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ORIGINAL ARTICLE

Comparison of efficacies of bovine immune colostral antibody and each immunoglobulin class against verotoxin 2, flagellum and somatic cells of *Escherichia coli* O157:H7 in mice

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Purpose: The efficacy of bovine immune colostral (colostral) antibodies against verotoxin (VT) 2, flagellum and somatic cells of *Escherichia coli* (*E. coli*) O157:H7 in mice was determined.
Methods: Three major immunoglobulin (Ig) classes were isolated from the colostral antibody against VT2 by affinity chromatography and were used for estimation. Mice inoculated with VT2 were administered each Ig class from the colostral antibody, colostral antibody (colostral whey containing antibody) or serum antibody against VT2 at 1 hour after VT2 inoculation.
Results: All control mice (20/20) died after administration of sterilized saline instead of the colostral antibody. The survival rate was 93.3% (14/15) after administration of S-IgA or IgM antibody, or colostral antibody. Survival rates for IgG antibody and serum antibody administration were 80% (12/15) and 60% (9/15), respectively. Serum concentrations of VT2, which was absorbed from the small intestine in mice after administration of VT2 and colostral antibody, were measured by fluorescence enzyme immunoassay (FEIA). Serum concentrations of VT2 after administration of colostral antibody were lower than those after administration of sterilized saline. Mice inoculated with VT2-producing *E. coli* 157:H7 were administered anti-flagellum or anti-somatic colostral antibodies. Survival rates for *E. coli* O157:H7-infected mice administered the anti-flagellum and anti-somatic colostral antibodies were 52.4% (11/21) and 22.2% (4/18), respectively. Furthermore, survival rates increased to 89.5% (17/19) with combined administration of anti-flagellum and anti-VT2 colostral antibodies.

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Conclusion: These results suggest that colostral antibodies against VT2, flagellum and somatic cells are effective against *E. coli* O157:H7 infection.

Introduction

A large outbreak of enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 occurred in Japan in July 1996,[1–4] and food poisonings caused by EHEC continue to be reported. Under the Japanese Infection Diseases Control Law, patients with EHEC infection are surveyed and 3878 symptomatic and asymptomatic cases were reported in 2010 (Infection Agents Surveillance Report. 2010. Enterohemorrhagic *Escherichia coli* infection as of May 2010, Japan: http://www.nih.go.jp. Accessed December, 2010). However, effective therapeutic approaches using antibiotics and the prevention of complications due to verotoxin (VT) released from *E coli* disrupted by antibiotics remain elusive.[5–12]

We previously reported that a bovine immune colostral (colostral) antibody against VT is able to neutralize the toxicity of VT[13,14]; the survival rates of mice infected with *E. coli* O157:H7 producing VT2 improved after colostral antibody administration and antibiotic treatment.[13] It has also been reported that bovine colostral antibody is resistant to proteases in beagle dogs, as compared with serum antibodies.[14] Furthermore, S-IgA is more resistant to proteases in beagle dogs than IgG or IgM.[14] Bovine colostral S-IgA and IgG antibodies thus resist proteases in the intestines of beagle dogs and exhibit an effective neutralizing activity against VT2 after oral administration. However, comparisons of neutralizing efficacy for each immunoglobulin class have yet to be performed.

In addition, we believe that adhesion of *E. coli* O157:H7 to the intestine would be more strongly inhibited by an antibody against the flagellum. Thus, we prepared a bovine colostral antibody against the flagellum, and investigated whether this antibody inhibits adhesion of *E. coli* O157:H7.

Materials and methods

Bacterial strain and VT2

VT2-producing *E. coli* O157:H7 isolated from a human patient was used in this study. Crude VT2 was obtained and VT2 levels in culture medium were determined in accordance with the method of Kuribayashi et al.[13]

Animals

A pregnant dairy cow (age = 8 years), 3 months prior to delivery, was used for the preparation of colostral antibody against VT2, and a pregnant dairy cow (age = 8 years), 4 months prior to delivery, was used for the preparation of colostral antibody against the flagellum or somatic cells of *E. coli* O157:H7. SPF ICR mice (Charles River Japan, Inc., Yokohama, Kanagawa, Japan) aged 3 weeks were used. All mice were deprived of food beginning at 18 hours before experiments.

All experiments conformed to Japanese regulations concerning animal care and use, as laid out in the Guidelines for Animal Experimentation (Japanese Association for Laboratory Animal Science, 1987), and were approved by the Institutional Animal Care and Use Committee of Azabu University.

Purification of VT2

VT2 was purified from culture medium by affinity chromatography using commercial mouse anti-VT2 A subunit monoclonal antibody (Capricorn Products LLC, Portland, ME, USA)-coupled Sepharose 4B (GE Healthcare UK Ltd., Amersham Place, Little Chalfont, Buckinghamshire, England).[15]

Quantitation of proteins

IgG, IgM and S-IgA concentrations in bovine immune colostrum were measured by single radial immunodiffusion using diagnostic kits (Ecos Institute Co., Inc., Miyagi, Japan). Protein concentrations of purified VT2 and isolated IgG, IgM and S-IgA from bovine immune colostral whey were measured using Coomassie Brilliant Blue G-250.[16]

Preparation of bovine colostral anti-VT2 antibody

Immunization was by daily administration of crude VT2 to cows, in accordance with the procedure of Kuribayashi et al.[13,14] Colostral whey containing antibody was prepared in accordance with the method of Kuribayashi et al.[13] Bovine serum antibody against VT2 was obtained from cows after 14 injections of purified VT2. Bovine anti-VT2 IgG antibody for fluorescence enzyme immunoassay (FEIA) was isolated from bovine serum using a protein G column (GE Healthcare UK Ltd.).

Neutralization test

Neutralization titers for colostral antibodies were measured by neutralization tests using vero cells.[17]

Isolation of bovine colostral immunoglobulin classes

Isolation of immunoglobulin classes from bovine immune colostral whey was carried out by affinity chromatography. Colostral IgG antibody was isolated from pre-treated bovine colostral whey using a protein G column. Colostral IgM and IgA antibodies were isolated from the non-adsorbed fractions on protein G column chromatography using an anti-bovine IgM (μ) antibody (VMRD, Inc., Pullman, WA, USA)-coupled sepharose 4B (GE Healthcare UK Ltd.) column and an anti-bovine IgA (α) antibody (VMRD, Inc.)-coupled sepharose 4B column, respectively.[15] Identification of Ig

classes isolated from bovine immune colostrum was carried out by the Ouchterlony method.

Identification of Ig classes isolated from bovine colostrum

Identification of Ig classes isolated from bovine immune colostrum was carried out by Ouchterlony agar diffusion, according to standard procedures, with 1% agar (Wako Pure Chemical Industries Ltd., Osaka, Japan) prepared with veronal buffer (pH 8.6).

FEIA for measurement of VT2 in mouse sera

FEIA plates (Matrix Technologies Corporation, Hudson, NH, USA) were coated with 100 μL (per well) of serially diluted standard VT2 of known concentration or mouse sera. After blocking with 1% ovalbumin in 0.05 M carbonate buffer (pH 9.6) at 37°C for 1 hour and rinsing with washing buffer, 100 μL of bovine anti-VT2 IgG antibody isolated from serum was added to all wells. After washing, the horseradish peroxidase-conjugated rabbit anti-bovine IgG (MP Biomedicals, Inc., Solon, OH, USA) in PBS was added at 100 μL/well. After washing, 100 μL of fluorescence substrate consisting of a 9:1 mixture of Quanta Blu substrate solution and Quanta Blu stable peroxidase solution (Pierce Biotechnology, IL, Rockford, USA) was added to all wells. Relative fluorescence intensity (RFI) was measured using a fluorometer (MTP-600F; Corona Electric Company, Ibaraki, Japan) at 360 nm (excitation) and 450 nm (emission).

Preparation of anti-flagellum antibody against *E. coli* O157:H7

Somatic cells of *E. coli* O157:H7 were obtained by centrifugation of culture supernatant at 1600*g* for 15 minutes. Somatic cells of *E. coli* O157:H7 were injected into cows in accordance with the procedure of Kuribayashi et al. *E. coli* O157:H7 producing VT2 was centrifuged at 1600*g* for 20 minutes and heated at 121°C for 15 minutes in order to obtain somatic antigen. This somatic antigen and *E. coli* O157:H7 were mixed with bovine immune colostrum and left to stand at room temperature for 15 minutes. The anti-flagellum antibody was obtained by centrifugation of this mixture at 1600*g* for 15 minutes.

Preparation of antibody against somatic *E. coli* O157:H7

The mixture of somatic antigen heated at 121°C for 15 minutes and bovine immune colostral antibody were left to stand for 1 hour at room temperature. The precipitates were obtained by centrifugation, and glycine-HCl buffer (pH 2.3) was added to the precipitates to yield anti-somatic antibody.

Indirect fluorescence antibody (IFA) titers and specifities for anti-flagellum and anti-somatic *E. coli* O157:H7

Indirect fluorescence antibody (IFA) for *E. coli* O157:H7 in the anti-flagellum and anti-somatic colostral antibodies were performed using the method of Killinger et al, with some modification, i.e., with fluorescein-conjugated goat anti-bovine immunoglobulin (Monosan, Uden, Netherlands).

Animal experiments

Efficacy of each immunoglobulin class: The neutralization efficacy of each immunoglobulin class was evaluated in 95 mice. The evaluation methods were based on the methods of Kuribayashi et al.[13]

Serum levels of VT2 in mice: Forty-eight mice were divided into two groups, and VT2 was administered to each group. At 1 hour after administration of VT2, colostral antibody was administered to the colostral antibody group and sterilized saline was administered to the control group. Blood was collected at 2, 4, 8, 12, 16 and 24 hours after VT2 administration. At each time point, four mice were sacrificed under anesthesia, and blood was collected.

Efficacy of anti-flagellum or anti-somatic colostral antibody: Eighty mice were divided into four groups. Mice in each group were inoculated with *E. coli* O157:H7 producing VT2 (2.5×10^8 CFU/mL). At 1 and 3 hours after inoculation, each antibody was administered to the respective groups of mice. Both the anti-flagellum antibody and anti-VT2 antibody were prepared for administration at equal volumes. Sterilized saline was administered to the control group. Beginning at 6 hours after inoculation 3 times a day for 3 days, mice were administered fosfomysin (FOM) at a dosage of 500 μ/g body weight (Meiji Seika Kiasha, Ltd., Tokyo, Japan) in order to disrupt *E. coli* O157:H7 present in the intestine. Survival rates in each group were observed for 2 weeks after inoculation.

Results

VT2 levels in culture medium

The agglutination titer of VT2 in culture medium on reversed passive latex agglutination test was 1:64. After the culture medium was concentrated, the agglutination titer was 1:512. This agglutination titer corresponded to approximately 800 ng/mL VT2, and the concentrated culture medium was administered to mice for evaluation of the neutralizing efficacy of each immunoglobulin class.

Neutralizing antibody titers of bovine colostra and sera

The neutralizing antibody titer of colostrum obtained from dairy cows immunized with VT2 was 1:128. The neutralizing antibody titer of sera obtained from the same cow was 1:32. These colostral and serum antibodies were administered to mice for evaluation of their neutralizing efficacy against VT2.

Identification of Ig classes isolated from bovine colostra

The antigenicities of Ig classes isolated from bovine immune colostrum were identified. The Ouchterlony patterns are shown in Fig. 1. The anti-bovine IgA, IgG and

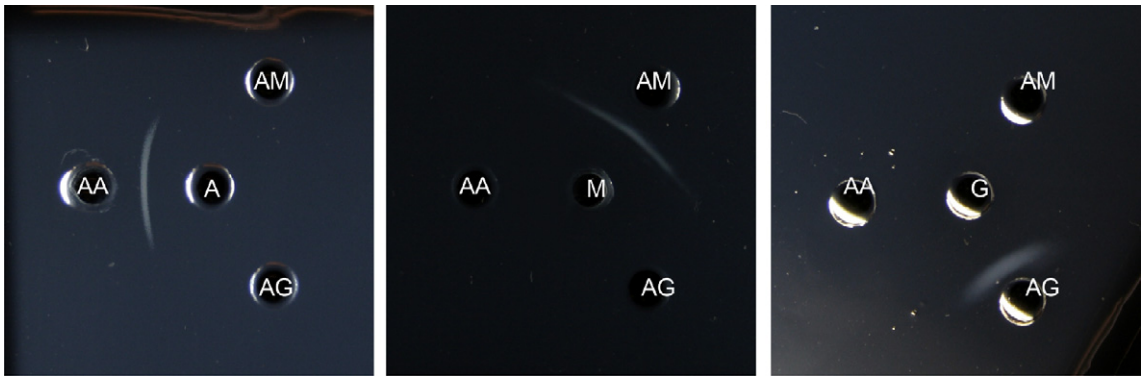

Figure 1. Ouchterlony analysis of S-IgA, IgM and IgG, isolated from immunized bovine colostrum. A = S-IgA; AA = rabbit anti-bovine IgA(α) antibody; M = IgM; AM = rabbit anti-bovine IgM(μ) antibody; G = IgM; AG = sheep anti bovine IgG(γ) antibody.

IgM sera, respectively, formed precipitation lines with the IgA, IgG and IgM isolated from bovine immune colostrum.

Concentrations of Ig classes isolated from bovine colostrum

The concentrations of IgG, S-IgA and IgM isolated from bovine colostrum were 94, 17 and 5 mg/mL, respectively. Each immunoglobulin antibody administered to mice was adjusted based on these concentrations.

Identification of anti-flagellum and anti-somatic antibodies against *E. coli* O157:H7

The specifities of anti-flagellum and anti-somatic *E. coli* O157:H7 antibodies are shown in Fig. 2. IFA titer for the anti-flagellum and anti-somatic antibodies against *E. coli* O157:H7 were 1:16 and 1:16, respectively. These antibodies were administered to mice after inoculation with *E. coli* O157:H7.

Efficacy of bovine anti-VT2 colostral immunoglobulin classes and anti-VT2 serum antibody

The survival rates of mice administered VT2 after treatment with bovine anti-VT2 colostral Ig classes and anti-VT2 serum antibody are shown in Table 1. Survival rates were 93.3% after treatment with colostral S-IgA and IgM antibodies and immune colostral whey groups. Survival rates in the groups treated with colostral IgG antibody and bovine serum antibody were 80.0% and 60.0%, respectively. On the other hand, all mice in the control group died.

The degree of intestinal bleeding with each immunoglobulin class is shown in Table 2. Intestinal bleeding was evaluated by visual inspection and was rated as follows: negative = 0; minimal = 1; moderate = 2; and severe = 3. Of dead mice treated with sterilized physiological saline, bovine immune colostral immunoglobulins and bovine serum antibody, 84.4% showed intestinal bleeding scores of 2 or 3, with 95.0% of dead mice in the control group showing scores of 2 or 3. In contrast, 33.3% of dead mice that had been treated with bovine serum antibody showed scores of 2 or 3. In addition, 15.6% of dead mice did not show any intestinal bleeding.

Serum levels of VT2 in mice

Serum levels of VT2 in mice after administration of VT2 are shown in Fig. 3. Serum levels of VT2 in the colostral antibody group were lower than in the control group, and significant differences were observed between the colostral antibody and control groups at 8 and 12 hours.

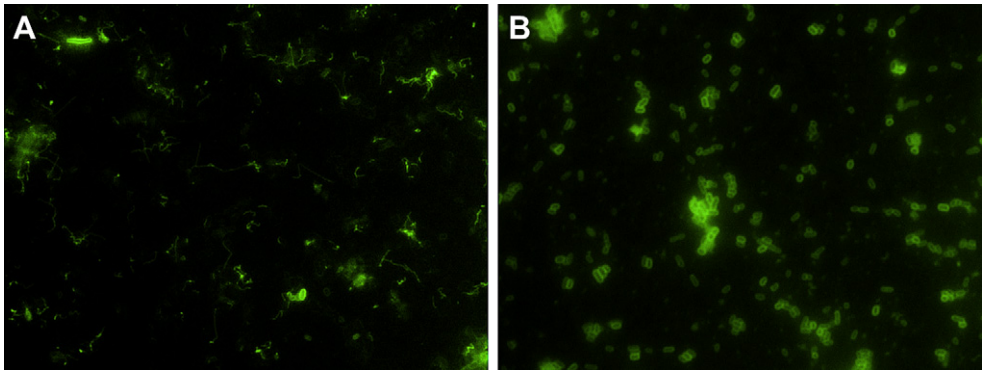

Figure 2. Specificity of anti-flagellum (A) and anti-somatic (B) antibodies against *E. coli* O157:H7 using the IFA method.

Table 1 Survival rates of VT2-administered mice treated with each immunoglobulin class of bovine anti-VT2 colostral and serum antibodies

Time after administration	Surviving mice treated with					
	S-IgA	IgM	IgG	Colostrum[1]	Serum antibody[2]	Sterilized saline
1 day	15	15	15	15	12	19
2 days	14	15	15	15	12	7
3 days	14	15	15	15	9	2
4 days	14	14	13	14	9	1
5—14 days	14	14	12	14	9	0
Survival rates	93.3% (14/15)	93.3% (14/15)	80% (12/15)	93.3% (14/15)	60.0% (9/15)	0% (0/20)

[1] Colostral whey containing antibody to VT2.
[2] Bovine serum containing antibody to VT2.
S-IgA, IgM and IgG antibodies were isolated from bovine immune colostrum.

Efficacy of anti-flagellum and anti-somatic antibodies against *E. coli* O157:H7

Survival rates of mice in each antibody group are shown in Table 3. Several accidental deaths in each group were observed after inoculation or administration of FOM. Survival rate in the anti-flagellum antibody group was higher than that in the anti-somatic antibody group. The anti-flagellum and anti-VT2 antibody group showed the highest survival rates among the four groups.

Discussion

E. coli O157:H7 infection is not generally treated using antibiotics,[8—11], as VT from *E. coli* O157:H7 disrupted by antibiotics leads to complications such as hemolytic uremic syndrome. A colostral antibody against *rotavirus* has been developed and its efficacy has been confirmed.[18] We therefore prepared a colostral antibody against VT by immunizing a cow with VT prior to pregnancy. This immune colostral antibody was expected to neutralize the toxicity of VT and inhibit VT absorption from the intestine. We demonstrated the neutralizing efficacy of immune colostral antibody against VT in weaning mice or beagle dogs. Furthermore, the present immune colostral antibody was confirmed to be resistant to proteases in the small intestines. However, efficacy was evaluated by administration of crude immune colostral antibody. The present study was conducted in order to clarify the efficacy of each immunoglobulin class against VT.

Table 2 Degree of intestinal bleeding in dead mice after administration of VT2

Treatment or after administration of VT2	Scores of intestinal				
	3+	2+	1+	0	Total
Sterilized (Control group)	18	1	0	1	20
Colostral antibody	0	0	0	1	1
Colostral S-IgA antibody	0	1	0	0	1
Colostral IgM antibody	0	1	0	0	1
Colostral IgG antibody	1	0	0	2	3
Serum antibody	4	1	0	1	6

Survival rates with S-IgA, IgM and colostral antibody were similar in mice administered VT2. S-IgA isolated from colostral antibody was found to have the highest resistance to proteases in the intestines of beagle dogs,[14] this may be the reason for the higher survival rate seen with S-IgA. On the other hand, we believe that IgM exists as a pentamer, and has greater affinity for VT2 than S-IgA,[19—21] despite its lower activity than S-IgA in beagle dogs,[14] and the concentration of IgM was one-third that of S-IgA.[14] The survival rate with IgM was therefore similar to that with S-IgA. The serum antibody showed the lowest survival rate, and this result was correlated with the resistance to protease *in vivo*. The differences in neutralizing efficacy on passive immune examination in the present mice, may have been due to differences in protease resistance *in vivo*. These results suggest that S-IgA or IgM is able to neutralize VT2 in the intestine. However, each immunoglobulin class was individually administered to mice. Only small amounts of antibody could be administered, as the mice used in the present study were small. Higher survival rates would be expected with repeated administration of each immunoglobulin class.

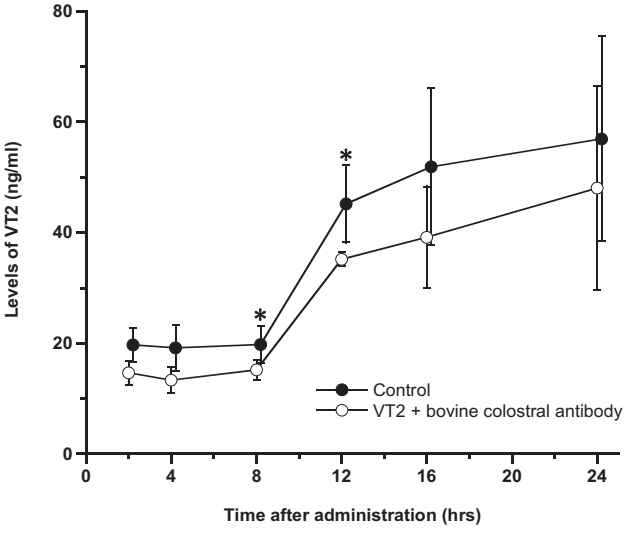

Figure 3. Serum levels of VT2 in mice after administration of VT2. Concentrations of VT2 in serum were measured by FEIA (* $p < 0.05$).

Table 3 Survival rates of *Escherichia coli* O157:H7-infected mice treated with anti-flagellum or anti-somatic cell antibody

Time after administration	Survived mice treated with			
	anti-flagellum antibody	anti-somatic antibody	anti-flagellum antibody and anti-VT2 antibody	sterilized saline
1 day	17	15	19	18
2 days	13	11	17	16
3 days	13	10	17	16
4 days	13	10	17	9
5—14 days	11	4	17	3
Survival rates	52.4 % (11/21)	22.2 % (4/18)	89.5 % (17/19)	15.0 % (3/20)

Furthermore, serum levels of VT2 decreased with administration of crude colostral antibody against VT2, as compared to control mice administered sterilized saline. This suggests that absorption of VT2 from the intestine is reduced by crude colostral antibody against VT2. This decrease in VT2 absorption was presumed to prevent injury to organs such as the kidney,[22,23] and was therefore considered to be one of the factors responsible for the improved survival rates.

The flagellum of *E. coli* O157:H7 is an important factor for adhesion to the intestine. We prepared a novel immune colostral anti-flagellum antibody, and we expected that *E. coli* O157:H7 infection would be prevented by inhibiting *E. coli* O157:H7 adhesion to the intestine. The anti-flagellum group showed an increased mice survival rate when compared to the anti-somatic group. This improvement of survival rate by administration of anti-flagellum antibody was presumed to inhibit *E. coli* O157:H7 adhesion to the intestine. Combined treatment with the anti-flagellum antibody and the anti-VT2 antibody further improved the survival rate, probably because simply inhibiting adhesion is only partially effective in limiting bacterial colonization, while the VT2 released by disrupted *E. coli* O157:H7 is neutralized by colostral anti-VT2 antibody. Thus, combination treatment with these antibodies is potentially useful against serious *E. coli* O157:H7 infections.

In conclusion, the immune colostral antibody against VT and the anti-flagellum antibody may be able to prevent serious complications in the treatment of *E. coli* O157:H7 infection.

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