Evidence of intravenous immunoglobulin as a critical supportive therapy against Clostridium difficile toxin-mediated lethality in mice

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Received 26 October 2010; returned 3 December 2010; revised 6 January 2011; accepted 17 January 2011

Objectives: Clostridium difficile produces toxins and is an aetiological organism of pseudomembranous colitis. Immunoglobulin is one of the treatment strategies against fulminant C. difficile infections, but the clinical evidence is still limited. We examined the efficacy of intravenous immunoglobulin (IVIg) in C. difficile toxin (CDT)-mediated lethality and cellular injury in mice.

Methods: Mice were intraperitoneally injected with 0.2 mL of filter-sterilized C. difficile culture supernatant (CDT preparation). The IVIg preparation was intravenously administered at several timepoints. We also examined alteration of intestinal permeability and an apoptosis marker in the gut. In vitro experiments, HEp-2 cells were incubated with a CDT preparation in the presence or absence of the IVIg preparation, after which cell viability and lactate dehydrogenase release were examined.

Results: All control mice died by day 2 after injection of the CDT preparation. The maximum effects of IVIg (100% survival) were observed when the mice were treated with IVIg at the same time as injection of the CDT preparation. The IVIg effects were closely associated with improvement of intestinal vascular permeability and mucosal damage in the gut. In addition, reduction of an apoptosis marker (histone-associated DNA fragments) was demonstrated in the mice treated with IVIg. Interestingly, a smaller increase in histone-associated DNA fragments was observed in FasL-deficient mice treated with the CDT preparation compared with wild-type.

Conclusions: These data demonstrated that IVIg may be protective against CDT-mediated lethality, when administered at the appropriate time. The present data also suggest an increase in intestinal permeability, probably through exaggeration of Fas/FasL-mediated apoptosis, as a key mechanism of C. difficile-mediated diseases.

Keywords: toxin A, FasL, apoptosis

Introduction

Clostridium difficile is a Gram-positive, anaerobic, spore-forming bacillus. C. difficile infection is becoming a worldwide problem as a cause of nosocomial diarrhoeal diseases, especially in North America and Europe. C. difficile produces several toxins, such as toxin A and toxin B, which are associated with the pathogenesis of C. difficile infections in the intestinal tract. These C. difficile toxin (CDT)-mediated physiological changes are well known to be associated with clinical symptoms in these patients, from mild diarrhoea to potentially deadly forms of diseases, including pseudomembranous colitis and toxic megacolon.

Intravenous immunoglobulin (IVIg) is a therapeutic preparation of normal human polyclonal IgG obtained from plasma pooled from a large number of healthy blood donors. Although IVIg is recommended to treat severe C. difficile infections, the effectiveness of this strategy has been variable and anecdotal. Further investigations, including animal model experiments and clinical research, are warranted, especially regarding the specific activity of IVIg for neutralization of CDT.

In this study, we have examined the efficacy of IVIg in a mouse model of CDT-mediated lethality. In addition, the mechanisms of pathogenesis of C. difficile infections, particularly toxin-mediated cellular permeability and involvement of apoptosis in intestinal cells, were explored.
Materials and methods

Experimental animals

BALB/c mice were purchased from Charles River Japan (Kanagawa, Japan). C57BL/6J mice and C57BL/6J Slc-gld−/gld− mice (FasL-deficient mice) were purchased from Sankyo Laboratory Service (Tokyo, Japan). Animal protocols were approved by the institutional animal care and use committee (approval number: 10-52-54).

IVIg

Human polyclonal IVIg was purchased from Genesys Corporation (Osaka, Japan).

C. difficile used and preparation of culture supernatant

A single colony of a clinical isolate of C. difficile grown on cycloserine/cefotixin/mannitol agar was transferred to 20 mL of brain heart infusion broth supplemented with yeast extract and L-cysteine, and incubated overnight at 37°C anaerobically. Then, the cultured broth was centrifuged at 3000 rpm for 20 min at 25°C. The supernatant was filter-sterilized through a 0.45 μm membrane, and used as a crude CDT preparation.

Assessment of intestinal vascular permeability

An aliquot of 200 μL of 1% Evans blue (EB) dye was administered intravenously into the tail vein of mice. One hour after the injection of EB dye, we sacrificed the mice. The gut was removed and washed three times with sterile water to remove feces. The intestines were homogenized and centrifuged for 20 min at 3000 rpm. The quantity of extracted EB dye in the supernatant was determined by measuring the absorbance at a wavelength of 640 nm.

Determination of histone-associated DNA fragments

To evaluate induction of apoptosis, the levels of histone-associated DNA fragments were determined in the serum, as described in the manufacturer’s instructions (Cell Death Detection ELISAplus; Roche Diagnostics).

Assessment of cell viability

Viability of cells was measured using TetraColor ONE (Seikagaku Kogyo, Tokyo, Japan), in accordance with the manufacturer’s instructions.

Statistical analysis

Statistical significance was determined using the two-tailed Student’s t-test. Survival curves were constructed by the Kaplan–Meier method and analysed by the log-rank test using GraphPad Prism (ver. 5.0). P < 0.05 was considered to indicate a significant difference.

Results

Effect of IVIg on survival and intestinal damage in mice treated with the CDT preparation

CDT preparation-injected mice started to die from 24 h after administration, and all mice died by 36 h (Figure 1a). In contrast, human polyclonal IVIg administration before or at the same time as injection of the CDT preparation significantly improved the survival of mice (P < 0.001). Notably, when IVIg was administered at the same time as the CDT preparation, complete protection was observed. Interestingly, survival of mice was significantly different (P = 0.02) between non-treated mice and mice treated with IVIg 6 h after administration of the CDT preparation. The effects of IVIg on survival clearly decreased if the timing of administration of IVIg was delayed. These data demonstrate that IVIg may be effective against CDT-mediated mortality, and further suggest that the timing of IVIg administration may be a critical factor.

Next, we examined the changes in the intestinal tract after injection of the CDT preparation. Histological examination of the intestinal tract clearly demonstrated disruption of mucosal architecture in both the small and large intestine of mice treated with the CDT preparation (data not shown). To assess the change in vascular permeability in the intestinal tract, EB dye was injected into the tail vein of mice and extravasation of EB dye was evaluated. As shown in Figure 1(b), IVIg treatment significantly suppressed the increase in vascular permeability induced by the CDT preparation. To evaluate the role of apoptosis in the pathogenesis of lethality of the CDT preparation in mice, histone-associated DNA fragments were measured. As shown in Figure 1(c), the increase in this apoptosis marker induced by the CDT preparation was significantly suppressed in mice treated with IVIg.

Protective effects of IVIg on cellular damage caused by the CDT preparation

As shown in Figure 1(d), the CDT preparation decreased the viability of HEp–2 cells to approximately 30% of the control, and cellular damage was reduced in the presence of IVIg in a concentration-dependent manner. Correlating with these data, the release of lactate dehydrogenase by the CDT preparation and its reduction in the presence of IVIg were demonstrated. These data confirmed the concentration-dependent protective effects of IVIg on the cellular damage caused by the CDT preparation.

Roles of Fas/FasL-mediated signalling in the pathogenesis of lethality caused by the CDT preparation

Finally, to examine the roles of Fas/FasL-mediated signalling in the pathogenesis of lethality in mice caused by the CDT preparation, we compared lethal sensitivity between wild-type and FasL-deficient mice. A trend towards delay of death was observed in FasL-deficient mice through the experimental period (Figure 2a). Consistent with these data, a smaller increase in histone-associated DNA fragments was observed in FasL-deficient mice (Figure 2b). These data suggested a contribution of Fas/FasL-mediated signalling and apoptosis to the pathogenesis of death of mice caused by the CDT preparation.

Discussion

It is well established that CDTs play a critical role in the pathogenesis of C. difficile disease, and several investigators have reported that patients with severe or recurrent disease have poor antitoxin antibody responses.7 Studies with animal models that demonstrate the importance of anti-CDT antibody in mitigating C. difficile diseases have been conducted mainly
in hamster and mouse models. Babcock et al.\textsuperscript{8} have reported that monoclonal antibodies directed against toxins A and B in combination significantly reduced mortality from 100% of the control to 45% ($P < 0.001$) in a hamster model of \textit{C. difficile} infection. In a gnotobiotic mouse model of \textit{C. difficile} infection, Corthier et al.\textsuperscript{9} have reported protective effects of anti-toxin A monoclonal antibodies. The present study evaluated the effects of IVIg on vascular permeability and apoptosis in the intestinal tract and lethality in mice challenged with a CDT preparation. The survival data of the present study are intriguing, in that a 100% difference in survival was observed if IVIg was administered at the same time as the CDT preparation. However, it may be important to carefully consider these data for their clinical relevance and applicability, because the model used in the present study was not infection by live bacteria but administration of the CDT preparation. In addition, we administered the CDT preparation by systemic injection, not via the intestine.

Previous reports have clearly demonstrated the involvement of cellular apoptosis, mainly through binding of CDT A, in the pathogenesis of \textit{C. difficile} infections.\textsuperscript{3,4} In the mouse ileal loop model, Kim et al.\textsuperscript{10} reported that CDT A strongly induced FasL in colonial epithelial cells, which was closely associated with exaggeration of apoptosis. The present data from FasL-deficient mice clearly demonstrate a critical role of Fas/FasL-mediated apoptosis signalling in the lethality in mice challenged with systemic administration of the CDT preparation.
Finally, in spite of several limitations, our data stress the timing of administration as a crucial factor for the efficacy of IVIg. This may pose another difficulty for IVIg therapy in the clinical setting, because it is impossible to predict *C. difficile* infection exactly in individual cases. Although groups of patients at high risk of *C. difficile* infections may be candidates for preemptive administration of IVIg, cost-effectiveness considerations and adverse reactions may be a barrier. More practically, there are no data available for titres of specific antibody against CDT A or B, which may vary between different manufacturing companies and from lot to lot. We believe that the data from the present study and the questions they raise may be important for future investigations to determine the appropriate positioning of IVIg for use in the treatment of life-threatening *C. difficile*.

**Acknowledgements**

We thank Yutaka Hirao (Benesis Corporation) for his cooperation in checking specific CDT antibody titres in IVIg. We would like to express our deep appreciation to Tse Hsien Koh for his critical comments and careful reviewing of the manuscript.

**Funding**

This study was supported by grant of the strategic basis on research grounds for non-governmental schools at Heisei 20th from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

**Transparency declarations**

None to declare.

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