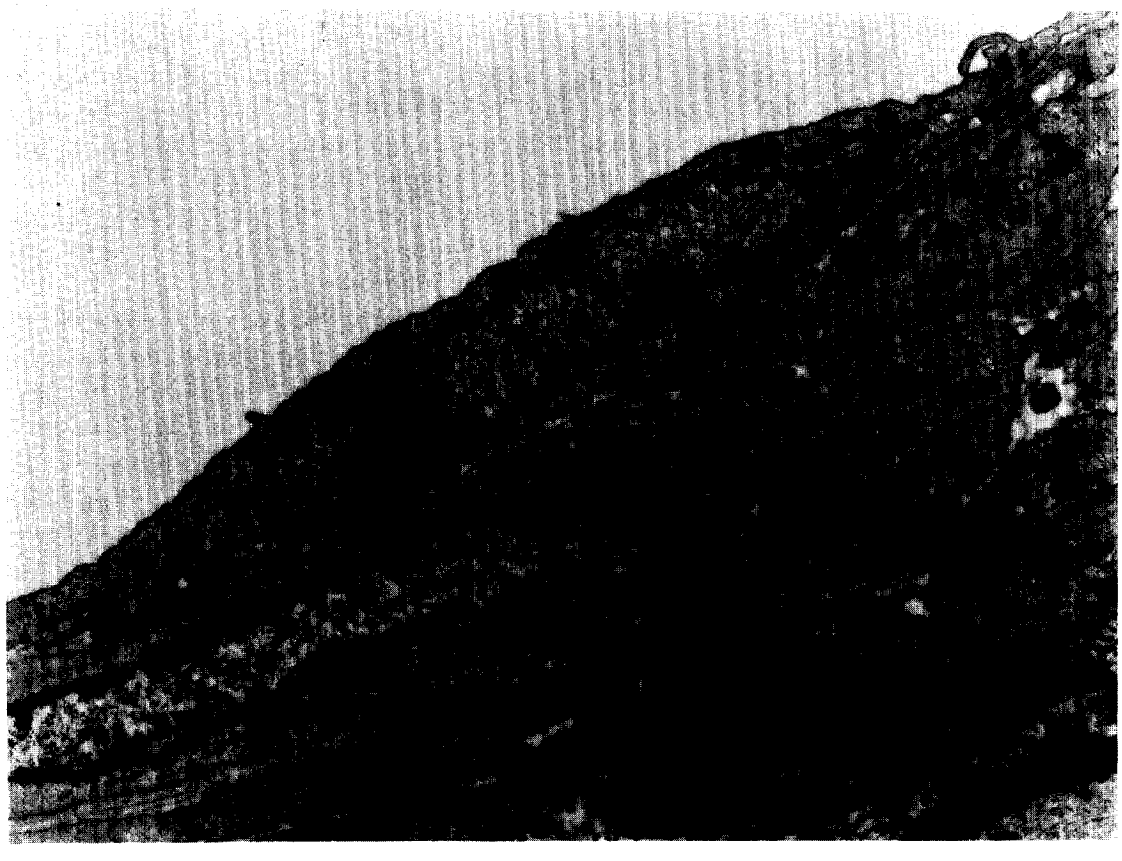

Fig 1. Electron micrography of a section from the brain stem of a piglet administered SLT-Ⅱ-neutralizing antiserum at birth, inoculated with SLT-Ⅱ-producing *E coli* 0157 : H7 when 1 day old, and euthanatized 198 hours later. Note normal flat endothelial cell and medial myocytes.

brain stem. No microscopic lesions were seen in any of the tissues of piglets in group 3.

Transmission electron microscopy : No degenerated of necrotic endothelial cells were seen in brain stem arterioles, capillaries, or venules of piglets in groups 1 and 3(Fig 1). Endothelial cells in capillaries and venules in the brain stems of piglets in group 2 were degenerated of necrotic. Arterioles had endothelial and medial myocyte degeneration and necrosis, but medial myocyte degeneration was not seen without endothelial degeneration(Fig 2).

Damaged blood vessels were not seen by TEM in the brain stem of the piglet in group 1 which had a focus of malacia in the cerebrum. Subsequently, fixed tissue in the cerebrum adjacent to the malacic area was examined by TEM, but no damaged blood vessels were seen.

Microbiology and serology : Pure growth of the respective inocula were isolated from the ceca of all inoculated piglets. The SLT-Ⅱ titer of the filtrates of strain B2387 cultured from the ceca of all inoculated piglets was 128. No SLT-Ⅱ titer(<2) was detected in the sera of piglets in groups 1, 2 and 3 at birth, 24 hrs post-immunization, or the terminal stage. Serum SLT-Ⅱ-neutralizing antibody titers were detected in samples of piglets in groups 1 and 2 at 24 hrs post-immunization and at the terminal stage. The 24 hr post-immunization and terminal geometric mean SLT-Ⅱ-neutralizing antibody titers(GMT) in piglets in group 1 were 11.31, compared to 64 for piglets in group 3. The 24 hour post-immunization and terminal GMT of piglets which were administered SLT-Ⅱ-neutralizing antiserum without milk in groups 1(GMT, 64 ; range 8 to 256) and 3(GMT, 256 ; no range) were higher than the GMT of piglets in the same groups (group 1 GMT, 5.38 ; range 2 to 16 ;

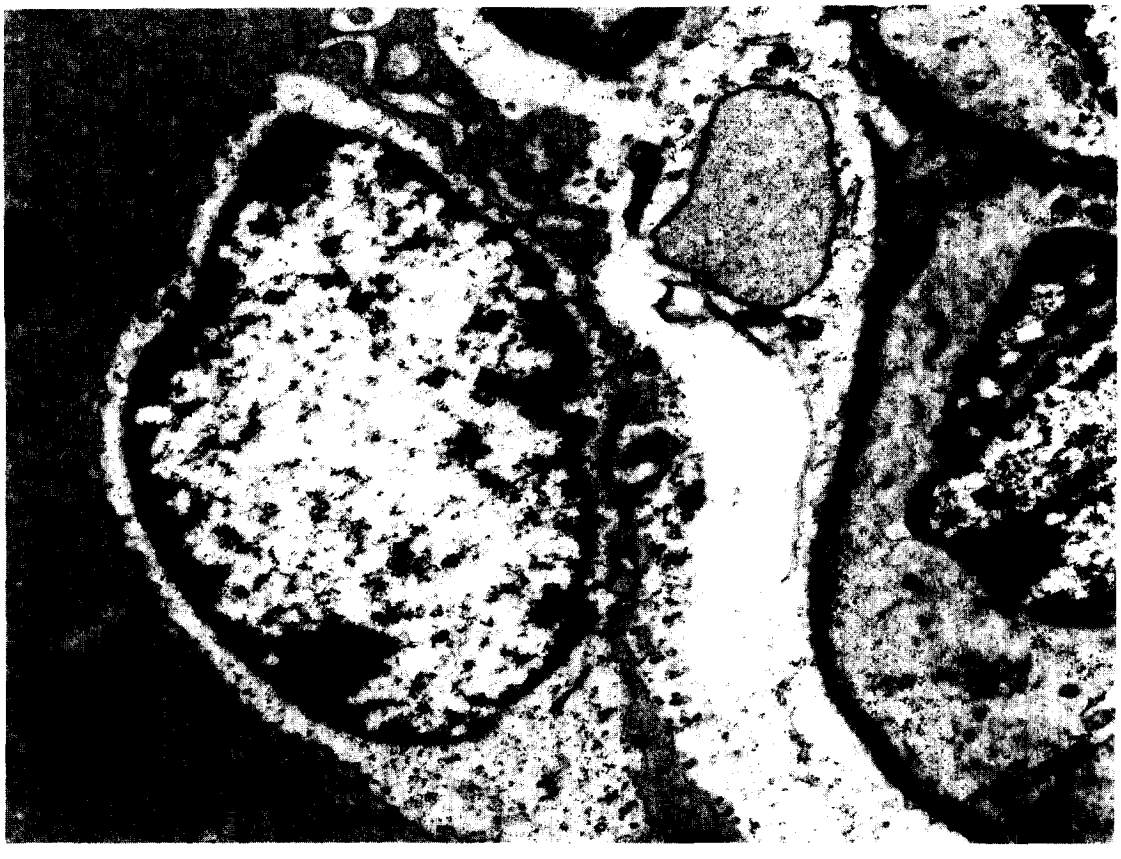

Fig 2. Electron micrography of a section from the brain stem of a piglet administered control antiserum at birth, inoculated with SLT-Ⅱ-producing *E coli* 0157 : H7 when 1 day old, and euthanatized 128 hours later at the onset of neurologic signs. Note endothelial cell and medial myocytes are necrotic. Subendothelial edema and detachment of endothelia cell from basement membrane is also seen.

vs group 3 GMT, 2.82 ; range 2 to 4) which were administered antiserum mixed with milk(Table 2).

Relation between serum SLT-Ⅱ-neutralizing antibody titers and protection : Although a relationship was seen between presence of a SLT-Ⅱ-neutralizing antibody titer and prevention of neurologic signs and brain lesions, there was no definite correlation between the level of titer and degree of protection. The piglets in group 1 which had a focus of malacia in the cerebrum had a SLT-neutralizing antibody titer of 8 ; 4 other piglets in group 1 had titers <8, but were completely protected.

Endotoxin, prostacyclin and cytokine assays : Endotoxin levels in terminal serum samples were not significantly different among the 3 groups($p>0.05$) and were <0.04 ng/mℓ. Plasma prostacyclin(6-keto $PGF_{1\alpha}$) levels in piglets in group 2(mean, 11.76 ± 5.22 pg/mℓ ; range, 6.2 to 17.3 pg/mℓ) were significantly higher ($p<0.05$) than in groups 1 (mean, 5.63 ± 1.18 pg/mℓ ; range 3.5 to 7.3 pg/mℓ) and 3(mean, 5.83 ± 0.38 pg/mℓ ; range, 5.4 to 6.3 pg/mℓ) (Table 3). The plasma prostacyclin levels in piglets in groups 1 and 3 were not significantly different ($p>0.05$). No(<2) TNF activity was detected in the terminal sera of any of the 3 groups(Table 3).

Clinical pathology : The only significant difference in clinical pathologic values among the 3 groups was in the neutrophil count and fibrinogen level (Table 4). The mean neutrophil counts of piglets in group 2($0.91\pm0.52\times10^6$/mℓ ; range, 0.39 to 1.60×10^6/mℓ) was significantly lower ($p<0.05$) than the mean count groups 1($2.82\pm1.43\times10^6$/mℓ ; range, 0.29 to 4.76×10^6/mℓ) and 3 ($2.60\pm1.45\times10^6$/mℓ ; range 0.91 to 4.05×10^6/mℓ), whereas, the mean counts of groups 1 and 3 were not significantly

Table 3. Endotoxin, prostacyclin, and tumor necrosis factor(TNF) data

Parameter	Group 1 (n=10)	Group 2 (n=6)	Group 3 (n=4)
Endotoxin(ng/mℓ)	0.038±0.077*	0.012±0.008	0.029±0.04
Prostacyclin(pg/mℓ)	5.630±1.18	11.76±5.22	5.830±0.38
TNF(titer)	<2	<2	<2

Note. group 1, piglets administered SLT-Ⅱ-neutralizing antiserum at birth and inoculated 24 hours later ; group 2, piglets administered control antiserum at birth and inoculated 24 hours later ; group 3 piglets administered SLT-Ⅱ-neutralizing antiserum at birth and not inoculated.

*Mean±standard deviation.

Table 4. Clinical pathology data

Parameter	Group 1 (n=10)	Group 2 (n=6)	Group 3 (n=4)
Cell Count			
Leukocytes(10^3/mℓ)	6.23±2.80*	4.06±1.37	5.70±2.75
Band cells(10^3/mℓ)	1.37±1.35	0.69±0.54	0.81±0.57
Neutrophils(10^3/mℓ)	2.82±1.43	0.91±0.52	2.60±1.45
Lymphocytes(10^3/mℓ)	1.88±0.63	2.31±1.10	2.10±0.74
Monocytes(10^3/mℓ)	0.08±0.08	0.14±0.16	0.16±0.15
Platelets(10^6/mℓ)	589±224.6	747±175.9	548±239.5
Erythrocytes(10^9/mℓ)	4.96±0.43	4.83±0.32	5.05±0.21
Hemoglobin(gm/dℓ)	9.50±0.91	9.74±0.47	9.53±0.57
Hematocrit(%)	31.1±2.59	29.8±1.10	31.0±1.55
MCV(fl)	63.6±2.95	61.8±2.68	61.3±2.63
MCH(pg)	19.4±0.48	20.2±0.80	18.8±0.66
MCHC(%)	30.5±0.76	32.6±0.73	30.7±0.62
Fibrinogen(mg/dℓ)	98.7±30.21	101.2±20.47	67.0±16,89

Note. group 1, piglets administered SLT-Ⅱ-neutralizing antiserum at birth and inoculated 24 hours later ; group 2, piglets administered control antiserum at birth and inoculated 24 hours later ; group 3 piglets administered SLT-Ⅱ-neutralizing antiserum at birth and not inoculated.

*Mean±standard deviation.

different ($p>0.05$). The mean fibrinogen levels of groups 1(98.7 ± 30.21 mg/dℓ ; range, 51 to 145 mg/dℓ) and 2(101.2 ± 20.47 mg/dℓ ; range, 81 to 135 mg/dℓ) were significantly higher ($p < 0.05$) than the mean of group 3(67.0 ± 16.89 mg/dℓ ; range, 47 to 81 mg/dℓ), whereas, the mean fibrinogen levels of groups 1 and 2 were not sifnificantly different ($p>0.05$).

Discussion

A lack of neurlogic signs and brain vascular lesions in 9 of 10 piglets administered SLT-Ⅱ-neutralizing antiserum and challenged with SLT-Ⅱ-producing *E coli* provides evidence that SLT-Ⅱ is directly or indirectly responsible for brain vascular lesions. As ednotoxin levels in the sera of these piglets were not significantly different from age-matched, germ-free piglets (group 3), it is suggested that endotoxin does not play a significant role in the pathogenesis of vascular lesions. These results support the findings of a recent *in vitro* study, whereby affinity purified SLT-Ⅱ from *E coli* 0157 : H7 was cytotoxic to human vascular endothelial cells, but purified endotoxin from the same strain was not only noncytotxic, but also non-synergistic with SLT-Ⅱ in the induction of cytotoxic activity.[40]

The lack of detection of SLT-Ⅱ in the sera of our experimental piglets does not rule out SLT-Ⅱ as the potential cause of endothelial lesions. Our results are similar to those of studies on HUS patients infected with Verocytotoxin-producing *E coli*.[19]

One possible explanation for the lack of detection of

SLT-Ⅱ in the serum is that there is rapid endocytosis of SLT-Ⅱ by endothelial cells upon entrance of the toxin into the circulation. In a study of the kinetics of Shiga toxin uptake, there was complete internalization of the toxin into the cytoplasm of HeLa cells within 15 to 30 min of binding to thegalactotriosyl ceramide (Galactose α 1-4 Galactose β 1-4 Glucose β 1-1 Ceramide ; Gb_3) toxin receptor.[35] Similar results for SLT-Ⅱ in our piglet model would be expected, based on the fact that the receptor and biological actions for the 2 toxins are identical, and that the receptor is located on the surface of endothelial cells.[8,17,29,37]

Since gnotobiotic piglets which were administered SLT-Ⅱ-neutralizing antiserum were not protected against attaching-effacing bacterial colonization or watery diarrhea that was induced by infection with SLT-Ⅱ-producing *E coli* 0157 : H7, the results support previous studies that indicate SLT is not responsible for attaching-effacing lesions as seen by TEM on intestinal epithelium.[12,42] However, based on our work alone, this conclusion could not be reached, since it is also possible that insufficient quantities of SLT-Ⅱ neutralizing antibodies were available on a continual basis to prevent attaching-effacing lesions. Similarly, mesocolonic edema in groups 1 and 2 could be due to non-SLT-Ⅱ-mediated inflammation or a manifestation of SLT-Ⅱ-mediated vascular damage, the latter especially if insufficient quantities of SLT-Ⅱ-neutralizing antibodies in group 1 piglets were available to prevent SLT-Ⅱ-mediated vascular leakage in the mesocolon. Although presence of an SLT-Ⅱ-neutralizing antibody titer in the serum was correlated with protection against brain vascular lesions, the finding of an area of malacia in the cerebrum of one piglet which had a titer of 8 also suggests that protection against brain lesions may be incomplete. Presumable, malacia in this piglet was secondary to vascular damage ; however, attempts to detect damaged blood vessels in adjacent brain parenchyma in this piglet were unsuccessful.

The finding of elevated prostacyclin and neutropenia in challenged piglets administered control antisera(group 2) is consistent with results of our previous studies on non-SLT-Ⅱ-immunized, strain B2387-challenged gnotobiotic piglets.

Increased prostacyclin levels are seen in HUS patients[15,39] and are interpreted to be a compensatory response to endothelial damage. Since prostacyclin levels in group 2 were significantly higher than in groups 1 and 3, the increase was due to vascular damage and not colitis. As vascular lesions by light microscopy were limited to the brain, presumably the prostacyclin increases were due to vascular lesions in the brain ; but, since TEM was limited to the brain, the possibility still exists that endothelial lesions not detectable by light microscopy could also have been present in other organs. The cause(s) and significance of the neutropenia is unknown, but may be due to excess tissue demand in the large intestine, coupled with immaturity of the piglets. Neutropenia can also be seen as an early response to endotoxin[18] ; however, serum endotoxin levels in experimental piglets were not significantly($p>0.05$) different from that of non-challenged (sterile) control piglets (group 3). The finding of neutropenia is in contrast to HUS and TTP patients[19,27] and to our knowledge there have been no reports of toxic effects of SLT-Ⅱ or other SLT on leukocytes other than lymphocytes.[6]

Piglets were given antiserum orally at birth since it is a natural, non-traumatic means of immunization.[32] Milk replacer was mixed with antiserum when the first litter of piglets was immunized to improve palatability of the antiserum, and enhance consumption by the piglets, thereby minimizing variability of immunization times. However, relatively low SLT-Ⅱ-neutralizing antibody titers were achieved by this technique, therefore antiserum was administered to the second litter by stomach tube, without the addition of milk replacer. presumable immunoglobulin absorption had been impeded in the piglets fed milk replacer because of heterologous (bovine origin) protein and lactose in the milk replacer.[44] Although SLT-Ⅱ-neutralizing antibody titers were lower in the piglets fed milk replacer, the titers were still protective against brain lesions and neurologic signs.

As TNF activity was not detected in terminal serum samples, this suggests that TNF is not involved in the pathogenesis of vascular lesions ; however, the lack of detectable serum TNF could be due to the short half-life of the cytokine. Peak levels of TNF are seen in the serum of conventional swine 60 min after injection of purified endotoxin, but are non-detectable 120 min after injection(M.J. Wannemuehler, personal observations). However, it is difficult to compare these results with the

present study since a continual supply of endotoxin would presumably be absorbed into the portal blood in our model, but removed by Kupffer cells in the liver.[8] Another possible reason for non-detectable TNF levels in the serum is that macrophages (including Kupffer cells) may not have been sufficiently activated in the gnotobiotic condition. Insufficient data in this study and insufficient supportive literature on cytokine responses in swine(especially gnotobiotes) are available at this time to draw conclusions. A recent *in vitro* study has provided some evidence for TNF induction by SLT-Ⅱ. Tumor necrosis factor titers in C3H/HeN mouse macrophage supernatants but not macrophage supernatants of C3H/HeJ mice, were enhanced by exposure to SLT-Ⅱ.[2] It was unclear whether SLT-Ⅱ actually induced TNF production or made the macrophages more sensitive to endotoxin, the levels of which were estimated to be < pg/mℓ. The authors commented that SLT-Ⅱ may render macrophages more sensitive to endotoxin rather than acting as an inducing agent itself. From the results of this study, it is also possible that endotoxin could have been physiologically active at such low concentrations in our piglets such that the role of endotoxin still cannot be ruled out, but it can at least be concluded that the levels in our piglets were much lower than in patients with septic shock[43] and no greater than the background levels in the assay as detected in the nonchallenged (sterile) controls (group 3).

Although polyclonal antiserum against SLT-Ⅱ holotoxin, containing antibodies to both the A and B subunits was used in this study, antibodies to the B subunit alone may have achieved the same level of protection. SLT-Ⅱ B subunit-specific monoclonal antibodies neutralized SLT-Ⅱ holotoxin in HeLa cell cultures, whereas, SLT-Ⅱ A subunit-specific monoclonal antibodies only partially neutralized cytotoxic effects.[7,31] In another study, immunization of mice with conjugates of 2 peptides of the B subunit of Shiga toxin led to partial protection against the lethal effects of shiga toxin.[13,14] similar results were achieved with SLT-I, in which polyclonal antibodies against the B subunit protected rabbits against SLT-I holotoxin challenge by injection.[4]

In summary, the results of this study provide evidence that SLT-Ⅱ is directly or indirectly responsible for vascular damage in the brain in the gnotobiotic piglet model. Transmission electron microscopy was limited to the brain, as brain blood vessels, especially endothelium, appears to be the major target in the piglet model. As levels of endotoxin in the sera of piglets with vascular lesions in the brain were no different($p > 0.05$) from the levels in sera of non-inoculated (sterile) piglets (group 3), a role for endotoxin in the pathogenesis of the vascular lesions, at least at the 7 pg/mℓ level, was ruled out. Similarly, the finding of attaching-effacing lesions in callenged piglets given SLT-Ⅱ antiserum supports the results of previous studies which suggest that SLT-Ⅱ is not associated with attaching-effacing lesions ; however, whether or not SLT-Ⅱ induces inflammatory or vascular changes in the colon could not be determined. Additional studies are needed to address the potential role of SLT-Ⅱ and vascular damage in various organs.

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