MODELS OF INFECTION

Experimental infection of germ-free mice with hyper-toxigenic enterohaemorrhagic *Escherichia coli* O157:H7, strain 6

HARUHIKO TAGUCHI, MOTOMICHI TAKAHASHI, HIROYUKI YAMAGUCHI, TAKAKO OSAKI, AKIO KOMATSU*, YASUNORI FUJIOKA* and SHIGERU KAMIYA

Departments of Infectious Diseases and *Pathology, Kyorin University School of Medicine, 6-20-2 Shinkawa, Mitaka, Tokyo 181-8611, Japan

A mouse enterohaemorrhagic *Escherichia coli* (EHEC) infection model was developed with a combination of germ-free (GF) mice and hyper-toxigenic EHEC (HT-EHEC) O157:H7 strain 6. The HT-EHEC strain 6 produced both Shiga-like toxin (SLT)-1 1.0 µg/ml and SLT-2 8.2 µg/ml *in vitro*. Eight-week-old germ-free mice were inoculated intragastrically with 5.0×10^7 cfu of HT-EHEC strain 6. All GF mice challenged with the HT-EHEC developed ruffled fur and convulsion of limbs or hindleg weakness within 3 days after the challenge, culminating in death within 5 days. The HT-EHEC colonised well at a level of 10^8 – 10^9 cfu/g of faeces 5 days after the challenge. Both SLT-1 and SLT-2 were demonstrated in the faeces of the mice for 5 days after challenge. Histological examination of the colons of the infected mice showed congestion of the lamina propria mucosa, mild inflammatory cell infiltration and goblet cell depletion. In proximal tubules of the renal cortex, epithelial swelling with scattered necrotic cells was recognised. Endothelial swelling and mononuclear cell infiltration were also observed in the glomeruli. The brain showed acute neuronal necrosis in the cerebrum and slight loss of Purkinje cells in the cerebellum. Passive immunisation with anti-SLT antisera prolonged the life of the mice without any neural symptoms. Microscopically, all tissue specimens from the passively immunised mice were not remarkable. These results indicate that the infection of GF mice with HT-EHEC is a useful animal model to study the pathogenicity of SLT-producing *E. coli* and the toxins.

Introduction

Enterohaemorrhagic *Escherichia coli* (EHEC) was first identified as a causative agent of haemorrhagic colitis [1]. Numerous studies have reported the association of certain serotypes of *E. coli* with outbreaks of diarrhoea [2], haemorrhagic colitis [1, 3, 4] or the haemolytic uraemic syndrome (HUS) [2, 3, 5–7]. *E. coli* strains of serotypes O157:H7, O157:H– and O26:H11 are the most common pathogenic agents responsible for outbreaks of haemorrhagic colitis and HUS [1, 4, 8–10]. These strains produce several toxins, one of which is virtually identical to Shiga toxin. Therefore, they are also called Shiga-like toxin (SLT)-producing *E. coli*

(STEC); two types of SLT are recognised, SLT-1 and SLT-2.

In outbreaks of EHEC infection, watery and mostly bloody diarrhoea is the predominant symptom. After several days, HUS and other systemic complications may develop [11]. Although HUS is marked by microangiopathic haemolytic anaemia, thrombocytopenia and acute renal failure, 20–30% of patients manifesting encephalopathy died before the development of HUS [12, 13]. Central nervous system (CNS) complication is newly recognised as a major determinant of mortality in the acute phase of infection and a major factor in chronic morbidity in EHEC infection [14–16].

Several animal models have been used to study the pathogenesis of EHEC infection. The role of SLT has been studied by injection of purified SLT [3, 17–19]. It has also been reported that EHEC caused gastrointestinal lesions, neurological disorders or death in gnotobiotic piglets [20–22], streptomycin-treated mice

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Corresponding author: Dr H. Taguchi (e-mail: taguchi@kyorin-u.ac.jp).

[23, 24] and infant rabbits [25]. However, haemorrhagic colitis and glomerular pathology were not observed in the animal models.

Therefore, this study attempted to establish an EHEC infection model with a combination of germ-free (GF) mice and a hypertoxigenic strain of EHEC (HT-EHEC). Gnotobiotic (i.e., GF) animals offer a well-defined model to study the pathogenicity of bacteria because it is possible to study the interaction of the bacterium and its toxins with the host without the influence of any other bacteria. Also, passively administered specific antibodies have been much more effective in prevention of toxin-mediated diseases than in protection against microbial infection. Thus, the efficacy of passive immunisation was examined in the GF animal model.

Materials and methods

Bacterial strain and culture

EHEC 0157:H7 strain 6, isolated from a patient with haemorrhagic colitis, was used in these experiments. It was kindly provided by Dr M. Tamura, National Institute of Infectious Disease (Tokyo, Japan). Strain 6 has an enhanced ability to produce both SLT-1 (1.0 µg/ml) and SLT-2 (8.2 µg/ml) *in vitro* compared with other strains. Thus, this strain was designated as HT-EHEC. It was subcultured on Columbia Agar (BBL, Cockeysville MD, USA) containing sheep blood 5% at 37°C for 18 h. For infection studies, the strain was grown in Mueller Hinton broth at 37°C for 6 h, and then washed once with phosphate-buffered saline (PBS, pH 7.2) by centrifugation (3000 g, 20 min). The bacterial suspension was adjusted to $c. 1.0 \times 10^8$ cfu/ml in PBS. The number of bacterial cells in the suspension was determined by a plate count on Sorbitol MacConkey Agar (SMA) (Oxoid).

Quantification of toxin produced in vitro

The bacterial strain was inoculated into CAYE medium (Nissui, Tokyo, Japan) and incubated at 37°C for 18 h with shaking (120 rpm). After centrifugation (1000 g, 15 min), titres of SLT-1 and SLT-2 in the supernate were determined with the VTEC-RPLA Kit (Denka Seiken, Tokyo, Japan), for which the limit of detection for SLT-1 and -2 was 1 ng/ml.

Experimental animals and infection

GF mice (IQI, 8 weeks old, female) were obtained from CLEA Inc. (Tokyo, Japan). The mice were housed in sterilised vinyl isolators and were fed with sterilised diet and water.

Seven GF mice were infected with HT-EHEC strain 6 to establish an animal model. The mice were inoculated intragastrically with 0.5 ml of the bacterial suspension

through a catheter tube (0.9 × 70 mm; Ikemoto, Tokyo, Japan). The condition of the mice was monitored daily for 10 days. Viable bacterial cells and the presence of toxins in the faeces were determined. The moribund or healthy control mice were killed by injection of sodium pentobarbitone. The intestine, kidney and brain were isolated from the mice for histological examination. This experimental infection was performed under the control of the ethical committee in accordance with the guidelines on animal experiments in Kyorin University and Japanese Government Animal Protection and Management Law (no. 105).

Passive immunisation schedule

The passive immunisation schedule is summarised in Fig. 1. Both rabbit anti-SLT-1 and anti-SLT-2 antisera were kindly provided by Denka Seiken. The antisera were diluted 1 in 100 in PBS; this dilution was able to neutralise 10 pg of SLT-1 and SLT-2 in a Vero cell assay and was used for passive immunisation. GF mice were treated intraperitoneally with 0.1 ml of diluted anti-SLT-1 or anti-SLT-2 antiserum, or both. The mice in group 1 received the antiserum mixture daily from the challenge day for 3 days. The mice in group 2 received the antiserum mixture daily from 2 days before the challenge and up to 2 days after the challenge. The mice in groups 3 and 4 received either anti-SLT-1 or anti-SLT-2 antiserum daily from 2 days before the challenge and up to 2 days after the challenge.

Quantification of EHEC and its toxins

The number of viable bacterial cells contained in 1 g of faeces was quantified on SMA. Then, the suspension of the faeces was centrifuged (46 000 g, 3 min) and supernate was used for titration of toxins. Titres of SLT-1 and SLT-2 in the supernates were determined with the VTEC-RPLA kit.

Histological examination

The intestine, kidney and brain were removed surgically immediately after the mice were killed and examined for pathological changes. The tissues were fixed in formaldehyde 10% in PBS and processed by standard procedures. Sections of paraffin-embedded tissue were stained with haematoxylin and eosin and observed by light microscopy.

Results

Infection of GF mice with HT-EHEC and the effect of passive immunisation

Two GF mice infected with HT-EHEC died on the third day, and all mice had died by the fifth day of infection (Table 1). These mice showed systemic symptoms including lethargy, ruffled fur and anorexia on the second to third day of infection, then developed neuro-

Fig. 1. Passive immunisation schedule: GF mice were treated intraperitoneally with 0.1 ml of rabbit anti-SLT-1 or anti-SLT-2 antiserum, or both. The mice in group 1 received the antiserum mixture daily from the challenge day for 3 days. The mice in group 2 received the antiserum mixture daily from 2 days before challenge up to 2 days after challenge. The mice in groups 3 and 4 received either anti-SLT-1 or anti-SLT-2 antiserum daily from 2 days before challenge up to 2 days after challenge.

Table 1. Experimental infection of GF mice with HT-EHEC and the effect of passive immunisation with anti-SLT-1 and anti-SLT-2

Group*	Passive immunisation	Number of dead mice/total number of mice at		
		3 days	5 days	10 days
C	—	2/7	7/7	7/7
1	+ (Anti-SLT 1 and 2, 3 times [†])	0/4	1/4	2/4
2	+ (Anti-SLT 1 and 2, 5 times)	0/5	0/5	0/5
3	+ (Anti-SLT 1, 5 times)	0/7	3/7	3/7
4	+ (Anti-SLT 2, 5 times)	0/7	1/7	2/7

*See immunisation schedule in Fig. 1. C, control group without passive immunisation.

[†]Number of immunisations performed.

logical symptoms including hindleg weakness or paralysis and convulsion of limbs, but diarrhoea was not observed. In contrast, mice passively immunised with 0.1 ml of rabbit anti-SLT-1 and SLT-2 serum mixture daily from 2 days before challenge and up to 2 days after challenge survived for 10 days without diarrhoea or convulsion of limbs (group 2). Of the mice passively immunised with 0.1 ml of anti-SLT-1 and anti-SLT-2 serum mixture daily from the challenge day up to 2 days after challenge (group 1), two of four mice had died by 10 days after the challenge. Of the mice immunised with 0.1 ml of anti-SLT-1 or anti-SLT-2 serum daily from 2 days before challenge up to 2

days after challenge (groups 3 and 4), three and two of seven mice had died by 10 days after the challenge, respectively.

Colonisation and production of SLTs by HT-EHEC in GF mice

Cell counts of viable HT-EHEC and the titres of SLTs produced by the organisms in faeces of the GF mice were determined (Fig. 2a and b). The HT-EHEC colonised well at a level of *c.* 10^8 – 10^9 cfu/g 5 days after inoculation. No other bacteria were detected throughout the experiment. Both SLT-1 and SLT-2

Fig. 2. Colonisation and production of SLTs by HT-EHEC in GF mice. (a) Number of HT-EHEC in the faecal contents of gnotobiotic mice. (b) Pattern of toxin detection in faeces of GF mice; □, SLT-1; ■, SLT-2.

produced by the colonising HT-EHEC were also detected in the faeces of the GF mice 5 days after inoculation.

Histological examination

Although intestinal haemorrhage was observed macroscopically in the GF mice challenged with HT-EHEC strain 6 (Fig. 3a), no pronounced haemorrhagic colitis was observed microscopically (Fig. 3b). However, the colons showed congestion of the lamina propria mucosa, mild inflammatory cell infiltration and goblet cell depletion. Microscopic examination of the kidneys from the GF mice challenged with HT-EHEC strain 6 showed epithelial swelling with scattered necrosis of the proximal tubules (Fig. 4a). Endothelial cell swelling, mononuclear cell infiltration, focal proliferation of mesangial cells and congestion were observed in the glomeruli (Fig. 4b). No microthrombi were seen in glomerular capillaries. Microscopic inspection of the cerebral cortex and cerebellum showed acute neuronal necrosis (Fig. 5a) and slight loss of Purkinje cell (Fig. 5b). However, no microscopic changes were observed in any tissue specimens from the mice that were passively immunised (data not shown).

Discussion

This study demonstrated that GF mice experimentally infected with HT-EHEC developed systemic and neurological symptoms. All the mice showed enteritis, renal failure with endothelial swelling and mononuclear cell infiltration in the glomeruli, and encephalopathy by histopathological examination.

It has been reported already that Shiga toxin [26] and SLT-2 [27] cause flaccid paralysis and death in mice. Neurological symptoms were observed in STEC infection models with streptomycin-treated [23, 24], mitomycin-treated [28] and malnourished mice [29], and the infected mice died by 10 days after infection. Furthermore, SLTs are cytotoxic to Vero cells and inhibit protein synthesis in eukaryotic cells [30], including endothelial cells of the kidney [31] and brain cells [32]. SLT-2 is also toxic to both endothelial cells and neurons in the central nervous system and causes fatal acute encephalopathy [28]. In this experiment, the GF mice challenged with EHEC O157:H7, which is a hyper-toxigenic strain, developed similar neural disorders and died within 5 days after the challenge. Furthermore, renal failure and acute neuronal necrosis in the cerebrum and slight loss of Purkinje cell in the cerebellum were observed microscopically. The appearance of histopathological changes of the kidney and the brain was attributed to the hyper-production of toxin by strain 6. On the other hand, passive immunisation with ant-SLT-1 and anti-SLT-2 antiserum mixture from 2 days before challenge up to 2 days after challenge was sufficient to prolong the life of the mice without any neural symptoms or typical histopathological changes. These results indicated that both SLT-1 and SLT-2 played a role in the pathogenicity of HT-EHEC, and the action of the toxin was prevented by passive immunisation. The colons of the mice challenged with HT-EHEC showed mild inflammatory cell infiltration and goblet cell depletion. Intimate bacterial attaching and effacing of the gut epithelium were demonstrated in tissue culture and animal models of EHEC infection [22, 33, 34]. The responsibility of the *eaeA* gene for intestinal lesions

Fig. 3. Histopathological changes in the colon of a moribund mouse. **(a)** Intestinal haemorrhage was observed by gross examination of tissues. **(b)** The colon showed congestion of the lamina propria mucosa, mild inflammatory cell infiltration and goblet cell depletion.

cannot be dismissed, as HT-EHEC strain 6 used in this study has a conserved *eaeA* gene (data not shown).

The characteristic renal histopathology of EHEC-mediated HUS in man includes thrombotic micro-angiopathy in the glomeruli, thrombotic micro-angiopathy with arterial involvement and cortical tubular

necrosis [35]. It has also been reported that the glomerular damage in man is a consequence of the cytotoxic effect of SLT on capillary endothelial cells [36]. Although the kidneys of mice in this model showed swelling of epithelial cells, endothelial swelling and mononuclear cell infiltration, the pathogenesis of EHEC-mediated HUS was not completely demon-

Fig. 4. Photomicrograph of the renal cortex and the glomeruli from a moribund mouse. (a) Swelling of epithelial cells with scattered necrosis was noted in the proximal tubules. (b) Endothelial swelling, mononuclear cell infiltration, focal proliferation of mesangial cells and vascular congestion were observed in glomeruli.

strated. Wadolowski *et al.* also reported that acute tubular necrosis without glomerular damage was observed in streptomycin-treated mice [23, 24]. They speculated that this might be due to the absence of functional SLT-receptor Gb3 in the mouse kidney compared with man.

However, the typical symptoms and disorders of this model were induced by only one oral challenge with HT-EHEC O157:H7 strain 6 without any treatment. This study has confirmed that the GF mouse challenged with HT-EHEC is a useful animal model to study the pathogenicity of SLT-producing *E. coli* and its toxins.

Fig. 5. Microscopic examination of cerebral cortex and cerebellum. Cortical acute neuronal necrosis (**a**) and slight loss of Purkinje cell (**b**) were observed.

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