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Stx2- but not Stx1-specific human monoclonal antibody protects piglets challenged with enterohemorrhagic *Escherichia coli* producing Stx1 and Stx2

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Abstract

Shiga toxin 2 (Stx2) producing *Escherichia coli* (STEC) strains are more frequently isolated from hemolytic-uremic syndrome cases than strains that produce Stx1 and Stx2, and rarely the strains that produce only Stx1. Studies have implicated Stx2 as the sole contributor to acute kidney failure and other systemic complications in humans, and our study adds further support to this assumption since Stx2- and Stx1-specific antibodies protected 100% and 0% of piglets, respectively, against an oral challenge with a Stx1- and Stx2-producing STEC strain. We conclude that Stx2-specific antibody is sufficient to protect piglets, and possibly humans, against STEC producing both toxins.

Keywords

Shiga toxin; Stx; antibody; enterohemorrhagic; piglet; hemolytic uremic syndrome; kidney failure; *E. coli*

Introduction

Hemolytic-uremic syndrome (HUS), characterized by hemolytic anemia, thrombocytopenia, acute renal damage, and variable degrees of central nervous system (CNS) complications can result in death or chronic, irreversible, renal dysfunction [1]. Infection with Shiga toxin (Stx) producing *Escherichia coli* (STEC) is the most significant cause of HUS, the leading cause of renal failure in children [1]. STEC strains produce either one or both of the immunologically distinct Stx1 and Stx2. However, epidemiological studies of STEC isolates from HUS sporadic and outbreaks implicate infections with strains that produce Stx2 alone, less frequently (5:1) with strains that produce both Stx1 and Stx2 (double producers), and rarely if ever, strains that produce only Stx1. [2]. Nonetheless, the double producer strains are >2.5 times more frequently associated with HUS than those that produce only Stx1 [2]. Experimental infections of gnotobiotic piglets using isogenic mutant strains of a double producer wild type *E. coli* O157:H7 strain 933, also suggests that Stx2 is the main cause of systemic complications [3]. In that study it was shown that a Stx2-producing isogenic mutant caused neurological symptoms and lesions in 90% of the piglets, whereas the wild type 933 strain producing both toxins caused neurological complications in only 33% of the piglets. The isogenic strain producing only Stx1 failed to induce any detectable neurological symptoms. However, that

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study did not investigate whether one or both toxins, when produced simultaneously, contribute to systemic complications in piglets infected with the double producer (wild type strain 933). The contribution of the individual toxin in inducing HUS and CNS complications in patients infected with the double producers is also not known. It is assumed that Stx2 and not Stx1 causes systemic complications in these cases.

In previous studies using the piglet model we have shown that human monoclonal antibody (HuMAbs) against Stx2, when administered systemically, even 48 hours after infection, completely protected piglets challenged with Stx2-producing STEC from developing fatal systemic complications manifested by CNS symptoms and lesions [4,5].

The aim of this study was to determine whether the Stx2-specific HuMAb which protected piglets challenged with a Stx2 producing STEC would be equally effective against strains that produce Stx1 and Stx2, as compared to piglets treated with Stx1-specific HuMAb. In addition, the results helped confirm the contribution of each toxin to the systemic lethal complications seen in piglets, and by inference, in patients infected with the double producers. To accomplish this, we challenged piglets orally with the double producer strain 933 and subsequently treated infected animals 48 h post-infection with either a Stx1- or a Stx2-specific HuMAb.

Materials and Methods

Bacterial strain

Enterohemorrhagic *E. coli* (EHEC) O157:H7 strain 933 which produces both Stx1 and Stx2 [3] was used.

Stx-specific HuMAbs

We have described the production of Stx1- and Stx2-specific HuMAbs elsewhere [6,7]. For this study we selected 5C12 and 5A4, our best Stx2- and Stx1-specific HuMAbs in protecting animals against Stx2- and Stx1-mediated lethal effects, respectively [4,8]. Both HuMAbs, recombinant 5C12 [9] and hybridoma 5A4 [7], were of human IgG1κ isotype. The HuMAbs were purified from cell culture supernatant by protein A affinity chromatography, quantified by UV spectrophotometry (ND-1000 Spectrophotometer, Nanodrop), aliquoted, and stored at -20°C. The HuMAbs were quantified prior to each experiment to confirm concentrations.

Gnotobiotic piglet model of *E. coli* STEC infection

The HuMAbs 5C12 and 5A4 were analyzed for their abilities to protect gnotobiotic piglets against the systemic complication of an oral challenge with EHEC strain 933 as described elsewhere [4,6]. A total of 31 piglets received a bacterial challenge of 1×10^{10} CFU (colony forming units) of the strain 933; after 48 h, 11 of these piglets received 5C12 (2 mg/kg), 6 received 5A4 (2 mg/kg) and 14 received PBS intraperitoneally (IP). The piglets were assigned to these groups randomly based on their body weight. Piglets were monitored several times daily for symptoms of diarrhea, dehydration, and CNS, which included ataxia, paresis, headpressing, paddling, convulsions, opisthotonos. Surviving animals were humanely euthanized if presenting severe CNS symptoms, or 14 days after challenge. Brain (cerebral cortex and cerebellum) and intestinal tissues were fixed in formalin and processed for histology.

Statistics

Fisher exact test was used to identify differences in survival rates among three groups (PBS, 5C12, and 5A4). Resulting *p*-values of less than 0.05 were considered significant.

Results

Table 1 summarizes the percent survival of piglets orally infected with the EHEC strain 933 and treated 48 h post-infection with HuMAbs 5C12 or 5A4 or PBS. Nine (64%) of the 14 control piglets that received PBS did not survive. Of these 9 piglets, 1 was found dead 88 h post-bacterial challenge, and the other 8 developed neurological symptoms of ataxia, headpressing and paddling, and were euthanized. All piglets that received Stx2-specific antibody 5C12 survived. In contrast, none of the 6 piglets that received Stx1-specific antibody 5A4 survived. Of these six piglets, 1 was found dead 111 h post-challenge, and the other 5 developed fatal neurological symptoms and were euthanized. The HuMAb 5C12 significantly protected piglets compared to PBS (p= 0.04) and 5A4 (p= 0.00008). Survival of PBS group was also significantly different from 5A4 (p= 0.01).

The neurological symptoms in piglets were accompanied histologically by cerebellar vascular lesions of petechial hemorrhages in the molecular and cortex layers, with evidence of infarction and extensive shrinkage of the neuronal nuclei, as previously described in more detail [3,4,6, 10]. The piglets developed fatal neurological complications within 88 to 216 h (about 3.5 to 9 days) post-infection. Antibody 5A4 did not have any effect on the time of onset of neurological symptoms since piglets in both PBS and 5A4 groups started to develop these symptoms at 88 h post-infection.

Piglets generally developed diarrhea within 23 to 47 h and rarely after 47 h of the bacterial challenge. All piglets developed diarrhea due to an intimate attachment of bacteria to the mucosal surfaces of the terminal ileum and the entire large bowel which include the colon and cecum. The nature, distribution and extent of the mucosal lesions in gnotobiotic piglets induced by STEC strain 933 were consistent with those amply described in the past [3,4,6,10]. The surviving piglets of the 5C12 and PBS groups continued to excrete strain 933 bacteria and had diarrhea but at reduced intensity at the time of euthanasia.

Discussion

We previously reported that Stx2-specific HuMAb 5C12 protects piglets [4,5] and mice [8] against systemic complications of *E. coli* infection and death when administered intraperitoneally 48 h after oral challenge with EHEC strains producing either Stx2 or Stx2 variants. In the present study we demonstrated that 5C12 also protects piglets against oral challenge with an EHEC strain that produces both Stx1 and Stx2. In contrast, Stx1-specific 5A4 was not protective. These findings support the assumption that Stx2, and not Stx1, causes systemic complications in individuals infected with the STEC producing both toxins. These results, together with the results of our previous studies [4], suggest that a Stx2-specific antibody could be sufficient to prevent HUS in patients infected with strains that produce only Stx2 or both Stx1 and Stx2.

In an earlier study, we showed that fatal systemic complications occurred in 31% of animals infected with the Stx1- and Stx2-producing EHEC strain 933 [3]. In the present study the mortality rate among the control piglets infected with the same EHEC strain was 64%. Furthermore, all 6 piglets in the Stx1-specific 5A4 antibody group died. The speculation that 5A4 blocks binding of Stx1 to its cell-surface receptor Gb3 (unpublished), and that this function of 5A4 may have provided more opportunity for Stx2 to bind to the same target sites (Stx2 and Stx1 share the same cell-surface receptor Gb3) should not be the reason for complete mortality in the 5A4 group since systemic administration of either Stx1 or Stx2 is lethal to piglets [11]. It is also possible that with a larger 5A4 group sample size we might have seen <100% mortality. However, the reasons for differences in toxicities of the toxins following oral infection seem to lie with how the two toxins are uptaken from the gut since Stx2 whether produced in the gut

[4] or administered systemically [11] is lethal but Stx1 is lethal only when administered systemically [3,7]. The complete mortality in 5A4 group could not have been due to 5A4 toxicity in piglets since the brain lesions were typical of those that are mediated by Stx2. Furthermore, isotype of 5A4 is same as that of 5C12, and 5C12 protected all piglets.

Diarrhea in the piglets in the present study occurred usually between 23 and 40 h and rarely after 40 h of bacterial challenge, which is consistent with our earlier reports [3,4,6,10]. In those earlier reports, the systemic complications occurred between 48 to168 h, whereas in the present study those complications occurred between 88 to 216 h post-bacterial challenge [3,4,6,10]. Although the reasons for the delay in appearance of the systemic complications are not clear, genetic variations among outbred piglets may be a factor. The clinical observations and histopathological brain lesions were consistent with our earlier reports on the use of oral STEC infection piglet model for systemic Stx complications [3,4,6,10].

In conclusion, we have demonstrated that Stx2, but not Stx1, contributes to systemic complications in piglets infected with *E. coli* strains which produce both toxins, which further confirms the notion that Stx2, and not Stx1, is largely responsible for the induction of HUS and CNS complications in individuals infected with strains that produce both toxins. In addition, the study shows that protecting piglets with specific antibody against Stx2 is sufficient to prevent systemic complications in piglets, and possibly in humans, even against STEC strains that produce both toxins.

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Table 1

Survival of piglets orally-challenged with 1 × 10^{10} CFU of EHEC strain 933 and treated IP 48 h after challenge with 2 mg of either Stx2-specific HuMAb 5C12 or Stx1-specific HuMAb 5A4

Treatment	Total no. of piglets	No. (%) of survivors[2]	No. (%) died
PBS[1]	14	5 (36)	9 (64)
5C12	11	11 (100)	0 (0)
5A4	6	0 (0)	6 (100)

[1] PBS group served as a placebo control.

[2] Survival rates were significantly different (*p* values: 0.04 for PBS vs 5C12, 0.01 for PBS vs 5A4, and 0.00008 for 5C12 vs 5A4).